METHODS IN VIROLOGY

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METHODS IN VIROLOGY

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Preface

In a letter to Dr. John L. Dorsey dated May 23, 1804, Benjamin Rush said that "A wide field opens for medical investigations in the United States. The walls of the Old School are daily falling about the ears of its masters and scholars. Come, and assist your Uncle and his friends in erecting a new fabric upon its ruins." In the seventeen years since the publication of the first volume of *Methods in Virology,* many "walls of the Old School" have fallen down to be replaced by "new fabric." The editors, being cognizant of these events, have tried in these two volumes to present the reader with up-to-date, modern revisions of techniques applied to animal, plant, and insect virology.

The early volumes in this series were published at the dawn of the era of molecular virology. Today, techniques applied to the study of molecular virology are becoming standard household techniques, and the contents of Volumes VII and VIII reflect the existence of the "new world" of virology. A series of books in existence for seventeen years may be regarded as a "venerable" one. We feel, however, that none of the preceding volumes can be classified as obsolete. New techniques and methods must be considered as improvements of previously described techniques, but not necessarily as their replacements. Moreover, one never knows what surprises the future holds for students of virology.

Volumes VII and VIII will be of considerable usefulness to all who are engaged in virus research, including graduate students interested in becoming familiar with modern techniques. Infectious disease specialists, bacteriologists, immunologists, vertebrate, invertebrate, and plant pathologists, parasitologists, biochemists, veterinarians, geneticists, and biotechnicians will find these volumes of interest. These two books are an important addition not only to the series but to the rapidly growing list of works dealing with viruses as well.

We express our thanks and appreciation to those who have contributed chapters to Volumes VII and VIII. The authors were chosen on the basis of their outstanding knowledge of given methods, as recognized authorities in

their specialized fields, or as creators of new techniques. We also wish to express our appreciation to the staff of Academic Press for continuous encouragement and advice throughout the planning and completion of these volumes.

KARL MARAMOROSCH
HILARY KOPROWSKI

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1 *Monoclonal Antibody Techniques Applied to Viruses*

Bellur S. Prabhakar, Martin V. Haspel, and Abner L. Notkins

I. Introduction

A polyclonal serum against a given virus may have the ability to neutralize the virus, fix complement, inhibit hemagglutination by the virus, or facilitate killing of virus-infected cells by macrophages and lymphocytes.

Whether these multiple abilities exhibited by the polyclonal serum are due to the same antibody molecules or to different molecules has been hard to evaluate. These questions could be answered if one could obtain large quantities of relatively homogeneous and pure antibody preparations. The main obstacle to this has been the inability of B lymphocytes to stay alive and replicate in culture over extended periods of time. This problem has been overcome by the development of hybridoma technology (Kohler and Milstein, 1975). The strategy for immortalizing specific immunoglobulin-producing B lymphocytes in culture is to fuse them with tumor cells. The ensuing hybrids are then subjected to a selection process in the presence of hypoxanthine, aminopterin, and thymidine (HAT) medium. The aminopterin blocks the de novo synthesis of DNA, which is a prerequisite for cell replication. However, normal cells are capable of utilizing hypoxanthine and thymidine as substrates in an alternative pathway to synthesize DNA. This alternative pathway requires two key enzymes, thymidine kinase (TK) and hypoxanthine guanine phosphoribosyltransferase (HGPRT). The myeloma cells used for fusion with lymphocytes have been selected for growth in the presence of either 5-bromo-2′-deoxyuridine (BUdR) or 8-azaguanine. Cells that grow in the presence of BUdR or 8-azaguanine lack the enzyme TK and HGPRT, respectively. Therefore, myeloma cells cannot synthesize DNA utilizing the alternative pathway in HAT selection medium. Although spleen cells contain both the enzymes, they still cannot survive *in vitro* for extended periods of time because of their inherent inability to grow in culture. However, the hybrids between lymphocytes and myeloma cells would grow in the HAT selection medium because of the availability of the key enzymes from the lymphocytes and the replicative machinery from the myeloma cells. Thus, the myeloma cells are actively prevented from replicating by the HAT medium, whereas the lymphocytes die out within 2–3 weeks.

The hybridoma technique has been used successfully to immortalize lymphocytes without compromising their ability to produce specific antibodies. By this technique, a large number of cells originating from a single hybrid cell producing a single type of antibody can be grown. Monoclonal antibodies against a large number of viruses have already been generated, and some of them have been characterized as to their biological properties. Monoclonal antibodies are useful in discerning the structural and functional properties of various viral proteins and have been instrumental in the identification of a large number of antigenic variants that were not previously known.

Table I lists a number of representative viruses against which monoclonal antibodies have been generated. Table I also includes the nature of antigens used for immunization, the assay for detection of antibodies, the

type of antibodies obtained, and a list of references. In this chapter, efforts will be made to give the reader an understanding of the principles of hybridoma techniques with a broad technical description. We will discuss the general strategy for immunizing animals, the fusion protocol, various methods for antibody screening, characterization of antibodies and viral antigens, and the generation of viral variants. An Appendix is also included to provide a specific protocol for preparing various reagents. For more details, the reader should refer to specific papers listed in Table I or to review articles (Kennet *et al.*, 1980; Milstein, 1980; Oi and Herzenberg, 1979a; Yelton and Scharff, 1980) that deal with this subject in detail.

II. Immunization Protocol

Viruses that are pathogenic for mice present both the advantages of a replicating antigen and the problem of morbidity and mortality. A number of strategies have been developed to overcome these problems. For example, monoclonal antibodies against bunyaviruses have been developed by inoculating mice with 1 LD_{50}, followed by a larger dose of virus given to the survivors (Gonzales-Scorano *et al.*, 1982). Alternatively, mice may be immunized with formalin- or β-propiolactone-inactivated virus, with subsequent immunizations with either inactivated or live virus (Balachandran *et al.*, 1981; Pereis *et al.*, 1982). Decreased pathogenicity may result when the route of inoculation is changed; for example, a very high dose (10,000 LD_{50}) of neurotropic mouse hepatitis virus may be inoculated with low mortality by the intraperitoneal route, as compared to the high mortality when administered by intracerebral inoculation (Collins *et al.*, 1982).

Mice may be immunized with viruses following procedures for other protein antigens. The immunogen may range from crude homogenates or intact virus-infected cells to highly purified single viral polypeptides. A crude lysate of infected cells would be the immunogen of choice where antibodies reactive with nonstructural viral proteins or virus-induced host proteins are required. For other studies, purified whole virions or fractions such as nucleocapsids or glycoproteins may be more appropriate antigens (Sweig *et al.*, 1979; Volk *et al.*, 1982). Purified viral antigens, at a dose of 50–200 μg, are usually administered in complete Freund's adjuvant, followed by subsequent immunizations in incomplete adjuvant. There is considerable diversity in the literature concerning the most appropriate schedule of immunizations. A list of references is provided in Table I. The most universally accepted procedure is that the final immunization be either intravenous or intraperitoneal, with removal of the spleen 3 days later.

TABLE I

REPRESENTATIVE VIRUSES AGAINST WHICH MONOCLONAL ANTIBODIES HAVE BEEN MADE

Virus	Immunogen	Assay	Type of antibodies	Reference
Alpha virus				
Sindbis virus	Purified virus	ELISA	Antistructural proteins	Roehrig *et al.* (1980); Schmaljohn *et al.* (1982)
Bunyavirus				
Tahyna	Live virus	ELISA	Antiglycoprotein and nucleocapside protein	Gonzales-Scarano *et al.* (1982)
Flavivirus				
West Nile	β-Propiolactone-inactivated or live virus	Solid-phase RIA	Antiglycoprotein	Pereis *et al.* (1982)
Tick-borne encephalitis	Purified glycoprotein	ELISA	Antiglycoprotein	Heinz *et al.* (1982)
Dengue type 2	Crude virus	RIA	HI-neutralizing CF	Gentry *et al.* (1982)
Dengue type 3	Crude virus	FA	Hemagglutination inhibition	Dittmar *et al.* (1980); Venkatesan *et al.* (1982)
Enterovirus				
Polio	Purified virus	Neutralization	Neutralizing	Emini *et al.* (1982); Icenogle *et al.* (1981)
Coxsackie B	Purified virus	Neutralization	Neutralizing	Prabhakar *et al.* (1982)
Foot-and-mouth disease virus	Purified virus	ELISA	Antiviral subunits	McCullough and Butcher (1982)
Rhabdovirus				
Rabies virus	Inactivated virus	RIA	Antiglycoprotein, nucleocapsid protein	Flamand *et al.* (1980a, b); Wiktor and Koprowski (1978)
Vesicular stomatitis virus (VSV)	Live virus	ELISA	Antiglycoprotein	Lefrancois and Lyles (1982)
VSV	Purified glycoprotein	RIA	Antiglycoprotein	Volk *et al.* (1982)
Paramyxovirus				
Measles	Live or disrupted virus	FA	Antihemagglutin, nucleoprotein, membrane and L protein	Bohn *et al.* (1982); Giraudon and Wild (1981)

Respiratory syncytial virus	Virus-infected syngenic cells	RIA	Neutralizing and antiviral glycoprotein	Cote *et al.* (1981); Fernie *et al.* (1982)
Mumps	Live virus	RIA	Hemagglutination inhibition	Server *et al.* (1982)
Orthomyxovirus				
Influenza virus	Live virus	RIA	Antihemagglutinin	Effros *et al.* (1979); Gerhard *et al.* (1981); Gerhard and Webster (1978)
Influenza virus	Purified virus	RIA	Antihemagglutinin	Koprowski *et al.* (1977)
Reovirus	Purified virus	RIA	Antiviral polypeptides	Lee *et al.* (1981)
Rotavirus	Purified virus	ELISA	Neutralizing and hemagglutination inhibition	Sonza *et al.* (1983)
Lymphocytic choriomeningitis virus	Crude or purified virus	FA	Antinucleoprotein and glycoprotein	Buchmeier *et al.* (1980)
Ecotropic murine leukemia virus	AKR K36 cells	RIA	Anti-MuLV antigens	Nowinski *et al.* (1979)
Xenotropic murine leukemia virus	Spleen cells from parental strain	FA	Anti-MuLV envelope polypeptides	Portis *et al.* (1982)
Adenovirus	Purified virus or soluble antigens	FA	Antihexon, penton, fiber, and glycoprotein	Russel *et al.* (1981)
Adenovirus	Adeno 5-transformed cells	Liquid-phase RIA	Anti-adeno type 5 Elb-58,000 tumor antigens	Sarnow *et al.* (1982)
SV40 tumor antigen	SV40-transformed cells	Liquid-phase RIA	Anti-SV40 tumor antigens	Harlow *et al.* (1981)
Mouse hepatitus virus	Live virus	FA	Antinucleocapsid and glycoproteins	Collins *et al.* (1982)
Hepatitis B	Purified HBe	Passive hemagglutination	Antihepatitis Be antigen	Imai *et al.* (1982)
Hepatitis B	HBs Ag	Solid-phase RIA	Antihepatitis Bs antigen	Shih *et al.* (1980)
Herpes virus				
Herpes simplex types 1, 2	Nucleocapsids	ELISA	Antinucleocapsids	Zweig *et al.* (1979)
Herpes simplex type 1	Live virus or virus-infected cells	Liquid-phase RIA	Antiviral proteins	Showalter *et al.* (1981)
Herpes simplex type 2	Inactivated lysates infected cells	RIA, FA, ELISA	Antiglycoprotein	Balachandran *et al.* (1981)
EB virus	Disrupted virus	RIA, indirect FA	Antimembrane antigens	Hoffman *et al.* (1980)
EMB (membrane antigen)	B95-8 cells	Membrane fluorescence	Antimembrane antigens	Strnad *et al.* (1982)

III. Preparation of Cells

A. MYELOMA CELLS

There are a number of murine myeloma cells that are HAT sensitive and do not secrete immunoglobulins. The nonsecreting myeloma cells are preferred because of the advantage of not having contaminating immunoglobulins in the culture supernatants. NS1 (P3-NS1/1-AG4-1) (Kohler and Milstein, 1975), SP2/0 (SP2/0-AG14) (Schulman *et al.,* 1978), and 653 (X63-AG8.653) (Kearney *et al.,* 1979) are the most commonly used myeloma cell lines. The cells are maintained in medium containing 8-azaguanine (20 μg/ml) to prevent them from reverting back from $HGPRT^-$ to $HGPRT^+$ cells. The cells should be maintained in log phase (passaged every 3 days). Three days prior to cell fusion, the cells should be passaged in medium free of 8-azaguanine to remove all the residual drug from the cytoplasmic pool.

B. FEEDER LAYER

Resident unstimulated peritoneal exudate cells (PEC) have been found to be extremely useful as a feeder layer, because in addition to being scavengers, they also seem to promote growth of hybrids. The PEC are collected by lavage of the peritoneal cavity. Briefly, the mice are exsanguinated (to minimize contamination with erythrocytes) and injected intraperitoneally (ip) with 6 ml of sterile phosphate-buffered saline (PBS). After gentle abdominal massage, the peritoneal cavity is aseptically opened and the saline is collected with a sterile Pasteur pipette. The cells are pelleted, washed once with medium, and resuspended in HAT medium. The large-sized cells are counted, and the PEC are plated into 96-well plates at a density of 5×10^3 cells (in 100 μl) per well. It is most convenient to prepare the feeder layer the day before the fusion.

C. SPLENIC LYMPHOCYTES

A single-cell suspension of splenic lymphocytes may be prepared by a variety of techniques. A convenient method is to fasten a piece of 93 μm Nitex mesh (J. E. Frankle Company, Philadelphia, PA) to the top of a 100-ml beaker and gently to force the spleen against the Nitex mesh with a plunger from a 3-ml plastic syringe. During this procedure, the spleen should be constantly bathed with 10 ml of serum-free medium. The process is continued until only the spleen capsule remains. The cells are centrifuged at 110 g_{max} for 10 min and resuspended in 5 ml of 0.83% ammonium chloride. After incubation at room temperature for 3–4 min, the cell suspension is

centrifuged again. The ammonium chloride lysis of erythrocytes should be repeated only if the pellet is still excessively contaminated. The spleen cells are resuspended in 5 ml of serum-free medium and layered over 5 ml of fetal bovine serum. The discontinuous gradients are centrifuged at approximately 110 g_{max} for 10 min. The viable spleen cells will have pelleted to the bottom of the 15-ml centrifuge tube. The cells are then resuspended in serum-free medium and counted, and viability is determined.

IV. Hybridization

The viability of both the lymphocytes and the myeloma cells should not be less than 90%. The spleen cells and myeloma cells are washed at least twice in warm serum-free medium and then mixed at a ratio of 10:1 and centrifuged together at 110 g_{max} for 10 min in a 50-ml centrifuge tube. All the supernatant medium is removed, and the cell pellet is resuspended gently in approximately 100 μl of medium. One milliliter of 50% polyethylene glycol (PEG) (molecular weight 1000), prewarmed to 37°C, is used for fusing 100×10^6 spleen cells with 10×10^6 myeloma cells. Over the course of 1 min, PEG is added dropwise to the cell pellet while gently agitating the tube. Then 1 ml of prewarmed serum-free medium is added dropwise in the course of 1 min, again while gently agitating the tube. During each ensuing minute, twice the previous volume of the medium is added to the cell suspension until the tube is filled. In this manner, the PEG is gradually diluted. After centrifugation at 110 g_{max} for 10 min, the cell pellet is gently resuspended in HAT medium to a concentration of 2.5×10^6 cells/ml, and 100 μl is added to each well containing PEC feeder layer (5×10^3 cells per well of a 96-well flat-bottomed microtiter plate). If the cells are left too long in PEG, severe damage will result. Conversely, if the cells are left for too short a period in PEG, the fusion may not be complete. Therefore, the fusion protocol should be followed with care.

V. Maintenance and Specificity Testing of Hybridomas

A. CARE OF HYBRIDOMAS

Every 3–4 days, remove one-half of the supernatant fluid from each well and replace with 100 μl of fresh HAT medium. Maintain the hybridomas in HAT medium for a minimum of 2 weeks and then grow them in medium containing hypoxanthine and thymidine (HT) for 2 weeks. The cells can then be maintained with normal growth medium.

B. SCREENING OF HYBRIDOMAS

To decrease the possibility of false negatives, screen the hybridomas for specific antibody when the cells have reached maximum saturation density. This may occur as early as 12 days or as late as 28 days after fusion. If the assay requires more than 1 day, expand the cells from each well of a microtiter plate into the well of a TC-24 plate containing 0.5 ml of HT medium because the viability drops precipitously once saturation density has been reached. Because of this, having a proper assay is very critical. The ideal assay is both sensitive and rapid, so that an immediate decision can be made as to whether or not to clone the hybridoma. The choice of the assay is also extremely crucial because it can greatly affect the selection of antibodies with different specificities. Table I lists some of the assays used for screening antiviral antibodies. For example, in order to obtain monoclonal antibodies useful for the selection and analysis of antigenic variants, viral neutralization assays were used as a screen (Icenogle *et al.*, 1981; Emini *et al.*, 1982; Prabhakar *et al.*, 1982). Immunofluorescence permits the detection of a wide range of surface and cytoplasmic antigens including nonstructural proteins (Bohn *et al.*, 1982; Buchmeier *et al.*, 1980; Russel *et al.*, 1981). Binding assays, both solid-phase and liquid-phase radioimmunoassays (RIA) as well as enzyme-linked immunosorbent assay (ELISA), are rapid means of screening for antiviral antibodies (Koprowski *et al.*, 1977; Wiktor and Koprowski, 1978; Effros *et al.*, 1979). If the fusion is carried out shortly after primary immunization without a boost, one gets predominantly an IgM antibody response. Repeated inoculations will give rise to hybridomas that make IgG antibodies. If the animals are properly immunized, a very high proportion (60–80%) of the hybrids may be positive for the antiviral antibody.

1. *Microneutralization Assay*

One hundred tissue culture infectious dose 50 ($TCID_{50}$) of virus in 50 μl of medium are mixed with an equal volume of hybridoma supernatant fluid in a microtiter well and incubated at 37°C for 1 hour. Susceptible cells (approximately 10^4) are then added, and the plate is incubated until appropriate viral cytopathic effect (CPE) is observed in the control wells. In some viral systems, freshly trypsinized cells may be less sensitive to virus infection. In these cases, the virus–antibody mixture should be transferred to a well containing a monolayer of susceptible cells (Icenogle *et al.*, 1981; Emini *et al.*, 1982; Prabhakar *et al.*, 1982).

2. *Immunofluorescence (FA)*

a. Surface. Virus-infected cells, grown on coverslips, tissue culture slides, or in suspension, are incubated with hybridoma supernatant fluid for 1 hr

at 4°C, washed with four changes of PBS (3 min per wash), and then incubated with fluorescein- or rhodamine-conjugated anti-mouse Ig. The cells are again washed, mounted under phosphate-buffered glycerol (10% PBS, 90% glycerol), and examined using a fluorescence microscope. Some viral surface antigens are well preserved by light fixation with 4% paraformaldehyde (in PBS, pH 7.4, for 3 min), allowing for the preparation of suitable target cells several days in advance (Flamand *et al.*, 1980b; Giraudon and Wild, 1981; Bohn *et al.*, 1982).

b. Cytoplasmic Antigens. The infected cells should be washed with PBS, air dried, and fixed with acetone at room temperature for 5–10 min, or fixed without air drying with acetone at −20°C for 10 min. The fixed cultures can be kept at −20°C with little or no loss of antigenicity. The incubations may be done at 37°C for 30 min or at room temperature for 1 hr. Background, owing to nonspecific binding of the anti-mouse conjugate, is a greater problem with cytoplasmic than with surface fluorescence. Consequently, more extensive washing may be necessary (Flamand *et al.*, 1980a; Buchmeier *et al.*, 1980).

3. *Enzyme-Linked Immunosorbent Assay (ELISA)*

Microtiter ELISA plates (Dynatech), plastic-coated metallic beads (Litton Bionetics), or other suitable substrates are coated with viral antigens in carbonate buffer (pH 9.6, 1.59 g of Na_2CO_3 + 2.93 g $NaHCO_3$/liter) by incubation for 16–24 hr at 2–8°C (Imai *et al.*, 1982; Sonza *et al.*, 1983). Although carbonate buffer works well for most proteins, some viruses attach better to the plates under different pH and buffer conditions (Katze and Crowell, 1980). Therefore, the optimal conditions for coating the plate and the concentration of antigen need to be determined for each experimental system, and a large number of different methods for this are available (Table I). The plastic surface is washed and blocked with a suitable irrelevant protein antigen, such as 1% bovine serum albumin or 10% normal goat serum, by incubation at room temperature for 30 min. After several washes, the antigen is incubated with hybridoma supernatant fluid for 60–90 min at either room temperature or 37°C. The wells are washed with at least three changes of PBS containing 0.05% Tween 20 and 1% bovine serum albumin. The antigen is then incubated as described above with anti-mouse Ig (IgA + IgG + IgM) conjugated with peroxidase. The wells are again washed at least six times and incubated with a suitable substrate, such as 0.01–0.05% 2,2′-azinodi-(3-ethylbenzothiazoline 6-sulfonate) (ABTS) (Litton Bionetics), for 20 min at room temperature. The reaction is then stopped by the addition of sodium fluoride (1.25%). The plates may be read either visually or with one of the commercially available ELISA plate readers.

4. *Solid-Phase Radioimmunoassay (RIA)*

The wells of polyvinyl microtiter plates are coated with either purified virus or virus-infected cell extract (Shih *et al.*, 1980; Pereis *et al.*, 1982; Flamand *et al.*, 1980a). The plates are incubated until the viral antigens are dried onto the wells. These wells are filled with PBS containing 10% γ-globulin-free horse serum (HS) and incubated for 30 min at room temperature. Buffer is removed, and 50 μl of hybridoma culture supernatant is added. The plates are incubated at 37°C for 1 hr and washed five or six times with PBS containing 1% HS. Fifty microliters of an appropriate dilution of ^{125}I-labeled anti-immunoglobulin antibody is added to each well and incubated for 1 hr at 37°C. The plates are washed five or six times with PBS containing 1% HS. The wells are cut off from the plate, and the radioactivity bound to the well is determined using a gamma counter (Koprowski *et al.*, 1977).

5. *Liquid-Phase Radioimmunoassay*

This type of screening procedure requires a radiolabeled preparation of purified virus. Various radiolabeling and purification procedures are available (Rueckert and Duesberg, 1966; McClintock *et al.*, 1980), and an appropriate method should be chosen for a given virus. In general, the viruses can be labeled using, e.g., [^{35}S]methionine, [^{3}H]uridine, or [^{3}H]leucine in the medium. The labeled virus is harvested and then can be purified on either sucrose or cesium chloride gradients, or both. Hybridoma supernatant fluid (50 μl) is mixed with labeled virus in 150 μl of a suitable buffer and incubated for 3–4 hr at room temperature. The immune complexes are precipitated by adding heat-killed, Formalin-fixed *Staphylococcus aureus* cells and centrifuging the tubes at 3000 g_{max} for 10 min. Both the pellet and the supernatant are counted separately in a liquid scintillation counter. Certain types of immunoglobulins are not precipitated effectively by the *S. aureus* cells. Therefore, a second anti-immunoglobulin antibody should be used to precipitate the immune complexes.

VI. Cloning of Hybridomas

Because a well may contain a mixed population of producer and nonproducer hybrid cells, it is extremely important to clone the cells as soon as possible; otherwise, the nonproducer cells may outgrow the productive hybridoma cells. Cloning by limiting dilution in liquid medium is a well accepted method (McKearn, 1980). The use of PEC feeder layers increases the efficiency of cloning. PEC feeder layers are prepared as previously described and are plated at 5×10^3 cells per well. Wells are seeded with an

average concentration of 10, 5, and 1 cells per well in HT medium. Plates are incubated at 37°C and left undisturbed for 5 days. At that time, the wells are examined for the presence of a single colony of hybrid cells. The hybrid cells can be readily distinguished from other cells because they grow in clumps. In addition, PEC feeder layer cells often will serve as "anchors," aiding in the recognition of a single colony. Single colonies observed at limiting dilution are probably monoclonal. It is preferable that antibody-positive clones be recloned a second time. When the clones have grown to confluence, the supernatant fluids are examined for the production of specific antibody. The positive clones are then expanded and an adequate supply of backup cells is stored in liquid nitrogen as early as possible. After extensive cell passage, the hybridomas may require recloning. Although there are other procedures available for cloning of hybrid cells, this is a very simple and effective method.

VII. Determination of Immunoglobulin Class

The procedures for purification of immunoglobulin vary with immunoglobulin isotype. The isotype may be determined by Ouchterlony double diffusion. Alternatively, there are very sensitive ELISA kits now available commercially for the determination of immunoglobulin isotype.

VIII. Preparation of Ascites Fluid

Hybridomas in culture typically produce 10–100 μg of immunoglobulin per milliliter. In contrast, when grown as ascites tumors, hybridoma cells may produce as much as 20 mg of immunoglobulin per milliliter of ascites fluid. When large quantities of immunoglobulin are desired, preparation of ascites fluid may be preferred. On the other hand, if highly purified immunoglobulin preparations are required, they can be readily obtained with relative ease from tissue culture supernatant fluid, especially when γ-globulin-free serum is used. For inducing ascites, syngeneic mice should be used. If this is not possible, one should consider various immunosuppressive measures in order to prevent host-versus-graft reaction before inoculating the hybrid cells into mice. Mice are injected intraperitoneally with 0.5 ml of Pristane (2,6,10,14-tetramethylpentadecane) (Aldrich Chemicals). Two weeks after priming, the mice are injected intraperitoneally with 5×10^6 viable hybridoma cells. At 10–14 days after injection, the ascites fluid is aseptically collected and clarified at 250 g_{max} for 20 min. After incubation at 56°C for 30 min to inactivate the complement, the ascites fluid should be tested. This can be used as is, concentrated, or purified.

IX. Concentration and Purification of Immunoglobulins

The immunoglobulins from culture supernatants or the ascites fluid can be concentrated by precipitating with saturated ammonium sulfate solution (50%, v/v) at 4°C. The precipitate is resuspended in PBS and dialyzed extensively against PBS. This can then be lyophilized and stored at 4°C.

The antibodies can be obtained in a pure form by passing the culture supernatant or ascites fluid through a protein A–Sepharose or an anti-immunoglobulin antibody-affinity column (Prowse and Jenkin, 1978). The columns are washed extensively with the buffer (0.1 *M* NaCl and 0.01 *M* Tris-HCl, pH 7.5) to remove unbound proteins. The antibodies bound to the affinity column are eluted using 0.1 *M* glycine–HCl solution with pH 2.5. The eluate is made neutral before dialysis, and eluted proteins are dialyzed extensively against PBS, lyophilized, retested, and stored at 4°C.

X. Characterization of Viral Antigens Using Monoclonal Antibodies

Monoclonal antibodies have become very useful in understanding the structure and biological function of viral subunits. Since these antibodies have unique specificities, they react with particular epitopes that may be expressed only on a certain protein. Taking advantage of these interactions, various structural proteins of viruses have been isolated in a relatively pure form. The most commonly used technique for this purpose is immunoprecipitation, followed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) (Lee *et al.*, 1981) and Western blotting (Towbin *et al.*, 1979).

A. ISOLATION OF VIRAL PROTEINS BY ELECTROPHORESIS

Purified virus or virus-infected cell lysate radiolabeled with either [^{35}S]methionine or [^{3}H]leucine (or with any other suitable radiolabeled substrate) can be prepared according to procedures already published (Burstin *et al.*, 1982; McCullough and Butcher, 1982; Prowse and Jenkin, 1978). Virus-infected cell lysate or the virus is solubilized in immunoprecipitation buffer (e.g., 0.2% SDS, 0.5% sodium deoxycholate, 0.5% NP-40 and 0.85% sodium chloride). Insoluble fraction is removed by centrifugation (12,000 g_{max} for 20 min). To this, monoclonal antibody is added, and the mixture is allowed to incubate at room temperature for 3 hr or at 4°C overnight. The immune complexes are then precipitated by using a 10% suspension of *S. aureus* (incubate for 30 min at room temperature). The pellet is washed with Tris-HCl buffer (e.g., 10 m*M* Tris-HCl and 0.15 *M* NaCl, pH 7.6) and resuspended in a small volume of buffer containing 1% NP-40.

The proteins are solubilized by adding an equal volume of 2× sample buffer (e.g., 0.15 *M* Tris, 4.6% SDS, 10% 2-mercaptoethanol, 20% glycerol, and 0.2% phenol red, pH 6.8). This solution is boiled for 10 min and centrifuged at 12,000 g_{max} for 20 min. The supernatant is loaded onto a 10–15% polyacrylamide gel for electrophoresis. After electrophoresis, the gel can be stained, destained, and autoradiographed. The viral polypeptides reactive with the monoclonal antibodies will be immunoprecipitated and thus appear as bands on the autoradiogram. Caution should be exercised, since all subclasses of immunoglobulins do not bind well to the *S. aureus*. This problem can be overcome by using a second anti-immunoglobulin antibody and then allowing reaction with *S. aureus*.

B. CHARACTERIZATION OF VIRAL PROTEINS BY THE WESTERN BLOT TECHNIQUE

Unlabeled virus-infected cell extract or the purified virus can be separated by SDS-PAGE (see above), and the separated proteins can be electrophoretically transferred to a nitrocellulose paper (Towbin *et al.*, 1979). After the transfer, the nitrocellulose paper is saturated with 3% bovine serum albumin in Tris-HCl buffer for at least 4 hr to prevent nonspecific binding of the subsequently added protein. The paper is then incubated with the antibody for 3–4 hr at room temperature and washed in at least six changes of buffer. To this one can add anti-immunoglobulin antibody or protein A labeled with either peroxidase or ^{125}I. This second antibody or protein A will react with the first antibody bound to the viral protein on the blot. After incubation for at least 2 hr at room temperature, blots are again washed as above to remove excess antibody or protein A. To visualize the reactive polypeptides, the blots can be autoradiographed. If the second antibody or protein A is conjugated to peroxidase, one should add the substrate mixture (5 mg of 3,3′-diaminobenzidine and 10 μl of H_2O_2 in 40 ml of distilled water) to the blot. The polypeptides that react with the antibody will be stained brown.

C. COMPETITIVE INHIBITION ASSAY FOR DETERMINING THE IMMUNOGLOBULIN SPECIFICITY

This will allow determination of the specificity of reaction of different monoclonal antibodies. The basic principle is to allow one antibody to react with the protein and then measure the inhibition of binding of a second antibody to the same protein. The ability of the first antibody to inhibit binding of the second antibody competitively will indicate whether the two antibodies in question are reacting with the same determinant (Lubeck and Gerhard, 1981). The most commonly used approaches are either RIA or

the ELISA. Wells of a microtiter or ELISA plate are coated with the antigen and made to react with an unlabeled monoclonal antibody. After incubating for 1 hr at 37°C, the wells are washed extensively. To the same wells, as well as to those to which the first antibody was not added, a second antibody labeled with either ^{125}I or peroxidase is added and incubated for 1 hr at 37°C. The wells are thoroughly washed. In the case of RIA, the wells are cut and the radioactivity is determined. In the case of ELISA, the substrate is added to each well and the optical density of the reaction is determined.

D. SELECTION AND CHARACTERIZATION OF ANTIGENIC VARIANTS

The ability of monoclonal antibodies to react with very small but well-defined portions of the virus enables one to determine the major antigenic domains of the virus, as well as to map the epitopes in a given antigenic site. Such studies require a large number of antigenic variants with minor differences. These variants can be obtained using monoclonal antibodies as a basis for their selection.

Tenfold dilutions of plaque-purified virus preparations are incubated for 1 hr at 37°C, either in the presence or the absence of monoclonal neutralizing antibody (ascites fluid containing a high titer of the antibody). The virus antibody mixtures are then added to monolayers of cells in petri dishes and absorbed for 60 min. The inoculum is removed, and cultures are overlaid with medium containing the same antibody used for neutralization and 2–3% methylcellulose or 1% agarose. This will prevent any residual virus from infecting the cells. After 3–7 days of incubation, the cells are stained with neutral red and plaques are counted. The plaques that arise in the presence of antibody are presumed to be variants. The frequency of mutation can be calculated by dividing the number of plaques in the presence of antibody by the number of plaques in the absence of the antibody (Prabhakar *et al.*, 1982).

In order to confirm that the presumed variants are true variants, the plaques are randomly picked, grown, and tested for the ability of the variant virus to resist neutralization by the selecting antibody. This approach is extremely useful to study the frequency of mutation. Analysis of these mutant viruses may also provide insights into the mechanisms of viral mutation that result in new antigenic variants.

XI. Prospectus

Hybridoma technology has created a revolution in the biomedical sciences, particularly in virology. Prior to the availability of monoclonal an-

tibodies, the production of monospecific sera required the use of highly purified immunogens and often necessitated exhaustive adsorptions. It is now possible to produce large quantities of antibodies specific for a single epitope on a single viral polypeptide using crude antigen for immunization. For example, monoclonal antibodies have been isolated that distinguish between herpes simplex types 1 and 2 (Holland *et al.*, 1983). Because of high specificity and low background, monoclonal antibodies will permit the rapid differential diagnosis of many viral infections. In addition, through the use of competitive inhibition, sensitive binding assays for antiviral antibodies will be readily available.

The ability of a monoclonal antibody to detect small antigenic variations is revolutionizing viral epidemiology. It is now known that viruses such as rabies and Coxsackie B4, which were thought to be homogeneous, actually show considerable variation. Monoclonal antibodies can be used to classify naturally occurring antigenic variants, and also to select variants in the laboratory. Therefore, it is now possible to ask fundamental questions concerning the relationship of antigenic variation, tissue tropism, and disease.

The capability of generating large quantities of monoclonal antibodies opens the possibility of passive immunotherapy of viral diseases. These antibodies might be useful therapeutics in life-threatening diseases such as rabies. Monoclonal antibodies have also facilitated the molecular study of viruses. The assignment of biological functions to particular viral polypeptides can readily be done through the use of monoclonal antibodies. For example, monoclonal antibodies that neutralize poliovirus react with VP1, suggesting that this polypeptide is the target of virus neutralization (Blondel *et al.*, 1983). Anti-idiotypic antibodies directed against monoclonal antiviral antibodies may permit the isolation and subsequent characterization of cell surface virus receptors (Nepom *et al.*, 1982).

Finally, more recent data suggest that certain viruses may share antigenic determinants with normal host antigens (Fujinami *et al.*, 1983; Dales *et al.*, 1983; Gould *et al.*, 1983). These studies would not be possible with the use of conventional polyclonal sera. The development of these cross-reacting antibodies in response to virus infection may be a factor in triggering autoimmune diseases. Thus, antiviral monoclonal antibodies may have significant impact upon allied fields such as immunopathology.

XII. Appendix: Preparation of Reagents

1. *8-Azaguanine Stock (250X).* Prepare stock to a final concentration of 5 mg/ml in water. A small amount of NaOH (approximately 0.5 ml 1 *M* NaOH/100 ml stock solution) should be added to bring the 8-

azaguanine into solution. The stock solution is sterilized by filtration (0.2 μm), aliquoted, and stored at $-20°C$.

2. *Hypoxanthine/Thymidine Stock (100X).* Hypoxanthine (136 mg) and thymidine (38 mg) are mixed with 50 ml of water; 5 *M* NaOH is added dropwise until they are dissolved, and the volume is brought up to 100 ml. After sterilization by filtration, the HT stock solution is aliquoted and stored at $-20°C$.

3. *Aminopterin Stock Solution (1000X).* Aminopterin (17.4 mg) is suspended in 50 ml of water; 5 *M* NaOH is added dropwise until the aminopterin dissolves, and the volume is brought up to 100 ml. The aminopterin stock solution is also sterilized by filtration, aliquoted, and stored at $-20°C$.

4. *Polyethylene Glycol (PEG) 50% (w/v).* There are variations in the toxicity and fusion efficiency of different batches of polyethylene glycol. It is therefore important that the PEG be pretested by the investigator. Melt PEG 1000 (J. T. Baker) at 60°C, mix 50 g with serum-free medium prewarmed at 45°C, and adjust the pH to 7.4. Bring the volume up to 100 ml, and sterilize the 50% PEG by filtration. Aliquot and store at $-20°C$.

5. *Growth Medium.* There is a variation among different batches of fetal bovine serum in their ability to support cell growth. Therefore, the serum should be pretested using myeloma cells to ensure optimal conditions for the growth of hybrids. The growth medium used for many murine myeloma cells consists of Dulbecco's modified Eagle's medium with high glucose (4.5 g/liter) supplemented with 10% fetal bovine serum, sodium pyruvate (10m*M*), L-glutamine (2 *mM*), gentamicin sulfate (20 μg/ml), and fungizone (0.1 μg/ml).

6. *Myeloma Medium.* Use growth medium containing a 1:250 dilution of 8-azaguanine stock.

7. *HT Medium.* The composition is the same as for HAT medium without the addition of aminopterin.

8. *HAT Medium.* Growth medium is supplemented with an additional 10% fetal bovine serum, HEPES buffer (25 m*M*), a 1:100 dilution of HT stock, and a 1:1000 dilution of aminopterin stock.

9. *Freezing Medium.* Use growth medium containing 50% fetal bovine serum and 7.5% dimethyl sulfoxide (DMSO).

10. *Freezing and Thawing of Cells.* Freeze 2×10^6 viable cells in 1 ml of freezing medium in vials in a deep freezer ($-70°C$). This helps to bring down the temperature gradually and minimizes damage to the cells. After overnight storage in the deep freezer, the cells can be transferred to the liquid nitrogen. To reconstitute, thaw the cells at once by immersing the vial in an alcohol bath. Spin down and resuspend the cells in 5 ml of growth medium. After 24 hr, the cells can be resuspended in fresh medium.

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2 Competition Radioimmunoassays for Characterization of Antibody Reactions to Viral Antigens

Howard R. Six and Julius A. Kasel

I. Introduction

The development of radioimmunoassays (RIA) in the late 1950s by Berson and Yalow represents a landmark in biochemistry because of the remarkable sensitivity with which antibodies and polypeptide antigens could be detected (Berson *et al.*, 1956; Berson and Yalow, 1958; Yalow and Berson, 1959, 1960). Initially the RIA methodology was utilized primarily in studies of peptide and nonpeptide hormones, and the general principles governing the development of RIA procedures were established during this time (Elkins, 1974; Goldsmith, 1975; Yalow, 1980). This methodology was later extended to the labeling of antibodies rather than the antigen, and the method was called immunoradiometric assay (Miles and Hales, 1968a,b). Since then numerous technological variations of the assay have been described, and the RIA methodology is now used in laboratories throughout

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the world with an application to virtually every area of biology and medicine.

Until recently, characterizations of viral antigens and antibody responses to these have been performed by a variety of precipitation, immunological, and biological tests such as double immunodiffusion, complement fixation, enzyme inhibition, and hemagglutination inhibition assays. Each of these methods has at least two potential disadvantages. First, only a portion of an antibody response to an antigen is measured; and second, no data on the nature of the antigenic determinants mediating the cross-reaction are provided. Because competition RIA assays can overcome both of these disadvantages, its utilization for assessments of antigenic relationships has increased dramatically over the last 10 years. In general, the solid-phase type of assay is not as versatile and quantitative as the radioimmunoprecipitation (RIP) method for assessment of an antibody–antigen reaction. This is mainly attributable to the use of nonpurified antigens and the technical problem in controlling the antigen concentration in the test system. In virology, hepatitis B virus provided the initial stimulus for the application of this type of methodology to the study of a viral agent (Feinstone *et al.*, 1979). The development of competition RIP assays led to the acquisition of a considerable body of knowledge on the definition of antigenic determinants of the hepatitis surface antigen (Budkowska *et al.*, 1977; Gerin *et al.*, 1975; Shih and Gerin, 1975; Shih *et al.*, 1978). With the advent of techniques to produce protein antigens in a highly purified form, the competition RIP method was adapted to the study of other viruses.

In this chapter, the methods of competition RIP assay as developed for immunological assessments of adenoviruses and influenza viruses are described (Scott *et al.*, 1975, 1979; Kasel *et al.*, 1978; Six and Kasel, 1978, 1979; Six *et al.*, 1981, 1982). Our goal is to provide a technical background that can be used for the performance of the same methods with other viral protein antigens and to illustrate the type of information that can be generated from their application. No attempt has been made to cite all the original publications leading to the current state of knowledge on RIA; rather, references to publications that describe alternative procedures and those that discuss the relative merits of different methodologies have been included.

II. Preparation and Characterization of Reagents

A. Protein Antigens

A purified protein preparation to be used as a tracer antigen is a critical requirement for the development of a highly specific and sensitive competition RIP (Berson and Yalow, 1968). The use of nonpurified proteins

can lead to serious errors (Henry, 1967). Antigen preparations such as those described for the capsid proteins of adenoviruses (Pereira *et al.*, 1968; Mautner and Pereira, 1971) and hemagglutinin (HA) proteins of H3N2 viruses (Laver, 1964) are ideal types of proteins acceptable for use in assays.

The purity of viral antigens can be assessed by numerous procedures, such as sodium dodecyl sulfate (SDS)–polyacrylamide gel electrophoresis molecular sieve chromatography, and in some cases by isoelectrofocusing (Cooper, 1977).

B. COMPETING ANTIGENS

The source of competing antigen used in RIP assays is usually determined by the purpose of the experiment. The critical considerations are that protein preparations be of a sufficient volume for the needs of the study and that the proteins be antigenically active. The specificity of the reaction is usually derived from the purity of the tracer antigen. Consequently, partially purified preparations of competing antigen can be employed. For example, influenza virions purified by rate zonal centrifugation in a sucrose gradient and a soluble adenovirus capsid protein preparation obtained by banding in a discontinuous cesium chloride gradient have been shown to be satisfactory reagents for immunological assessments. However, as amply documented by previous studies (Hunter, 1973; Suda *et al.*, 1978) interference in the assays can come from unexpected sources, e.g., tissue cell receptors for the viral antigen, cross-reactions with cellular or blood proteins, and nonspecific inhibitors of antigen–antibody reactions.

C. SPECIFIC ANTIBODY POPULATIONS

Three different immunoglobulin preparations are required for the performance of competition RIP tests. These include a serum containing antibodies to the tracer antigen, serum from an animal of the same species that is free of antibody to the tracer antigen (carrier serum), and an antiserum raised in another species to immunoglobulins of the first antiserum (second antibody).

1. *Antibody to the Viral Antigen*

Many different procedures have been used in the preparation of an antiserum for a RIA, and some investigators claim that success is more an art than a science. This is due in part to a wide variety of substances that have been used as immunogens (e.g., steroids, peptides, lipids, and nucleic acids). Many of these substances behave as haptens and are in general poor immunogens or nonimmunogenic. Also, some are normally found in animals and humoral immune responses to them are strictly regulated to prevent

autoimmune diseases. Neither of these restrictions severely limits the production of an antiserum to viral protein antigens, and, in general, the choice of animal species and the method of immunization reflect practical rather than theoretical considerations. However, as noted by several investigators a highly avid antiserum will increase the sensitivity of antigen detection and reproducibility of test results (Elkins, 1974; Hunter, 1973; Rodbard *et al.*, 1971). For comparison of antigens it is preferable to have as many antibody specificities as possible represented in the antiserum. For these reasons some type of adjuvant is generally employed for preparation of antiserum (Hunter, 1973; Vaitukaitis, 1981). While a purified protein is not a prerequisite for preparation of an antiserum, the quantity of protein and the need to be in "native" form are essential considerations.

For human serum specimens, the availability of appropriately documented specimens is the major factor. If one wishes to characterize a serum antibody response following a viral infection, isolation of the etiological agent, in addition to a serological rise, is an advantage. Also, some knowledge of the individual's past exposure to the antigen is helpful, since this may influence the response.

Of a more practical nature, the serum specimen should not contain particulate material, as this may increase the background in RIP assays. Since many viruses can hemagglutinate human red blood cells, hemolyzed serum specimens may create a problem. In such cases, one may wish to separate the immunoglobulin fraction by ammonium sulfate precipitation (Heide and Schwick, 1978) to ensure that the antibody level determined by RIP assay actually reflects antibody to viral antigen, rather than a receptor for the tracer antigen.

2. *Carrier Serum*

The purpose of a carrier serum is to maintain a constant concentration of immunoglobulin in the assay system. This is essential for two reasons. First, as a test antiserum is diluted, the immunoglobulin concentration is reduced, thereby necessitating the addition of different quantities of second antibody. Second, antibody can usually be detected at very high dilutions of serum where the amount of precipitate formed even with addition of second antibody is insufficient to be separated by physical means (e.g., centrifugation). Carrier serum is added to maintain equivalency and to provide a bulk precipitate. Since the carrier serum is added at a relatively low dilution, usually 50 μl of a 1:200 dilution, it needs to be free of antibody to the tracer antigen. For RIP assays with animal serum, a suitable carrier serum can usually be obtained from nonimmunized animals. Because the equivalency of the second antibody is adjusted to the carrier immunoglobulins, large volumes of a single carrier serum reagent will minimize the need

continually to restandardize the second antibody for test purposes. Obtaining a suitable carrier serum for use in assays with human serum specimens is sometimes difficult because individuals have experienced infections with a variety of viruses. When possible, a suitable carrier serum should be sought by screening a large number of specimens from adults. The advantage of this approach is that a sufficient volume of serum can be obtained to perform a great number of assays with the same reagents. For respiratory viruses, it is usually necessary to screen a battery of specimens from children about 9–12 months of age by the RIP method, the rationale being that they will have lost maternally acquired antibody and have had a limited opportunity for exposure to the causative agents. Even under these conditions of selection the number of sera that are free of antibody may be relatively few.

3. *Second Antibody*

Several qualifications govern the selection of a second or precipitating serum antibody; these are the availability in large volumes to minimize restandardization of equivalency, the capability of quantitatively binding all the specified immunoglobulin in the carrier serum and forming a suitable matrix within a reasonable incubation period, and the ability to exhibit a lack of interference with the formation of stable complexes between the tracer antigen and the first antibody. The latter can occur when a precipitating antiserum contains antibody directed toward the Fab′ portion of the first antibody. This effect can be overcome by adsorption of the serum with Fab′ fragments covalently attached to Sepharose beads (Cuatrecasas, 1970; Rowe, 1962) or to prepare an alternative antiserum with Fc fragments as the immunogen.

A second antibody prepared in goats generally meets these criteria. When ordering commercially, samples of several different lots should be tested prior to purchasing batch volumes of the reagent. It is essential to characterize reagents by using the RIP assay, particularly for immunoglobulin class-specific reagents, because cross-reactions can occur under these conditions that are not evident in less sensitive tests, such as immunodiffusion (Kasel *et al.*, 1978).

D. BUFFERS AND DILUENTS

The high sensitivity achieved with RIP assays necessitates manipulation of antigens and antibodies at extremely dilute concentrations. Accordingly, formulations of buffers used in assays are designed to enhance the stability of these proteins, minimize their nonspecific adsorption to the surfaces of pipettes and containers, and reduce protein aggregration in solutions. The buffer preparations presented in Table I take into account these require-

TABLE I
COMPOSITION OF BUFFERS USED IN RADIOIMMUNOPRECIPITATION (RIP) ASSAYS

Abbreviation	Composition and uses
PBS	150 m*M* NaCl, 20 m*M* potassium phosphate, pH 7.2
RIP	PBS supplemented with BSA (1 mg/ml), Triton X-100 (0.03%), EDTA (0.1 m*M*). Used as a diluent for antisera, second antibody, and as the basic buffer for RIP assays
RIP (0.3%)	RIP with the concentration of Triton X-100 increased to 0.3%. Used as a diluent for competing antigens and for development of Sepharose 6B columns
RIP mix	RIP buffer supplemented with 10% fetal calf serum and carrier serum (usually 0.125%). Used as the basic mixture for antibody titrations. For competition assays to assess antigenic relationships, antiserum is also added to the mixture at a dilution that will precipitate approximately 50% of the ^{125}I-labeled antigen

ments. Bovine serum albumin (BSA) is added to assist in stabilizing the more diluted antigens and antibodies, and, with the addition of 0.03% Triton X-100, it minimizes nonspecific adsorption to glass or plastic surfaces. A highly purified preparation of BSA is recommended because less expensive grades may contain immunoglobulins that can contain antibody to viral antigen. Ethylenediaminetetraacetic acid (EDTA) is added to chelate heavy metals that might catalyze oxidation of the sulfhydryl or disulfide bonds in viral proteins and to prevent interference by complement components of antigen–antibody reactions. For storage of ^{125}I-labeled antigens, the buffer is supplemented with large amounts of protein; usually antibody- and inhibitor-free horse or fetal calf serum is employed for this purpose. For some viral proteins, it is necessary to add a reducing reagent, dithiothreitol, to achieve stability during prolonged storage (Scott *et al.*, 1975).

III. Basic Methodologies

A. RADIOLABELING OF PROTEIN ANTIGENS

1. *Labeling Procedures*

Radioactive tracer viral antigens suitable for competition RIP can be prepared by different techniques using any of several radioisotopes (Chard, 1978; Dalrymple *et al.*, 1972; Hunter, 1978; O'Dell and Daughaday, 1971; Tack and Wilder, 1981). The principal criteria in selection of the labeling procedure and of the isotope to be used are the specific activity and antigenicity of the final product. Techniques commonly employed are the bio-

synthetic incorporation of a β-emitting isotope (^{14}C-, ^{3}H-, or ^{35}-labeled amino acid or carbohydrate) and the chemical addition of a γ-emitting isotope (^{125}I or ^{131}I) into a protein. A drawback with the use of β emitters is that their long half-lives limit the specific activity of the resulting product, thereby requiring high concentrations of labeled antigen for the performance of assays. While this may not be of any importance in comparisons of antigenicity of viral proteins, it does severely limit the usefulness of assays because of decreased sensitivity for biological tracking and detection of antibody. The need for high sensitivity has limited the isotopes available for this purpose of ^{131}I and ^{125}I. However, since introduction of these isotopes into viral proteins involves the direct chemical modification of tyrosyl residues, the potential for alterations of biological and immunological properties of the antigen exists. While the longer half-life of ^{125}I as compared to ^{131}I represents a theoretical disadvantage, from a practical standpoint this is not the case. First, the relative abundance of ^{131}I in commercial preparations is not nearly as high as it is for ^{125}I (usually about 100%), and the counting efficiency is greater for ^{125}I than for ^{131}I. Second, the very short half-life of ^{131}I (8 days) limits the period of time that an antigen preparation can be used. For these reasons, virtually all methods utilizing protein antigens have used ^{125}I as the tracer label.

Iodination of viral proteins can be accomplished by several procedures. Iodine can be allowed to react directly with tyrosyl residues by electrolysis and addition of oxidizing agents, such as lactoperoxidase with hydrogen peroxide, chloramine-T, or iodogen (1,3,4,6-tetrachloro-3α,6α-diphenylglycoluril). Alternatively, a compound such as the *N*-hydroxysuccinimide ester of 3-(*p*-hydroxyphenyl)propionic acid can be iodinated and used to derivatize the protein (Bolton and Hunter, 1973). Use of this reagent can result in a protein having a high specific activity (SA), since it modifies the ϵ-amino group of lysine residues, which are usually more abundant on proteins than the phenolic rings of tyrosyl residues. Iodogen offers the potential advantage that protein or polypeptide antigens are exposed to minimal concentrations of this reagent because of its limited solubility (Salacinski *et al.*, 1981). An oxidizing reagent can alter the chemical integrity of the antigen and reduce biological and immunological reactivities. Despite the fact that chloramine-T is a relatively strong oxidizing reagent, this method has been most widely used essentially as first described by Greenwood *et al.* (1963) or with minor modifications therein.

2. *Chloramine-T Procedure*

For the past 7 years, we have utilized a modified chloramine-T procedure (Greenwood *et al.*, 1963; Hunter, 1973) for iodination of viral protein antigens from type 5 adenovirus and HAs of H3N2 influenza viruses. A sil-

iconized 1.0-ml Reacti-Vial (Pierce Chemical Company) is used as a reaction vessel. An appropriate amount of protein to be labeled, usually 10–20 μg of protein in 50–80 μl of 0.2 *M* phosphate buffer, pH 7.4, is added to the reaction vessel, and the volume is brought to 100 μl with buffer. After addition of 1 mCi of NA^{125}I (10 μl) and 10 μl of chloramine-T (50 μg), the reaction is allowed to proceed at ambient temperature for 30 sec. Sodium metabisulfite (100 μg in 10 μl) and potassium iodide (KI) (200 μg in 100 μl) are added, and the solution is applied to a Sepharose 6B column to separate the radiolabeled antigen from the reaction milieu.

Prior to iodination, the tracer protein is dialyzed against 0.2 *M* phosphate buffer, pH 7.4. To maintain constancy in ionic strength and pH, this buffer is added to adjust the volume to 100 μl prior to addition of the chloramine-T. This ensures that the final concentrations of chloramine-T and sodium metabisulfite are the same in every experiment. The 30-sec reaction time is based on practical considerations. It requires approximately 20 sec to open the reaction vessel, add chloramine-T, reseal the vessel, mix, and reopen the vessel before adding sodium metabisulfite. The 30-sec time period is sufficient to reproduce the exposure to the chloramine-T reagent. Thus, any chemical damage resulting from exposure to the oxidizing agent is consistent. Because exposure to metabisulfite can reduce disulfide bonds in proteins and modify chemical structure, the practice of adding the KI immediately and applying the reaction mixture as quickly as technically possible to the Sepharose column is recommended. Under these procedural conditions 40–70% of the ^{125}I molecules will be covalently attached to the protein. The SA of the labeled antigen can be altered as needed by adjusting the amount of protein or ^{125}I used in the reaction.

3. *Separation of Iodination Products*

Molecular sieve chromatography is the method routinely used to separate iodinated protein antigens from nonbound isotope. However, there is an advantage in using larger columns prepared with resins of sufficient pore size so that proteins are actually chromatographed rather than being excluded by the resin. This procedure in a single step separates protein aggregates and degradation products that may be generated during storage or during the radiolabeling procedure from other reaction products (Salacinski *et al.,* 1981). The effectiveness of the approach can be appreciated by the results obtained with the HA of A/Hong Kong/68 (H3N2) influenza virus, as shown in Fig. 1. The elution profile shown in Fig. 1A was developed with a Sepharose 6B column and a 15-μg sample of HA iodinated by the chloramine-T procedure. The HA protein had been recently purified and had a hemagglutination end point of 1:120,000 against 0.5% chick red blood cells. The Sepharose column (1 × 15 cm) had been coated with protein by passage

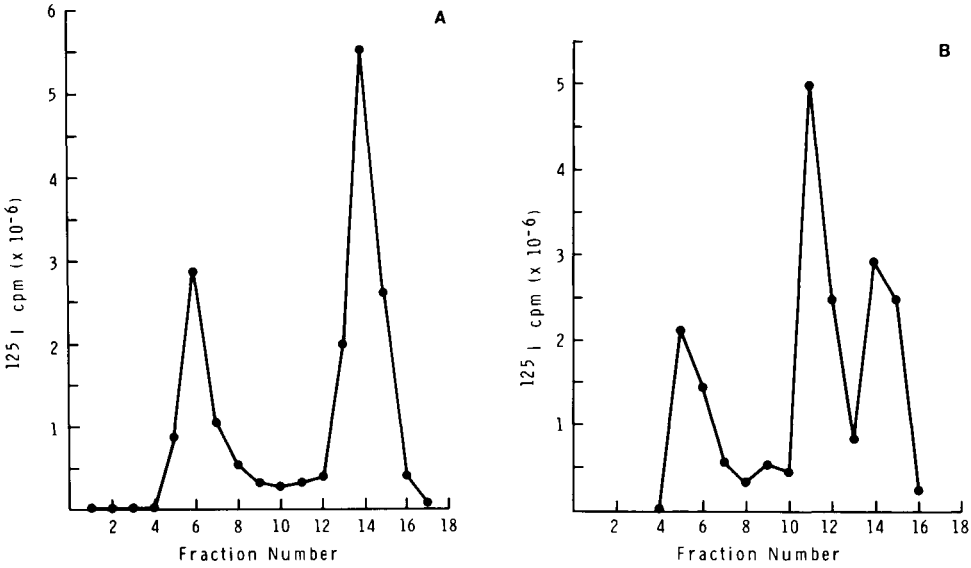

FIG. 1. Elution patterns of iodination reaction products from a Sepharose 6B column. Purified hemagglutinin derived from A/Hong Kong/68 (H3N2) influenza virus was iodinated by the chloramine-T procedure as described in the text. The reaction mixture was applied to a 1 cm × 20 cm column equilibrated and eluted with RIP buffer containing 0.3% Triton X-100; 0.7-ml fractions were collected. The elution profiles were obtained with recently purified hemagglutinin (panel A) and after storage (panel B).

of 1.0 ml of fetal calf serum through the resin. After equilibration of the column with RIP buffer containing 0.3% Triton X-100, the sample was chromatographed using the same buffer. The iodinated HA eluted in a symmetrical peak around fraction 6, and unbound ^{125}I eluted at the total volume of the gel bed (around fraction 14).

The elution profile shown in Fig. 1B was obtained after iodination of 15 μg of the same HA preparation that had been stored at $-70°$ for 3 months. The hemagglutination end point had decreased to approximately 1:50,000 during the storage interval. In addition to the radioactive peaks representing ^{125}I-labeled HA and unbound ^{125}I, a large portion of the isotope was recovered in a new peak that chromatographed with an apparent molecular weight substantially less than that expected for the HA (fractions 10–12). Screening of the radioactive peaks from the two iodination columns for precipitability with trichloroacetic acid (TCA) indicated that the larger molecular weight peaks contained ^{125}I-labeled protein (Table II); however, only the first protein peak demonstrated antigenic activity, as indicated by the

TABLE II
IDENTIFICATION OF ^{125}I-LABELED HEMAGGLUTININ AFTER MOLECULAR SIEVE CHROMATOGRAPHY ON SEPHAROSE 6B

Column	Fraction No.	Counts per minute precipitated by TCA (%)[a]	Counts per minute precipitated by antibody (%)[b]
I	5	98.9	91.2
	6	98.7	89.0
	7	98.0	87.0
	13	6.0	8.3
	14	6.8	7.7
	15	5.9	8.0
II	5	98.8	89.3
	6	97.5	87.2
	7	98.0	95.0
	10	91.8	10.1
	11	89.0	11.0
	12	91.2	8.9
	14	7.8	9.9
	15	7.0	9.5

[a]Samples (10 μl) of the indicated fractions were added to 200 μl of RIP buffer containing 10% fetal calf serum, and 800 μl of 10% trichloroacetic acid (TCA) was added. After incubation at 4°C for 30 min, the tubes were centrifuged at 3000 *g* for 30 min and radioactivity in the pellet and supernatants was determined.

[b]Samples of the indicated fractions were diluted in RIP buffer to contain 10,000 cpm per 50 μl, and 50-μl aliquots were added to assay tubes containing 200 μl of RIP buffer containing 0.125% carrier serum, 10% fetal calf serum, and 50 μl of 1:2000 dilution of guinea pig anti-HA antiserum. After incubation at 37°C for 2 hr, goat anti-guinea pig 7 S immunoglobulin in equivalence with the carrier serum was added and then incubated at 37°C for 2 hr and at 4°C for 16 hr. After addition of 0.5 ml of PBS, the tubes were centrifuged as described above.

high level of precipitability of these fractions with antibodies raised to the HA.

An expression of biological activity may be useful in predicting the elution pattern of iodinated preparations during storage. Experience with the HA of H3N2 viruses suggests that a correlation exists between the ability of a preparation of antigen to hemagglutinate red blood cells and its reactivity with antibodies (H. R. Six and J. A. Kasel, unpublished results).

4. *Specific Activity of Labeled Antigen*

Calculation of the SA of an iodinated preparation requires a determination of the amount of the isotope and protein content in column eluates. In practice, neither of these is completely recovered from the column, for the reasons previously discussed. While the quantity of ^{125}I in each fraction can be determined easily in a gamma counter, quantitation of the protein presents more of a problem. Methods are currently available that are in

most cases sensitive enough to measure small quantities of protein present in the eluates, but direct determination would require placing relatively large amounts of ^{125}I in a spectrophotometer or fluorimeter (Lowry *et al.*, 1951; Udenfriend *et al.*, 1972). To avoid this situation, a procedure that estimates protein recovery from "blank" iodination runs can be used. The reagents and procedure are exactly the same, but NaI is substituted for $Na^{125}I$. The reaction mixture is chromatographed on a Sepharose 6B coated with fetal calf serum and equilibrated with RIP buffer from which the BSA has been omitted. The quantity of protein recovered in each fraction can then be determined. Our method utilizes the Fluorescamine procedure (Udenfriend *et al.*, 1972). For a freshly prepared preparation of HA (i.e., preparation that has full biological activity), about 70% of the protein and about 70% of the hemagglutinating activity can be routinely recovered in the expected molecular weight peak. For this type of preparation, protein is usually not recovered in eluates for other portions of the column and is assumed to be lost owing to nonspecific binding to the resin, column, and tubing. For preparations having less hemagglutinating activity, the amount of protein recovered in the correct position can be substantially less, but the peak will contain approximately 70% of the initial hemagglutinating activity. If the HA preparation has lost considerable hemagglutinating activity, an additional peak of protein may be recovered at a position of lower molecular weight, and generally the total amount of protein recovered from the column will be lower. The total amount of ^{125}I recovered after molecular sieve chromatography is not a critical consideration. The amount of ^{125}I recovered in the antigen peak is the essential information, and this is obtained by counting samples of each fraction (usually 10 μl). The SA of the ^{125}I antigen preparation is then calculated as follows:

$$\text{SA} = \frac{\mu\text{Ci of } ^{125}\text{I recovered in antigen peak}}{\mu\text{g of protein recovered}} \tag{1}$$

$$\text{SA} = \frac{\Sigma \text{ cmp in 10 } \mu\text{l of appropriate fractions} \times 700\ \mu\text{l}/10\ \mu\text{l}}{0.72 \text{ (efficiency of counter)} \times 2.2 \times 10^6 \text{ dpm}/\mu\text{Ci}} \Bigg/ \left(10\ \mu\text{g protein} \times 0.70 \text{ (expected recovery)}\right) \tag{2}$$

To increase the reproducibility of this determination, the practice of keeping a set of micropipettes reserved exclusively for assaying iodination columns should be followed. Thus, errors due to inaccuracy of pipettes are reproducible and minimized. The only assumption necessary for the calculation is that the concentration of protein recovered during the iodination procedure is equal to that obtained in the "blank" runs. Although the SA is generally expressed as microcuries per microgram of protein, in considerations of experimental design a more meaningful expression is counts per

minute (cpm) per nanogram of protein because the data are acquired on a counts per minute basis.

Calculation of the SA for each preparation of radiolabeled antigen provides several useful applications. It serves as a control on the reproducibility of iodination procedure and is an indicator of the sensitivity that can be expected for detection of antibodies or unlabeled antigen. Occurrences of higher SA for a given purified antigen may be indicative of a protein decay during storage. If a portion of protein becomes denatured during storage, this may result in an exposure of tyrosyl residues that are hidden in the native protein. Thus, the denatured form will be preferentially iodinated, resulting in an apparently high SA, but the portion of antigen that is capable of reacting with antibody may be of a lower value. Unless the two forms of radiolabeled protein can be separated by some means (e.g., chromatography, as illustrated in Fig. 1A and B), expression of antigen concentration from SA can be misleading. The portion of labeled antigen that can be precipitated by homologous antibody also indicates when this occurs. Since the only antigen molecules that can be measured during the assay are those that carry ^{125}I, there is no advantage to labeling a protein to an SA where the ratio of ^{125}I to protein is less than one on a mole for mole basis. For a protein having a molecular weight of 240,000, such as the HA trimer of an influenza virus, the SA will be approximately 7 $\mu Ci/\mu g$ protein if every protein molecule has one atom of iodine. In practice, this does not mean that a HA preparation with SA of 7 that all the molecules actually carry an ^{125}I atom, nor that all the ^{125}I molecules are on a given tyrosine residue in the primary structure. It does, however, provide a starting point for determining the extent of iodination that can be attained (i.e., higher SA) before the integrity of the protein is sacrificed.

B. Storage of Labeled Proteins

Since proteins vary in their inherent stability, it is worthwhile to determine the most suitable conditions for storage of labeled antigen preparations. Usually most of the deterioration of viral protein is attributable to radiation damage. Thus, any condition minimizing this effect will prolong viability of the tracer antigen. In general, supplementary protein (25% fetal calf or horse serum) is necessary, $-70°C$ is better than $-20°C$ or $4°C$, and high SA preparations are less stable than those with a lower activity. With adenoviral and influenza viral preparations, SAs with a range of 15–20 and 7–10 $\mu Ci/\mu g$ protein, respectively, are satisfactory. Labeled preparations should be rechromatographed immediately before use, regardless of storage time, to remove any denatured or degraded protein.

C. ANTIBODY TITRATIONS

To establish conditions for use in competition RIP assays, an antibody titration is a prerequisite. Technically the titration can be performed by the following method (Scott *et al.*, 1975, as modified by Six and Kasel, 1978).

Fourfold dilutions (usually starting with 1:200) of serum (50 μl) prepared in RIP buffer are added to duplicate assay tubes (12 × 75 mm polystyrene) containing 200 μl of a solution composed of 10% (v/v) horse or fetal calf serum and 0.125% (v/v = 1:800 dilution) carrier serum free of antibody to the tracer antigen. Iodinated viral protein after passage through a "cleanup" (Sepharose 6B) column is adjusted to a concentration of 10,000 cpm in 50 μl and added in this volume to each serum dilution. After mixing, the tubes are incubated at 37°C for 4 hr followed by 18 hr at 4°C. Goat anti-IgG (or the immunoglobulin of choice) of the desired species (in this example, 50 μl of a 1:8 dilution) in equivalence with the carrier serum is added to all tubes, and the reaction mixture is incubated at 4°C for 16 hr. Fifty microliters of RIP buffer in place of serum is added to eight tubes containing the same volume of each of the other assay reagents to serve as controls. After the last incubation step, 1.0 ml of PBS is added to all serum dilution and four control (background) tubes. One milliliter of 15% (w/v) of trichloroacetic acid (TCA precipitation) is added to the remaining four controls. After centrifugation at 3000 *g* for 30 min, radioactivity in the pellet and supernatant fluid of each tube is counted for 1 min and the counts per minute for each set of serum dilutions and controls are averaged.

When the background level is 5% or less, the percentage of antigen bound by each serum dilution is calculated as follows:

$$\%\ \text{of antigen precipitated} = \frac{\text{cpm precipitated by antiserum}}{[\text{total cpm (supernatant + pellet)}] \times (\text{ratio of TCA-precipitable cpm})} \times 100 \tag{3}$$

When technical competence is established in performance of the test, the need to count supernatant fluids can be eliminated and the percentage of antigen precipitated by a test specimen is determined as follows:

$$\%\ \text{of antigen precipitated} = \frac{(\text{cpm precipitated by antiserum}) - (\text{cpm in background})}{(\text{cpm precipitated by TCA}) - (\text{cpm in background})} \tag{4}$$

This method of determining antigen precipitation is also employed when the background level in an assay is high (6–12%). Equation (4) eliminates the contribution of nonspecific binding to assessments of antibody titers.

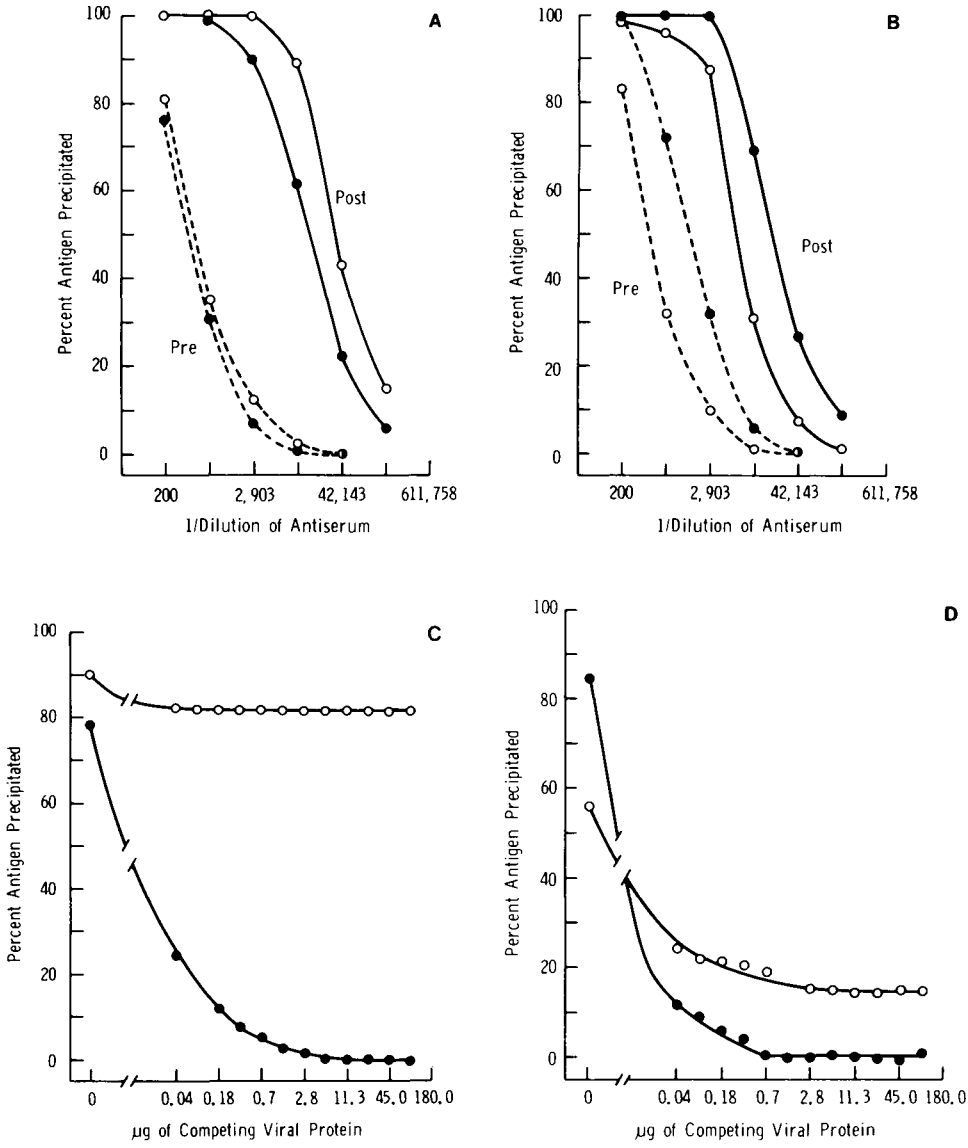

FIG. 2. Results of a competition RIP assay for determining the concentration of competing antigen required to inhibit the binding of cross-reactive antibody. Serum antibody levels were determined by RIP assays in which ^{125}I-labeled A/Port Chalmers/73 hemagglutinin (○) and ^{125}I-labeled A/Hong Kong/68 hemagglutinin (●) were used. Antibody titration curves for prevaccination and postvaccination serum specimens from volunteers 1 and 2 are shown in panels A and B, respectively. A 1:3000 dilution of postimmunization antiserum from individ-

The carrier serum, the second antibody, and the incubation conditions, in addition to the tracer antigen, influence the background levels of precipitation in RIP assays. Since the sensitivity for detection of antibody is dependent on this value, it is necessary to minimize nonspecific binding. The use of an appropriate quantity of carrier serum and second antibody is important. Two hundred microliters of RIP mix containing a 1:800 dilution of carrier serum contains a sufficient quantity of IgG (approximately 2.5 μg) for separation of free from bound antigen when second antibody is added. This amount of precipitate does not contribute to background values. However, when it is necessary to use lower dilutions of carrier serum and second antibody, the precipitate may become large enough to entrap antigen and elevate the level of nonspecific binding. The length of incubation periods can also result in high background values. When these are shortened, background levels may be reduced, but sensitivity is usually sacrificed.

An antibody titration curve is then constructed by plotting percentage of antigen precipitated versus dilution of antiserum. It has several applications to competition RIP assays (see Fig. 2A).

For antibody end points, the highest dilution that precipitates a prescribed percentage of tracer antigen is selected as the titration end point. Although sensitivity is increased when a titer is based on the lower end of the linear portion of the curve, reproducibility of test results can be a problem. For our assays, a 35% precipitation level is generally used. This level of precipitation is high enough for detection of significant titers of antibody, but low enough so that antigen is in excess. Moreover, it ensures a high degree of reproducibility.

In this example antibody titrations were performed with iodinated preparations of HA from A/Hong Kong/68 (H3N2) and A/Port Chalmers/73 influenza viruses on serum specimens collected before and after immunization with A/Port Chalmers/73 whole-virus vaccine. Using an antibody end point of 35% precipitation, the titers prior to vaccination were 665 and 762 to A/ Hong Kong/68 and A/Port Chalmers/73, respectively, and the postimmunization titers to these same antigens were 27,940 and 62,244, respectively. If a 20% level had been chosen, these prevaccination titers would be 1138 and 1818 and the postimmunization would be 46,903 and 107,492, respectively. When individual titers performed by this procedure vary by

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ual 1 (panel C) and individual 2 (panel D) was tested for its ability to precipitate the iodinated hemagglutinins in the presence of increasing concentrations of competing antigen as described in the text. Unlabeled A/Hong Kong/68 antigen completely inhibited reactivity of prevaccination sera to both hemagglutinin proteins. From Six *et al.* (1982).

less than 40%, a 2-fold rise in a postvaccination specimen can be considered to be a significant response (Six and Kasel, 1978; Kasel *et al.*, 1978).

The titration curve is used to obtain an appropriate antibody concentration for assays designed to assess a response or characterize antigenic relationships. This is generally selected from the linear portion of the slope of the curve (50–60%).

If the SA of the antigen is accurately determined, the test results can also be expressed in terms of antigen binding capacity. The advantage of expressing the results in this manner is that binding capacities determined at different antigen concentrations (accomplished by adding unlabeled protein to tracer antigen) can be used to assess the relative avidity of different sera (Minden and Farr, 1973). Based on the specific activity of the tracer antigen and the counts per minute of ^{125}I in the reaction mixture, the quantity of viral protein in each assay tube can be calculated. The nanograms of antigen represented by 35% precipitation multiplied by the serum dilution at which this level occurs gives antigen binding capacity in nanograms of antigen per 50 μl of undiluted serum.

IV. Applicability to Identification of Antibody Populations

Results of experiments shown in Fig. 2 and Table III demonstrate the applicability of competition RIP assays for measuring a selected antibody response in a human serum raised in response to an immunogenic stimulation with a viral protein (Six *et al.*, 1982). An antibody titration curve for the immunogen, A/Port Chalmers/73 (H3N2), and an antigenically related protein antigen, A/Hong Kong/68 (H3N2), was constructed to identify a serum dilution that can quantitatively precipitate each viral protein (Fig. 2A and B). That dilution of serum (1:3000 in each instance) was reacted with increasing concentrations of unlabeled heterologous competing antigen. The competing antigen, a purified whole-virus preparation, was diluted in RIP buffer containing a detergent. The latter was added to disrupt virus, thus freeing HA, and to prevent aggregation. Dilutions of competing antigen were incubated for 120 min at 37°C with serum dilutions before labeled antigen was added. The procedure as described for the direct RIP was followed to complete the assay. As shown in Fig. 2C and D, 5 μg of competing A/Hong Kong/68 viral protein inhibited all reactivity with iodinated A/Hong Kong/68 HA, but each postvaccination antiserum retained the ability to react with labeled A/Port Chalmers/73 (82 and 15%, respectively). The failure of an 18-fold greater concentration of competing

TABLE III
ASSESSMENT OF THE A/PC/73 (H3N2) SERUM AHAB RESPONSE BY DIRECT AND COMPETITION RADIOIMMUNOPRECIPITATION (RIP) TESTS

	Serum AHAB titers to the following HAs[a] Postvaccination			
	Without competing antigen		With competing antigen[b]	
Volunteer	A/HK/68	A/PC/73	A/HK/68	A/PC/73
1	27,940	62,244	<200	15,322
2	31,931	9,603	<200	762
3	7,353	11,061	<200	3,771
4	17,511	18,719	<200	2,211
5	12,542	26,136	<200	2,903
6	13,408	24,448	<200	2,903
7	9,603	15,322	<200	3,772
8	17,511	14,333	<200	6,878
9	4,926	6,018	<200	2,702
GMT[c]	13,608	16,763	100	3,380

[a]RIP tests were performed by using iodinated preparations of the indicated hemagglutinins (HAs). Titers are expressed as the reciprocal of the serum dilution that precipitated 35% of the test antigen. Data from Six *et al.* (1982).

[b]A disrupted preparation of A/HK/68 (H3N2) purified virus was used as the competing antigen, and 90 μg of viral protein was added to each assay tube.

[c]Geometric mean titer. A titer of 100 was assigned to sera with undetectable antibody (titer, <200).

antigen to reduce reactivity with A/Port Chalmers/73 HA indicates that the remaining antibody population had no measurable affinity for the heterologous protein. Competition RIP assays were then performed using 90 μg of competing antigen to inhibit the heterologous response to identify strain-specific, i.e., anti-A/Port Chalmers/73, HA serum response. The results obtained from a panel of test serum from individuals are shown in Table III. This method revealed that a portion of the antibody response of each individual was specific for the immunogen. Since such assays measure antigen-binding capacity, the titers provide estimates of strain-specific responses in proportion to the total response. As seen for this assessment, the proportion of strain-specific antibody ranged from 8 to 48%, and the mean was 20% of the total antibody response.

Another method for the selective measurement of type-specific responses to viral proteins is the use of an immunological probe to block cross-reactive antigenic sites on the tracer protein. This approach has been applied to the study of hexon and fiber proteins of adenovirus type 5 (Scott *et al.*, 1979). The type-specific antigenic determinants of these proteins are im-

munologically distinct, and each induces protective serum antibody following infection and immunization that cannot be differentiated by neutralization tests. In this RIP design the principal feature involves the use of Fab′ fragments from a rabbit antiserum raised against a closely related serotype to block group and subgroup antigenic determinants on iodinated tracer proteins (Fig. 3). As shown for the fiber system, saturating concentrations

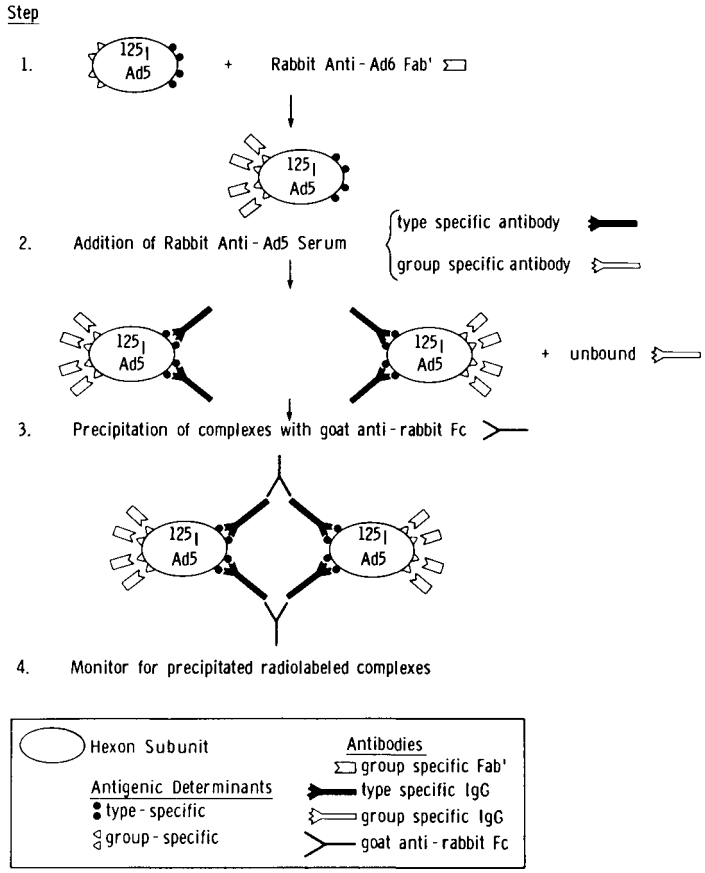

FIG. 3. Schematic illustration of the basic reaction design used to develop a radiolabeled immunological probe for measurement of antibody to the type-specific antigenic determinants on the Ad5 hexon. Group and subgroup antigenic determinants on the labeled hexon were blocked with Fab′ fragments derived from a heterologous anti-Ad antiserum. From Scott *et al., J. Immunol.* **122,** 1881–1885, © 1979, The Williams & Wilkins Co., Baltimore.

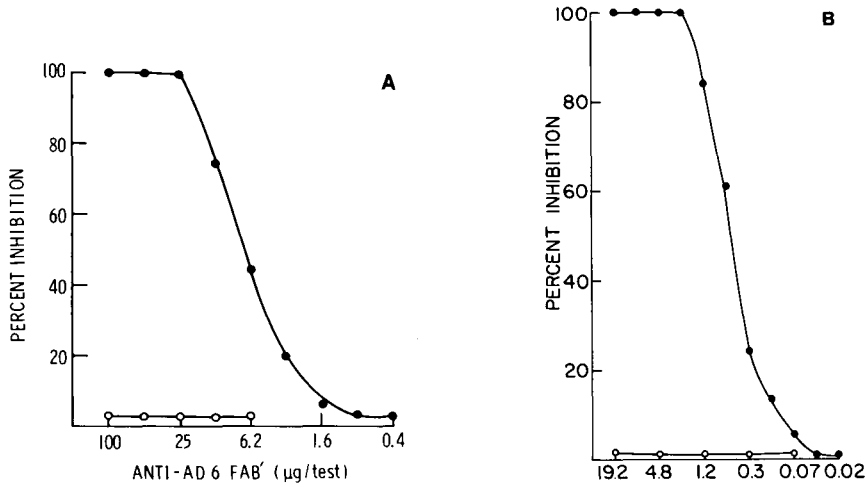

FIG. 4. (A) Titration of Fab' Ad6 fragments with ^{125}I-labeled Ad5 fiber for maximum inhibition of fiber subgroup antibodies in rabbit anti-Ad5 fiber (●) and rabbit anti-Ad6 (○) sera diluted 1:400 and 1:1600, respectively. (B) Competitive inhibition of ^{125}I-Ad5 fiber by SA Ad6 using the same antisera dilutions as in panel A. Antiserum dilutions bound 100% of iodinated fiber in the absence of Fab' Ad6 or SA Ad6. From Scott *et al., J. Immunol.* **122,** 1881–1885, © 1979, The Williams & Wilkins Co., Baltimore.

of Fab' to adenovirus type 6 used to block all cross-reactivity of the adenovirus 6 serum did allow detection of type-specific antibody in the type 5 serum (Fig. 4). Data obtained from assessments of antifiber antibody with serum specimens before and after immunization of individuals with type 5 fiber vaccine are shown in Table IV. For comparison, results of direct RIP and competition RIP tests are included. By comparison of titers by direct RIP with those by the indirect assays, each vaccine recipient was shown to have responded to cross-reactive and type-specific determinants. As can be seen, both methods—saturation of cross-reactive sites with Fab' fragments or competing antigens—can permit detection of small quantities of type-specific antibody. In this instance, the relative mean filters of type-specific antibody to the fiber protein were 4.0 and 4.1% of the total antibody population in postvaccination specimens. Specificity of each RIP assay in measuring fiber antibody was shown by the lack of a response to hexon vaccine (data not shown). Results of the same type were obtained with assessments of antihexon antibody (Table V). A potential application of the method in virology is that RIP assays can be designed with viral proteins with varying

TABLE IV
MEASUREMENT OF TOTAL AND TYPE-SPECIFIC ANTIBODY TO Ad5 FIBER IN HUMAN SERA

Participant No.	Antibody titers[a] obtained by RIA using					
	^{125}I-Ad5 fiber		Fab′ Ad6-^{125}I-Ad5 fiber[b]		SA Ad6/^{125}I-Ad5 Fiber[c]	
	Pre	Post	Pre	Post	Pre	Post
11	800	76,800	<50	1600	<50	2400
12	3200	150,000	100	3200	100	3200
13[d]	2000	38,400	<50	1600	50	2400
14	1600	102,400	<50	1000	<50	1000
15	1400	170,000	125	9600	150	9600
16	2400	120,000	250	7200	250	9600
17	1200	48,000	75	3200	100	4800
18	800	6,400	<50	200	<50	300
19	600	20,000	<50	800	<50	800
20	800	18,800	50	4800	100	4800
Mean titer	1289	50,448	51	2062	62	2496

[a]Reciprocal of serum dilution that precipitated 20% of the labeled antigen, determined as an average of three assays. Data from Scott *et al.* (1979).

[b]Fab′ Ad6 (50 μg per test).

[c]SA Ad6 (4.8 μg per test).

[d]Coefficient of variation on 10 replicate assays of this serum (No. 13) for each RIA described was 5%.

antigenic relationships as blocking agents so that the role of cross-reactive antibodies in host immunity can be evaluated.

V. Applicability to Characterization of Viral Antigens

The competition RIP procedure also provides a means of characterizing the nature of antigenic relationships between viral proteins not possible by other types of assays. It elaborates on the composition of determinants recognized by antibody populations and permits an evaluation of the relative avidity of antibody populations for viral antigens.

To perform an assay for this purpose, the procedure is identical to that described above for determination of strain-specific responses using competing antigens except that a single dilution of antiserum that precipitates 50–60% of iodinated tracer protein as predetermined by a direct RIP antibody titration is used. In addition to the usual background and TCA controls, four tubes containing RIP buffer with 0.3% Triton X-100, but no

TABLE V
MEASUREMENT OF TOTAL AND TYPE-SPECIFIC ANTIBODY TO Ad5 HEXON IN HUMAN SERA

Participant No.	Antibody titers[a] obtained by RIA using					
	^{125}I-Ad5 hexon		Fab′ Ad6-^{125}I-Ad5 hexon[b]		SA Ad6/^{125}I-Ad5 hexon[c]	
	Pre	Post	Pre	Post	Pre	Post
1	1,600	4,800	<50	<50	<50	<50
2	12,800	76,000	100	300	75	400
3	19,200	102,400	75	300	100	300
4	38,400	102,400	100	400	100	400
5	76,800	307,200	200	600	200	600
6	52,200	204,800	150	600	100	500
7	25,600	153,700	<50	500	<50	500
8	22,400	102,400	<50	300	<50	300
9[d]	25,600	204,800	100	400	150	600
10	12,800	51,200	<50	300	<50	400
Mean titer	20,211	74,425	62	300	62	325

[a]Reciprocal of serum dilution that precipitated 20% of the labeled antigen determined as an average of three assays. Data from Scott *et al.* (1979).

[b]Fab′ Ad6 (25 μg per test).

[c]SA Ad6 (12.5 μg per test).

[d]Coefficient of variation on 10 replicate assays of this serum (No. 9) for each RIA described was 5%.

competing antigens, are included as controls to determine the amount of ^{125}I-labeled antigen precipitated. The percentage of inhibition for each concentration of competing antigen is computed as follows:

$$\% \text{ inhibition} = \frac{\begin{array}{c}(\text{cpm precipitated by antibody alone}) \\ -\ (\text{cpm precipitated in competing antigen}) \\ -\ (\text{cpm in background})\end{array}}{\begin{array}{c}(\text{cpm precipitated by antibody alone}) \\ -\ (\text{cpm in background})\end{array}} \times 100 \quad (5)$$

Inhibition curves for homologous and heterologous competing antigens are constructed by a semilog plot of the percentage inhibition versus the concentration of competing antigen. The displacement curves are a function of the average association constant of antibodies with all the reactive sites on viral proteins. Thus, the degree of displacement by competing antigen reflects the composition of antigenic determinants recognized by an anti-

body population. The slope of a competition curve can also provide a measurement of antigenic relatedness between antigens. A numerical value for a slope is obtained by trendline analysis. For this method, the lowest concentration of competing antigen that produces sufficient inhibition to fall on the linear portion of the curve is assigned an X value of 1, and for each 2-fold increase in concentration the X value is increased by 1. The Y values are equal to the percentage of inhibition for each datum point. The slope is then determined by linear regression analysis. An "antigenic relatedness" can then be estimated by the following expression.

$$\frac{\text{slope of the heterologous curve}}{\text{slope of the homologous curve}} \times 100 \tag{6}$$

In determinations of antigenic relatedness by competition assays, one is really characterizing the different specificities and avidities for cross-reactive antigens of antibody populations found in an antiserum and extrapolating this finding to the antigenic structure that induced the humoral response. Thus, it is essential that several antisera to each antigen be used in these assessments to ensure the validity of these interpretations, and antisera raised by hyperimmunization are preferable because they usually will contain a wide spectrum of antibody specificities. However, the use of such antisera conveys two properties to the RIP assay that need to be considered when interpreting the results. Mathematical treatments of RIP data are based on mass-action equations. In the simplest form,

$$[\text{Ag}] + [\text{Ab}] \underset{k_2}{\overset{k_1}{\rightleftharpoons}} [\text{Ag Ab}] \tag{7}$$

$$K = \frac{k_1}{k_2} = [\text{Ag Ab}] \,/\, [\text{Ag}]\,[\text{Ab}] \tag{8}$$

where K is the equilibrium constant, and k_1 and k_2 are the rate constants for association and dissociation, respectively.

First, for multivalent protein antigens reacting with spectrum of antibody specificities, k_1 is much greater than k_2 and equilibrium is never obtained. In fact, very little dissociation of antigen–antibody complexes occurs during the incubation conditions used by us. If the addition of unlabeled competing protein is delayed by as much as 4 hr, much of the antigen cannot be displaced from the complexes already formed. For this reason, it is necessary to add the competing protein and the labeled antigen to the antiserum simultaneously.

Second, because protein antigens are multivalent, a large number of an-

tibodies with different specificities and affinities are participating in the binding reaction at one time. Therefore the amount of inhibition seen at a given concentration of competing antigen reflects its ability to associate with many different antibodies, and rate constants calculated from these data would not necessarily reflect the binding functions of any of the individual reactions (Berzofsky and Schechter, 1981; Creighton, 1980). For this reason, we have chosen to use the slopes of the competition curves for comparison of the relative avidity of an antiserum to an antigen. A shallower slope of a competition curve represents a lower average association rate over a range of concentrations for one antigen than for another.

Competition RIP assays designed to determine antigenic relatedness of fiber proteins of adenoviruses belonging to the same (C) and different subgenera (A, E) illustrate the type of information that can be obtained by a homologous competition RIP system (Fig. 5). For these evaluations, competing antigen preparations consisted of fiber proteins partially purified by banding in discontinuous cesium chloride gradients (Pereira *et al.*, 1968). The inhibition curve of the homologous reaction serves as a reference point for identifying heterologous antigenic relationships. In this instance, it is relatively steep, and at a protein concentration above 1000 μg/ml the reaction is quantitatively inhibited. With competition curves developed by

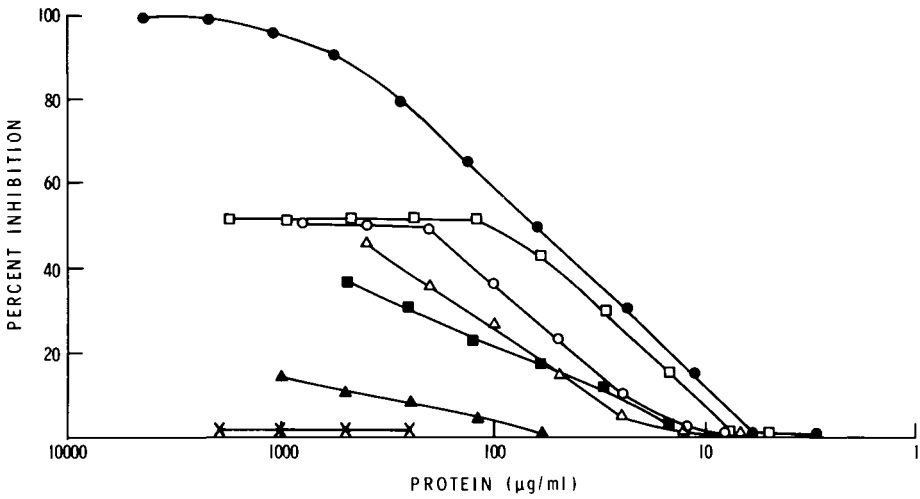

FIG. 5. Competition radioimmunoassays of ^{125}I-labeled Ad 5 fiber with soluble antigen preparations from the following serotypes: Ad 5 (●), Ad 1 (□), Ad 6 (○), Ad 2 (△), Ad 31 (■), Ad 18 (▲), and Ad 4 (×). Assays were initiated with a 1:9,600 dilution of rabbit anti-Ad 5 fiber antibody. Unpublished data of J. V. Scott, J. A., Kasel, and G. R. Dreesman.

reactions with fiber proteins of other adenoviruses within the same subgenus (types 1, 2, and 6), several different antigenic events are discernible. Each protein exhibits binding of approximately 50% of the homologous reaction, and the plateau portion of the curve reflects the presence of unique determinants that are not expressed on other subgenus serotypes. A comparison of the slopes of the inhibition curves show that those of types 1 and 6 were the same as that of type 5. This demonstrates that half of the antigenic determinants for the latter are completely identical with those of types 1 and 6. The lack of a parallel slope with adenovirus 2, the other virus belonging within the same subgenus that was evaluated, indicates only a partial identity with cross-reactive determinants of type 5 fiber protein. The relatively shallow slopes developed with types 18 and 31 fiber protein antigens (subgenus A) and the absence of any inhibition by type 4 (subgenus E) indicate that these have determinants with a lower affinity for antibody to the fiber protein. Using the above expression to estimate the degree of antigenic relatedness between viral proteins, the relationships of types 1, 6, 2, 31, and 18 fiber proteins to that of type 5 are 0.98, 0.97, 0.69, 0.46, and 0.19, respectively.

Competition RIP assays as described for HA proteins of major variant viruses of the H3N2 demonstrate approaches for obtaining a more extensive analysis of cross-reactive antigenic determinants (Six and Kasel, 1979). This was accomplished by assessing the binding capacities of heterologous HA proteins in homologous and heterologous antigen and antibody competition RIP systems. The type of antisera employed for assays are of importance. The experimental design of the assays included employment of hyperimmunized sera raised to purified HA proteins and the use of disrupted whole virus preparations as competing antigens.

A presentation of the types of results obtained by the homologous systems is shown in Figs. 6 and 7. The percentage inhibition of the homologous reaction by competing antigens at maximal concentration levels provided information on the relationships of the HA proteins recognized by each antibody population. It is seen that this ranged from <10 to 75%. Moreover, the range of slope values in these assays was indicative of marked differences in the avidity of cross-reactive antibodies. Using estimations of the degree of antigenic relationships between the H3 proteins computed from the displacement curves, it was possible to identify a relatively consistent pattern of relationships among the H3 HA viruses. The antigenic intimacy with the HA of the original variant (A/Hong Kong/68) and those of succeeding variants (A/England/72, A/Port Chalmers/73, and A/Victoria/74) progressively decreased by approximately 50% (Figs. 6 and 7). This difference in antigenic sharing between variants was confirmed in other competition RIP experiments and by examining additional antisera

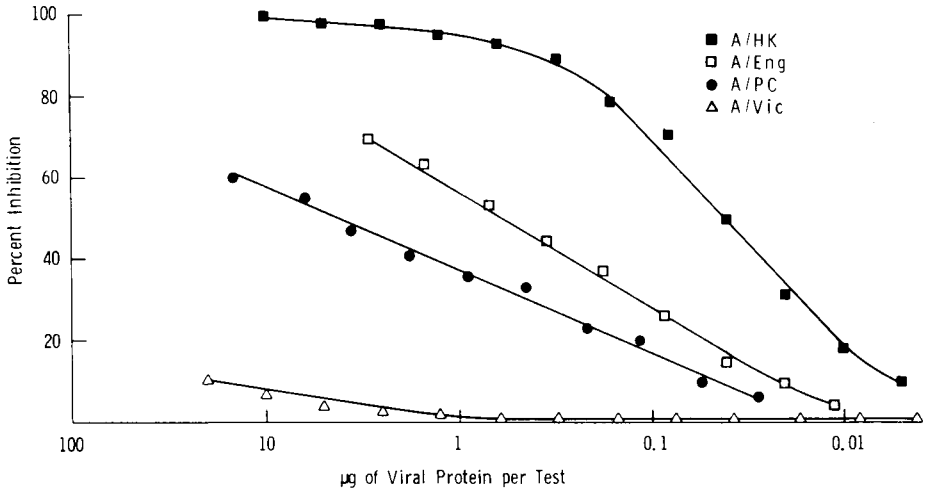

FIG. 6. Analysis of the antigenic relationship of the A/HK/68 HA to similar subunits of the H3N2 subtype. This competition radioimmunoassay was performed with ^{125}I-labeled A/HK/68 HA (10,000 cpm) and 1:100,000 dilution of a guinea pig antiserum raised to purified A/HK/68 virus. The slopes for the competing antigens A/HK/68, A/Eng/72, and A/PC/73 were 16.0, 9.7, and 6.0, respectively. The slope for A/Vic/75 could not be determined. From Six and Kasel (1979).

produced to the variants (Six and Kasel, 1979). From the results of the homologous competition RIP assays, it can be inferred that, because of the different expressions of avidity, the antigenic determinants of the variants were similar but not identical.

Because the homologous competition assays are performed using high dilutions of antiserum, the reactions observed are representative of the dominant or major populations of serum antibodies; antibody specificities that represent a small portion of the humoral response may not effect these reactions.

Further segregation, if present, of subpopulations of antibodies can be investigated by RIP assays using heterologous antisera and iodinated HA proteins rather than homologous systems. The procedure for the performance of the assay is the same as that described for the homologous system, except that an antibody titration needs to be performed with the heterologous viral protein to be used to select a dilution of test serum that will precipitate 50–70% of the tracer protein antigens. As shown in Fig. 8, in this system with the same HA proteins of H3N2 virus as used in the homologous competition RIP assays, another subpopulation of antibodies could be identified. The slopes of the inhibition curves produced by varying

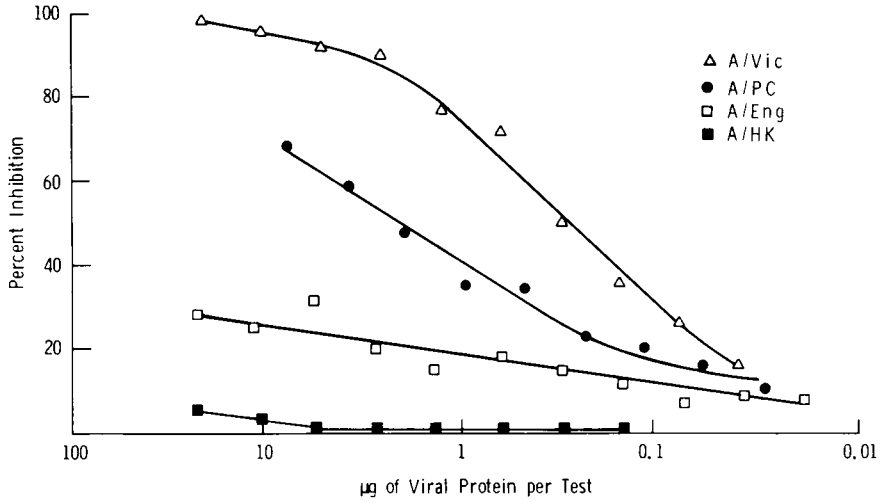

FIG. 7. Analysis of the antigenic relationship of A/Vic/75 HA to other H3 variants. This competition radioimmunoassay was performed with ^{125}I-labeled A/Vic/75 HA (10,000 cpm) and a 1:50,000 dilution of guinea pig antiserum raised to purified A/Vic/74 virus. The slopes for the competing antigens A/Vic/75, A/PC/73, and A/Eng/72 were 14.3, 8.7, and 2.5, respectively. The slope for A/HK/68 could not be determined. From Six and Kasel (1979).

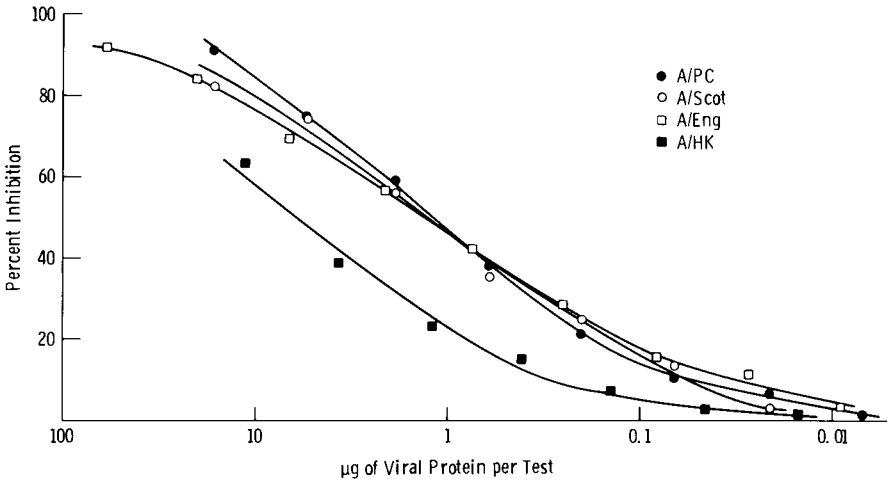

FIG. 8. Analysis of the cross-reactive antigenic determinants of A/PC/73. This competition radioimmunoassay was performed with ^{125}I-labeled A/PC/73 HA and a 1:6000 dilution of guinea pig antiserum raised to purified A/HK/68 hemagglutinin. A/Scot indicates that A/Scotland/74 (H3N2) disrupted virus was used as a competing antigen. From Six and Kasel (1979).

concentrations of each unlabeled competing HA protein preparation were the same. This result suggests that this portion of the antibody population recognizes an identical structure on each antigen.

From the homologous and heterologous competition RIP experiments, several conclusions are evident. A major portion of the antibody population raised to an HA protein is directed against cross-reactive antigenic regions, and reactivity with heterologous HAs are of a lower avidity. Thus, some of the antigenic structures carried by HA proteins of H3N2 subtype viruses are similar but not identical. This sharing between HAs indicates either that the primary sequences of the shared antigenic sites are different or that the same sequence is presented in different stereochemical configurations. The heterologous competition assays showed the existence of a minor antigenic region that is present in an identical form (both primary sequence and conformation) on several closely related HAs. It cannot be inferred, however, that the identical structure is a unique site on the molecule that can be physically separated from other antigenic regions. Antibodies directed against the same antigenic region may exhibit partial or complete cross-reactivity depending on the individual epitope (portion of an antigenic determinant) that they recognize. The results of the RIP assays are consistent with current concepts of the immunochemical structure of the HA protein, which suggests that the molecule possesses four distinct antigenic regions and that amino acid substitutions occur in the primary sequence of each of the four sites on each new variant (Gerhard *et al.*, 1981; Russ *et al.*, 1981; Russell *et al.*, 1979; Wiley *et al.*, 1981).

VI. Biological Tracking of Viral Proteins

Competition RIP assays provide a sensitive means for quantitation of specific viral proteins in biological specimens. For the adenovirus and influenza virus antigens described herein, concentrations of less than 1 ng/ml are readily detectable; assays with proteins from other viruses have reported comparable sensitivities (Barker, 1975). Moreover, it is likely that this level can be significantly reduced by lengthening the reaction of unlabeled protein and antiserum prior to addition of the tracer antigen (Hales and Randle, 1963; Rodbard *et al.*, 1971) and by appropriate selection of a high-avidity antiserum (Hunter, 1973). Despite these advantages and the excellent examples of the usefulness of competition RIP assays for quantitation of biologically active substances in endocrinology and pharmacology, this technique has not been widely used in virology. Notable exceptions have been the use of solid-phase RIA for antigen detection in blood of hepatitis carriers (Feinstone *et al.*, 1979) and detection of viral antigens in stool specimens for rotavirus infection (Kapikian *et al.*, 1979).

The impetus for the development of these assays stems from inability to propagate these viruses by standard virological techniques. The establishment of tissue cell culture procedures for diagnosis of human infections by detection of infectious virus particles and the availability of the necessary reagents including defined cell lines from commercial sources probably account for the lack of utilization of RIP technology in this area. However, applications of RIP methodology for diagnosis and studies of disease processes in viral infections will undoubtedly increase (Forghani, 1979). Areas that appear particularly promising are situations in which antigens rather than intact virions may be found or virus particles may be noninfectious. These applications may include detection of viremic or antigenic states during acute illness or identification of carriers of the virus, identification of viral antigens in persistent or chronic infections, quantitation of viral protein synthesis in degenerative diseases or "slow virus" infections, and diagnosis of repeat viral infections with similar viruses where the specimens may contain antibody.

VII. Concluding Comments

In principle, the methods described in this chapter are applicable to development of RIP assays for any viral protein. The use of a purified protein as the tracer antigen has several inherent advantages. It allows for direct characterization of the tracer antigen by biochemical and biophysical techniques so that quantitative assessments of antibody levels to specific antigen rather than to the virus can be obtained. In general, competition RIP tests are more quantitative than other RIA techniques for detection of antigen in biological specimens. The ability to compare antigenic structures of cross-reactive proteins and to quantitate antibody levels to selected antigenic determinants illustrates the applicability of these assays to understanding immunological responses to viruses. However, the versatility that is provided by the use of a purified antigen also carries the disadvantage that methods must be available for purifying the protein in an antigenically active configuration. This limitation is likely to be overcome by utilization of recombinant DNA technology to produce protein antigens (Lai *et al.*, 1981; Palese and Young, 1982) and by direct synthesis of polypeptides representing antigenic portions of proteins (Green *et al.*, 1982; Jackson *et al.*, 1982; Dreesman *et al.*, 1982).

The development of hybridoma methodology for production of monoclonal antibodies (Galfre and Milstein, 1981) should further increase the utilization of RIP assays. First, it provides a means of obtaining large amounts of homogeneous antibody, which should further increase the re-

producibility of the method for antigen quantitation. This would also provide reproducible antibody populations that could be used in different laboratories to standardize the assay system. Second, using a battery of monoclonal antibodies prepared to the same viral protein, i.e., the HA of influenza virus (Gerhard, 1976; Gerhard *et al.*, 1981), it should now be possible to define completely all the parameters that influence competition curves with complex protein antigens. As discussed earlier, calculation of the rate and affinity constant cannot be done because the influence of antibodies to multiple antigenic determinants on the same molecule and antibodies with different affinities to the same site is not presently known. However, theoretical models based on mathematical treatments have been described (Berzofsky and Schechter, 1981; Creighton, 1980), and monoclonal antibodies should provide a means for validating these interpretations.

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tions" (E. H. Lennette and N. J. Schmidt, eds.), pp. 171–189. American Public Health Association, Washington, D.C.

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3 *Enzyme Immunosorbent Assays in Plant Virology*

M. F. Clark and M. Bar-Joseph

I. Introduction

The antigenic nature of a plant virus, tobacco mosaic virus, was first reported more than 50 years ago (Beale, 1928). Since then the development of serological methods for identifying and assaying plant viruses has progressed erratically, mainly by adopting techniques derived from basic im-

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munology research and from the serodiagnosis of human and animal pathogens. The introduction of enzyme-linked immunosorbent assay (ELISA) (Engvall and Perlmann, 1971; van Weemen and Schuurs, 1971) followed the same pattern, although it seems likely that its acceptance as a practical diagnostic method is today more widespread with diseases of plants, particularly virus diseases, than with those of man. Advantages of ELISA over alternative diagnostic assay methods for plant viruses include the following:

1. Sensitivity for detecting very small amounts of virus, typically detecting concentrations as low as 1–10 ng/ml.
2. Speed of reaction—results are usually available within 6–24 hr.
3. Scale of operation—several hundred samples may be handled readily, either individually or in groups of samples.
4. Use with plant extracts and purified preparations.
5. Specificity, for differentiating serotypes.
6. Suitability for both intact and fragmented virions of different size or morphology.
7. Possibility of obtaining quantitative measurements.
8. Possibility of automation and of standardizing tests by the production and use of kits.
9. Low cost and long shelf life of reagents.
10. Basic requirement for simple equipment.
11. Economical and efficient use of antibodies and antisera.

Other techniques may have specific advantages over ELISA in certain respects; e.g., immunosorbent electron microscopy (Derrick, 1973) is probably more suitable for detecting virus in very small samples such as individual insect vectors, whereas latex flocculation tests give very rapid results. However, the combined advantages offered by enzyme immunosorbent assay have made it the preferred method for many field and laboratory applications where sensitivity and specificity of antigen detection together with economy and scale of operation are factors of prime importance.

In this chapter we include protocols for various methods of enzyme immunosorbent assay in current use in plant pathology. For a consideration of the theoretical aspects of enzyme immunosorbent assay, see relevant research papers or published reviews (Wisdom, 1976; Schuurs and van Weemen, 1977; Koenig, 1978; O'Sullivan *et al.*, 1979; O'Beirne and Cooper, 1979; Voller *et al.*, 1979; Bar-Joseph and Garnsey, 1981; Clark, 1981a,b; Koenig and Paul, 1982).

II. Principles of Enzyme Immunosorbent Assay

Enzyme immunosorbent assays represent a departure from the classical procedures based on immunoprecipitin reactions in that recognition of immunospecific activity is through the action of the associated enzyme "label" rather than by observing the formation of an insoluble antigen–antibody complex. By using an enzyme marker, usually linked to the virus-specific antibody, the detection of a specific reaction can be augmented several hundredfold relative to the threshold of visibility of an immune precipitate. Essentially the same principle is utilized also in methods involving radio- or fluorescent labels and in serological electron microscopic techniques in which antibodies are "tagged" with electron-opaque markers.

A significant feature of heterogeneous immunoassays, i.e., those in which reacting and nonreacting components are separated, is the immobilization of antigen on a polystyrene or polyvinyl surface and the sequential presentation of the various reactants at the reaction site. The removal of irrelevant substances from the reaction site is advantageous, as potentially inhibitory compounds or host-associated enzymes that could hydrolyze the test substrate do not accumulate but are washed away after each stage in the procedure, leaving only the specifically immobilized reactants. Consequently, assays can be carried out as effectively with crude plant extracts as with purified virus preparations of equivalent virus content.

Most applications of enzyme immunosorbent assay for the detection of plant viruses have utilized the double antibody sandwich (DAS) method of ELISA (Clark and Adams, 1977) in which specifically immobilized viral antigen is allowed to react directly with enzyme-labeled antibody, the resultant complex being revealed by the addition of enzyme substrate. Protocol N lists the sequence of operations for a typical routine assay of virus in field-collected samples, using this form of ELISA. Various modifications to this basic procedure have been reported, protocols for some of which are also given below.

Procedures currently in use in plant pathology fall into one of two main categories: "direct" procedures, in which antigen immobilized on the solid phase is detected with an enzyme-labeled, specific antibody; "indirect" procedures, in which the immobilized antigen is the target for unconjugated, specific antibody, which in turn is detected by an enzyme-labeled, anti-immunoglobulin molecule. The last may be either an anti-species antibody conjugate or a protein A conjugate. Antigen may be adsorbed to the solid phase directly (antigen coated) or selectively trapped by specific antibody or antibody fragments (double-antibody sandwich, DAS) previously adsorbed to the solid phase. Alternatively, antigen and antibody in solution

may be incubated together and the resultant immune complex then immobilized by the complement component Clq adsorbed to the solid phase.

The relationship of these variants is represented schematically in Fig. 1.

High specificity of serotype detection is reported to be a property more of direct than of indirect procedures, but the dependence of direct procedures on enzyme-labeled, antigen-specific conjugates necessitates the production of separate conjugates for each antibody to be evaluated. In contrast, indirect procedures can utilize a single "all-purpose" enzyme conjugate, based either on an antiglobulin antibody or on a protein A molecule. Most indirect DAS procedures require the use of specific antibodies from two different animal species to enable the antiglobulin conjugate to discriminate between "trapping" and "detecting" antibodies. The $F(ab')_2$ ELISA method (Barbara and Clark, 1982) was devised to circumvent the requirement for separate antisera. In this procedure enzymatic removal of the Fc portion from the antibody used for trapping the antigen allows the use of a protein A or anti-Fc antibody conjugate, which will react only with intact antibodies used to "detect" the antigen.

Choice of a particular procedure will be governed by the availability of reagents and facilities, as well as by the type of investigation to be carried out. For large-scale, routine indexing of virus in crops, the standard DAS

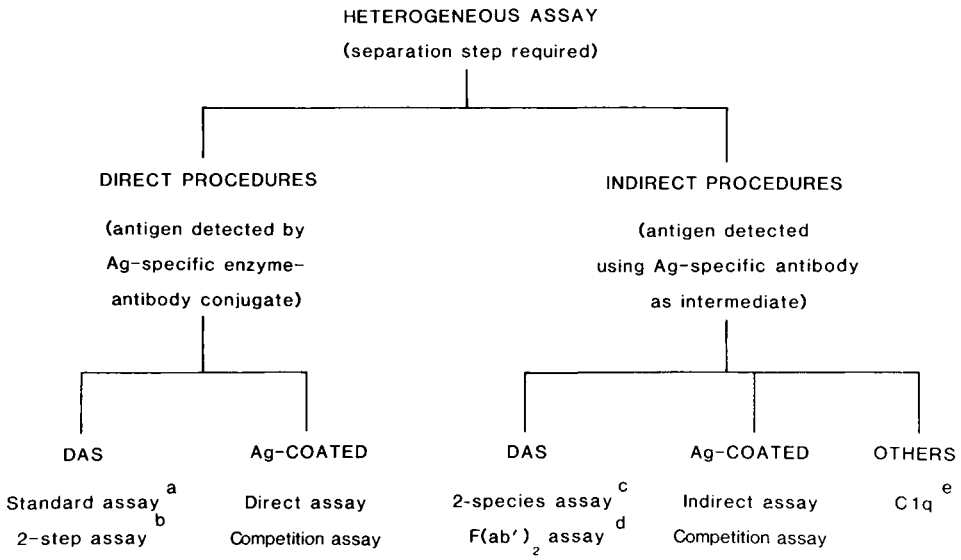

FIG. 1. Schematic relationship of variants of the ELISA procedure. (a) See protocol N; (b) see protocol O; (c) see protocol Q; (d) see protocol P; and (e) see protocol R.

"direct" procedure is usually convenient and effective. When greater flexibility is required, as in studies of serological relationships, in comparisons of antisera, or when it is not feasible to produce specific conjugates, an indirect procedure should be considered. For a comparative evaluation of various procedures, see Koenig and Paul (1982).

Regardless of the type of assay to be used, a satisfactory result will depend on the suitable selection and careful preparation of the reactants and on a comprehension of the capabilities and limitations of the procedure to be used. In the following sections brief consideration will be given to some of the more important of these factors. A fuller discussion of individual topics is available in specific research reports and in the reviews listed previously.

III. Preparation of Sample

Assays may be made with samples taken directly from plant extracts or with purified virus preparations. For tests with purified virus diluting the preparation directly into PBS–TPO buffer (see Appendix) is usually satisfactory. This buffer has been found to be generally useful for extracting a wide range of viruses from many plant hosts, with some exceptions, e.g., the closterovirus, apple chlorotic leafspot virus (CLSV), whose virions are unstable in the presence of salt. For this virus a special buffer can be used (Detienne *et al.*, 1980) or a different procedure (e.g., protocol O) may be employed to maintain the morphological integrity of the virus.

The intentional use of degraded virus or virus subunits as the antigen may sometimes be useful. For such tests it is necessary to make sure that the antiserum to be used reacts adequately with this type of antigen as there have been several reports of serological discrimination between virus subunits and intact virions (Shepard and Shalla, 1970; Hiebert and McDonald, 1976; van Regenmortel, 1978; Clark, 1981b). Subunits can be prepared by a variety of methods, some of which may be unsuitable for enzyme immunosorbent assay because the virus-degrading compounds employed can destroy the effectiveness of the immunoassay. Thus, residual amounts of sodium dodecyl sulfate (SDS) or pyrrolidine, often used to degrade potyviruses, will probably interfere with the detection of the degraded viral fragments by ELISA, although such procedures are effective for gel diffusion tests (Shepard, 1972; Purcifull and Batchelor, 1977; Garnsey *et al.*, 1978; Carroll *et al.*, 1979). Antisera made against labile viruses, such as the closteroviruses, ilarviruses, some potyviruses, frequently contain a variable proportion of antibodies directed specifically against virus subunits. For these viruses it may be advantageous to encourage further virus fragmentation

by employing buffers or conditions known to accelerate degradation, e.g., mechanical homogenization, high salt concentrations (Chairez and Lister, 1973), or pH adjustment. However, it is advisable to carry out the ELISA test itself under conditions of moderate salt concentration and near-neutral pH.

Samples obtained from plants may be extracted directly into PBS–TPO. However, many plants contain compounds that may interfere with the antibody–antigen interaction or can inhibit or inactivate one or more of the reagents. The polyvinylpyrrolidone (PVP) component of PBS–TPO was originally included in this buffer to counteract the inhibitory effects of tannins, frequently present in extracts made from woody perennial hosts such as fruit trees. Polyethylene glycol (PEG, M_r 6000) may be used instead but is probably less effective. On their own, PVP or PEG cannot adequately neutralize the effects of all the detrimental compounds found in plant extracts, and other countermeasures may be necessary. The simplest method is to dilute the plant extract to eliminate any inhibitor effects. This course of action is possible only if prior investigation has shown that there is sufficient concentration of virus in the plant to allow the extract to be diluted without reducing the reliability of the assay. As a general rule samples should be tested at the greatest dilution of extract that permits reliable detection in order to minimize any deleterious effects of the extract constituents. Dilutions of tissue extracts of the order 0.2–1.0 g fresh weight per 10 ml of buffer usually are satisfactory. Postextraction treatments may also be effective in reducing background reactions, e.g., storage of potato tuber extracts for some hours before testing (Tamada and Harrison, 1980).

Different extraction buffers and various additives have been reported to facilitate detection of virus in specific virus–host combinations (Lister and Rochow, 1979; Beijersbergen and van der Hulst, 1980; Albouy and Poutier, 1980; Detienne *et al.*, 1980; McLaughlin *et al.*, 1981). However, we suggest that PBS–TPO should be regarded as the basic or standard buffer with which to compare the effectiveness of other extraction media.

Samples are extracted directly into buffer by one of several methods, according to individual preference and the prevailing circumstances. Mortar and pestle extractions are simple and efficient but may be impractical where many samples are to be processed. Overhead dispersion homogenizers are effective and are easily cleaned providing there is little plant fibre in the sample. Extracts of soft tissues, such as potato leaves and sprouts, may be obtained using a roller press, and an ingenious drilling device has been described for making preparations from potato tubers (Gugerli, 1979). Viruses that occur in high concentrations may be assayed directly from leaf disks without the need for prior homogenization (Marco and Cohen, 1979; Romaine *et al.*, 1981).

As extraction and preparation of the sample are probably the most time

consuming of the ELISA procedures, it is worth giving careful consideration to the various alternative methods before deciding which is most appropriate for a specific application.

IV. Production of Antiserum

The production of a satisfactory antiserum is the basis of a successful ELISA procedure. Although various animal species may be used to raise the antiserum, it is likely that rabbits will continue to be the preferred animal for most applications. However, as ELISA is remarkably economical in its use of reagents, including antiserum, it may be that using smaller animals, such as guinea pigs or rats, would be more convenient for producing the few milliliters of antiserum necessary for small-scale investigations.

Before embarking on a virus purification and injection schedule, careful consideration should be given to the purpose for which the antiserum will be required. For indexing natural infections in large populations of plants, the requirement will be for a high-titer antiserum having broad spectrum specificity, whereas for discriminating among serotypes, e.g., in tests of identification or in epidemiological investigations, an antiserum possessing considerable serotype specificity will be called for. As a general rule, antisera with high specificity should not be used for field indexing operations in case some naturally occurring serotypes are not detected. A prior knowledge of the likely field occurrence and cross-reactivity of different strains or isolates is a prerequisite to the establishment of a satisfactory indexing scheme.

Such factors as the method of preparing the immunogen, the use of protein subunits rather than intact virus or, conversely, the use of formaldehyde-stabilized virus, the number of injections administered, and the time between primary immunization and bleeding can all be manipulated to enhance the usefulness of the antiserum for its designated purpose. For specific requirements, the production and use of monoclonal antibodies might be considered. Protocols for preparing antisera appear in several general texts on plant virology (e.g., Matthews, 1967; Bercks *et al.*, 1972; Gibbs and Harrison, 1976) or on immunology (e.g., Williams and Chase, 1967; Campbell *et al.*, 1970).

A. CROSS-ABSORPTION OF ANTISERA

Every effort should be made to prepare highly purified antigens for injection, which retain their specific antigenic activity. However, this is not always possible, and it may be necessary to remove unwanted antibodies

from the antiserum by cross-absorption with a preparation of the contaminating antigen, such as fraction 1 proteins or other antigens derived from the host plant from which the specific antigen was prepared.

Cross-absorption may be carried out by sequentially adding aliquots of a preparation of the contaminating antigen, e.g., crude plant extract or resuspended pellet from a viruslike preparation made from a healthy plant, to the antiserum and removing by centrifugation or filtration any immunoprecipitate formed after suitable incubation of the mixture. This approach is usually satisfactory for macromolecular contaminants, especially if the treated antiserum is to be processed subsequently to purify the γ-globulins. However, for some purposes it may be undesirable to contaminate the antiserum with an excess of host plant material, in which case cross-absorption by means of an insoluble immunoadsorbent may be preferable (Avrameas and Ternynck, 1969). The following method has proved to be satisfactory both in removing antibodies to one virus from an antiserum raised against a preparation from a mixed infection, (Clark, unpublished results) and in eliminating residual antihost activity in several antivirus antisera (D. J. Barbara, personal communication).

General reagents and equipment

Antiserum to be processed
Distilled water
Glassware: pipettes, beakers, flasks, tubes, etc.
Dialysis tubing, prepared by boiling for 10 min in 0.01 *M* EDTA
UV photometer
HCl, 0.1 *N*
NaOH, 0.1 *N*

Specific requirements

Antigen preparation (protocols A and D)
Bovine serum albumin, fraction V (protocol A)
Glutaraldehyde, 25%, electron microscope grade (protocols A and D)
Kieselguhr, acid-washed (protocol A)
Glass-in-glass homogenizer (protocol A)
Chromatography column, e.g., a 20-ml glass syringe barrel (protocols A and B)
Ammonium sulfate, saturated solution (protocol B)
DEAE-cellulose, prepared according to manufacturer's instructions (protocol B)
Medium-speed centrifuge (protocol B)
Protein A–Sepharose CL-4B (protocol C) (Pharmacia Fine Chemicals AB, Sweden)

Waterbath at 37°C (protocols D and E)
Ultracentrifuge (protocol D)
Pepsin, e.g., Sigma 1:10,000 (protocol E) (Sigma Chemical Co., St. Louis, MO)
Sephadex G-25 or G-75 (protocol E)
Polyethylene glycol, M_r 6000 (protocol F)

Buffers

PBS (see Appendix)
PBS–T (see Appendix)
Glycine, pH 2.7 (see Appendix)
Sodium acetate, 0.07 *M*, pH 4.0, containing 0.05 *M* NaCl
Tris-HCl, 0.5 *M*, pH 7.8, containing 0.15 *M* NaCl

Protocol A. Preparation of immunoadsorbent

1. Prepare the antigen to be used for cross-absorbing antiserum by a suitable procedure. Suspend or dissolve the preparation in 4 ml of PBS.
2. Add 50 mg of bovine serum albumin (fraction V) and dissolve in the antigen preparation.
3. Add 0.8 ml of 25% glutaraldehyde. Mix and leave to gel at 30°C overnight.
4. Transfer gel to a glass-in-glass homogenizer, and homogenize in PBS–T.
5. Mix with approximately 3 g of acid-washed kieselguhr and transfer to a small chromatography column. Ensure that the gel remains dispersed throughout the kieselguhr during loading of column.
6. Wash the material in the column with PBS–T until absorbance of effluent (OD_{280}) is zero or remains constant.
7. Load the column with 1–5 ml of antiserum and allow to filter through column slowly. Monitor the effluent at 280 nm and collect nonabsorbed antiserum. This step should take about 30–60 min.
8. Process the antiserum as normal (e.g., protocol B).
9. Regenerate the column if required by washing with 0.2 *M* glycine-HCl, pH 2.7, containing 0.1 *M* NaCl, followed by a preequilibration wash with PBS–T as in step 6.

B. PURIFICATION OF IMMUNOGLOBULINS FROM ANTISERUM

Immunoglobulins may be prepared from whole antiserum by any of several published methods. The following protocols are basic procedures in common use in plant virology and do not preclude the use of other variants or methods.

Protocol B. Preparation of immunoglobulins by salt precipitation and DEAE-cellulose filtration

1. To 2.0 ml of whole antiserum, add 8 ml of distilled water.
2. Add 8 ml of saturated ammonium sulfate solution and mix at room temperature for 30–60 min. A flocculent precipitate should develop.
3. Centrifuge at 8000 *g* for 10 min. Discard the supernatant.
4. Dissolve precipitate in 10 ml of water and repeat steps 2 and 3.
5. Dissolve precipitate in 2 ml half-strength PBS (1:1 PBS:H_2O).
6. Transfer to prewashed dialysis tubing and dialyze against at least three changes of 500 ml of half-strength PBS over a period of 24 hr.

The procedure may be terminated at this stage. However, such preparations frequently contain considerable amounts of lipid compounds (giving a bluish haze to the preparation), and it may be advantageous to remove these by a filtration step as follows:

7. Prepare a column of 5- to 10-ml bed volume of preequilibrated DEAE-cellulose.
8. Wash the DEAE-cellulose in the column with at least five bed volumes of half-strength PBS or until no UV-absorbing material can be detected in the washings.
9. Pipette 2 ml of immunoglobulin preparation on top of the cellulose.
10. Wash the immunoglobulin through the column with half-strength PBS, collecting the eluate in approximately 1-ml fractions.
11. Monitor the fractions at 280 nm and combine fractions containing the first protein peak to be eluted.
12. Measure the OD_{280} of the combined fractions and adjust the concentration of the γ-globulin with half-strength PBS to read approximately 1.4 (about 1 mg/ml). The ratio OD_{280}:OD_{252} should be about 2.5–2.6, and the preparation should be water-clear to transmitted light.

Protocol C. Preparation of immunoglobulins by affinity adsorption with protein A–Sepharose

1. Rehydrate 0.5 g of protein A–Sepharose CL-4B according to manufacturer's directions and pack into a 5-ml disposable syringe.
2. Wash with several bed volumes of PBS.
3. Pipette 1 ml of rabbit antiserum into the column and wash it through the column with several bed volumes of PBS.
4. Elute adsorbed IgG with 0.1 *M* glycine-HCl buffer, pH 2.7; monitor eluate at 280 nm and collect 1-ml fractions into tubes containing 0.5 ml of 0.5 *M* Tris-HCl + 0.15 *M* NaCl buffer, pH 7.8.

5. Combine fractions containing the eluted IgG and adjust the protein concentration as described in protocol B, step 12.

Protocol D. Isolation of specific antibodies by affinity methods

Various procedures for obtaining plant virus-specific antibodies by affinity methods have been reported (Hardie and van Regenmortel, 1977; McLaughlin *et al.*, 1980). However, such procedures have rarely been employed for practical applications, probably because plant viruses tend to be good immunogens, enabling antisera of sufficiently high titer and specificity to be produced without further purification. Nevertheless, it may sometimes be desirable to use purified virus-specific antibodies for which an affinity purification method will be required.

Usually the interaction of virus and antibody and removal of the immune complex are easily achieved through the use of affinity columns, centrifugation, or immunoadsorbents (see protocol A). However, the subsequent dissociation and separation of virus from specific antibody requires fairly severe conditions, e.g., low pH treatment or the action of chaotropic agents such as sodium thiocyanate. Consequently, only viruses or antigens capable of withstanding such treatments are suitable candidates for these procedures, although immobilization on a solid phase or fixation by glutaraldehyde or formaldehyde may stabilize the virus sufficiently to overcome problems of antigen dissociation. The following protocol has proved to be satisfactory for the isolation of antibodies specific for tobacco mosaic virus.

1. Prepare a purified preparation of virus or antigen, as appropriate, in PBS adjusted to pH 7.8.
2. Dialyze antiserum to be treated against PBS at pH 7.8.
3. Mix the virus and antiserum using conditions of slight antigen excess; incubate at 37°C for 4 hr with occasional agitation, or at 4°C overnight.
4. Centrifuge at 40,000 *g* for 60 min.
5. Pour off the supernatant and resuspend the pellet in 5 ml of water.
6. Adjust the pH of the resuspended pellet to 2.9 using 0.1 *N* HCl.
7. Centrifuge at 40,000 *g* for 75 min.
8. Collect the supernatant and adjust the pH to 7.5 with 0.1 *N* NaOH.

An alternative method is to use an insoluble immunoadsorbent prepared by glutaraldehyde polymerization of virus and bovine serum albumin, as described in protocol A. The immunoadsorbent is then washed alternately in PBS-T and 0.2 *M* glycine-HCl buffer, pH 2.7, in 0.1 *M* NaCl, until the washings are free from UV-absorbing compounds. The final wash should be in PBS-T. Antiserum is mixed with the immunoadsorbent as in step 7 of protocol A, and the complex is washed extensively with PBS-T to remove

unbound antibodies. Specifically bound antibodies are then recovered by treatment of the immunoadsorbent with glycine-HCl buffer, the eluted antibody solution being readjusted to pH 7 with 0.1 *N* NaOH as soon as possible.

Protocol E. Preparation of F(ab')$_2$ fragments of immunoglobulin for use in F(ab')$_2$ indirect ELISA (Protocol P)

F(ab')$_2$ fragments may be obtained by pepsin digestion of IgG prepared by any of the above methods or partially purified according to protocol B, steps 1–5, inclusive. The procedure is essentially that described by Campbell *et al.* (1970).

1. Transfer IgG solution at 2.5 mg/ml to a dialysis tube and dialyze against 1 liter of 0.07 *M* sodium acetate buffer, pH 4, containing 0.05 *M* NaCl. The pH of the solution must be lowered as inactivation of the enzyme may occur above pH 5. After dialysis, transfer to a clean glass tube.
2. Dissolve 5 mg of pepsin in 1 ml of acetate buffer as above. For each 1 mg of IgG to be digested, add 10 μl of pepsin solution, i.e., 50 μg of pepsin per milligram of IgG.
3. Incubate at 37°C for 18 hr.
4. *Either* dialyze digested product against three changes of 1 liter of PBS to remove the low-molecular-weight products of enzyme hydrolysis
   Or separate F(ab')$_2$ fragments from polypeptides and other hydrolysis products by exclusion chromatography on Sephadex G-25 or G-75 in PBS.

Protocol F. Preparation of immunoglobulin from egg yolk

Hen egg yolk is a rich and convenient source of immunoglobulins that may be used as detecting antibody in the HADAS (Bar-Joseph and Malkinson, 1980) indirect ELISA procedure (protocol Q). For this purpose a crude egg yolk preparation is usually sufficient; this may be obtained by the following steps.

1. Transfer each egg yolk to a 50-ml centrifuge tube and add to each tube 20 ml of PBS; shake thoroughly to mix.
2. Centrifuge at 10,000 rpm (12,000 *g*) for 20 min and collect the supernatant.
3. Add an equal volume of glycerol and store at −20°C.

If required the immunoglobulins may be partially purified by the following method (Polson *et al.*, 1980).

4. Mix 1 volume of yolk with 2 volumes of PBS containing 0.01% NaN_3.
5. Add PEG 6000 to 3.5% (w/v) and stir to dissolve.
6. Centrifuge at 14,000 *g* for 10 min.
7. Collect the clear supernatant and filter through a loose cotton plug to remove any remaining lipid material.
8. Add additional PEG 6000 to bring the concentration to 12% (w/v).
9. Centrifuge at 14,000 *g* for 10 min to sediment the precipitated immunoglobulins.
10. Dissolve the pellets in the original volume of PBS and repeat steps 8 and 9.
11. Dissolve the final pellets in 7–8 ml of buffer. The protein concentration of the solution will normally be in the range 6–12 mg/ml.

C. STORAGE OF IMMUNOGLOBULINS AND IMMUNOGLOBULIN FRAGMENTS

For long-term preservation of purified immunoglobulins, it is probably best if the preparation is freeze-dried in small aliquots and stored in glass vials under vacuum. Additives such as dextran or bovine serum albumin should be avoided, as these may interfere with some aspects of any ELISA tests performed subsequently (e.g., coating of the solid phase). Immunoglobulins may be stored for several months at 4°C with a suitable preservative such as 0.02% sodium azide, or for longer at 4°C or at −18°C in 50% glycerol. Glycerol when diluted to concentrations of less than 1% appears to have little or no significant effect on the adsorption of IgG to polystyrene surfaces, and therefore it may be used for storing preparations to be diluted to coat ELISA plates.

Immunoglobulin preparations from egg yolk are best stored at −20°C after adding glycerol to 50% (v/v).

V. Choice of Enzyme and Preparation of Conjugate

Of the various enzyme labels suitable for use in immunosorbent assays, only two have been employed to any extent for the serodiagnosis of plant viruses. These are alkaline phosphatase (ALP) (EC 3.1.3.1) and horseradish peroxidase (HRP) (EC 1.11.1.7). A discussion of the relative merits of various possible enzyme labels can be found elsewhere (Wisdom, 1976; Schuurs and van Weeman, 1977; O'Beirne and Cooper, 1979; O'Sullivan *et al.*, 1979; Voller *et al.*, 1979).

For the majority of applications in plant pathology, ALP has been the preferred enzyme as it is stable, is simply and conveniently linked to protein

by a glutaraldehyde bridge, and exhibits essentially linear reaction kinetics with its substrates, e.g., *p*-nitrophenyl phosphate (PNP). Drawbacks to the use of this enzyme are its high cost relative to HRP and the inefficiency of its conjugation to protein A by gluteraldehyde.

Horseradish peroxidase is a less expensive enzyme to purchase but is not as efficiently linked to proteins by the one-step glutaraldehyde procedure as ALP. However, it is a more versatile enzyme in that other conjugation methods are possible, owing to its steric conformation and to the presence of carbohydrate moieties in the molecule. Also there is a wide range of possible chromogenic substrates from which to choose, although most of these are likely to be insufficiently sensitive for use in ELISA. The reaction kinetics of HRP with its substrates are nonlinear, the enzyme apparently being progressively inhibited during the course of substrate hydrolysis. However, similar levels of sensitivity of antigen detection can be achieved with both HRP conjugates and ALP conjugates. To label protein A with HRP we recommend linkage by periodate oxidation of its carbohydrate moieties (protocol I; Wilson and Nakane, 1978).

General reagents and equipment

Purified IgG
Alkaline phosphatase, e.g., Sigma type VII
Horseradish peroxidase, e.g., Sigma type VI
Glutaraldehyde, 25%, electron microscope grade
Bovine serum albumin, fraction V
Glassware; pipettes, beakers, flasks, tubes, etc.
Dialysis tubing, prepared by boiling for 10 min in 0.01 *M* EDTA
Sodium azide (*N.B.* Poisonous, handle with care; do not leave in contact with metal surfaces)
Distilled water
Glycerol

Specific requirements

NaCl, 0.15 *M* (protocol H)
Lysine, 0.2 *M* (protocol H)
Sodium metaperiodate solution, 0.1 *M* (protocol I)
Acetic acid (protocol I)
Protein A (protocol I)
Sodium borohydride solution, 4 mg/ml (protocol I)
Ammonium sulfate, saturated solution (protocol I)
Medium-speed centrifuge (protocol I)

Buffers

Phosphate, pH 6.8 (see Appendix)
PBS (see Appendix)
Sodium carbonate, 0.2 *M*, pH 9.6

Protocol G. One-step procedure for conjugation by glutaraldehyde of ALP or HRP to IgG (Avrameas, 1969; Avrameas *et al.*, 1978)

1. Dissolve 2 mg of purified IgG in 2 ml of PBS or dispense 2 ml of a 1 mg/ml solution of IgG into a glass tube.
2. Dissolve 5 mg of enzyme, either as dry powder or as the centrifugally collected ammonium sulfate precipitate, directly in the IgG solution.
3. Dialyze at least three times against 1 liter of PBS; this is necessary only for preparations with residual ammonium sulfate, as failure to remove traces of this salt will encourage glutaraldehyde linkage to irrelevant NH_4^+ groups of the salt rather than to requisite amino groups on the protein.
4. Add 50 μl of freshly prepared 2.5% glutaraldehyde solution and mix gently.
5. Incubate for 4 hr at 30°C or at room temperature. A very pale yellow-brown color may slowly develop.
6. Transfer to a dialysis tube and dialyze at least three times against 1 liter of PBS to remove glutaraldehyde.
7. If necessary, centrifuge at low speed to remove any precipitate that may have formed.
8. Add BSA to about 5 mg/ml for storage.
9. Store ALP conjugates at 4°C with 0.02% sodium azide (up to 6 months), or at −20°C for long-term storage after adding an equal volume of glycerol.

The HRP conjugates should not be stored with azide, as this interacts with the heme group of the peroxide molecule and inhibits its activity. The conjugates may be stored mixed with 50% glycerol at 4°C or at −20°C, or they may be freeze-dried and stored in glass vials under vacuum.

Protocol H. Two-step procedure for conjugation by glutaraldehyde of HRP to IgG (Avrameas and Ternynck, 1971)

This method produces homogeneous conjugates of low molecular weight, in which antibody and enzyme combine in approximately equimolar ratio, but the efficiency of coupling is rather low. Antibodies labeled with HRP by this method are suitable for immunohistochemistry and for other applications where tissue penetration is required. However, such conjugates

do not perform as well in conventional microplate ELISA tests as do conjugates produced by the one-step procedure (protocol F) or by periodate oxidation (protocol I).

1. Dissolve 10 mg of HRP in 0.5 ml of 0.02 *M* phosphate buffer, pH 6.8.
2. Add 25 μl of 25% glutaraldehyde, mix, and incubate for 18 hr at room temperature.
3. Dialyze three times against 500 ml of 0.02 *M* PBS.
4. Transfer to a glass tube and add 0.5 ml of 0.15 *M* NaCl containing 5 mg of specific IgG.
5. Add 0.5 ml of 0.2 *M* carbonate buffer. Check that the pH is approximately 9.6; if not, add more carbonate buffer.
6. Incubate for 2–4 hr at 4°C.
7. Add 0.1 ml of 0.2 *M* lysine solution and incubate for 2 hr at 4°C.
8. Dialyze three times against 500 ml of PBS.
9. Add an equal volume of glycerol and store at 4°C.

Protocol I. Conjugation of immunoglobulins or protein A with HRP by periodate oxidation (Wilson and Nakane, 1978)

Horseradish peroxidase can be linked to proteins by a periodate oxidation reaction. The technique is based on the principle that active aldehyde groups produced by oxidation of the carbohydrate moieties are able to react with the amino groups of the protein to be conjugated, forming Schiff bases. The nascent bases are labile but may be stabilized by reduction with sodium borohydride. Conjugates produced by this procedure have similar properties to those made by the one-step glutaraldehyde procedure, being composed of a heterogeneous collection of molecules of high molecular weight. The yield of conjugate is higher with this procedure than with the one-step glutaraldehyde method for peroxidase conjugates, and the product is very well suited for use with both direct and indirect ELISA procedures. Typically, conjugates made with protein A are used at an equivalent concentration of the protein A of about 10 ng/ml, i.e., diluted 1:15,000 to 1:30,000 from the preparation made according to the following protocol.

1. Dissolve 4 mg of HRP in 1.0 ml of distilled water.
2. Add 0.2 ml of 0.1 *M* sodium metaperiodate. *N.B.* The periodate must be freshly prepared: the HRP solution should change to a greenish color; if not, fresh periodate is needed.
3. Shake for 20–30 min at room temperature.
4. Transfer to dialysis tubing and dialyze against distilled water adjusted to pH 4.4 with acetic acid at 4°C for several hours or overnight.

5. Transfer to a glass tube and add 50 μl of 0.2 *M* sodium carbonate buffer, pH 9.6.
6. Immediately add
   Either (a) 8 mg of IgG in 1.0 ml of 0.01 *M* carbonate buffer
   Or (b) 2.5 mg of protein A + 5 mg of BSA in 1.0 ml of 0.01 *M* carbonate buffer.
   Check that the final pH is approximately 9.6.
7. Incubate for 2 hr at room temperature with occasional shaking.
8. Add 0.8 ml of freshly prepared sodium borohydride solution and incubate for 2 hr at 4°C.
9. Add an equal volume of saturated ammonium sulfate and leave at room temperature for 30 min or until the precipitate has formed.
10. Collect the precipitate by centrifugation, dissolve in 4 ml of PBS, and dialyze three times against 500 ml of PBS.
11. Store freeze-dried under vacuum or at 4°C after adding an equal volume of glycerol.

Each of the above protocols gives preparations consisting of a mixture of conjugated and unconjugated molecules. In our experience there is no advantage to be gained in assaying plant viruses by ELISA by separating the unconjugated reactants. However, if required this is simply achieved by molecular sieve column chromatography using Sepharose 6B, Ultrogel 22, or other chromatographic material of similar molecular porosity.

VI. Substrates

Substrates for ELISA must provide a sensitive, quantitative measure of the amount of enzyme conjugate specifically reacting with antigen immobilized on the solid phase. Ideally the substrate and its reaction products should be easily prepared, stable, soluble, and have a high turnover rate giving a colored or fluorescent product whose intensity is proportional to the amount of enzyme trapped. The substrate should also be nontoxic and inexpensive. For alkaline phosphatase a popular and convenient substrate that meets most of these requirements is *p*-nitrophenyl phosphate (PNP). This substrate is available as a powder or, more conveniently, as tablets that can be dissolved directly in the substrate buffer to give a colorless solution. The reaction product, *p*-nitrophenol, is a yellow compound, which is easily visualized and can be measured colorimetrically at 405 nm. There is a high rate of substrate turnover, which progresses more or less linearly with time. The reaction may be terminated, if necessary, with NaOH (the usual method) or with chelating agents such as EDTA (Brauner and Fridlender, 1981).

Fluorogenic substrates are possible alternatives to the colorimetrically determined PNP. Two such substrates are 4-methylumbelliferyl phosphate and 3-*o*-methylfluorescein phosphate. Fluorescence assays are potentially more sensitive than colorimetric assays (Torrance and Jones, 1982) but require the use of a fluorimeter to measure the reaction, as the product is less easily visualized than is the colored *p*-nitrophenol. For those assays in which extreme sensitivity of antigen detection is not essential, the easy preparation and use of PNP probably more than compensate for any loss of sensitivity in comparison with a fluorogenic substrate.

Various substrates have been used to assay HRP. These include (1) 5-aminosalicylic acid (5-AS); (2) *o*-dianisidine; (3) 2,2-azinodi-(3-ethylbenzothiazoline sulfone-6) (diammonium salt) (ABTS); (4) *o*-phenylenediamine (OPD); (5) 3,3′,5,5′-tetramethylbenzidine (TMB).

o-Dianisidine and 5-AS are not particularly sensitive and give products that are partially insoluble. ABTS and OPD exhibit much higher sensitivity, and both have been widely used although ABTS is reported to have a poor dose–response curve (Voller *et al.*, 1979), and solutions of OPD are unstable to light and atmospheric oxygen. OPD is also a suspect mutagen (Voogd *et al.*, 1980) and should therefore be handled with caution. TMB is a recently described substrate (Bos *et al.*, 1981) that is reported to be nonmutagenic (negative in the Ames test) (Holland *et al.*, 1974; Garner *et al.*, 1975). In addition, solutions of this compound are light stable and less sensitive to autodegradation than are solutions of OPD. According to Bos *et al.* (1981) the color yield is superior to that obtained with OPD. However, both substrates gave similar color yields in tests at East Malling, but TMB gave cleaner backgrounds and has now been adopted as the preferred substrate.

PREPARATION OF SUBSTRATES

It is essential that all glassware and other containers to be used for preparing substrate solutions be scrupulously clean. A good policy is to dedicate a set of glassware to this purpose so that there is no risk of contamination by enzyme residues.

Protocol J. p-Nitrophenyl phosphate (PNP)

Prepare a stock solution of diethanolamine buffer. Make up the substrate by dissolving *p*-nitrophenyl phosphate (powder or tablets) to a concentration of 0.67 mg/ml and add 200 μl of substrate to each well. Incubate at room temperature or at 30°C if required. Reactions involving this substrate should be allowed to proceed for a minimum of 30 min, but preferably for

at least 1 hr. Autodegradation of this substrate should occur only slowly, and satisfactory reactions have been achieved with incubation periods of several hours or even overnight. If necessary stop the reaction with 3 *N* NaOH (50 μl per well), at the same time shaking or stirring to ensure thorough dispersion of NaOH throughout the reaction volume.

Measure absorbance at 405 nm (not stopped); 405 nm (stopped with NaOH).

Protocol K. 4-Methylumbelliferyl phosphate (4-MP) (Torrance and Jones, 1982)

Prepare a stock solution of diethanolamine buffer. Make up a stock solution of 4-MP in water to a concentration of 2 mg/ml and store frozen in 0.5-ml aliquots at −20°C. Prepare the substrate by diluting the stock solution of 4-MP in diethanolamine buffer to a final concentration of 15 μg/ml (0.5 ml of stock solution diluted with 66 ml of buffer). Add 200-μl aliquots to each well and allow the reaction to proceed for 1–2 hr at room temperature. Stop the reaction with 3 *M* K_2HPO_4 adjusted to pH 10.4 with KOH (50 μl per well).

Fluorescence: emission = 448 nm; excitation = 368 nm.

Protocol L. o-Phenylenediamine (OPD)

Prepare a solution of 0.025 *M* sodium acetate buffer, pH 5.5. Dissolve OPD in buffer to a concentration of 0.5 mg/ml (caution: possible mutagen). Add hydrogen peroxide to a final concentration of 0.03% (by volume) and dispense immediately. Allow the reaction to proceed for 20–30 min at 30°C, or at room temperature, in the dark. Stop the reaction with 3 *M* H_2SO_4 (50 μl per well). This substrate autodegrades rapidly and must be prepared fresh immediately prior to use.

Measure absorbance at 450 nm (not stopped); 492 nm (stopped).

Protocol M. 3,3′, 5,5′-Tetramethylbenzidine (TMB)

Prepare a stock solution of 1.0 *M* sodium acetate buffer, adjusted to pH 5.8 with citric acid. Prepare a stock solution of 10 mg of TMB per milliliter in dimethyl sulfoxide (caution: rapidly absorbed through skin), and store frozen at 4°C. To prepare the substrate, dilute 2 ml of stock solution of buffer and 0.2 ml of stock TMB solution in 20 ml of distilled water; add 0.02 ml of 6% hydrogen peroxide (final concentration 0.006%); dispense in 200-μl aliquots immediately. Allow reaction to proceed for 20–30 min at room temperature (incubation in the dark is not necessary). Stop the reaction with 3 *M* H_2SO_4 (50 μl per well).

Measure absorbance at 655 nm (not stopped); 450 nm (stopped).

VII. Choice of Solid Phase

Several different types of solid phase have been used for enzyme immunosorbent assays. These include polystyrene tubes and beads, polyvinyl chloride and polystyrene microagglutination plates, polystyrene cuvettes, nylon tubing, glass rods, and cellulose powder. Of these, polystyrene microtiter plates are well suited to most applications and have been used for the majority of immunosorbent assays in plant pathology. Plates from different manufacturers vary in quality and uniformity. Even different batches of plates from the same manufacturer may respond differently, necessitating the testing of each batch before it is accepted for routine use. Plates catalogued as being specifically manufactured for enzyme immunosorbent assay are usually more expensive than standard, nonsterile plates, but may perform no better. Tissue culture grade plates should not be used. Newcomers to this field are advised to evaluate for themselves plates or other types of solid support from different sources before deciding on a particular batch or brand.

VIII. Types of ELISA Procedure

The following protocols represent the types of procedure, both direct and indirect, in most common use in plant virology. Competition assays are not included, as they have not been employed to any extent. The details given for each procedure provide a basic sequence of operations that may be modified according to specific requirements. Variations for some of these procedures are noted and explained subsequently under *Notes* at the end of Section VIII,B.

General reagents and equipment

Antiserum
Purified γ-globulin to be used for coating plates and/or as detecting antibody
Microtiter plates (see Section VII)
Plastic plate covers, or sandwich wrap, cling film, or similar material to cover plates
Substrate solution, freshly prepared
3.0 *M* H_2SO_4 or 3.0 *M* NaOH
Wash bottle or aspirator bottle for washing plates
Large incubator at 30°C
Light box or photometer for recording results

Specific requirements

$F(ab')_2$ fragments of specific IgG (protocol P)
Enzyme-labeled, antigen-specific immunoglobulin conjugate (protocols N and O)
Protein A–HRP conjugate (protocols P and R)
Antiserum or γ-globulin preparation from second animal species (protocol Q)
Enzyme-labeled, antispecies immunoglobulin conjugate (protocols Q and R)
Bovine C1q (freeze-dried) (protocol Q)
Gelatin (protocol Q)

Buffers (see Appendix)

Carbonate coating buffer
PBS-T
PBS-TPO
Substrate buffer

A. DIRECT PROCEDURES

Protocol N. Standard DAS ELISA (Clark and Adams, 1977)

1. Add 200-μl aliquots (see Note a, below) of purified γ-globulin (protocols B–D) or $F(ab')_2$ (protocol E), appropriately diluted in coating buffer, to each well of the microtiter plate. Cover the plate to prevent evaporation.
2. Incubate at 30°C for 2–4 hr or at 4°C overnight (see Note b).
3. Empty plates, then wash by flooding wells with PBS-T (see Note c). Leave to soak for 3 min. Repeat wash and soak operations twice, then empty plate and shake out residual liquid.
4. Add 200-μl aliquots of test sample extracted or diluted in PBS–TPO (see Note d) to duplicate wells. Cover plate and incubate at 4°C overnight.
5. Repeat wash procedure as in step 3.
6. Add 200-μl aliquots of specific conjugate (enzyme-labeled γ-globulin; protocols G and I) appropriately diluted in PBS–TPO to each well (see Note d). Cover the plate and incubate at 30°C for 3–6 hr (see Note b).
7. Repeat wash procedure as in step 3.
8. Add 200-μl aliquots of appropriate enzyme substrate (protocols J–M) to each well.

9. Incubate at room temperature for 1 hr or until color has developed to desired intensity (see Note e).
10. Terminate reaction (if necessary) with 50 μl of appropriate stop solution (protocols J–M). Agitate to ensure thorough mixing.
11. Record results by visual observation (see Note f) or by measuring absorbance or fluorescence, as appropriate (protocols J–M).

Protocol O. Two-step (modified) DAS ELISA (Flegg and Clark, 1979)

1. Add purified antibody to the plate (protocol N, step 1).
2. Incubate (protocol N, step 2).
3. Wash the plate (protocol N, step 3).
4. Add 100-μl aliquots of test sample (see Note g) extracted or diluted in PBS-TPO (see Note d) to duplicate wells.
5. Add 100-μl aliquots of specific conjugate (see Note g) to each well. Agitate the plate to mix contents of wells thoroughly.
6. Cover the plate to prevent evaporation, and incubate at 4°C overnight (see Note b).
7. Wash the plate (protocol N, step 3).
8. Add substrate (protocol N, step 8).
9. Incubate (protocol N, step 9).
10. Stop reaction (protocol N, step 10).
11. Record results (protocol N, step 11).

B. INDIRECT PROCEDURES

Protocol P. F(ab')$_2$ ELISA (Barbara and Clark, 1982)

1. Add 200-μl aliquots (see Note a) of F(ab')$_2$ fragments of specific antibody (protocol E), appropriately diluted in coating buffer, to each well of the microtiter plate. Cover plate to prevent evaporation.
2. Incubate at 30°C for 2–4 hr or at 4°C overnight.
3. Empty the plates, then wash by flooding with PBS-T (c). Leave to soak for 3 min. Repeat the wash and soak operations twice, then empty the plate and shake out the residual liquid.
4. Add 200-μl aliquots of test sample, extracted or diluted in PBS–TPO (see Note d) to duplicate wells. Cover the plate, and incubate at 4°C overnight.
5. Repeat wash procedure as in step 3.
6. Add 200-μl aliquots of purified γ-globulin or whole antiserum (see Note h), appropriately diluted in PBS–TPO (see Note d), to each well. Cover the plate, and incubate at 30°C for 3 hr.
7. Repeat wash procedure as in step 3.

8. Add 200-μl aliquots of protein A-HRP conjugate (protocol I), appropriately diluted in PBS-TPO (see Note d), to each well. Cover the plate, and incubate at 30°C for 3 hr.
9. Repeat the wash procedure as in step 3.
10. Add 200-μl aliquots of OPD (protocol L) or TMB (protocol M) substrate. Incubate at room temperature (see Note i) for 30 min.
11. Terminate the reaction with 50 μl of 3 *M* H_2SO_4. Agitate to ensure thorough mixing.
12. Record the results by visual observation or by measuring absorbance at 492 nm (OPD) or 450 nm (TMB).

Protocol Q. Indirect ELISA (including HADAS-ELISA) using antibodies from different animal species (Bar-Joseph and Malkinson, 1980; van Regenmortel and Burckhard, 1980; Koenig, 1981)

1. Add 200-μl aliquots (see Note a) of purified γ-globulin from animal species 1, appropriately diluted in coating buffer, to each well of the microtiter plate. Cover the plate to prevent evaporation.
2. Incubate the plate (protocol N, step 2).
3. Wash the plate (protocol N, step 3).
4. Add the test sample (protocol N, step 4).
5. Wash the plate (protocol N, step 3).
6. Add 200-μl aliquots of purified γ-globulin or whole antiserum from animal species 2, or of hen egg yolk preparation (protocol F), appropriately diluted in PBS-TPO (see Note d), to each well. Cover the plate and incubate at 30°C for 3 hr.
7. Wash the plate (protocol N, step 3).
8. Add 200-μl aliquots of enzyme-labeled antibody conjugate specific for immunoglobulins of animal species 2, appropriately diluted in PBS-TPO (see Note d), to each well. Cover the plate and incubate at 30°C for 3 hr. *N.B.* The conjugate may be collected and re-used up to six times for routine tests (Bar-Joseph *et al.*, 1979).
9. Wash the plate (protocol N, step 3).
10. Add 200-μl aliquots of substrate appropriate for the enzyme being used (protocols J-M). Incubate for 30 min (HRP substrate) or 1 hr (ALP substrate).
11. Terminate the reaction with 50 μl of 3 *M* H_2SO_4 (HRP substrate) or 50 μl of 3 *M* NaOH (ALP substrate). Agitate to ensure thorough mixing.
12. Record the result by visual observation or by measuring absorbance or fluorescence as appropriate (protocols J-M).

The above procedure may be adapted for use in "antigen-coated" indirect ELISA by substituting the requisite antigen preparation for the coating

γ-globulin in step 1 and by omitting steps 3 and 4. The reactions of the solid-phase antigen with selected antisera are then determined after the use of these antisera in step 6 of the protocol.

Protocol R. C1q ELISA (Torrance, 1980)

1. Add 200-μl aliquots (see Note a) of 10 μg of bovine C1q per milliliter in PBS to each well of the microtiter plate. Cover the plate to prevent evaporation.
2. Incubate at 34°C for 3 hr.
3. Wash the plate (protocol N, step 3).
4. Add 200-μl aliquots of 0.05% gelatin in PBS to each well. Cover the plate and incubate at room temperature for 2 hr.
5. Wash the plate (protocol N, step 3).
6. Add 200-μl aliquots of test sample extracted or diluted in PBS–TPO to duplicate wells (see Note g).
7. Add 50-μl aliquots of purified specific antibody (see Note g) (protocols B–D), appropriately diluted in PBS–TPO (see Note d), to each well. Mix well.
8. Cover the plate and incubate at 4°C overnight.
9. Wash the plate (protocol N, step 3).
10. Add 200-μl aliquots of antiglobulin antibody conjugate diluted in PBS–TPO (see Note d) to each well.
11. Cover the plate and incubate at 30°C for 4 hr.
12. Wash the plate (protocol N, step 3).
13. Add the substrate (protocol N, step 10).
14. Terminate the reaction (protocol N, step 11).
15. Record the results by visual observation or by measuring absorbance or fluorescence, as appropriate (protocols J–M).

Notes a–i.

a. 200-μl aliquots are recommended, but other volumes may be used provided that the same volume is kept throughout the procedure.

b. Various authors use different incubation conditions, depending on the antibody or antigen preparation used, and according to individual preference or circumstance. However, for incubation temperatures above ambient it is important to avoid the development of thermal gradients across the plate, e.g., by using a large incubator with a high thermal capacity and by incubating plates singly rather than stacked.

c. Some authors find that washing with water + Tween 20 or with salt solution + Tween 20 is satisfactory. Reproducibility is impaired, however, if Tween is omitted. Plates should not be left to soak for extended periods in wash solution, as this may lead to partial desorption of reagents (Dr. M. Cambra, personal communication).

d. PBS–TPO is the recommended standard buffer for diluting the samples, the detecting antibody preparation, and the enzyme conjugate. However, other buffers may be as good or even better for particular assay conditions.

e. Substrate conversion by ALP conjugates should be allowed to progress for a minimum of 30 min to offset the time taken to fill the plate. Also, a very rapid rate of substrate conversion will probably indicate that the conjugate is too concentrated or is being used uneconomically.

f. A slide-viewing box in a darkened room is suitable both for viewing and for making photographic records.

g. Sample and conjugate dilutions should be adjusted to allow for subsequent dilution on mixing.

h. Either can be used, as only the specific antibodies will interact with antigen immobilized on the solid phase.

i. Incubation can also be carried out at 30°C. OPD substrates should be incubated in the dark to prevent light-associated autodegradation.

IX. Evaluation of Reagents and Analysis of Results

A. SETTING UP THE PLATE

Having prepared the reagents and selected an appropriate procedure, it is necessary to ascertain the optimum combination of reagent dilutions for performing the assay. This is best done by means of a test plate using a "checkerboard" format (Fig. 2) in which the different components of the assay interact in various combinations. Selection of appropriate reagent dilutions is then made by choosing the combination that gives maximum discrimination between the negative (healthy) test samples and the weakest positive (infected) sample that is likely to be encountered in practice. The results may be recorded either visually or with a photometer. For visually recorded results the aim should be to select a combination in which all negative samples remain colorless but all positive samples show some degree of color. Selection of the optimum combination for photometrically recorded assays can be more difficult, especially if the negative samples are not completely free of nonspecific or background reaction. Nevertheless, the aim should again be to select a combination in which negative sample reactions are minimal, or at least show minimal variation, but which gives an adequate and distinguishable response with the positive samples. When extreme difficulty is encountered in selecting an appropriate combination, it may be necessary to carry out a number of trial assays with a selection of samples before a final decision is made. As a general rule it is better to err on the side of weak negative reactions rather than to attempt to achieve

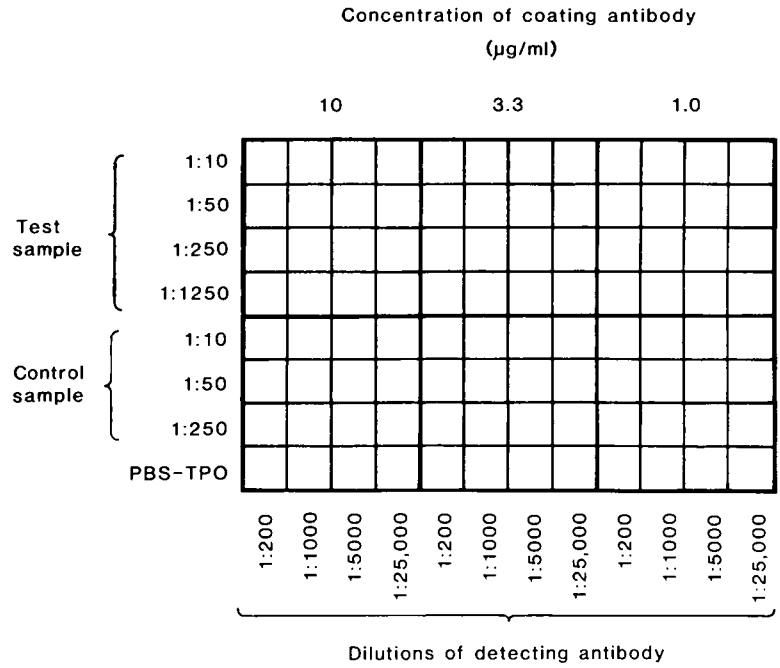

FIG. 2. Scheme for determining optimum concentration of coating and enzyme-labeled antibodies (direct procedures), or detecting antibody (indirect procedures). Indicated concentrations are suggestions and should be amended as appropriate.

the strongest possible positive reactions. This applies whether the assay is to be assessed visually or photometrically.

B. QUALITATIVE TESTS

Recording the results of an assay merely as positive (infected) or negative (healthy) is generally adequate for the majority of applications in which field populations of plants are indexed for the presence of one or more viruses. The standard form of DAS ELISA (protocol N) is well suited to this type of application. This is because the number of samples to be tested is usually sufficiently great to justify the production of a specific antivirus conjugate, and the assay procedure and visual scoring of results are readily carried out by field staff whose laboratory experience may be limited. Conjugates made with ALP are often used for this purpose, as the substrates and reaction products are reasonably stable and not subject to such drawbacks as autodegradation or nonlinear reaction kinetics, as found with some HRP substrates. It is the overall suitability of the standard DAS ELISA

for this type of application that has primarily been responsible for the widespread acceptance of the principle of serodiagnosis by enzyme immunosorbent assay both by research scientists and by agencies responsible for operating eradication schemes or for monitoring the health status of planting stocks.

Field-collected samples may be assayed individually, but when very large numbers are to be examined, or where the incidence of infection is expected to be low, it may be advantageous to combine samples for testing as a composite. Such a procedure is practicable only when prior investigation has established unequivocally that the test will reveal the presence of a single infected sample in the group. Individual infections may be identified subsequently by reassaying only samples of those composites giving a positive reaction. Frequently, significant savings in materials and reagents can be achieved by this approach, especially when the proportion of infected plants in the population is very low. Thus, in a survey for raspberry bushy dwarf virus in *Rubus* cultivars, Barbara and Wilson were able to locate 63 individual infections in a population of 5525 plants, using a total of 1105 initial tests and 110 retests (D. J. Barbara, personal communication).

When it is desired to estimate the proportion of infected plants in the population rather than to identify individual infected plants, this may also be achieved by group testing, and the results can be analyzed according to the formula

$$L = 100(1 - \sqrt[n]{1 - G/100}) \quad (1)$$

where L is the proportion of the plants infected, n is the number of individuals tested as a group, and G is the percentage of groups giving a positive reaction. Table I provides figures for such an analysis for groups of various sizes and levels of infection. Similarly, it is possible to calculate the sample size (n) necessary to indicate, with a given degree of reliability (β), populations whose proportion of infected individuals is higher than a specified maximum (α) [Eq. (2)].

$$n = \log(1 - \beta/100)/\log(1 - \alpha/100) \quad (2)$$

Such a calculation is useful both in determining the feasibility of a proposed indexing operation and in establishing the experimental parameters for such an investigation. For example, it may be calculated that to identify with 95% reliability seed lots of lettuce with as little as 0.01% lettuce mosaic-infected seed, it is necessary to test a total of 30,000 seeds (Table II).

C. QUANTITATIVE TESTS

The potential of ELISA for providing quantitative data has by no means been fully exploited. Moreover, the complexities of the multiple interactions involved in producing the overall response have not yet been fully compre-

TABLE I
CALCULATED PROPORTION OF PLANTS INFECTED FROM ELISA TESTS MADE WITH GROUPS OF SAMPLES

Groups infected %	Number of plants tested per group			
	2	5	25	100
2	1.0	0.4	0.08	0.02
5	2.5	1.0	0.20	0.05
10	5.1	2.1	0.42	0.11
25	13.4	5.6	1.14	0.29
50	29.3	12.9	2.73	0.69
75	50.0	24.2	5.39	1.38

TABLE II
MINIMUM SAMPLE SIZE NECESSARY TO ENSURE PERMITTED LEVEL OF INFECTION IS NOT EXCEEDED

	Reliability (%)		
	90	95	99
Infection level (%)	Number of seeds		
<5.0	45	60	99
<1.0	230	300	460
<0.1	2,300	3,000	4,600
<0.01	23,000	30,000	46,000

hended and, so far, ELISA has been used only for relatively uncomplicated analytical assays and investigations. Such studies fall mainly into one of two principal categories; (1) comparative evaluations of antisera, mainly for serotyping purposes; (2) assays for antigen, e.g., in studies on host–parasite relations and antigen distribution in plants, and for monitoring the efficiency of purification schedules. Although "direct" assays using enzyme-labeled specific antibody conjugates have been used, particularly for those studies in (2), "indirect" assays are probably better suited to this purpose. This is because indirect assays employ only "native" antibodies, that is, antibodies whose avidity for specific antigens has not been masked or altered by their incorporation into giant conjugate molecules or impaired by the action of polymerizing agents such as glutaraldehyde. In "direct" procedures, the best concentration of antigen-specific conjugate is dictated not only by the optimum activity of the specific antibody, but also by the amount of enzyme incorporated in the conjugate. The concentration chosen is often the highest possible compatible with an acceptable level of non-

specific activity. On the other hand, the concentration of detecting antibody in an "indirect" ELISA can be optimized at different levels for different situations; the concentration of the "all-purpose" conjugate is selected independently. Thus, in the example illustrated in Fig. 3 antiserum to the strawberry strain of AMV (AMV-S) detected only homologous virus when diluted 1:10,000, but detected both the homologous virus and the grapevine fanleaf isolate of AMV (AMV-GFL) when used at 1:100. Likewise, antiserum to AMV-GFL detected both viruses when diluted 1:100, but detected only the homologous virus at an antiserum dilution of 1:1000.

The dose-response curve is sigmoidal for most antigens and antibodies. The curve can be divided into three main regions, which may be termed proportional response, linear response, and plateau regions (Fig. 4). Experimental conditions that approach the plateau region should be avoided, as values obtained under such conditions do not solely reflect the interaction of antibody and antigen but reflect also such factors as saturation of binding sites, substrate limitation, intermolecular competition, and inter-

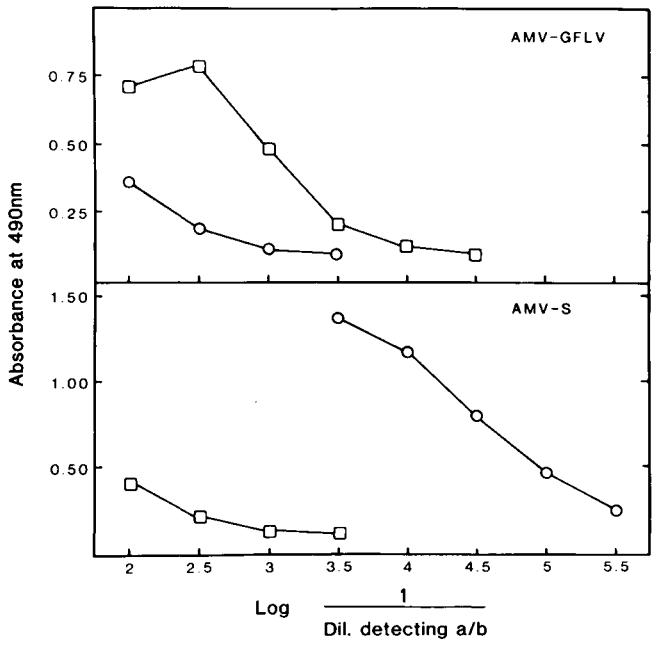

FIG. 3. Effect of concentration of detecting antibody on detection of two isolates of AMV using homologous and heterologous antisera. (□) Antiserum to grapevine fanleaf virus; (○) antiserum to a strawberry isolate of AMV.

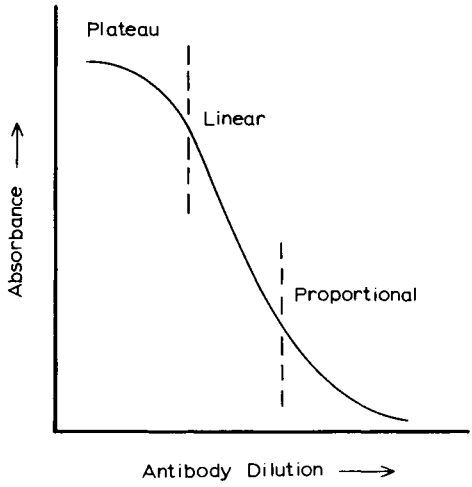

FIG. 4. Representative response curve for the reaction between solid-phase antigen at fixed concentration and dilutions of detecting antibody.

ference by unrelated external influences such as inhibitors present in plant extracts. Regions of linear response may be satisfactory for comparisons of samples exhibiting similar dose–response curves, but in certain cases are subject also to some of the limitations applying to the plateau region.

At East Malling we have found experimental conditions giving responses in the proportional region to be most satisfactory for comparative evaluations of antisera and antigens as well as for determining antigenic relationships using reciprocal homologous and heterologous comparisons (Clark and Barbara, unpublished data). Such conditions are conducive to establishing an interaction equilibrium according to the formula

$$K_a = [AbAg]/([Ab]\ [Ag]) \quad (3)$$

The use of higher concentrations of reagents tends to push the reaction more toward completion, whereas conditions in which antigen is limiting produce assays that reflect the activity of high-avidity antibodies rather than being representative of the total specific antibody population.

D. CONTROLS AND CALIBRATION

Adequate controls are an absolute requirement for every ELISA test carried out. For qualitative tests minimal controls should consist of known positive and negative samples whose purpose is to provide an internal check

of the satisfactory performance of the procedure. Such checks are necessary to guard against operator mistakes and random errors as well as to ensure that any undue variation in binding capacity of plates will be recognized. Samples should always be tested at least in duplicate, as "rogue" wells sometimes occur and occasional spillages can contaminate individual wells. For indexing field populations, it is also important to know the extent to which variation in ELISA values can occur among the negative samples. As with any biological test sample, variability often gives rise to a continuous spectrum of reaction intensity so that discrimination between high negative and weak positive values becomes more subjective. It may be necessary to reintroduce a measure of objectivity into the analysis by measuring the intensity of reaction with a photometer and classifying samples as positive or negative by relating individual ELISA values to the observed range and variation of negative sample values. Samples may then be recorded as infected if the observed ELISA value is greater than that of the mean of the negative values plus 2 or 3 standard deviations, depending on whether it is more important to reject all positive samples or to retain all negative samples.

For quantitative investigations it is necessary to include a set of standards on each plate, usually consisting of a dilution series made from a reference sample. Each plate must be calibrated separately to offset any errors arising from plate variability.

The appropriate dilution range must be determined by prior experimentation, but the factors discussed in Section IX,C should be considered when deciding the precise experimental conditions. It is best to choose a reference sample that can be relied on to behave reproducibly between different experiments so that assays carried out over a protracted time period can be compared and accurate estimates of relative antigen concentrations obtained. Probably the most satisfactory method of preparing such a standard is to freeze-dry a reference sample in aliquots of a size suitable for the reconstitution of one vial per plate or per experiment.

E. AUTOMATION AND COMPUTER ANALYSIS OF RESULTS

The repetitive nature of many of the operations involved in enzyme immunosorbent assay makes some aspects of this technique well suited to automation. Equipment for dispensing reagents, washing plates, and reading and recording results can be obtained from several manufacturers, and its purchase may be justified even when relatively sporadic use is made of the technique. In particular, the current boom in microcomputer technology and the linking of such machines to automatic plate readers makes the acquisition and handling of ELISA data extremely easy. However, few micro-

computer programs suitable for the manipulation and analysis of ELISA data are as yet available commercially.

Nevertheless, a growing number of diagnostic laboratories are performing ELISA in which data are being handled by computer programs specifically written for the purpose by scientists who are also microcomputer enthusiasts. Not only is the recording and analysis of results facilitated by such programs, but the ease with which data can be stored and retrieved for further manipulation has implications in other areas of plant pathology, such as in the construction of mathematical models for disease forecasting and in epidemiological investigations. This newfound ability to obtain vast quantities of data rapidly and to analyze and manipulate the data with inexpensive but powerful and sophisticated laboratory equipment will undoubtedly have far-reaching effects.

X. Conclusions

Serodiagnosis of plant disease has undergone a revolutionary change in the past few years with the introduction of a variety of new and powerful techniques. Enzyme immunoassays, in particular, offer many advantages over the more traditional immunoprecipitation-based procedures. With growing demands being placed on researchers and plant health agencies to provide solutions to practical problems rapidly, the adoption of internationally acceptable methods for the speedy acquisition and interpretation of disease-related data is becoming increasingly important. The widespread acceptance of enzyme immunoassays in plant pathology further extends the opportunity to advance toward this goal.

XI. Appendix: Buffer Formulations

Phosphate buffer, pH 6.8

Na_2HPO_4, 4.45 g
KH_2PO_4, 3.4 g
Distilled water to 1 liter

Check the pH of the final solution.

PBS, pH 7.4

NaCl, 8.0 g
$Na_2HPO_4.2H_2O$, 1.44 g
KH_2PO_4, 0.2 g
KCl, 0.2 g
Distilled water to 1 liter

Check the pH of the final solution. It may be convenient to prepare a stock solution at 10× concentration. *N.B.* The pH of concentrate will be lower than that of working strength solution.

PBS-T

Add 0.5 ml of Tween 20 to 1 liter of PBS.

PBS-TPO

Polyvinylpyrrolidone, M_r 44,000, 20.0 g
Egg albumen powder, technical grade, 2.0 g
PBS-T to 1 liter

A precipitate may form on storage of this solution. This is due to the gradual denaturation of egg albumen at the air-liquid interface.

Coating buffer, pH 9.6

Na_2CO_3, 1.59 g
$NaHCO_3$, 2.93 g
Distilled water to 1 liter

Check the pH of the final solution.

Diethanolamine substrate buffer, pH 9.8

Diethanolamine, 97 ml. *N.B.* Store warm to prevent solidification
Distilled water, 800 ml

Adjust the pH to 9.8 with 1.0 *N* HCl, about 67 ml. Make up to 1 liter with distilled water.

Glycine-HCl buffer, pH 2.7

Glycine, 15.0 g
NaCl, 5.8 g
Distilled water, 900 ml

Adjust the pH to 2.7 with 1.0 *N* HCl. Note that the pH of glycine buffer is temperature dependent and pH adjustment should be made at the appropriate temperature.

N.B. Sodium azide may be added at 0.2 g/liter to these buffers (except glycine-HCl), if required. Note that this compound is highly toxic and that it binds to metals, forming compounds that are explosive when dry.

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4 *Electron Microscopy for the Identification of Plant Viruses in in Vitro Preparations*

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I. Introduction

Electron microscopy of *in vitro* preparations is used on a large scale in diagnosis, screening, and taxonomy of plant viruses, and as a backup to other approaches, such as serology and symptomatology. Nevertheless, there is no recent review of the methods involved, directed at the plant virologist. It must also be said that as microscopes have improved, and are therefore left increasingly in the hands of assistants or of virologists who dabble in microscopy (as opposed to microscopists dabbling in virology), so the electron micrographs of *in vitro* preparations of plant viruses presented at meetings or in journals have tended to become worse, with consequent loss of information or introduction of ambiguity.

Poor use of the microscope does not end with poor micrographs. There may proliferate a dangerous unawareness of levels of impurity or contamination existing in virus preparations. This can lead, for example, to the production and use of Jekyll-and-Hyde antisera that react nicely with their required antigens—and also lead a second life of their own of which the virologist may be unaware.

We must also not underrate the difficulty of keeping a microscope running efficiently and operated by an intelligent, experienced, and sympathetic microscopist. Some laboratories must do without a microscope, and their recourse is to develop links with another institute, perhaps overseas, that can examine samples. The techniques and problems of electron microscopy by airmail are also important.

There are people practicing good electron microscopy on plant virus material who are able to do so in sophisticated and well-founded laboratories. For them, many of the ideas and techniques described here are familiar. From the not too distant viewpoint of the animal virologist, a good review of advanced methods is that of Nermut (1982), and Field (1982) has illustrated the more traditional approach.

For discussions that include surveys of the use of electron microscopy in the identification and characterization of plant viruses, the reader is referred to Hamilton *et al.* (1981), Kurstak (1981), Bock (1982), and Francki *et al.* (1984). For a thorough but wider treatment of electron microscopy, the series "Principles and Techniques of Electron Microscopy. Biological Applications," edited by M. A. Hayat, is recommended, as is the equally competent alternative series, "Practical Methods in Electron Microscopy," edited by A. M. Glauert.

II. The Electron Microscope

Assuming that one is setting up a new facility, the best choice seems to be to buy, if possible, a new medium-resolution (say 5 Å) microscope of

proven quality and reliability, not necessarily adorned with refinements such as a tilting stage, scanning facilities, or darkfield imaging unless these are wanted for particular purposes. If funds for a new microscope are not available, a second-hand instrument, such as a Philips EM 300 or a JEM 100B, corresponding to the best of the penultimate generation, will do very well. The second-hand market is often ignored, but sometimes very good instruments are available for about a third of their original price. Microscopes of a still older generation, such as the once excellent Siemens Elmiskop I or IA are not generally recommended because of increasing difficulties of maintenance and limited availability of spare parts.

The important requirement for all microscopes is a crisp image available on the screen and on the negative. This is not only measured by the manufacturer's specification (in ångstrom units), but achieved through a combination of good theoretical resolution, minimal contamination and beam damage, stability, reliability, ease of on-the-spot maintenance, and availability of prompt servicing by competent engineers.

Excellent discussions on electron microscope operation and on the electron microscope laboratory are found in Meek (1970), Agar *et al.* (1974), and Alderson (1975).

III. Support Films

Literature on making support films is widely available (see, e.g., Kay, 1965; Hayat, 1970; Meek, 1970; Milne, 1972; Baumeister and Hahn, 1978; Nermut, 1982); as neither principle nor practice has changed much recently in regard to routine microscopy, it seems to be unnecessary to describe the various procedures once again.

Suffice it to say that for negative staining it is generally best to use 400-mesh grids (apertures approximately 40 μm) covered either with carbon alone or with a plastic film that is later reinforced with carbon. If a vacuum coating unit is not available, support films made of plastic alone may be used, but they are likely to drift when irradiated and so make photography difficult. There may also be problems in obtaining a good spread of negative stain. I use Formvar films having a gray interference color; before use, a light (just visible) layer of carbon is evaporated onto the Formvar, and the specimen is subsequently placed on the carbon face. Collodion (e.g., Parlodion, pyroxylin, or Necoloidine) films strengthened with carbon are about equally good, and carbon alone, though rather brittle for routine purposes, can give excellent results. For high resolution, very thin support films have advantages. However, in my experience, these become apparent only after other problems such as that of beam damage have been adequately controlled (see Section V, D,2).

Support films made in different laboratories (or in different rooms or at different times of year) may differ in performance not only because of variation in controllable factors, such as type of plastic or quality of carbon. Variables such as back-streamed pump oil, the vacuum attainable, or the humidity may also have notable effects. The best procedure is to try several systems and to settle for the one that works best under local conditions.

Often, best results are obtained by using support films immediately after they have been carboned. Films carboned some time previously usually become hydrophobic, with the result that specimen adhesion and negative staining are less satisfactory. Subjecting the films to a high-voltage glow-discharge (Choppin and Stoeckenius, 1964; Reissig and Orrel, 1970; Nakasone *et al.*, 1978; Nermut, 1982; van Balen, 1982) in air or argon makes the films hydrophilic, apparently by coating them with a negative surface charge. The grids are bathed in the discharge for around 30 sec at a pressure of 0.1 torr, then used immediately.

Positive charges can be deposited on support films by glow-discharge in amylamine vapor (Dubochet *et al.*, 1971), but precautions must be taken because amylamine is very poisonous and also attacks the oil in the rotary pump. Alternatively, positive charges can be obtained on films by (a) subjecting them to glow-discharge in air, then floating them for a few seconds on 1 μg/ml polylysine ($M_r \sim 2000$) (Williams, 1977; Fisher and Williams, 1979); (b) floating for 5 min on 1% Alcian blue 8GX (Nermut, 1982); (c), floating for 15 min on ethidium bromide (30 μg/ml) (Sogo *et al.*, 1979; van Balen, 1982). (Note that ethidium bromide is a carcinogen.) These methods have yet to be compared critically as aids to trapping plant virus particles and nucleic acid molecules, although it seems clear that a layer of positive charges on the film surface is helpful in trapping nucleic acids (Fisher and Williams, 1979).

IV. Calibration of Magnification

A. INITIAL REMARKS

Important taxonomic characteristics, such as the size of virus particles or the pitch of their primary helices (when they are rod shaped), can be measured only using images presented at accurately known magnifications. However, the nominal magnification of the instrument may be in error by 6%, and there also may be a drift in magnification of the order of 4%. A further error of around 1–5% is easily introduced due to variation in specimen height between the pole pieces of the objective lens. In practice this means that significant magnification change is introduced if the specimen has to be substantially refocused.

Because of these problems it is important to run calibration checks when photographing (or otherwise imaging) virus particles for size measurement. Several calibration methods exist; for a comprehensive discussion, see Dunn (1978), and for a simpler treatment, see Milne (1972). There are two basic approaches, using either external or internal size standards. (An internal standard is one that is mixed with the virus preparation, negatively stained with it, and photographed with it, preferably on the same negative.) If used with great care, external standards are admissible, though accurate calibration is always best achieved with internal standards.

B. MATERIALS USED AS STANDARDS

Of the various standards available, three can be recommended as being reasonably accurate and easy to use. Calibration should preferably involve at least two of these systems in combination.

1. *Diffraction Grating Replicas*

The best replica to use, easily obtained from suppliers, is a square grating of 2160 lines/mm. It can be used as an external standard to calibrate magnifications up to ×40,000, but at the top of the range so few lines appear on one negative that it is best to photograph the replica in five or more overlapping places and, after pasting the negatives together in a collage, to measure a continuous span of not fewer than 20 lines. The procedure should be applied twice, at the beginning and at the end of a series of exposures, with the virus in question photographed in between, without changing magnification, without switching off the high tension, and making sure that only minimal changes of focus are necessary.

2. *Beef Liver Catalase*

This crystal lattice, with or without fixation in glutaraldehyde, can be negatively stained and used as an external or internal standard. Wrigley (1968) suggested that the repeat distance used (actually half the true lattice spacing) should be taken as 8.6 nm, though he noted that different workers had in the past produced a disappointingly wide scatter in values for the lattice constant. The value determined by him was 8.75 nm, but that obtained by Hills, cited in Meek (1970), was 8.44 nm. As Wrigley also noted, other crystal habits with different spacings may be present, so some familiarity is required before the correct lattice is recognized with certainty.

3. *Tobacco Mosaic Virus (TMV)*

The particles of the particular isolate used should first be carefully checked against at least one other standard to see where their modal length falls (presumably, near 300 nm). Thereafter, the particles can be used as

an external or, preferably, internal standard (Milne, 1972; Bos, 1975) which may also reveal, on good photographs, the 2.3 nm repeat of the primary helix that can be used as a second standard.

C. COMPARISONS BETWEEN VIRUSES

Often, a useful question to ask is not so much, what is the size of this virus?, but what is the difference in size (if any) between this virus and another (e.g., the type member of the group)? Individual measurements are subject to errors that are hard to estimate, whereas internal comparisons between two viruses are subject to identical systematic errors and are therefore more useful in establishing difference or identity (Francki *et al.*, 1984). A good procedure is to mix the two kinds of particle, adsorb them to the grid, and see whether histograms can detect a bimodal distribution of particle sizes.

V. Negative Staining

A. INITIAL REMARKS

Negative staining of virus particles is simple in principle, but the subject is one where many seemingly unimportant factors can have critical effects and where the skills of the operator may be decisive in producing good results. Perhaps for these reasons there exist different schools of thought on the best approaches to negative staining, and the stains themselves are the subject of strong brand loyalties. There are no recent reviews of negative staining as applied to plant virus diagnosis, but the papers by Hitchborn and Hills (1965), Haschemeyer and Myers (1972), Nermut (1972, 1982), and Horne (1967, 1979) are enlightening.

The perfect universal negative stain has yet to be formulated, so the best policy may be something like the following. First, in any new or unknown situation, use at least two different negative stains—preferably a battery of four or five—and also vary the staining technique (Section V,C). Second, always be aware that any one negative stain used under given conditions brings with it a particular set of artifacts that may include distortion of virus structure, removal or generation of certain components, destruction of the particles, and deemphasis or destruction of certain host components such as ribosomes. The medium in which the virus is suspended may also influence the appearance and dimensions of virus particles; for example, Govier and Woods (1971) found that certain potyviruses were longer (900 nm) and straighter in the presence of Mg^{2+} or Ca^{2+} but became shorter

(800 nm) and more flexible if these ions were sequestered with EDTA. Duncan and Robinson (1981) noted that pea early-browning tobravirus particles had modal lengths of 196 and 82 nm in ammonium molybdate or sodium phosphotungstate, whereas in uranyl acetate the modal lengths were 213 and 94 nm.

One problem that has never been satisfactorily overcome is that, frequently, both upper and lower surfaces of the particle are represented in the negative-stain image, making interpretation of the real "one-sided" structure difficult. Techniques have been described for producing particles of certain viruses that are stained either from above, or below (see Nermut, 1982). These should be tried if structural analysis is required, but a one-sided image is not needed for routine diagnostics.

B. NEGATIVE STAINS

1. *Phosphotungstate (PTA)*

The stain is usually made up as a 2% solution in water of sodium or potassium phosphotungstate adjusted to pH 7 with NaOH or KOH. For a long time, neutral PTA was the chief, or indeed only, stain used by plant virologists; for many workers this is still true, mainly because neutral PTA is generally an excellent stain that is easy to use. It is now clear, however, that neutral PTA damages or destroys the particles of a number of plant viruses, among which are cucumoviruses, some geminiviruses, some ilarviruses, alfalfa mosaic virus, rhabdoviruses, fijiviruses, tomato spotted wilt virus, and some closteroviruses (Francki *et al.*, 1984). If damage or loss of particles due to the PTA is suspected, the preparation can be either mounted in a different negative stain or prefixed in suspension with 1% glutaraldehyde for 15 min at room temperature. Alternatively, the grid bearing the adsorbed virus can be floated on a drop of 0.1% glutaraldehyde for 5 min before staining.

Glutaraldehyde itself damages the structures of some viruses, e.g., fijiviruses. In such cases, bifunctional cross-linking reagents such as dimethyl adipimidate, dimethyl suberimidate, and dithiobis(succinimidyl) propionate should be tried as fixatives (Bancroft and Smith, 1975; Schaffer and Soergel, 1976; Boccardo and Milne, 1981; Carpenter and Pierpoint, 1981).

The reason why neutral PTA is damaging to some viruses is not apparent, but such viruses may possess capsids stabilized predominantly by protein–nucleic acid interactions (Kaper, 1975) and are often also labile in, say, 0.2 *M* NaCl or CsCl. At pH values from 3 to 4.5, PTA no longer causes damage, and the stain at this pH might therefore seem very promising. However, again for reasons not clear, the staining is "muddy" and the level of structural detail revealed is disappointing.

Phosphotungstate at low pH is in fact a useful stain for revealing the presence of suspected virus particles of unknown type; once the particles are detected, other stains or staining techniques may be applied to improve the image detail (Bos and Benetti, 1979). At pH values much above 7, PTA no longer forms a satisfactory negative stain.

2. *Uranyl Acetate (UA)*

The stain is made up as a 2% (or as little as 0.1%) solution in water; the pH is not adjusted and has a value around 4.2. It is best to use a dark bottle as UA is unstable in strong light. Otherwise, the stain may be kept at room temperature for about 2 weeks, after which it is best made up fresh. It should be noted that UA is slightly radioactive, and though extreme precautions seem unnecessary, care should be taken to minimize exposure.

When used properly, UA has advantages over PTA in giving higher contrast and sometimes higher resolution. With the exception of one class of viruses, it does not, in my experience, damage the types of virus that are labile in neutral PTA. Rhabdoviruses, however, are often damaged and may be stripped of their envelope leaving the helical nucleocapsid. UA also has disadvantages that have perhaps prevented it from becoming as popular as it deserves. The first is that the stain precipitates at pH values above 5.5 or in the presence of moderate concentrations of phosphate ions or of plant sap. This means that once the virus preparation is on the grid it should be rinsed carefully with water before the stain is applied. Poor results are obtained if rinsing is not thorough or if the forceps remain contaminated. Having to rinse the grid entails the disadvantage that some virus particles may be washed off, and that what remains may no longer faithfully represent the original sample. [However, with any negative staining method except spray droplet deposition, and perhaps gel filtration (Section V,C), what remains on the grid is in any case unlikely to represent faithfully the population of particulate matter in the original suspension.]

Conversely, rinsing brings advantages in terms of cleaner preparations that are more easily scanned and that contain finer detail. Simple rinsing also makes possible the direct observation of material such as bands from density gradients of sucrose or cesium salts. This avoids lengthy and wasteful (but sometimes useful) alternatives such as dialysis or ultracentrifugation.

Instead of rinsing the grid with water just before staining, it is possible to rinse with neutral 0.01 *M* (even 0.05 *M*) phosphate buffer (see Craig *et al.*, 1980). This may in certain cases limit the flattening of particles that usually occurs and may provide better preservation of fine structure. In other cases, a phosphate rinse makes little difference to the image. Rinses with up to 0.1 *M* $CaCl_2$, $MgCl_2$, or other salts are also feasible and may

alter the image in useful or interesting ways (see, e.g., Maw and Rowe, 1980).

A sometimes severe problem with UA (especially with highly purified and dilute virus preparations) is that the stain may not adhere to the support film if this is not freshly prepared or rendered hydrophilic. When the stain fails to form a background film, virus particles appear positively stained and have about half the diameter seen in negative stain. Glow-discharge of the support films (Section III) usually solves this problem. If difficulties persist, a protein such as bovine serum albumin or, better, bacitracin (Gregory and Pirie, 1973) at a concentration of 0.01 – 0.1% can be added to the virus preparation. The stain is then keyed to the support film although the protein molecules remain completely or nearly invisible. Some workers use bacitracin routinely, but this is not advised unless it appears to be absolutely necessary. Müller (1972) advocated the incorporation of 1% dimethyl sulfoxide into negative stains to promote better spreading, but he did not experiment with UA.

3. *Uranyl Formate (UF)*

This stain, made up and used in the same way as UA, has much the same properties, but, being unstable, it must not only be kept in the dark but be made up fresh each day. Notably with helically structured viruses and with alfalfa mosaic virus, it gives better resolution of structure than UA and is the stain of choice. As with UA, prestain rinses with, e.g., 0.01 *M* phosphate buffer, pH 7, or with 0.1 *M* $CaCl_2$ may be beneficial in supporting or preserving certain virus structures.

4. *Ammonium Molybdate (AM)*

A 2% solution of AM made up in water has a pH around 5.5 and may be adjusted between pH values 4 and 9 with HCl or ammonia. The AM is stable at room temperature. The main defect of the stain is its relatively poor contrast, but it is used quite extensively because it may be mixed directly with virus preparations (like PTA but unlike UA and UF) while causing less damage than PTA in certain cases. In our hands, with plant viruses representing 13 different groups, AM generally gave better results at pH 4 than at pH 5.5, 7, 8, or 9, but in only one case was it superior to both PTA and UA when these were used appropriately. The exception was barley yellow striate mosaic rhabdovirus.

5. *Methylamine Tungstate (MT)*

This stain is usually made up as a 2% solution in water and then has a pH of 6.5 (Oliver, 1973; Fabergé and Oliver, 1974; Hills and Gay, 1976; Dobos *et al.*, 1979; McNulty *et al.*, 1979). In our laboratory, the stain has

generally given disappointing results with "muddy" images of poor contrast, and rather frequent stain precipitation. (If precipitation is encountered, washing of leaves to remove possible external residues of fumigants, inoculation buffers, etc., is advised before the material is homogenized.) Bos and Benetti (1979) have discussed some problems encountered with MT.

The stain cannot therefore be recommended for normal routine use or for high-resolution studies. Nevertheless, it can be mixed directly with the virus sample and, in a number of cases tested (M. J. W. Webb and R. G. Milne, unpublished results) using cucumber mosaic virus in White Burley tobacco, cauliflower mosaic virus in Chinese cabbage, and bean common mosaic virus in *Phaseolus vulgaris,* MT revealed 10–20 times more virus particles than were found after a water rinse followed by UA. This is a very useful increase that could be vital in diagnosis. In other cases, such as alfalfa mosaic virus and tobacco rattle virus in White Burley, bean yellow mosaic virus in *Nicotiana clevelandii,* and tomato bushy stunt virus in *N. glutinosa,* the two types of staining gave about equal numbers of particles. Here, no advantage was gained by using MT.

Methylamine tungstate should be considered as one of the standby stains to be tried with unknown samples.

6. *Sodium Silicotungstate, Sodium Tungstate, Lithium Tungstate*

All these stains are usually made up as 2% solutions in water. Sodium silicotungstate is generally adjusted to pH 7 with sodium hydroxide; the other two stains have unadjusted pH values above 7 and are brought to pH 7 with formic acid. Alternatively, pH values of 4 or 4.5 can be used for all these stains, sometimes with advantage.

While all three stains have their specialist uses, they do not seem to possess overall merits that justify their routine use alongside PTA, UA, UF, AM, and possibly MT.

7. *Other Negative Stains*

Many other heavy metal salts have been used as negative stains (e.g., sodium zirconium glycolate; Vernon *et al.,* 1976), and the possibilities are in fact very wide. But for routine diagnosis the stains recommended above should suffice. It is probably more important to concentrate on a few stains, learning how to get the best from them, than to range widely but superficially.

C. NEGATIVE-STAINING PROCEDURES

1. *Initial Remarks*

It should be noted that, whenever possible, a support film bearing virus particles must not be dried except in the presence of negative stain. This is because virus particles are supported to some extent by the negative stain

during drying. If they are dried in its absence (critical point, and perhaps freeze-drying excepted) only to be rewet and redried, they suffer much more from flattening and distortion.

It is also worth amplifying a point raised in Section V,B,2 about the extent to which the contents of the test tube come to be faithfully represented on the negatively stained grid. Where a highly purified preparation containing one kind of particle is adsorbed to a support film and negatively stained, there is little to go wrong. But where there is more than one kind of virus particle, or a mixture of virus particles plus impurity, selection can occur for two reasons. There is competition for adsorption sites on the support film, and there may also be selective removal of particles (or impurities) during rinsing and staining. Usually, virus particles do not compete well with impurities for adsorption sites, so that particle counts may easily be lower in a dirty preparation full of virus than in a clean preparation containing a considerably smaller virus concentration. Crude preparations of sap containing virus particles sometimes give equal or higher particle counts if they are diluted with water or buffer up to 10 times. Lesemann *et al.* (1980) confirmed that plant sap constituents notably inhibit the adsorption of virus particles to support films. They also showed that when purified preparations of several different tymoviruses, at standard concentrations, were allowed to adsorb to support films, certain viruses, such as belladonna mottle, adhered in greater numbers than other viruses, such as turnip yellow mosaic. Such differences in affinity for the support film are likely to be the rule rather than the exception, both among different viruses in a group, and perhaps also between virus groups. The surface charge on the support film (Section III) may be influential, especially where the virus is suspended in water or a low-molarity buffer. For diagnostic purposes, these considerations may not be important, but they need to be borne in mind.

2. *Mixing and Placing on the Grid*

In the simplest approach, the virus preparation and the stain are mixed in equal volumes and placed on the grid, which is then drained and dried. A variant of the method is to place a small amount of the virus–stain mixture in a spray gun or airbrush run on compressed air. The grid is sprayed so that small drops land on it and spread out, often with the virus particles concentrated round the edge. The method can be quantitative provided the drops are small enough for their entire circumference to be visible. The technique was originally developed by Williams and Backus (1949), with contrast supplied by metal shadowing, and its aim was to determine the absolute numbers of particles in virus preparations, using an admixture of precisely known quantities of polystyrene microspheres (see Milne, 1972).

The advantages of these methods are simplicity and the fact that all types

of nonvolatile material are likely to be represented on the grid. However, there are several drawbacks.

First, the stain must be compatible with the preparation, and so UA and UF would be excluded. Second, the preparation is best first clarified by low-speed centrifugation, and it should contain only the lowest concentrations of salts and sugars. Because of these problems, high-quality images of particles are not to be expected from this technique unless the virus is purified and resuspended in a low molarity buffer. Useful buffers, if higher molarity is desired, are those based on ammonium acetate or carbonate, as they evaporate leaving no residue.

3. *The Brandes Dip Method* (Brandes, 1957; Hitchborn and Hills, 1965)

This early method had considerable success and is still used because it is simple and gives a clean result. A freshly cut leaf or an epidermal strip is drawn through the suface of a drop of water on a glass slide. Part of the contents of the ruptured cells flows into the drop and part forms a film on its surface (see Section V,C,6). A filmed grid is touched to the surface, and then negatively stained; alternatively, the cut leaf is drawn directly through a drop of negative stain on the grid, which is then drained and dried. In the first variant, a sample of the surface film is obtained, whereas in the second variant, draining is likely to remove at least part of this film, and the image may therefore largely represent the contents of the drop.

4. *Adsorption to the Grid Followed by Rinsing and Staining*

In the simplest of this family of techniques, the virus preparation is first placed on the support film. After adsorption, which apparently takes only a matter of seconds, the grid is rinsed with a few drops of stain and is drained and dried. The aim is to produce reasonable grids starting from preparations that would contain too much electron-dense, gummy, or crystallizing matter if prepared by the methods described in Section V,C,2. With this version of the technique, PTA or AM are often employed as stains; UA and UF generally cannot be used, as contact with the plant materials or buffers in the preparation would probably cause stain precipitation.

To avoid precipitation when using uranyl salts, the grid can be rinsed carefully with water or various aqueous solutions (Section V,B,2) before applying the stain. As noted, rinsing carries the advantage that virus particles and their fine structure are generally revealed more clearly. The risk, to be evaluated in each case, is that material of interest may be washed off.

5. *Loading of the Grid Followed by Filtration and Staining*

We have seen that mixing of virus preparations with stain, application of the mixture to the support film, and drying gives a good chance that the

electron microscope image will be representative of the original preparation, but that the image may be poor and the range of possible stains restricted. We have also seen that some kind of rinsing will tend to give superior images and allow the use of UA and UF, but that an element of selection is introduced.

Kellenberger and colleagues (see Kellenberger and Bitterli, 1976) attempted to get the best of both worlds by developing a filtration method that, briefly, goes as follows. A petri dish containing agar is prepared and a thin film of collodion is spread over the agar surface. The virus suspension is then spread over the collodion and left for some time, during which the water and small molecules such as sugars and salts pass through into the agar. The collodion film is then floated off onto a rather high concentration of negative stain (e.g., 10% PTA or 8% UA), collected from below on grids, dried, and examined. With care the results can be good, with the virus and other particles well imaged and quantitatively retained, but it is essential that the support film not be flooded during the negative staining step so that the quantitative nature of the method is lost. The collodion film is not stabilized by carbon, but a carbon layer can presumably be evaporated onto the completed preparations.

Webb (1973) described a simpler method in which the grid, bearing the virus preparation, is dried and then placed virus side up on a filter paper wick through which flows water or an appropriate buffer or negative stain. After elution of the low-molecular-weight substances (about 15 min) the grid is dried and, if necessary, negatively stained using a spray gun. The method effectively removes salts and sugars but may not quantitatively retain all the particulates if there are broken squares in the support film through which the eluting liquid can flood. The need to dry the grid before staining is also a problem.

6. *Spreading Procedures*

Apart from their use with nucleic acid preparations (Section VIII), spreading methods can be useful for examining preparations of infected plants (Milne, 1970, 1972). When a crushed piece of leaf or epidermal strip, 2–4 mm^2, or a few microliters of a crude plant homogenate are touched to a clean water surface (the hypophase), the proteins and lipids naturally present cause spreading and the establishment of a surface monolayer in which virus particles, ribosomes, and other materials are trapped. Sugars, salts, and other interfering substances disperse in the water. The limits of the spread can be marked with talc. Samples of the film are collected by touching a specimen support to the surface, and the grid is then negatively stained as usual.

The hypophase can be of water or buffer, or if a labile virus like tomato spotted wilt is being handled, 1% buffered glutaraldehyde can be used

(Milne, 1970). The degree of spreading can be moderated by adding 0.01–0.001% sodium dodecyl sulfate or other detergent to the hypophase; with purified preparations, spreading may be enhanced by adding a protein such as cytochrome *c* to the preparation to be spread.

The advantages of the method are that very clean preparations result, and the transfer time from the living plant to the stabilized (and if necessary, fixed) monolayer can be very short—a matter of seconds.

D. Observation, Photography, and Particle Measurement

1. *Observation*

It is rare that all squares on a grid are uniformly negatively stained, and it often happens that different areas on one square are more, or less, suitable. Two grids prepared ostensibly in the same manner can also turn out to be different. Thus the first step when examining a grid is to survey a number of squares at low power. Once a suitable area is chosen, a good magnification for observation is ×30,000–45,000. At higher magnifications the beam has to be excessively bright and therefore damaging, whereas at lower magnifications it may be hard to recognize individual virus particles. The binocular is used for close scrutiny.

Some of the newer microscopes have an inferior or poorly positioned pair of binoculars. The older, classical instruments had the best binoculars and viewing arrangements, and the newer models show erosion of binocular specification and viewing geometry. Inferiority of the binoculars can take the form of (a) poorly corrected optical astigmatism; (b) small objective aperture (e.g., 30 instead of 50 mm); and (c) low magnification (×7 or ×8 when it ought to be ×12 or ×15). Large objective apertures and high magnifications allow an image of given brightness and magnification to strike the user's retina, but with reduced beam intensity and lower electron-optical magnification. Poor geometry can take the form of (a) placement of the binocular viewing screen high in the projection chamber, thus reducing magnification, and (b) immovable fixture of the binoculars just where one wants to put one's forehead or nose when scrutinizing the main screen.

2. *Photography and Particle Measurement*

It is much easier to observe virus particles than to photograph them. First, the microscope should be well aligned and accurately corrected for astigmatism. Stigmation is not so easy to achieve because in practice each square on a grid may require its own correction—with further correction necessary if the subject lies near a grid bar. It is good practice, every few minutes when taking photographs, to increase the magnification to, say, ×300,000

and check the symmetry of the grainy structure in the support film, when passing through focus. With practice, this is much more convenient and accurate than checking fringes around holes in a film mounted on a separate grid. Moreover, the image can then be checked for stability and sharpness.

A second rigorous requirement for a good photograph is that it should be taken fast, before beam damage and contamination have degraded the structure too severely (see Isaacson, 1977; Glaeser, 1979). This means that the field just corrected for astigmatism should not be photographed; one close by is chosen, that has not yet been in the focused beam. Beam damage and contamination can also be controlled to some extent by using a liquid nitrogen trap and by always operating the microscope under the best possible vacuum.

Quite good photographs can be obtained by choosing the field under low illumination, brightening the beam for rapid focusing, then quickly defocusing the beam once more for immediate exposure. If the particular structure is unique or uncommon, there is no alternative to this procedure. Where the structure of interest occurs fairly frequently and uniformly over the grid, the technique of minimal exposure (Williams and Fisher, 1970) should be used. Here, the microscope is set up (astigmatism correction, focus, illumination setting, film transport), and a field close by, previously unexamined, is then photographed. On older microscopes, the switch can be achieved by displacement of the projector lens or by using the specimen shifts if these are not subject to creep following a rapid movement. Modern instruments have built-in facilities for beam deflection. The majority of photographs will be substandard for such reasons as poor focus, drift, or poor subject matter, but a few should reveal fine structure not otherwise obtainable. A good test is to be able routinely to obtain convincing pictures of the 2.3-nm primary helix of tobacco mosaic virus.

Wrigley *et al.* (1983), noting that the simple minimal exposure technique is rather hit-or-miss, have proposed a method whereby 80% of the photographs can combine the virtues of selected subject matter, low dose, and accurately chosen focus. The method is sufficiently interesting to warrant description in outline.

1. The object of interest is found at low magnification (×2000) using minimal beam intensity.
2. A shutter above the object plane is actuated to protect the specimen while the microscope is set up for high magnification (the normal camera shutter is below the specimen).
3. A beam deflector system is switched on so that an area to one side of the object can be imaged.
4. The upper shutter is opened, and the image is focused very carefully at ×300,000 to obtain the minimum grain structure (i.e., exact focus).

5. The correct amount of defocus, and also the predetermined amount of focus correction for a magnification jump to, say, ×20,000, are applied. The upper shutter is closed.
6. The magnification is reduced to ×20,000, the condenser is set for a predetermined illumination level, the beam deflectors are switched off, a photographic plate is advanced, the screen is raised, and the camera shutter is opened.
7. The exposure is now made, using the shutter above the specimen.

The heart of the system is the protection of the specimen while it is handled at three very different magnifications: very low, for search and positioning; very high, for accurate focusing; and medium, for the actual exposure. Routinely (as noted later in this section), a single compromise magnification is used for all three functions, and the specimen gets burned.

Further refinements, such as the use of very high vacuum, extremely thin support films, or cold stages, may assist in attaining high-resolution results, but these fall outside the scope of our discussion.

Routinely, the best compromise magnification for photographing virus particles is generally the same as that used for observation. Below ×30,000, it may be hard to focus accurately, whereas above ×45,000, beam damage is likely to be too severe, and the required further enlargement is in any case much better obtained in the photographic enlarger than in the microscope. The best magnification for prints intended for publication and showing diagnostic detail is ×200,000–300,000. Much smaller magnifications, though often used, must fail to reveal fine detail of diagnostic value possibly present in the negative, whereas ×300,000 is about the highest magnification that can usefully present the detail at around 2.0 nm present in the best negatives of a routine nature. For elongated viruses, it is clear that at ×300,000 only a segment of the particle image can be presented for publication. A field at lower magnification can be used, with the highly magnified segment inset in one corner.

For measuring the sizes of isometric viruses, a magnification of ×40,000 is convenient, but for elongated viruses, a magnification of around ×20,000 may be better. In both cases, at least 100 particle images should be measured for each preparation considered. For elongated viruses, there may be more than one modal length, and breakage or aggregation may create a severe "noise" background. Chiko (1975) noted, for example, that for resolution of the length classes of hordeiviruses, the class interval chosen had to be not greater than 7 nm (preferably 2.5 or 3.5 nm), with a sample size of 600 or more particles.

Crude extracts rather than purified preparations should be used for size measurements, as there is then less chance that the particles will be broken or aggregated, or that certain sizes will have been artificially selected. Where

particle numbers are very few, it may be legitimate to use immunosorbent electron microscopy (ISEM; see Section VII), which traps increased numbers of particles on the grid, probably without great distortion of a modal length. However, Pares and Whitecross (1982a) have noted that, with tobacco mosaic virus, ISEM traps a greater proportion of small sizes than is obtained by direct adsorption to untreated support films.

One further problem with the measurement of "isometric" viruses arises if the particles are distinctly angular. Here it may be best to quote both the diameter corner-to-corner and the diameter edge-to-edge on particles resting on their 3-fold axes and exhibiting a regular hexagonal silhouette.

E. OPTICAL DIFFRACTOMETRY

This is a very useful technique for measuring accurately the distances between repeating structures, such as the turns in the primary helix of a rod-shaped virus particle. Determination of the pitch in this way is more accurate than simple counting and measuring from the negative, a projection, or a print, and very often the pitch can be determined by optical diffraction in cases where this is not possible by visual inspection. For details, the reader is referred to the treatments of Beeston *et al.* (1972), Johansen (1975), and Horne (1979).

F. IDENTIFICATION OF VIRUSES WITH THE AID OF NEGATIVE-STAIN IMAGES

Individual viruses of course cannot usually be identified from simple negative-stain images, but often one can get some idea of the group to which the virus belongs. Together with other data such as those on host range or type of vector, the field of possibilities can often be narrowed enough to begin making specific serological tests. For good photographs of virus particles on which to base diagnosis, the atlases of Williams and Fisher (1974), Maramorosch (1977), and Francki *et al.* (1984) should be referred to, together with the handbook edited by Kurstak (1981) and the series of CMI/AAB Descriptions of Plant Viruses edited by Harrison and Murant.

1. *Rod-Shaped Viruses*

The classification of rod-shaped viruses according, in part, to their modal length, is well established (Matthews, 1982). Other morphological markers are diameter, rigidity, visibility of the axial canal, visibility and pitch of the primary helix, and visibility of higher-order helices or longitudinal files of subunits. Below are given short descriptions highlighting the morphological differences between some of the rod-shaped viruses.

a. Tobamoviruses. These are highly characteristic rigid rods about 300 nm long and 18 nm wide. The axial canal is visible. The primary helix is visible, pitch 2.3 nm, but easily degraded in the electron beam.

b. Viruses Such as Beet Necrotic Yellow Vein, Soilborne Wheat Mosaic, Potato Mop Top, and Peanut Clump (see Tamada, 1975; Brakke, 1971; Wiese, 1977; Harrison, 1974; Thouvenal and Fauquet, 1981). Soil-borne viruses of uncertain affinity (perhaps near tobamoviruses) having rigid rods of two or three modal lengths between 390 and 65 nm, and about 20 nm wide. The axial canal is visible. The primary helix is usually not measured and is not seen in the generally poor photographs available. In potato mop top the helix is visible, with a pitch of 2.4–2.5 nm. The pitch of peanut clump virus is probably close to 2.6 nm (J. C. Thouvenal, personal communication). The primary helix, at least of soilborne wheat mosaic virus, remains visible in the electron microscope long after that of tobacco mosaic virus has disappeared. The pitch of the helix is near 26.5 nm in uranyl formate (Milne, unpublished).

c. Tobraviruses. These are rigid rods of at least two modal lengths, 180–215 nm and 46–114 nm, and are about 22 nm wide. The axial canal is visible. The primary helix is easily seen and rather stable in the electron beam; pitch 2.5 nm.

d. Hordeiviruses. These are rigid rods about 20 nm wide. The axial canal is visible. The primary helix is prominent and stable; pitch about 2.5 nm. Two-, three-, or four-particle-length classes (depending on the strain) are found in the range 150–100 nm.

e. Potexviruses. These are slightly flexuous rods of one modal length, 470–580 nm, and about 13 nm wide. The axial canal usually is not visible. The primary helix is visible; pitch about 3.4 nm. Four longitudinal files of subunits also visible. The transverse and longitudinal patterns combine to give the characteristic appearance of a square lattice.

f. Carlaviruses. These are slightly flexuous rods of one modal length, 620–700 nm long, and about 13 nm wide. The axial canal is usually not visible. The primary helix is generally not visible, but about four longitudinal files of subunits are clearly seen on good images.

g. Potyviruses. These are flexuous rods of one modal length, 680–900 nm long, and about 12 nm wide. The axial canal is not visible. Subunits and pitch generally are not visible except on the very best images; even here, the subunits have very low contrast. Longitudinal files of subunits are not seen.

h. Barley Yellow Mosaic Virus (Inouye and Saito, 1975). This is a currently ungrouped virus with flexuous rods 13 nm wide and of two modal lengths, 550–600 nm and 250–300 nm. No good photographs are available, but this virus produces "pinwheels" in infected cells, and in structure the particles may resemble those of potyviruses.

i. Closteroviruses. These are very flexuous rods of one modal length, from 600 to 2000 nm long, according to the virus, and 10–12 nm wide. The axial canal is not visible. The primary helix is very prominent and "loose-jointed," stable in the electron beam; pitch about 3.7 nm.

j. Potato Virus T, Apple Stem Grooving Virus, and Lilac Chlorotic Leafspot Virus (Salazar and Harrison, 1978; Lister, 1970; Brunt, 1979). These are ungrouped viruses with flexuous rods of one modal length, 620–640 nm long (potato T, apple stem grooving) or 1540 nm (lilac chlorotic leafspot), and 12.5–13 nm wide. The axial canal is not visible. The primary helix is evident; the pitch is 3.4 nm, except for lilac chlorotic leafspot, pitch 3.7 nm. Lilac chlorotic leafspot virus has been grouped with the closteroviruses (Brunt, 1979), but its fine structure differs from theirs considerably and seems nearer to that of potato T and apple stem grooving viruses (Conti *et al.*, 1980; Lisa, 1980).

2. *Small Isometric Viruses*

The enormous number and variety of small isometric viruses makes identification to group level, on morphological grounds alone, impractical. Different methods of specimen preparation may also alter the appearance and apparent diameter of the particles. Nevertheless, certain characters are useful. These are diameter, particle outline, visibility of morphological subunits, the presence or absence of penetrated particles, and lability in neutral PTA. A few examples are given below.

a. Luteoviruses. These are somewhat angular particles 25 nm in diameter, with a very smooth sharp outline. No substructure is visible; no penetrated particles. They are resistant to neutral PTA.

b. Cucumoviruses. Particles are 29 nm in diameter with a circular outline. The substructure is granular, often with a central dimple. There are no penetrated particles. These viruses are labile in neutral PTA.

c. Tymoviruses. Particles are 30 nm in diameter, with near-circular outline. Prominent subunits are grouped in fives and sixes. Both penetrated and unpenetrated particles are present. The viruses are resistant to neutral PTA.

a. Comoviruses. These are particles with a strongly angular outline, about 28 nm in side-to-side diameter, 32 nm corner-to-corner. Usually no substructure is visible except on good photographs. Penetrated and unpenetrated particles are present. The viruses are resistant to neutral PTA.

e. Tombusviruses. These are particles with a circular but "knobbly" outline, 30–31 nm in diameter. Subunits are visible around the edge and across the face of the particle. Penetrated particles occasionally are seen. The viruses are resistant to neutral PTA.

f. Ilarviruses. Particles are oval or irregularly circular in outline, 26–35 nm in diameter, with distinct size classes sometimes detectable. The sub-

structure is granular. Particles are often penetrated. They are labile in neutral PTA.

3. *Viruses with Particles of Other Morphologies*

The following viruses or virus groups are easily identified by their morphology in negative stain.

a. Alfalfa Mosaic Virus. This comprises a series of particles, 18 nm in diameter, and from 18 to 58 nm long, with rounded ends. There are affinities with ilarviruses. The fine structure is distinct. It is labile in neutral PTA.

b. Geminiviruses. The particles are geminate (i.e., twinned), each unit being 18–20 nm in diameter, with a pentagonal outline. Some single units also seen. Some members, e.g., maize streak virus, are labile in neutral PTA.

c. Caulimoviruses. These are isometric particles about 50 nm in diameter, often with a central electron-dense spot. They are stable in neutral PTA.

d. Fijiviruses. These are double-shelled isometric particles 65–70 nm in diameter with rarely seen external knobs (A spikes) and distinct subunits. Particles frequently degrade to subviral particles 50 nm in diameter with prominent B spikes, three, five, or six of which are usually seen in profile. The outer shell and all spikes are labile in neutral PTA, leaving a smooth 50-nm isometric particle, sometimes penetrated.

e. Phytoreoviruses. These are double-shelled isometric particles 70 nm in diameter without spikes but with prominent subunits. There are no subviral particles. The viruses are resistant to neutral PTA.

f. Cacao Swollen Shoot and Rice Tungro B Viruses. These are bacilliform particles 23–35 nm in diameter and 110–350 nm long, with parallel sides and rounded ends but not resembling alfalfa mosaic virus or rhabdoviruses. No substructure is visible. The viruses are resistant to neutral PTA. Cacao swollen shoot virus (Brunt, 1970) has particles 28 nm in diameter and 120–130 nm long. Two kinds of particles are associated with rice tungro disease (Gálvez, 1971; Hibino *et al.*, 1978): a small isometric (I) particle and a bacilliform (B) particle. The B particles are of various lengths (110–350 nm); they have a diameter of 35 nm in neutral PTA (Hibino *et al.*, 1978) and 23 nm in UA (Milne *et al.*, 1981).

g. Rhabdoviruses. These are bacilliform or bullet-shaped particles 60–100 nm in diameter and up to 350 nm long. The morphology is highly subject to variation according to method of preparation (see Francki, 1973). In neutral PTA, particles are generally broken into short segments or damaged at one end; PTA at pH 4 preserves the particles, but without good fine structure. UA often strips off the lipoprotein envelope, leaving the helical nucleocapsid, which may unravel into threads or spirals.

h. Tomato Spotted Wilt Virus. This has approximately spherical enveloped particles about 80 nm in diameter, best seen by crushing host material and immediately spreading it on a hypophase of glutaraldehyde (see Section V,C,5). Particles are often observed in groups, sometimes within a common enveloping membrane. The virus is labile in neutral PTA. The particles can be confused with host materials, so diagnosis is best confirmed by thin sectioning.

i. Maize Stripe and Rice Stripe Viruses (Gingery *et al.*, 1981; Gordon *et al.*, 1981; Kogunezawa *et al.*, 1975; Toriyama, 1982). Kinked or helical filaments are of uncertain length and about 3 nm in diameter. Pairs of filaments may be associated to form thicker pseudobranched rodlike structures. The viruses are resistant to neutral PTA.

VI. Metal Shadowing

A. FOR VIRUS PARTICLES

As a diagnostic or taxonomic aid in determining the dimensions, shapes, or fine structures of virus particles, shadowing has so generally been displaced by negative staining that it will not be discussed in detail here. That is not to say that shadowing does not play an important part in certain investigations of fine structure (see, e.g., Hatta and Francki, 1977; Nermut, 1975, 1982). For general treatments of shadowing technique, see Henderson and Griffiths (1972) and Martin and Rowe (1979); for more specialized applications, see Nermut (1982). The points noted in the next section also largely apply to the metal shadowing of virus particles.

B. FOR NUCLEIC ACID MOLECULES

Here, the problem is to give adequate contrast to slender molecules that may be lying in any orientation in the plane of the support film. Metal is evaporated from a source about 6° above the plane of the support film and 10–15 cm distant (see, e.g., Evenson, 1977; Inman and Schnös, 1974). Alternatively, grids secured from above may be shadowed from below. During shadowing, a turntable on which the specimens lie is rotated by a small motor inside the chamber or may be driven from outside with a magnetic drive or via a spindle passing through a gasket.

For evaporation, it is common to use a tungsten filament 0.5 mm in diameter, bent when hot into a V shape and having 3 cm of Pt–Pd (80:20 alloy) wire of 0.2 mm diameter wound round the tip. The filament is clamped between the high-current terminals; after a good vacuum has been reached, the filament is gently heated to outgas it. The current is then shut

off while a good vacuum is reestablished, and is then slowly increased until the charge melts. Increasing the current a little further evaporates the charge over a period of about a minute and finally breaks the filament.

During evaporation, it is important for good resolution to operate under the best possible vacuum, better than 10^{-5} torr if possible, but in any case better than 10^{-4} torr.

VII. Immunoelectron Microscopy

A. Initial Remarks

As we have seen, simple negative staining is an indispensable diagnostic tool, but it does suffer from two major weaknesses. First, there are viruses that look similar but may be taxonomically (and pathogenically) distinct. Second, rather many (at least 10^8/ml) particles have to be present before even one is seen in the microscope, so that very often particles may be present but go undetected. Immunoelectron microscopy offers techniques that can greatly increase the specificity and sensitivity of diagnostic electron microscopy, provided that suitable antisera are available.

For reviews and general remarks on immunoelectron microscopy, the reader is referred to Almeida and Waterson (1969), Brown and Smale (1970), Milne and Luisoni (1975, 1977), Milne and Lesemann (1978), Roberts and Harrison (1979), Roberts *et al.*, (1982), Torrance and Jones (1981), and van Regenmortel (1981, 1982).

B. Immunosorbent Electron Microscopy (ISEM)

The rationale behind this technique is that antibodies are adsorbed to the support film and then used to trap any antigenically closely related virus in the suspension with which the support film is incubated (Derrick, 1973). The method is used to detect viruses present at concentrations too low to be found by ordinary negative staining. It has also been used to compare serological relationships between viruses and virus strains. The procedure is discussed in this series (Milne and Lesemann, 1984) and will not be further treated here.

C. Decoration

This term is used both for a result and a method (Roberts *et al.*, 1982). The result is the specific coating of virus particles with a layer of antibody molecules; the best method involves the adsorption and immobilization of

the virus on the support film, followed by incubation with antiserum, rinsing, and negative staining so that the antibody is directly detectable around the particles (Ball and Brakke, 1968; Yanagida and Ahmad-Zadeh, 1970; Luisoni *et al.*, 1975; Milne and Luisoni, 1975, 1977). Sometimes, for reasons not yet clear, the virus particles do not remain immobile on the grid but migrate into clumps (Almeida *et al.*, 1980).

The decoration method is very simple and, unlike ISEM, there seem to be only the following few important variables to consider.

1. Adsorption of the virus to the support film follows the normal practice used for negative staining and, as noted by Milne and Lesemann (1984), can also be boosted using ISEM.
2. After virus adsorption, the grid is rinsed with buffer (generally 0.1 *M* phosphate, pH 7) and is then incubated with the antiserum. This is used at a dilution about 10 times less than the gel-diffusion (or slide precipitation) titer (e.g., with a titer of 1/1024, a dilution of 1/100 would be suitable). Either a small drop (1–2 μl) of serum is incubated on the grid, or the grid is floated on the antiserum.
3. An incubation time of 15 min at room temperature is quite long enough for antibody attachment, and 1 min is enough in many cases.
4. After incubation with antiserum, the grid is rinsed with water and stained as for normal negative staining.

The principal uses of decoration are (a) establishment of the identity of a virus; (b) measurement of antiserum titers; (c) estimation of the degree of relationship between viruses; (d) localization of particular antigens on the virus particle; and (e) increase of the size and contrast of virus particles as an aid to identification.

1. *Establishment of Virus Identity*

Generally, the decoration test will give a simple positive or negative result. Occasionally, a mixed population of particles is unexpectedly revealed (see, e.g., Milne *et al.*, 1980, or Lisa *et al.*, 1981). The best demonstration of a specific positive or negative reaction is made using an artificial mixture of the unknown virus and a morphologically similar one whose serological status is well established (see Milne *et al.*, 1979; van Regenmortel, 1982).

When particles of a single kind of virus are decorated with the homologous (or a related) antiserum, all particles should, in principle, react positively and in a uniform manner. It may happen, however, especially where there are numerous virus particles on the grid, that a few (less than 1%) may escape decoration, and remain "clean." This is almost certainly because these particles, during incubation with the antiserum, are somehow protected (behind a grid bar, in a fold of the support film) but are displaced

onto the film surface during rinsing and negative staining. When such "clean" particles are more numerous or more consistently found, the presence of more than one virus should be suspected.

2. *Measurement of Antiserum Titers*

Grids bearing adsorbed virus are incubated under standard conditions with a 2-fold dilution series of the antiserum. The highest dilution is determined that gives a consistent positive decoration.

This is a useful method because the total operation takes no more than about 40 min including reading the grids. This last is easy because it involves no counting. Moreover, only very small amounts of virus (in both volume and concentration) suffice for the determination. Experience in our laboratory suggests that the decoration titer of a given serum is about one step higher than the gel-diffusion or slide-precipitation titer, and correlates well with titers established by these methods.

The sensitivity of the system depends on the visibility of the antibody halo, and it is clear that sensitivity might be increased by labeling the antibody in some reliable way that did not damage the antibody. Ferritin is an obvious possibility, but in practice it is difficult to obtain consistent and specific results without loss of antibody avidity. A promising alternative is to label the antibody with gold particles (see Frens, 1973; Geoghegan and Ackerman, 1977; Horisberger and Rosset, 1977; Bendayan *et al.*, 1980; Müller and Baigent, 1980; Craig and Millerd, 1981; Ochs and Stearns, 1981; Giunchedi and Langenberg, 1982; Pares and Whitecross, 1982b), though, at least with plant virus preparations, the results do not yet appear to combine high specificity and good resolution.

A further choice is to perform a first decoration step with, say, rabbit antivirus serum, and follow this, in what has been termed double decoration, with a second step using, say, goat anti-rabbit serum (Kerlan *et al.*, 1981, 1982). This increases the detectability of the original antibody and is said to result in an increase in sensitivity of several 2-fold steps, without a corresponding increase in background antibody levels.

3. *Estimation of Degree of Relationship*

A crude first approach is to compare the density of the antibody coat around the homologous and heterologous virus particles, but it is better to titrate the serum against each virus isolate and find the number of 2-fold steps by which the titers differ (van Regenmortel, 1975; Koenig, 1976). To obtain comparable conditions, it is best to purify the viruses in question, mix them to obtain about equal amounts on the grid, and titrate the antiserum against the mixture, noting the decoration end point (titer) for each component.

When a distant relationship is suspected, the decoration test may be performed using undiluted antiserum, but difficulties can arise due to partial disruption or digestion of the virus particles, or formation of nonspecific haloes around them. A first solution to this problem is to heat the sera to 60°C for 10 min, or to use only sera that have been stored liquid at 4°C for several months. Alternatively, the IgG fraction can be purified using ammonium sulfate fractionation or a resin column such as DEAE-Affi-Gel blue (Bio-Rad Laboratories) that is claimed to give, in one step, an IgG fraction free from proteolytic activity.

4. *Localization of Antigens*

[See Brown and Smale (1970), Yanagida and Ahmad-Zadeh (1970), Tosi and Anderson (1973), Luisoni *et al.* (1975), Milne and Luisoni (1977), Otsuki *et al.* (1977), Otsuki and Takebe (1978), and Fukuda *et al.* (1980).] Immobilization of well-separated virus particles on the grid, followed by incubation with antiserum, usually preabsorbed with certain of the viral antigens, offers a direct method of visualizing the sites of the remaining antigens on virus particles having a complex antigenic structure. Localization of the initiation site of tobacco mosaic virus RNA encapsidation by coat protein (Otsuki *et al.*, 1977; Fukuda, *et al.*, 1980) offers a nice example of the method, though it should be said that the microscopy in these papers is at a technically poorer level than the rest of the work.

5. *Increase in Size and Contrast*

An isometric particle, for example, may have a diameter of 30 nm; after decoration the particle's diameter will be increased to about 50 nm. During negative staining, the antibody layer acts like a sponge, so that the level of contrast against the background is greatly increased. These effects combine to render a reacted particle highly visible on the grid.

D. CLUMPING

In this essentially simple technique, the virus preparation and the antiserum are mixed and incubated together, after which the material is collected on grids (perhaps after concentration by centrifugation), negatively stained, and observed for the presence of clumped rather than separated virus particles. Clumping occurs because of complexes formed between the virus particles and the bridging antibody (see Almeida and Waterson, 1969; Milne and Lesemann, 1978).

Although clumping is a classical immunoelectron microscopic method, it has been largely superseded by the techniques (separated or combined) of ISEM and decoration. It must also be said that clumping is unreliable (a)

if the virus preparation contains virus aggregates to start with or develops them nonspecifically; (b) if there is great antibody excess, which tends to inhibit clumping but produce strong decoration; or (c) if there is very little virus present, making virion-to-virion encounters too rare to produce detectable clumps. Because of the possibility of nonspecific aggregation, it may be better, with this technique, to rely more on the detection of the antibody bridges between the particles than on the clumping itself (Brown and Smale, 1970); the technique is then interpreted as a kind of decoration.

E. Mailing Grids between Laboratories

Any immunoelectron microscopic process is best carried through as a whole in one laboratory, from preparation to observation. However, negatively stained grids are stable for long periods if kept dry, and it is therefore possible to send them through the post in, say, small gelatin capsules surrounded by silica gel in a sealed polyethylene envelope.

Derrick and Brlansky (1976), Milne and Luisoni (1977), and Paliwal (1977) have noted that, in the ISEM procedure, grids can also be dried and stored after the antiserum coating step, and can be used up to 6 weeks later for trapping appropriate virus particles. The best method is probably to rinse the already coated grids with water before drying them, store them desiccated, and rewet them with buffer before they are used for trapping. It is clear that in this procedure the grids might, for example, be prepared and coated at point A, mailed, incubated with virus at point B, negatively stained, and returned to A for examination.

Another possibility (Dr. G. I. Mink, personal communication) is that grids bearing virus particles (routinely negatively stained in UA, dried, and stored) can be rewetted with water and then buffer, and decorated with antiserum. The grids are then restained in UA. Even grids that have been examined in the electron microscope can be decorated subsequently, but only on those grid squares that have not been directly in the electron beam.

Where quarantine problems might arise through mailing grids bearing virus particles from one country to another, the particles should be fixed, while on the grid, with glutaraldehyde (0.1% in 0.1 *M* phosphate buffer, pH 7, for 15 min at room temperature) before mailing.

VIII. Imaging of Nucleic Acids

A. Initial Remarks

This subject can here be discussed only in outline. The reader is referred to reviews by Evenson (1977) and Fisher and Williams (1979). A general background on plant virus nucleic acids is given by Hall and Davies (1979),

particularly the chapters in Volume II by Hull, Zaitlin, Lane, and Dickson. Bozarth (1977), Murant *et al.* (1981), Haugli *et al.* (1982), and Junghans *et al.* (1982) provide good examples of work on characterizing viral nucleic acids by electron microscopy.

For the purposes of diagnosis and taxonomy, the main questions concerning nucleic acids posed to the microscopist are the following: Is the nucleic acid DNA or RNA? Is it single stranded (ss) or double stranded (ds) or partially base paired? Are the molecules linear or do they form closed loops? What is the distribution of length classes? An important supplementary question might be, what is the state of contamination or degradation of the sample?

Various preparative approaches are available and can be divided into protein monolayer techniques and nonprotein techniques. The protein monolayer techniques, originated by Kleinschmidt and colleagues, are generally the more suitable for our purposes. The nucleic acid preparation (at, say, 2–5 μg/ml in 0.5 *M* ammonium acetate, pH 8) is mixed with cytochrome *c* (0.01%) and run down a ramp on to the cleaned surface of a liquid (the hypophase) such as 0.15 *M* ammonium acetate. The mixture spreads on the surface to produce a film in which the nucleic acid molecules are trapped, and the cytochrome *c* also binds to the nucleic acid, increasing its thickness and visibility. Grids bearing support films are touched to the surface, withdrawn, positively stained in dilute uranyl acetate in ethanol or acetone, rinsed in the solvent, dried, and rotary shadowed (Section VI).

It should be noted that nucleic acids, particularly single-stranded ones, are susceptible to contaminating nucleases derived from sources such as bacteria, fingerprints, poorly cleaned glassware, or host plant materials. All solutions and glassware should therefore be sterile, and steps (such as thorough purification of the virus, use of bentonite or diethyl pyrocarbonate) should be taken to remove or inhibit nucleases—particularly ribonucleases if ssRNAs are being examined (see Fraenkel-Conrat *et al.*, 1961; Brakke, 1967; Solymosy *et al.*, 1968; Evenson, 1977; Hall and Davies, 1979).

B. DISTINGUISHING BETWEEN DNA AND RNA

There is no direct way to distinguish DNA from RNA in the electron microscope. The simplest approach, therefore, is to treat the preparation, under the appropriate conditions and with proper controls, with DNase and RNase (see Evenson, 1977) and observe whether the molecules remain intact.

For dsRNA, antisera can be prepared, and so ISEM (Section VII,B) can be applied to trap increased numbers of molecules (Derrick, 1978; French *et al.*, 1982). This test can form the basis for distinguishing between dsRNA, which should be trapped, and other kinds of nucleic acid, which should

not. The antiserum-coated grid is incubated with the nucleic acid, rinsed with buffer, floated on cytochrome *c* (to thicken the molecules), then rinsed, stained, and shadowed in a routine manner. Unfortunately, it appears that decoration (Section VII,C) cannot be applied to the identification of dsRNA because serum proteins other than the specific antibodies adsorb to the nucleic acid (Luisoni *et al.*, 1975).

C. Distinguishing between Single-Stranded and Double-Stranded Molecules

The difference in thickness or contrast between these two types of structure is not a reliable guide to strandedness, especially after coating with a layer of cytochrome *c*. Therefore, again, various selective treatments must be applied before the preparations are examined in the microscope. Some examples are outlined below.

Pancreatic DNase digests both dsDNA and ssDNA, whereas nuclease S1 from *Aspergillus oryzae* digests only single-stranded molecules. (It also digests ssRNA.) However, the dsDNAs of caulimoviruses contain single-stranded breaks that allow S1 to cut the molecules so that three or four smaller but resistant pieces result from each originally intact genome (see Hull, 1979). The ssDNAs of geminiviruses are completely digested. Pancreatic RNase in low-molarity salt solutions digests both ssRNA and dsRNA, whereas in high-molarity salt the enzyme digests ssRNA but not dsRNA (see Hatta and Francki, 1978).

When a mixture of ssRNAs and dsRNAs is mixed with cellulose powder in the presence of 15% ethanol, the dsRNAs are selectively bound, and can later be recovered by washing the cellulose with ethanol-free buffer (Morris and Dodds, 1979). A different procedure (see Diaz-Ruiz and Kaper, 1977; Luisoni *et al.*, 1979) uses 2 *M* LiCl to precipitate ssRNAs while dsRNAs remain in solution.

A third approach is to compare the behavior of the preparations when spread under aqueous nondenaturing conditions (such as those indicated in Section VIII,A) or under denaturing conditions involving the use of formamide, urea, and possibly also formaldehyde (see Evenson, 1977). Denaturing, in this context, indicates the breakage of noncovalent bonds, and it is useful to consider two kinds of treatment. Partial denaturation retains entirely base-paired structures intact but opens out and extends poorly base-paired or entirely single-stranded regions. Complete denaturation separates both double-stranded and single-stranded structures into extended single strands. Nondenaturing conditions or partial denaturation are therefore best for distinguishing between double- and single-stranded structures.

If a molecule is partly double-stranded but contains non-base-paired regions, these, under nondenaturing conditions, will appear as "puddles,"

and under partially denaturing conditions will be opened out as single-stranded loops along the still predominantly double-stranded molecule. This kind of image forms the basis of heteroduplex analysis where, in its simplest form, dsDNA of one virus is denatured in the presence of one of the strands of another virus. The mixture is then renatured and spread. Nonhomologous unpaired regions then declare themselves; precise maps can be made, and the degree of homology can be estimated (see, e.g., Chattoraj and Inman, 1972, 1973). The method is also applicable to RNA viruses if these have dsRNA or if DNA complementary to the viral ssRNA can be made (see Taylor *et al.,* 1976; Gould *et al.,* 1981).

D. LINEAR MOLECULES OR CLOSED LOOPS?

This is a straightforward determination, but it must be made on denatured or partially denatured preparations in the case of ss molecules because, when not denatured, the closed loops collapse and become at least partially base-paired. Good examples of this condition are viroids (see, e.g., Randles and Hatta, 1979; Palukaitis *et al.,* 1979), where the linear structures seen under nondenaturing conditions open out into circles when denatured.

E. LENGTH MEASUREMENT AND MOLECULAR WEIGHT ESTIMATION

A rough estimate of molecular weight is easy to obtain from measuring the contour lengths of nucleic acid molecules as photographed in the microscope. However, accurate estimation is less simple for the following reasons: (a) the microscope requires accurate calibration; (b) molecular lengths must be carefully measured and histograms produced; (c) nucleic acids of known molecular weight must be used as standards; and (d) allowance must be made for the fact that different procedures (ionic strength of hypophase, pH, addition of cytochrome *c,* presence of denaturants, and so on) induce shortening or lengthening of the molecules. These problems are discussed by Evenson (1977) and Fisher and Williams (1979). Evenson suggests that as a general rule of thumb, the molecular weight per unit length is very approximately 2×10^6 per micrometer for double-stranded molecules and 1.2×10^6 for single-stranded ones. An interesting comparison of ssRNA molecular weights obtained in various ways has been made by Murant *et al.* (1981).

F. VIROIDS

Native viroid molecules are ssRNAs of around M_r 125,000 that are extensively base-paired to form a dumbbell shape. This is the form seen as a

short rod about 50 nm in length under nondenaturing conditions. When the molecules are denatured, they open out into circles about 110 nm in circumference, with generally a proportion of linear molecules of about the same length. Electron microscopy has been used extensively to investigate viroids; examples in the literature appear in the papers of Sogo *et al.* (1973), Sänger *et al.* (1976), McClements and Kaesberg (1977), Riesner *et al.* (1979), Randles and Hatta (1979), Palukaitis *et al.* (1979), Haseloff and Symons (1981), and French *et al.* (1982); the reviews by Diener and Hadidi (1977), Dickson (1979), and Diener (1981); and the book by Diener (1979).

The viroid concept has been complicated by the finding of viroidlike RNAs encapsidated within the particles of certain small isometric plant viruses (Randles *et al.*, 1981; Gould, 1981; Gould and Hatta, 1981; Francki *et al.*, 1983). Electron microscopy has also played a significant part in these investigations.

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5 *Cloning and Expression of Viral Antigens in Escherichia coli and Other Microorganisms*

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I. Introduction

In the last several decades considerable advances have been made in the prevention of viral disease by vaccination. In many cases the material of choice for a vaccine is a killed or otherwise attenuated form of the virus itself. This approach, although effective, has several disadvantages. First and foremost, the risk that pathogenic variants will escape the inactivation or attenuation processes is of considerable concern. Second, some viruses are impossible to grow under commonly used culture conditions, and in such cases no vaccine of import exists.

The techniques of genetic engineering are of particular utility for the production of viral antigens and, it is hoped, viral vaccines. In brief, these techniques permit the production of proteins derived from one organism in a second, often unrelated, organism. Thus it is now possible to produce antigens from several pathogenic viruses in organisms such as *Escherichia coli, Bacillus subtilis,* and the yeast *Saccharomyces cerevisiae.* Such an approach eliminates all risk, and even potential risk, of reversion of the virus to its virulent form. Further, innocuous fragments of DNA from viruses that it is impossible to propagate under laboratory conditions can be transferred from primary biological material to a microbial vector and subsequently propagated in the host organism of choice.

In this review we describe the general techniques used for isolating, propagating, and expressing, in microorganisms, those segments of viral genomes coding for major antigens. In our selection of material we have restricted ourselves to a discussion of the better studied cases; however, it should be noted that similar studies have been performed upon other important viruses, in particular those of the herpes and paramyxovirus groups. We do not aim at giving precise experimental details of the now conventional technologies of genetic engineering; these can be found in the original literature, in one of the excellent technical manuals (e.g., Maniatis *et al.,* 1982), or in a recent review (see Lathe *et al.,* 1983; Harris, 1983). Instead, we use the viral antigen as a model for the discussion of the technical aspects of cloning viral nucleic acids and the problems related to expression of foreign proteins in microorganisms.

II. Expression of Foreign Coding Sequences in Escherichia coli

In this review we concentrate on the techniques that have been used to isolate and identify DNA sequences coding for viral antigens. Techniques for cloning RNA in the form of a DNA copy (cDNA cloning) have been extensively reviewed elsewhere (Williams, 1981; Efstradtiadis and Villa-Komaroff, 1979) as have the techniques for direct cloning of DNA genomes (Dahl *et al.,* 1981). The isolation of an antigen-coding sequence is an essential step toward the production of the corresponding vaccine. Nevertheless, the efficient expression of this sequence as an antigenic polypeptide is a further hurdle that must be surmounted. For this reason we will briefly discuss strategies that may be employed for expressing a foreign gene in *E. coli.* Other host microorganisms are described in Sections VIII and IX.

A. VECTOR COPY NUMBER

The rate of synthesis of a given protein depends, by and large, on the number of copies of the cognate gene that exists in each cell. Thus, rather than integrating cloned genes into the bacterial chromosome, most workers have elected to use high-copy-number episomal vectors based on plasmids such as pBR322 (Bolivar *et al.,* 1977) and bacteriophages such as M13 (Messing and Vieira, 1982) or λ (Murray, 1983). This strategy has disadvantages in that high copy numbers of certain DNA sequences may confer a selective disadvantage, and populations then suffer from high rates of rearrangement of such DNA sequences. In order to offset this problem, several vectors have been developed whose copy number may be maintained at a low level and amplified when required by, for instance, a temperature shift (Uhlin *et al.,* 1979; Rao and Rogers, 1978; Sninsky *et al.,* 1979). Of particular interest are those vectors that have the capacity to integrate into the bacterial chromosome (where they are thought to be stable), from which they may be excised at a later stage and induced to replicate (Howard and Gottesman, 1982; Nakano and Masuda, 1982).

B. SIGNALS FOR GENE EXPRESSION

Gene expression in *E. coli* requires signals that specify transcription into RNA and translation of this message. Much work has been carried out in recent years to identify such signals, and this is described briefly below.

1. *Transcription Initiation*

Promoters for transcription in *E. coli* comprise at least two sequence elements, the "Pribnow box," a short AT-rich zone lying about 10 nucleotides

prior to the first transcribed nucleotide, and a second sequence, the −35 region, lying somewhat upstream (see Gilbert, 1976, for discussion). Transcription initiation is, in many cases, subject to further controls, exemplified by the *lac* (lactose utilization) operon. Here, expression is controlled both positively and negatively. Positive control is effected by a protein activator, catabolic activator protein (CAP), which interacts with DNA sequences upstream of the promoter and enhances RNA polymerase binding. Negative control is effected by the *lac* repressor, which binds to the promoter region and sterically blocks subsequent binding of the RNA polymerase (see Reznikoff and Abelson, 1978, for a review of transcription control at the *lac* promoter). Negative control of bacterial promoters has proved to be of enormous utility in the controlled expression of foreign genes.

2. *Translation Initiation*

It is not known exactly what elements are required for the efficient initiation of translation at a given ATG. Certainly the Shine–Dalgarno (S/D) sequence, a short purine-rich sequence a number of nucleotides upstream of the initiation codon, is of essential importance (see Steitz, 1979). This sequence is thought to hybridize to the 3′ terminus of the bacterial 16 S ribosomal RNA and specify translation initiation at the appropriate ATG. Other factors are involved, such as the mRNA secondary structure and other as yet unidentified sequence motifs. In an extensive analysis of translation initiation sites, Gold *et al.* (1981) and Scherer *et al.* (1980) demonstrated that such regions show nonrandom base distributions both upstream of the initiating ATG (e.g., the S/D sequence) and downstream, within the coding sequence. This result has important consequences for the expression of foreign protein-coding sequences in *E. coli.*

3. *Termination of Transcription and Translation*

Transcription termination sites in *E. coli* are, by and large, identified by a G-C-rich duster, accompanied in some cases by a stem-loop structure, immediately followed by a poly(T) sequence (for a general review, see Adhya and Gottesman, 1977).

Translation termination is thought to be brought about by the simple presence, in phase, of one of the three termination codons, but additional parameters are likely to be involved.

C. FUSED AND UNFUSED PROTEINS

As mentioned earlier, sequences necessary for efficient translation initiation may lie on both sides of an ATG initiation codon. Thus positioning

of a foreign coding sequence immediately beyond the ATG may compromise expression. For this reason, many groups have chosen to fuse foreign genes, in phase, at a site distal to the initiation codon of an endogenous bacterial gene. This strategy may also have unexpected benefits in terms of stability of the hybrid protein, but in certain instances the presence of an extended covalently bound polypeptide of bacterial origin may mask antigenic sites in a hybrid protein produced for vaccine purposes (Lathe *et al.*, unpublished data).

D. INDUCIBLE AND NONINDUCIBLE SYSTEMS

It has been clearly demonstrated that the expression of certain proteins in *E. coli* is severely detrimental to the survival of the host. Of particular note is the surface antigen of hepatitis B virus (see Section III). For this reason a better choice is to employ expression systems in which transcription of the coding sequence in question may be blocked, when necessary, by a repressor protein. Although early work employed uncontrollable ("constitutive") expression systems deriving from, for instance, the β-lactamase gene of plasmid pBR322, we restrict our discussion here to systems subject to regulation *in vivo*.

1. *The lac System*

Transcription from the *lac* promoter of *E. coli* is normally blocked by the presence of the *lac* repressor, the product of the *lac*I gene. Addition of isopropylthiogalactoside (IPTG) to the culture medium results in repressor inactivation and the induction of transcription and translation of the *lac*Z gene or coding sequences fused in phase with this gene. One note of caution: the presence of the *lac* promoter on a multicopy plasmid is sufficient to out-titrate the available repressor protein, and expression may ensue even in the absence of IPTG. To surmount this problem, strains containing the *lac*I^q allele (which generates elevated levels of *lac* repressor) are often used.

As mentioned earlier, expression from the *lac* promoter additionally requires the presence of an activator factor CAP. The activity of this factor is in turn controlled by the metabolic state of the cell and, in particular, by the level of glucose. Thus a mutant *lac* promoter (*lacUV5*) that has lost its dependence on the activator protein (see Reznikoff, 1978) is often employed.

2. *The trp System*

The synthesis of tryptophan in *E. coli* requires the presence of five enzymes encoded by the *trp* operon, which is normally repressed by the *trp* repressor in the presence of tryptophan. Several plasmid-borne systems have

been described in which protein coding sequences may be fused in phase with the first (*trp*E) or second (*trp*D) genes of the operon (Tacon *et al.*, 1980; Hallewell and Emtage, 1980) or, indeed, with the leader polypeptide *trp*L (Edman *et al.*, 1981). Here, tryptophan starvation or the addition of indoleacrylic acid (IAA) may be used to lift repression and allow production of the relevant protein (see Platt, 1978, for a general discussion of *trp* regulation). In contrast to the *lac* system, a single copy of the *trp*R (repressor) gene seems to be sufficient to block transcription from the *trp* promoter present on a multicopy plasmid.

3. *The λ P_L System*

The use of the major leftward promoter of phage λ, P_L, has a number of advantages. Under normal conditions, a single copy of the λ repressor gene (*cI*) present on the host chromosome is able completely to block expression from the P_L promoter located on a multicopy plasmid. Further, by using the *cI857* allele, a temperature shift from 30 to 42°C can be used to inactivate the mutant repressor and allow transcription to proceed. Note that systems based on the rightward promoter of phage λ are also available, and these have similar properties.

The use of P_L has, in addition, a further advantage. In phage λ the first gene downstream of P_L, the *N* gene, codes for a protein that interacts with the RNA polymerase in such a way as to prevent transcription arrest at most termination signals. Plasmids incorporating both P_L and *N* may thus be used with confidence to express foreign protein-coding sequences, since transcription termination within the foreign gene can be eliminated.

The *cI857*/P_L/*N* regulon, as it stands, lacks signals for initiating translation of a foreign DNA sequence. For this reason, a number of groups have incorporated sequences from other well-translated genes, such as the MS2 replicase gene (Küpper *et al.*, 1981) or the λ *cII* gene (Shatzman and Rosenberg, 1982). The DNA segment containing the powerful translation initiation signals associated with the membrane lipoprotein (*lpp*) gene of *E. coli* (Nakamura and Inouye, 1982) is a good candidate for combination with the λ P_L system.

E. HYBRID EXPRESSION SYSTEMS

1. *Tac*

De Boer *et al.* (1982, 1983) describe the construction of hybrid expression control elements. As mentioned earlier, transcription initiation signals in *E. coli* comprise at least two recognition elements, the −10 (Pribnow box) and −35 sequences. The *trp* promoter has been shown to possess an optimal (consensus) −35 transcription recognition region but a poor sequence at −10. In contrast, *lacUV5* comprises an optimal −10 sequence and a

poor −35 region. Thus two different fusions, *tac*I and *tac*II, were constructed in which the optimal elements of both promoters were combined. Both constructs retain their sensitivity to repression by the *lac*I protein (and thus inducibility by IPTG) characteristic of the *lac* promoter. When combined with a synthetic transcription translation region, these promoters were shown to direct the expression of a foreign gene 11 and 7 times (*tac*I and *tac*II) more efficiently than the parental *lacUV5* block (De Boer *et al.*, 1982, 1983). Similar constructs have been described by Russell and Bennett (1982).

2. *Rac*

In a further construction, De Boer *et al.* (1982) fused the upstream (−35) control elements of the powerful ribosomal RNA (*rrn*) genes with downstream sequences from *lac*. This system, which is as active as the *tac* constructs, may take advantage of a termination override mechanism associated with *rrn* promoters (Siehnel and Morgan, 1983).

3. *Other Systems*

Hybrids broadly similar to those described above have been constructed by Russell and Bennett (1982), who exchanged sequence elements between *trp, lac,* and the tetracycline resistance (*tet*) gene from pBR322. Note must be made of the synthetic consensus promoter and translation-initiation sequences (e.g., Soberon *et al.*, 1982; Ohtsuka *et al.*, 1981; Jay *et al.*, 1982) which, in combination, may supplant the other systems described earlier.

As a broad guideline, the transcription of a foreign DNA sequence mediated by a powerful promoter is, in many systems, a matter of routine. In contrast, the efficient translation of the messenger so produced, and the concomitant accumulation of the product, is a matter of delicacy. Particular factors that affect the yield are the precise sequence of the translation-initiation region (both before and after the initiating ATG), the secondary structure of the messenger RNA around the initiation codon, and the stability of the protein product. No general rules have so far been found that can adequately portray these parameters.

In the following sections we describe the isolation and cloning of viral genomes and, in particular, how the systems described above have been adapted to the production of viral antigens.

III. Hepatitis B Virus

Owing to its considerable clinical interest, hepatitis B virus (HBV) was one of the first main targets for recombinant DNA technology. Between 3 and 15% of healthy blood donors in Western Europe and the United States show serological evidence of exposure to HBV, and about 0.1% are carriers

of the virus. In many African and Asian countries a much increased prevalance has been observed. Indeed, the majority of the adult population can show signs of previous exposure and 5–10% of the population may be chronically infected.

Hepatitis B virus infection is normally subclinical, and the development of virus-specific antibodies is associated with remission of symptoms. However, a significant number of infections (1–5%) may produce chronic pathogenic effects such as chronic hepatitis of various types (including fulminant hepatitis), cirrhosis, and primary liver cancer (hepatocarcinoma) (Redeker, 1975; Szumuness *et al.*, 1978; Burrel *et al.*, 1979). It has been estimated that there are at least 120 million so-called "healthy" carriers of HBsAg throughout the world (Szumuness, 1975).

Because of its narrow host range (the hepatitis B virus infects only in humans and chimpanzees *in vivo*) and its inability to be propagated in tissue culture, investigation of the structure and mechanism of infection of HBV has been severely restricted.

Hepatitis B virus (Vyas *et al.*, 1978; Zuckerman, 1979; Prince, 1981) consists of a 42-nm particle (the Dane particle) containing the viral genome (ca. 3200 base pairs of partially single-stranded circular DNA) (Summers *et al.*, 1975; Robinson, 1977) bound to the core protein and the viral polymerase (Landers *et al.*, 1977; Robinson and Greenman, 1974; Lutwick and Robinson, 1977; Hruska *et al.*, 1977). The viral core is surrounded by a phospholipid-containing envelope that carries the major surface antigenic determinants. These seem to reside mainly in a single protein that occurs in both glycosylated and nonglycosylated forms (M_r 27,000–29,000 and 23,000–25,000, respectively; Shih and Gerin, 1977; Peterson, 1981). Hepatitis B infection leads not only to production of Dane particles, but also to a dramatic overproduction of 22-nm particles and filaments (the HBsAg particles) that contain the elements of the surface envelope (Cabral, 1978). In addition to HBsAg, two other antigens have been identified in the plasma of patients (Magnius and Espmark, 1972; Takahashi *et al.*, 1976): HBcAg, which corresponds to the core of the virus, and HBeAg, whose location within the virion has been demonstrated (Werner *et al.*, 1977; Ohori *et al.*, 1979).

Recombinant DNA technology has permitted a considerable increase in our knowledge of the hepatitis B virus. Preliminary restriction mapping of double-stranded circular HBV DNA generated *in vitro* was described in 1975 (Summers *et al.*, 1975). Using these data, in combination with complementary analyses, several groups succeeded in cloning the entire HBV genome in bacteria (Burrel *et al.*, 1979; Sninsky *et al.*, 1979; Charnay *et al.*, 1979; Valenzuela *et al.*, 1979; Galibert *et al.*, 1979; Pasek *et al.*, 1979). Such clones have been very useful for studying the expression of proteins encoded by

the virus and, as we shall discuss later, for large-scale production of viral antigens suitable for diagnostic and vaccine use (for alternative approaches, see Zuckerman, 1982). The cloning methods used were essentially the same, with slight modifications introduced by the various groups. A typical protocol is described below.

A. PURIFICATION OF DANE PARTICLES

The presence of Dane particles in serum may be detected by a DNA polymerase assay (Robinson and Greenman, 1974, Hruska *et al.*, 1977, Summers *et al.*, 1978; Valenzuela *et al.*, 1979), but it is important to note that only 10–20% of HBsAg-positive sera contain Dane particles. HBsAg-positive plasma is defibrinized by $CaCl_2$ precipitation and clarified by filtration or low-speed centrifugation. This preparation is then subjected to high-salt sucrose gradient centrifugation (typically 10–20% sucrose in buffer containing 1 *M* NaCl, 100,000 *g* for 15 hr at 4°C).

The pellet obtained by this procedure is recentrifuged through a sucrose gradient as before and resuspended into buffered CsCl solution ($\rho = 1.22$ g/cm^3). Recentrifugation at 300,000 *g* for 15 hr at 4°C generates two visible bands; the heavy band near the top contains HBsAg particles. This preparation can be assayed directly for DNA polymerase activity by following the incorporation of radiolabeled deoxynucleoside triphosphates (dNTP) into filter-retained material.

B. CLONING OF HBV DNA

Double-stranded DNA is synthesized in Dane particles by the endogenous DNA polymerase. Incubation is typically for 3 hr at 37°C in a buffered reaction mixture containing intact Dane particles and the dNTP precursors. DNA is isolated by phenol extraction and ethanol precipitation after digesting the Dane particles for 60 min at 56°C with proteinase K (0.8 mg/ml) (Valenzuela *et al.*, 1979).

This preparation of Dane-particle DNA may be directly digested with *Eco*RI endonuclease and cloned, via the unique HBV *Eco*RI site, into a suitable plasmid vector (this technique has been used by most groups).

C. THE HBV GENOME

The cloned genome comprises 3182 base pairs; the complete nucleotide sequence of HBV has been determined. Some discrepancies among sequences have been observed mainly between the different serotypes (Valenzuela *et al.*, 1979; Pasek *et al.*, 1979; Fritsch *et al.*, 1978; Siddiqui *et al.*,

1979), although differences between separate independent isolates of the same serotype have also been observed (Siddiqui *et al.*, 1979; N. Harford, T. Cabezon, M. Dewilde, and J. P. Lecocq, unpublished data). It should be noted that some isolates of HBV lack the characteristic unique *Eco*RI site (Harford, Dewilde, Cabezon, and Lecocq, unpublished data).

Eight open reading frames, each coding for polypeptide chains longer than 100 amino acids, have been located by Galibert *et al.* (1979) on a sequence corresponding to a *ayw* serotype. It appears that only four of these are read *in vivo,* those directing the synthesis of HBsAg, HBcAg, the viral DNA polymerase, and the X protein.

The HBsAg gene was located by comparison of the DNA sequence with partial amino acid data (Valenzuela *et al.,* 1979; Galibert *et al.,* 1979). The HBcAg gene was identified within a region of DNA that programmed, in *E. coli,* the synthesis of serologically active HBcAg (Pasek *et al.,* 1979). Neither the coding regions of the HBcAg gene nor those of the HBsAg gene appear to contain intervening sequences (Galibert *et al.,* 1979).

It has been suggested that the largest open reading frame, which occupies more than 75% of the genome and overlaps the HBc gene and encompasses the HBs gene, encodes the viral DNA polymerase (Galibert *et al.,* 1979; Valenzuela *et al.,* 1980; Tiollais *et al.,* 1981). A fourth open translational coding frame, the so-called X gene, has been identified within the viral sequence (Tiollais *et al.,* 1981), but no function has so far been associated with this gene; the expression of the X protein in *E. coli* may permit specific antibodies to be raised against this protein with the potential of detecting protein X in human serum.

The structure of the HBV genome is presented in Fig. 1.

D. EXPRESSION OF HBV SURFACE ANTIGEN IN *E. coli*

As mentioned earlier, the inability of HBV to grow in tissue culture cells has hampered the development of a conventional vaccine. This has led to attempts to find other preparations that might produce active immunity, including the use of inactivated HBsAg purified from plasma of asymptomatic human carriers. The safety, immunogenicity, and high protective efficiency of such preparations have been demonstrated (see Zuckerman, 1982, for a brief review). This type of subunit vaccine, however, suffers two considerable disadvantages: large quantities of human pooled plasma, with high titers of HBsAg, are necessary, and the manufacturing process is expensive. Containment facilities and strict safety testing of the vaccine are required, including a test for residual infectivity of HBV in susceptible chimpanzees. Thus the use of genetic engineering technology has become an attractive approach for producing HBsAg in quantity.

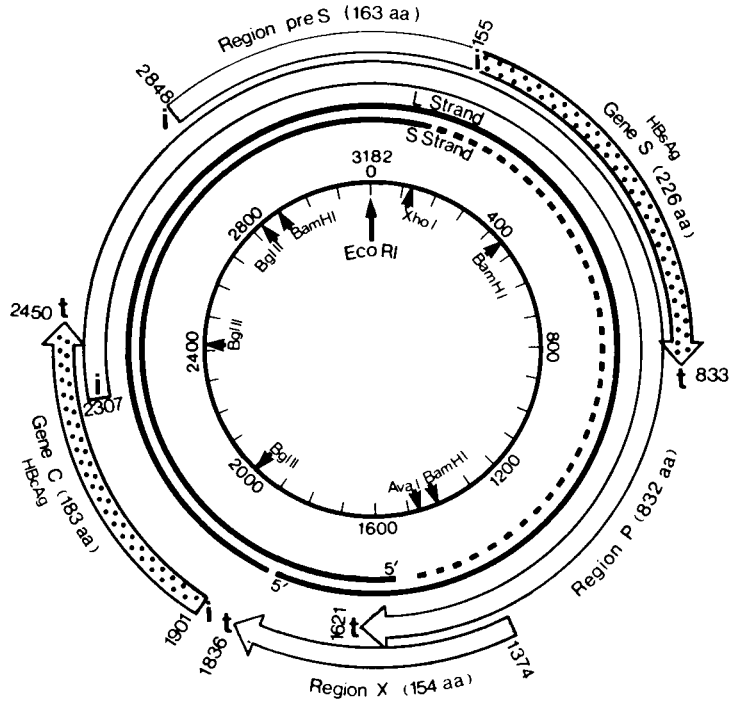

FIG. 1. Genetic organization of the hepatitis B virus (HBV) genome. The dashed line indicates the variable single-stranded region. The four potential coding regions, namely, S (divided into pre-S and gene S), C, P, and X surround the genome. The numbers of amino acids (aa) correspond to the lengths of the hypothetical polypeptides. **i**, Translation initiation codon; **t**, termination codon. After P. Tiollais, personal communication.

A first attempt to express HBV antigens in *E. coli* was described by Burrel *et al.* (1979) in a so-called shotgun cloning experiment. Complete double-stranded HBV DNA (generated *in vitro*) was digested with different restriction enzymes, 3′-oligo(dC) sequences were added to the fragments using polynucleotide terminal transferase (Roychoudhury *et al.*, 1976), and the fragments were annealed to plasmid pBR322 to which oligo(dG) sequences had been attached at the *PstI* site. These DNA preparations were then used to transform competent cells of *E. coli*. Transformants were subsequently screened by colony hybridization (Grunstein and Hogness, 1975) with radiolabeled Dane-particle DNA.

Colonies carrying recombinant plasmids were screened for the production of HBV antigens. The technique developed by Broome and Gilbert (1978) was applied using both anti-HBc (see later) and anti-HBs (human

and hyperimmune animal sera) antibodies. This experiment revealed only a small number of faint positive colonies.

After identification of the nucleotide sequence of the HBV genome corresponding to the surface antigen, it became possible to perform precise constructions in order to express the HBs antigen. McKay *et al.* (1981) inserted different fragments of the HBV genome containing all or nearly all of the coding sequence for the surface antigen into the *Pst*I site within the β-lactamase gene of plasmid pBR322. These constructions were designed to result in the synthesis of HBs peptides, either as a fusion with the N terminus of the β-lactamase protein or by reinitiation of translation after termination at a TGA stop codon lying upstream of the natural HBs initiation codon.

In all clones tested, solid-phase radioimmunoassay (Broome and Gilbert, 1978), gel electrophoretic analysis of proteins made in minicells (Reeve, 1977a), or an assay using microtiter wells coated with anti-HBsAg (Purcell *et al.*, 1973) all gave weak, variable, and, in general, inconclusive results. Nevertheless, some extracts prepared from these bacteria induced a low titer of anti-HBV antibodies in rabbits, but only after a long period of repeated booster injections.

Edman *et al.* (1981) also integrated the HBs gene into the *Pst*I site of a plasmid-borne β-lactamase gene but used a system in which the β-lactamase gene was under the control of the *trp* promoter (Fig. 2). A 744 base pair *Hin*dII fragment of HBV DNA containing the coding sequence for 204 amino acids of HBsAg was purified by polyacrylamide gel electrophoresis, tailed with d(C) using terminal transferase, and hybridized with dG-tailed plasmid p*trp*L1 prior to transformation of *E. coli* HB101.

The DNA sequence of this HBV DNA fragment predicted that, when the HBsAg sequence was in the proper orientation and the same reading phase as β-lactamase, a 43-kilodalton polypeptide would be produced. This hybrid protein should contain 183 amino acids of the pre-β-lactamase (21 kilodaltons), 5–10 glycine residues from the G/C tail (ca. 600 daltons), and 204 amino acids (22.6 kilodaltons) deriving from HBsAg. If the β-lactamase "signal" presequence were cleaved, the fusion protein would have a predicted molecular weight of 41,000. Colonies containing recombinant plasmids were examined, and a number were shown to produce the 41-kilodalton polypeptide. After immunoprecipitation with anti-HBs IgG, it was found that the levels of 41-kilodalton polypeptide produced in *E. coli* varied widely among different experiments and in some cases was barely detectable.

Charnay *et al.* (1980) described recombinant λ phage derivatives in which part of the HBsAg gene was fused to the β-galactosidase gene. The resulting fusion polypeptide (138 kilodaltons) comprised almost the entire β-galactosidase polypeptide followed by HBsAg lacking its first 29 amino acid res-

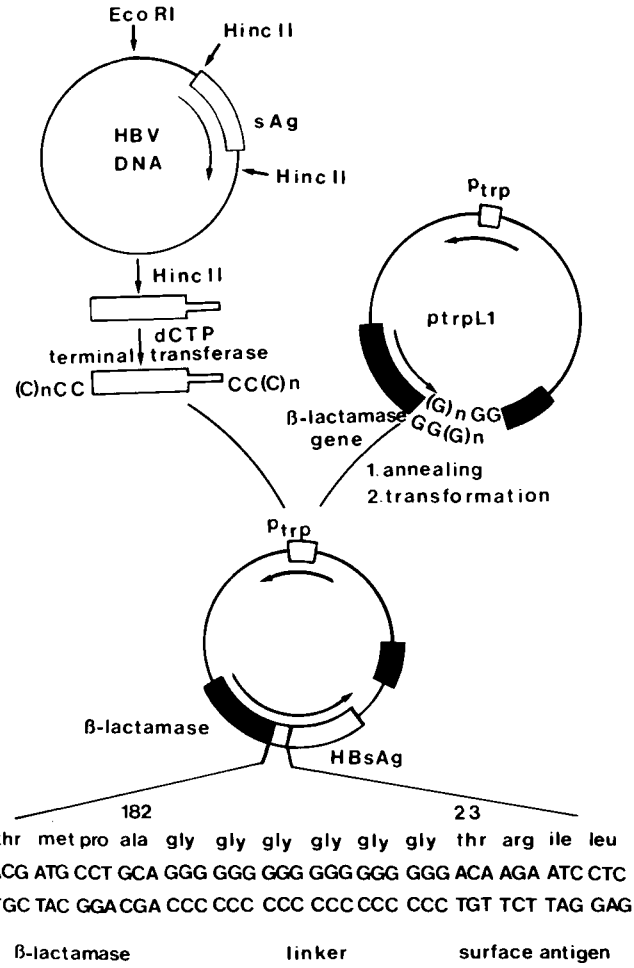

FIG. 2. Construction of plasmids expressing a β-lactamase:HBsAg fusion. Adapted from Edman *et al.* (1981), with permission.

idues. This polypeptide could be precipitated with anti-HBsAg antibodies, but reacted poorly in radioimmunoassays. Note that the structure of this fused polypeptide differs considerably from the construction of Edman *et al.* (1981) and McKay *et al.* (1981), in which few foreign amino acids were fused to HBsAg.

Numerous explanations for the poor expression of HBsAg in *E. coli* have been proposed (McKay *et al.*, 1981), but it is widely believed that the prob-

lem is due to a deleterious effect of the HBsAg protein on the growth and metabolism in *E. coli*. This phenomenon is well illustrated in an experiment performed at Smith-Kline-RIT (N. Harford, T. Cabezon, M. Dewilde, and J. P. Lecocq, unpublished data). Here, an attempt was made to express in *E. coli* the surface antigen derived from the two main serotypes, adW2 and ayW2. When these sequences were fused downstream of the *lac*Z initiation codon present on vector pPCψ3 (Charnay *et al.*, 1978), induction of the *lac* promoter with IPTG resulted in marked retardation of cell growth. Furthermore, cultivation in the presence of inducer resulted in extensive loss of the recombinant plasmid. These results indicate that production of a polypeptide containing HBsAg may be lethal to *E. coli*. To analyze this possibility further, a set of recombinant plasmids was constructed in which all or part of the HBsAg gene was fused to the 5′ end of either the *lac*Z or *trp*E genes. These plasmids were used to transform strains containing either normal or substantially elevated levels of the cognate repressor protein (*lac*I$^+$, *lac*I^q, or *trp*R$^+$) or, alternatively, strains lacking an active repressor ($\triangle$*lac* or *trp*R$^-$). It was observed that strains lacking repressor were transformed 10- to 100-fold less efficiently than strains having normal or elevated levels of repressor. None of the phenomena described above was observed when analogous plasmids were used in which HBV sequences were present in an orientation unsuitable for expression. Such experiments demonstrate that expression of HBsAg sequences is deleterious to the *E. coli* cell. It should be noted that removing either the N-terminal or both the N- and C-terminal sequences from the constructs did not enhance expression.

E. EXPRESSION OF HBV CORE ANTIGEN IN *E. coli*

In contrast to the surface antigen, the hepatitis B core antigen can be expressed in large amounts in *E. coli*, where it seems to be relatively stable. The expression of HBc antigenic determinants in *E. coli* was first described by Burrel *et al.* (1979). Clear, positive, and reproducible results were obtained. Nucleotide sequence analysis showed that the HBV core antigen was synthesized by a "translation restart" phenomenon on the mRNA (Pasek *et al.*, 1979).

Fragments carrying the core antigen gene have been placed under the control of the *lac* promoter of *E. coli* (Stahl *et al.*, 1982). Several of the recombinants direct high levels of synthesis of the antigen, the exact level depending on the precise structure of the plasmid and the mRNAs synthesized. Note that bacterially synthesized antigen is a satisfactory diagnostic reagent for the presence, in human sera, of antibodies directed against the core antigen.

As described in Section II, the *trp* promoter very efficiently enhances the expression of genes linked to its coding sequence. Edman *et al.* (1981) placed the core antigen under the control of the *trp* promoter in plasmid p*trp*L1. Here, the HBV DNA contains two potential initiation codons for HBcAg. These authors focused their efforts on a construction involving the first *met* codon, but there is now strong evidence that the second *met* codon is the natural initiation codon used *in vivo* (Tiollais *et al.*, 1981). Thus, the core antigen resulting from the construction of Edman *et al.* (1981) carries an additional 29 amino acids at its N terminus.

A 1005 base pair (bp) *Hha*I fragment of HBV DNA containing the entire HBcAg gene was purified by gel electrophoresis; in this fragment the first *met* codon for HBcAg lies only 15 bp from one extremity. After treatment with *HPa*II methylase to prevent cleavage of the fragment during subsequent restriction enzyme digestion, the fragment was subsequently treated with T4 polymerase in the absence of dNTPs (permitting $3' \rightarrow 5'$-nuclease activity) for 30 sec at 37°C. Under these conditions, between 0 and 10 nucleotides were removed from each 3′ end. The digested fragments were then treated with S1 nuclease, and dodecameric *Bam*HI linkers (CCGGATCCGG, which contain the *Hpa*II recognition sequence, CCGG) were ligated to the termini using T4 ligase. After digestion of the linkers with *Hpa*II, the linker fragments were removed and the DNA was ligated with *Cla*I-digested and calf intestinal alkaline phosphatase-treated p*trp*L1. The ligation mixture was used to transform *E. coli* strain HB101 (Cohen *et al.*, 1972), and transformants were screened for recombinant plasmid DNA. These were then tested for the production of HBcAg using a double-antibody radioimmunoassay (RIA) with human anti-HBcAgIgG (Ling and Overby, 1972).

Sequence analysis of positive clones confirmed that the initiation codon of HBcAg was in close proximity (13–16 bp) to the Shine–Dalgarno sequence of the *trp* leader peptide. The structure of the plasmid responsible for the highest level of expression is illustrated in Fig. 3.

When cells containing this plasmid were treated with the inducer 3-β-indolylacrylic acid, the level of HBcAg approached 10% of newly synthesized protein.

The DNA sequence of the construction predicts a protein of 22,000 daltons, whereas immunoprecipitation of induced extracts revealed two specific bands: 22 and 19 kilodaltons. The latter may result from restart synthesis at the second (natural) *met* codon.

In the preceding section we described how the core antigen of HBV can be expressed at high levels in *E. coli*. In contrast, the poor expression of the surface antigen in *E. coli* indicates that this organism may not be a suitable host for the elaboration of a vaccine against HBV.

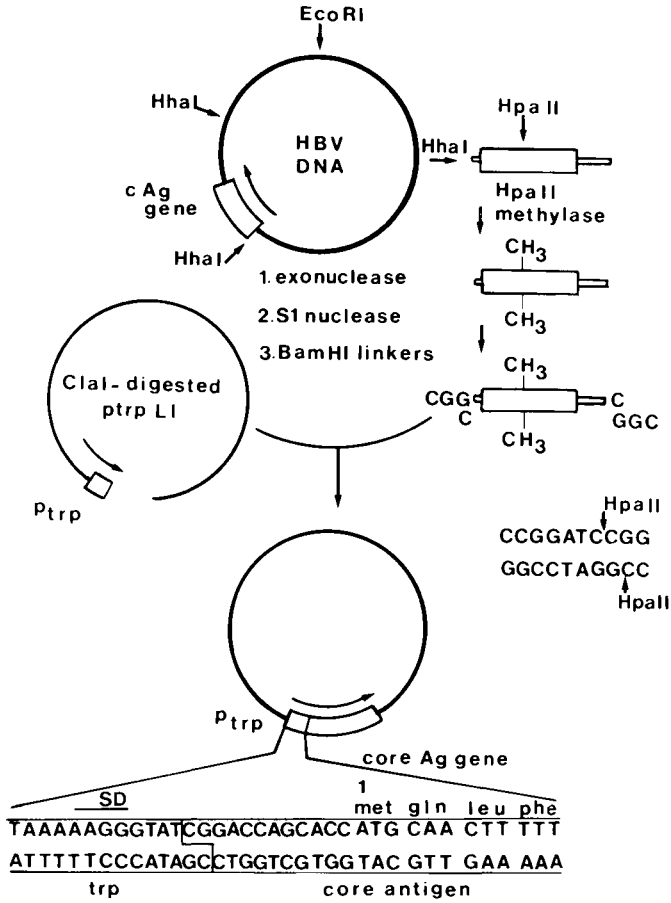

FIG. 3. Construction of plasmids expressing a *trp*:HBcAg fusion. Adapted from Edman *et al.* (1981), with permission.

IV. Foot-and-Mouth Disease Virus

Another prime target for recombinant DNA technology has been the foot-and-mouth disease virus (FMDV). It is perhaps useful to note that FMDV was the first animal disease to be described as being of viral origin, but, worldwide, it remains the most serious disease of commercial animal stocks.

Present vaccines are prepared from virus cultivated either in tongue epithelial tissue collected from slaughterhouse animals or in cell lines grown

in suspension in large vessels. Problems that arise in the production of the viral antigens are commonly related to certain strains of the virus and especially to the multiplicity of serotypes, which change regularly (Brooksby, 1982). Although much has been achieved with available vaccines, there still is a need for new methods of producing the viral antigens. The technology of genetic engineering could be considered to be a promising alternative to traditional methods.

FMDV is a picornavirus, consisting of a linear single-stranded RNA about 8000 nucleotides in length. It is polyadenylated at its 3′ end, has a poly(C) tract close to the 5′ end and a protein covalently attached to the 5′ terminus. Translation is initiated to the 3′ side of the 150-nucleotide poly(C) tract and yields a single polyprotein, which is subsequently processed (see Fig. 4) to generate the structural proteins of the virus: VP1, VP2, VP3, and VP4 (Sangar, 1979; Bachrach, 1977). It has been shown that the immunizing activity of FMDV particles is associated primarily with VP1*; this protein alone can elicit seroneutralizing antibodies (Laporte *et al.*, 1973; Bachrach *et al.*, 1975).

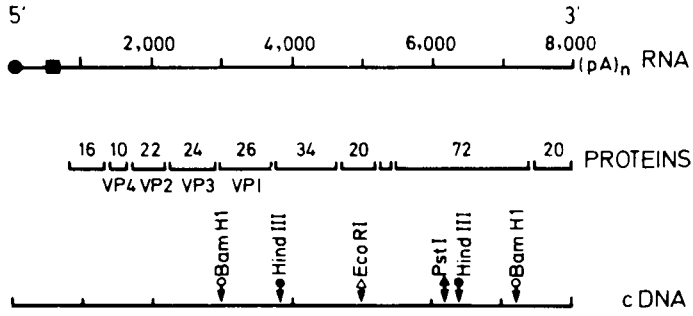

FIG. 4. Organization of the FMDV genome and location of restriction sites on the cDNA. For convenience, the size of the RNA is given as 8000 bases instead of the 7800 bases estimated by physical analysis and the *Bam*HI site is referred to as map position 3000. The molecular weights ($\times 10^{-3}$) of the proteins are as indicated. Within the RNA molecule, (■) indicates the internal poly(C) stretch close to the 5′ end, and (•) indicates the viral genomic protein (VPg) covalently linked to the 5′ end of the viral RNA. Reprinted by permission from Küpper *et al.*, *Nature* **289**, 555–559. Copyright © 1981, Macmillan Journals Limited.

*The fourth FMDV capsid polypeptide translated has been designated VP3, VP1, or VPthr owing to its variable migration in different gel electrophoretic systems. This protein will be referred to as VP1, as recommended at the 1982 American Society of Virology Meeting, Ithaca, New York.

A. PURIFICATION OF FMDV RNA

Virus, usually isolated during an epizootic outbreak, is typically isolated a few times from single plaques and passaged about 50 times in cell culture. Note that the identity of passaged virus must always be confirmed by complement-binding assays with the original antibodies.

Grubman *et al.* (1979) infected roller cultures of baby hamster kidney (BHK) cells with purified virus (10 PFU/cell) and harvested both intracellular and extracellular virus 3–6 hr after infection. Intracellular virus was released by swelling the cells in reticulocyte standard buffer + heparin and Dounce homogenization. Nuclei were removed by low speed centrifugation, and virus was purified by centrifugation through a 10–50% (w/v) linear sucrose gradient at 60,000 *g* for 17 hr at 4°. Extracellular virus was concentrated by polyethylene glycol (PEG) precipitation and similarly purified by sucrose gradient centrifugation as described above.

Purified virus in buffer was digested for 60 min at 37° with 500 μg of proteinase K per milliliter in the presence of 1% sodium dodecyl sulfate (SDS), and the viral RNA was further purified by centrifugation on a 15–30% (w/v) linear sucrose gradient containing 0.5% SDS (typically 46,000 *g* for 17.5 hr at 23°C).

B. CLONING OF FMDV cDNA

Küpper *et al.* (1981) prepared single-stranded cDNA by priming AMV reverse transcriptase with oligo(dT) hybridized to the polyadenylate tract present at the 3′ end of the virion RNA. The RNA–DNA hybrid so formed was heat denatured (3 min at 100°C) and quickly cooled. The single-stranded cDNA was rendered double-stranded by treatment with *E. coli* DNA polymerase I in the presence of the four deoxyribonucleoside triphosphates for 2 hr at 15°C. The reaction was arrested with phenol and double-stranded cDNA purified by Sephadex G-150 gel filtration.

To insert the double-stranded cDNA molecules into plasmid vector pBR322 , the cDNA was treated first with single-strand specific S1 nuclease and oligo(dC) tails subsequently added to the 3′ termini using terminal transferase. The dC-tailed DNA molecules were annealed to pBR322 to which 3′-oligo(dG) sequences had been added at the *Pst*I site and used to transform competent cells of *E. coli* HB101. Tetracycline-resistant transformants were transferred to microtiter plates, and replicas of each plate were used for hybridization with ^{32}P-labeled fragmented viral RNA. Colonies displaying strong hybridization with the probe were selected for analysis. A similar protocol was developed by Boothroyd *et al.* (1981).

C. CHARACTERIZATION OF THE VP1 GENE

Restriction enzyme analysis of all cDNA inserts generated a restriction map of the FMDV genome aligned relative to the 3′ end of the RNA. Approximate correlations between the different viral proteins and the viral genome were deduced from the order of translation of the different polypeptides and from their apparent molecular weights (Doel *et al.*, 1978). This correlation has then been used to predict the position of viral genes within specific restriction fragments of the FMDV genome. Although approximate, this analysis did locate the VP1 gene. Clones predicted to contain the VP1 gene were sequenced and compared with the known sequence of NH_2-terminal 40 amino acids of VP1 (Strohmaier *et al.*, 1978); this allowed the unambiguous identification of the VP1 coding region.

The use of ^{32}P-labeled fragments of FMDV RNA as probes to align the restriction map with the physical map was reported by Boothroyd *et al.* (1981). The sequence of the complete VP1 gene of FMDV is of particular interest since VP1 protein harbors the main antigenic determinant of the virion. Moreover, changes in its amino acid sequence are responsible for the high antigenic variability of FMDV. The complete nucleotide sequences of the VP1 gene corresponding to different serotypes have been reported (Kürz *et al.*, 1981; Kleid *et al.*, 1981).

A rapid method for selective cloning of the VP1 gene from different viral serotypes was necessary to characterize the new amino acid changes found after each new outbreak. Such a method was set up by Yansura *et al.* (1983).

Purified RNA from the new strain is annealed to three synthetic deoxyoligonucleotide primers, each 10 nucleotides long, which hybridize to RNA sequences approximately 200 nucleotides 3′ to the VP1 gene. These sequences were chosen because of homology noted in this area when comparisons are made of FMD virus types A12, 01, A27, and C3 (see Fig. 5). In the presence of reverse transcriptase enzyme and deoxynucleotide triphosphates, the RNA is copied into DNA (cDNA) beginning with the primers. After denaturation, this is converted into double-stranded cDNA (ds-cDNA) using the Klenow fragment of DNA polymerase (Klenow and Henningsen, 1970) and treated with nuclease S1 to digest single-stranded regions. Double-stranded cDNA longer than about 2000 base pairs is isolated by gel electrophoresis, tailed with dCTP using terminal transferase, and annealed to linearized pBR322 carrying poly(dG) tracts at the *Pst*I site. This material is then used to transform *E. coli*. Plasmids with incorporated ds-cDNA are isolated, and those containing inserts larger than about 1500 base pairs are analyzed by filter hybridization to radiolabeled DNA from the VP1 clone of virus type A12 (Kleid *et al.*, 1981). Plasmids containing the VP1 gene may then be analyzed by restriction mapping and the nu-

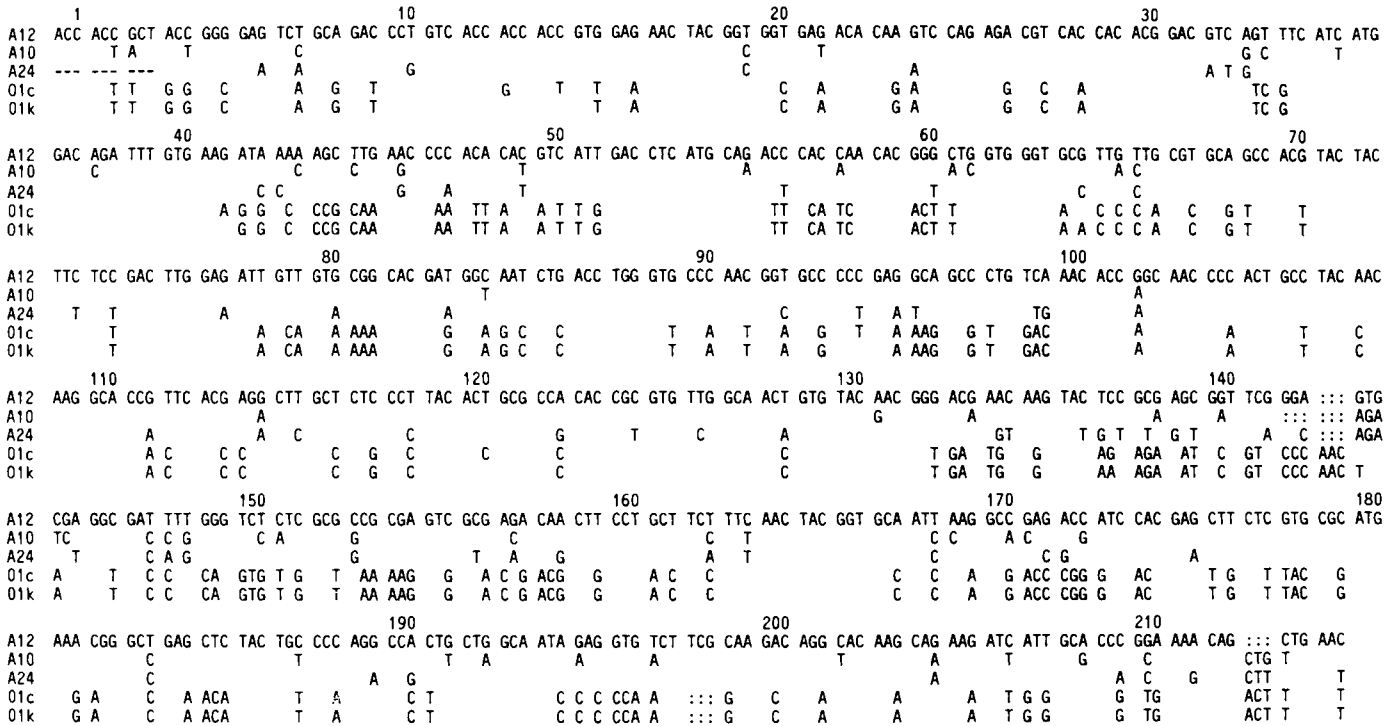

FIG. 5. Nucleotide sequences of the VP1 gene of FMDV type A12 and nucleotide substitutions present in types A10, A24, O1c, and O1k. Deletions introduced for purposes of alignment are represented by :::. The hypervariable region described in the text extends from amino acid positions 130 to 157. Adapted, with permission, from Yansura *et al.* (1983).

cleotide sequence of the VP1 variant gene determined using standard procedures.

D. EXPRESSION OF THE FMDV VP1 GENE IN *E. coli*

As described in Section II, the strong leftward promoter (P_L) of phage λ is a good candidate to promote expression of eukaryotic genes in *E. coli*; the activity of this promoter can be controlled by using host strains that carry the gene for a temperature-sensitive λ repressor. A vector (pPLc24) carrying the P_L promoter plus the SD region of the MS2 replicase gene was constructed by Remaut *et al.* (1981). This vector, which has been successfully used to express the gene for human β interferon (Derynck *et al.*, 1980), carries a unique restriction site for *Bam*HI at a position corresponding to amino acid 99 of the MS2 replicase gene and a unique site for *Hin*dIII 10 nucleotides downstream. A *Bam*HI-*Hin*dIII fragment of the FMDV cDNA which carries nearly all the VP1 coding sequence (the *Bam*HI site is located at the ninth amino acid) was inserted in the pPLc24 vector (Küpper *et al.*, 1981). A fusion polypeptide of 396 amino acids comprising the N-terminal 99 amino acids of MS2 replicase, 284 amino acids of FMDV, and 13 amino acids coded by the vector was predicted to be synthesized from the recombinant plasmid. Such a "tribrid" protein has been identified in induced *E. coli* extracts, at a yield of about 1000 molecules per cell.

Fusion of the VP1 gene (corresponding to the VP1 protein from serine 7, *Pst*I site, to glutamine 211, *Pvu*II site) with the *trp*E protein has been reported by Kleid *et al.* (1981). The yield of fused protein obtained in this expression system was about 17% of the total protein. Moreover, the fusion protein appears to be stable in the cell extract and can be used as a effective vaccine.

Enormous efforts to localize precisely the major antigenic site on the VP1 protein have been attempted. Examination of VP1 amino acid sequences from various types and subtypes revealed that there exists a particular region in the protein that is highly variable (Fig. 5). Studies indicated that this region of the VP1 protein is exposed on the virus surface and possesses a major antigenic site (Yansura *et al.*, 1983). A 13,000-dalton peptide that contains the variable region, isolated from CNBr-treated protein, has been shown to induce neutralizing antibodies (Kaaden *et al.*, 1977) and protect swine from FMD (Bachrach *et al.*, 1979).

Synthetic peptides from this region and linked to keyhole limpet hemocyanin carrier have been shown to induce neutralizing antibodies in guinea pigs and rabbits (Bittle *et al.*, 1982). Such peptides have been proposed as the basis of synthetic vaccines (see Lerner, 1982); indeed, a single inoculation of a synthetic peptide corresponding to amino acids 141–160 can elicit

neutralizing antibodies with a titer greater than that obtained with the purified capsid protein VP1, irrespective of whether this is produced by disruption of virus particles or by expression in *E. coli*. Thus the prospects for a totally synthetic vaccine against FMD are encouraging.

In an alternative approach, Yansura *et al.* (1983) elected to express this main antigenic site of the VP1 protein in *E. coli* in a form of a fused protein (see Fig. 6).

The coding sequence for amino acids 130–157 of the VP1 protein was recovered as an 83 bp *Alu*I-*Rsa*I fragment and ligated to plasmid pUC9 (Vieira and Messing, 1982), which had been cleaved previously with *Sma*I. The pUC9 plasmid, when used to transform *E. coli* JM83 to ampicillin resistance, codes for the amino-terminal portion (*lacZ'*) of β-galactosidase. This complements the carboxyterminal β-galactosidase protein expressed in the *E. coli* JM83 host and the reconstituted β-galactosidase can be detected by a suitable chromogenic substrate (e.g., X-gal). Introduction of the 83 base pair VP1 gene fragment into the *Sma*I site introduced a termination codon in the same reading frame with the initial ATG codon; thus derivatives containing the 83 base pair insert were easily identified by the absence of staining with X-gal.

In pUC9 the *Sma*I site in the *lacZ'* gene is flanked by *Pst*I or *Eco*RI sites, and one derivative containing the 83 base pair insert was treated with *Pst*I and *Eco*RI to recover the formerly blunt-ended fragment, now flanked by these sites. This fragment was introduced into the expression plasmid pNCV to give the derivative pFMB-42. In plasmid pNCV the *trp*P promoter lies upstream of a coding sequence generated by fusing the leader polypeptide (*trp*L) to the carboxyterminus of the *trp*E gene (the *trp* LE gene product, which has 190 amino acids, is extremely useful as part of a fusion protein, since it appears to be both insoluble inside the bacteria and resistant to proteolytic degradation). A *Pst*I site had been introduced into the *trp* LE fusion at the *trp* E termination codon of pNCV. When linked to the *Pst*I site of the modified VP1 gene fragment described above, a fused gene was created with the codons of the LE protein and the antigenic region of the VP1 protein in the same reading frame. The pNCV derivative, pFMB-42, was used to transform *E. coli*. *Escherichia coli*/pFMB-42, when grown under derepressed conditions, was found to express high levels of the desired fusion protein. Like the *trp*LE gene product, the fusion protein was found to be stable in *E. coli* and gave rise to insoluble intracellular inclusions visible by phase contrast microscopy. Levels of up to 20% of total cellular protein were obtained by this method. The product could be recovered from disrupted cells by centrifugation and the fusion protein be purified by a modification of a method similar to that described for the FMD virus VP1 protein (Bernard *et al.*, 1974).

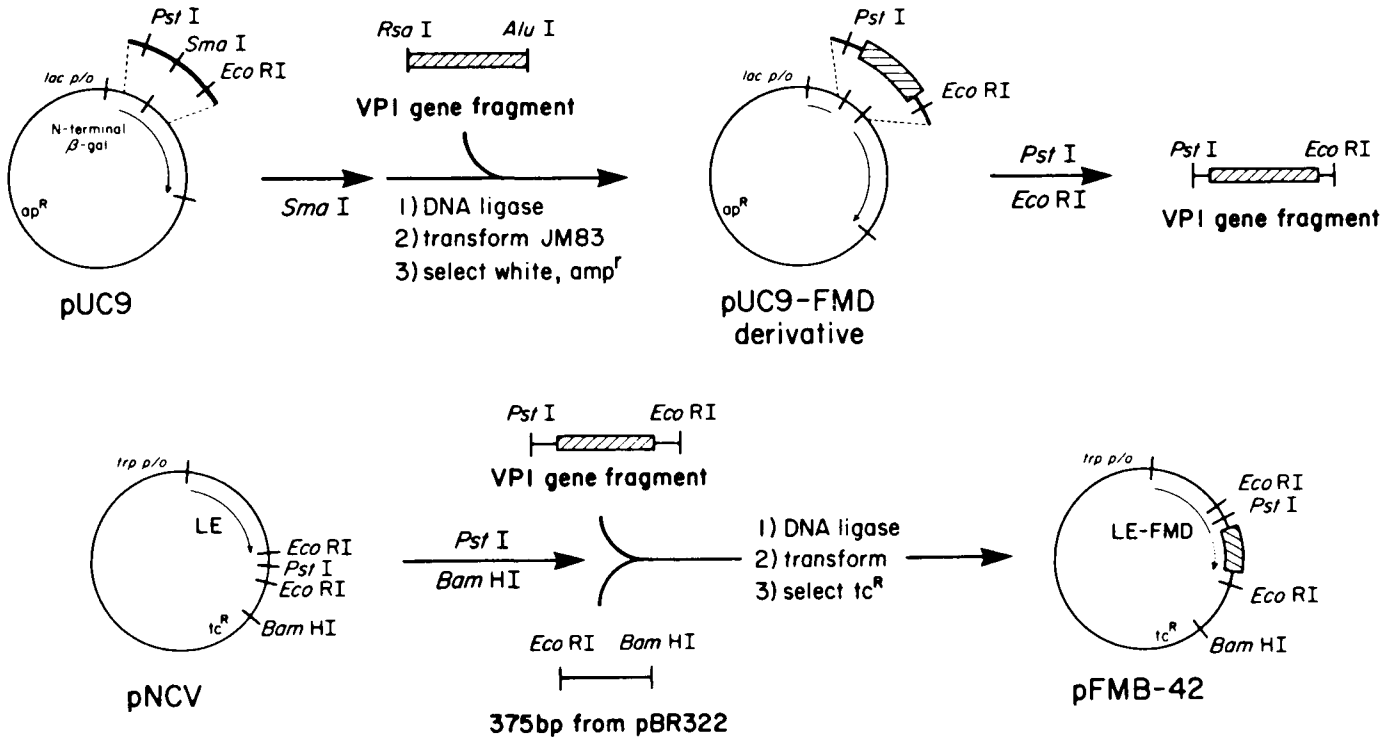

FIG. 6. Construction of the fusion protein expression plasmid, pFMB-42, containing codons 130–157 of the VP1 gene from FMDV A24 Cruzeiro. Reproduced from Yansura *et al.* (1983), with permission.

We have described how a single major epitope, either as a chemically synthesized oligopeptide or expressed in bacteria as a fusion protein, can elicit the production of seroneutralizing antibodies. Thus the prospects for a new FMDV vaccine are encouraging.

V. Poliovirus: Purification of Polio RNA and Cloning of Polio cDNA

Although the development of both killed and live vaccines (Salk and Salk, 1977; Sabin and Boulger, 1973) has been spectacularly successful in controling poliomyelitis in industrialized nations, polio remains a serious threat in much of the world. In spite of intensive research, the precise mechanisms by which poliovirus replicates and causes cytopathic effects remain unknown. The molecular basis for viral attenuation has also not been solved. Poliovirus, like FMDV, is a member of the picornavirus group. A variety of experimental results suggest that the poliovirus genome is also translated into a single polyprotein from which the functional viral proteins are derived by proteolysis (Jacobson and Baltimore, 1968; Holland and Klein, 1968; Jacobson *et al.*, 1970; Taber *et al.*, 1971; Saborio *et al.*, 1974).

The complete sequence of poliovirus cDNA has been reported (Kitamura *et al.*, 1981; Racaniello and Baltimore, 1981a). The structure of the viral genome is presented in Fig. 7.

The RNA molecule contained in the Mahoney strain is 7433 nucleotides long, polyadenylated at the 3′ terminus, and covalently linked to a small

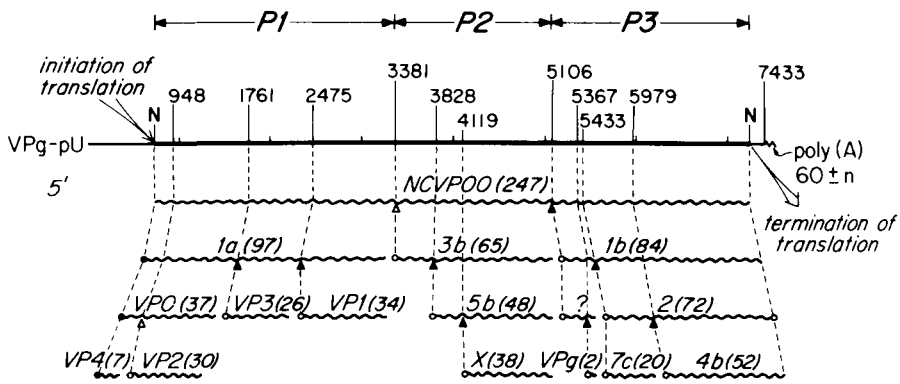

FIG. 7. Gene organization of poliovirus RNA. Reprinted by permission from Kitamura *et al.*, *Nature* **291**, 547–553. Copyright © 1981, Macmillan Journals Limited.

protein (VPg) at the 5′ terminus. An open reading frame of 2207 consecutive triplets spans over 89% of the nucleotide sequence and codes for the viral polyprotein NCVPOO. Twelve viral polypeptides have been mapped by amino acid sequence analysis and were found to be proteolytic cleavage products of the polyprotein, cleavage occurring predominantly at Gln–Gly pairs (Kitamura *et al.*, 1981).

Poliovirus RNA may be purified by the technique of Lee *et al.* (1979). In brief, the method involves the release of virus particles from infected HeLa cells by disruption in hypotonic medium. Virions are recovered by high speed centrifugation (78,000 *g*, 3 hr), and viral RNA is purified by serial phenol/chloroform extraction in the presence of 0.2% SDS followed by precipitation.

Polio RNA contains a poly(A) tract at its 3′ end (Armstrong *et al.*, 1972; Yogo and Wimmer, 1972), and this structural element has permitted copying the poliovirus RNA genome into cDNA using an oligo(dT) primer and reverse transcriptase. Cloning of the genome as a cDNA copy is usually a prerequisite for determining the primary structure of poliovirus genome; however, restrictions imposed by recombinant DNA guidelines led Kitamura *et al.* (1981) to sequence polio cDNA by a modification (Kitamura and Wimmer, 1980) of Sanger's chain termination method (Sanger *et al.*, 1977) without recourse to cloning.

Polio cDNA was synthesized *in vitro,* and chains of 7000–7400 deoxyribonucleotides were selected by zonal centrifugation. In turn, polio RNA was digested exhaustively with RNase T1 or with RNase A and large oligonucleotide products separated by two-dimensional gel electrophoresis. These were eluted from the gel, the 3′-phosphate group remaining after RNase treatment was removed with phosphatase, and the 5′ termini labeled by phosphorylation with [γ-^{32}P]ATP and polynucleotide kinase. The cDNA and specific 5′-^{32}P-labeled primer oligonucleotides were annealed and incubated with *E. coli* DNA polymerase I (Klenow fragment) in the presence of unlabeled dNTPs and one of four 2′,3′-dideoxynucleotide (chain-terminating) triphosphates. The products of this reaction were separated by gel electrophoresis and visualized by autoradiography; the sequence was determined from the position of chain termination with each of the four dideoxytriphosphates.

Racaniello and Baltimore (1981a) first reported the cloning of polio cDNA. Double-stranded poliovirus cDNA was synthesized by a classical method (Bothwell *et al.*, 1981) and inserted in the *Pst*I site of plasmid pBR322. The entire poliovirus genome sequence could be determined from three clones. One of these clones was generated from primer-extended DNA to contain cDNA corresponding to the 5′ end of RNA. Because the ds-cDNA used to generate the clones included a "snap-back" step for second-

strand synthesis, it was expected that the sequences at the very 5′ end of the viral RNA would not be found in the longest clones. To determine the number of bases by which the longest cDNA clones fell short of the 5′ end of poliovirus RNA, a restriction fragment comprising nucleotides 149–220 of the longest clone was prepared and subsequently 5′ end-labeled. This fragment was hybridized to poliovirus RNA and extended with reverse transcriptase by using classical procedures (Casey and Davidson, 1977; Lamb and Lai, 1980). In this experiment, the longest extended product that was synthesized, in high yields, was 220 bases long. Sequence analysis (Maxam and Gilbert, 1977) showed that the primer extension had proceeded up to and included the 5′-terminal U of the viral RNA. This result showed that the longest clone was missing the 5′-end 115 bases of poliovirus RNA. For cloning, the extended fragment was purified by gel electrophoresis and tailed with oligo(dC). The fragment was then made double-stranded by the Klenow fragment of *E. coli* DNA polymerase I in the presence of $(dG)_{12-18}$, tailed again with oligo(dC), and inserted at the *Pst*I site of pBR322.

Cloning of poliovirus cDNA using direct transformation of *E. coli* with RNA–cDNA hybrids annealed into the *Pst*I site of pBR322 was described by Van der Werf *et al.* (1981). cDNA was prepared using an oligo$(dT)_{10}$ primer and reverse transcriptase as described by Kitamura and Wimmer (1980). Hybrid cDNA–RNAs were purified by neutral 15–30% sucrose gradient centrifugation and trimmed with RNase A and RNase T1. Poly(dC) was added to the 3′ ends of the molecules using terminal transferase as before, and poly(dG) was similarly added to the cloning vector pBR322 at the *Pst*I site. These were hybridized and used to transform *E. coli*. Recombinant colonies were screened for the presence of polio-specific sequences by a modification of the Grunstein and Hogness (1975) colony filter-hybridization technique using as hybridization probes individual radiolabeled RNase T1 oligonucleotides.

To determine the molecular basis for the biological differences between virulent and attenuated polio strains, the cDNA corresponding to the LSc, 2 b stain [Sabin 1 strain, PV1 (Sab)] was cloned by Nomoto *et al.* (1982). Sequence analysis revealed that the virion RNA, 7441 nucleotides long, is also polyadenylated at the 3′ terminus. When the sequence is compared with that of the genome of the viral parent strain (Mahoney), 57 base substitutions, scattered throughout the genome, are observed. Of these, 21 result in amino acid substitutions in a variety of viral proteins. Nevertheless, amino acid alterations tend to be concentrated in the viral coat protein genes, more specifically in the amino-terminal half of the viral capsid protein, VP1. These results may imply that the mutations in the VP1 coding region contribute to attenuation.

Racaniello and Baltimore (1981a) have presented evidence suggesting that

cloned cDNA of the PV1 (M) genome is infectious in mammalian cells. An attractive approach to the identification of those mutations responsible for viral attenuation would be the study of recombinant viruses, which may be recovered by mixed transfection of the infectious cloned recombinant cDNAs derived from PV1 (M) and PV1 (Sabin) (Nomoto *et al.*, 1982). Site-directed mutagenesis is also likely to prove to be an extremely powerful tool for analyzing the relationship between the structure of the poliovirus genome and its biological significance.

VI. Influenza Virus

Influenza is unique among the viruses in its capacity for antigenic variation, and for this reason it has proved to be impossible to control it by vaccination (for reviews, see Webster *et al.*, 1982; Palese and Young, 1982). This variability of influenza viruses is in marked contrast to the antigenic properties of other viral agents, such as poliovirus or measles virus, which appear to remain essentially unchanged. The situation with influenza viruses also differs from that of herpes or rhinoviruses, which coexist as a number of variants in the population but do not undergo the rapid changes characteristic of in influenza viruses.

Influenza epidemics have been relatively mild over the past decade. However, it is useful to remember that during the winter of 1918–1919, the influenza A virus, which killed at least 20 million persons, was almost certainly antigenically related to swine virus. Influenza viruses have also long been known to cause diseases in other animals besides humans. For example, fowl plague virus causes high mortality in chickens and is of great commercial significance. Also of interest are influenza virus strains that affect turkeys, pigs, or race horses. These agents are important not only to veterinarians and others concerned with animal husbandry, but also to virologists and epidemiologists who must attempt to understand the interaction of these viruses in different species and their potential for causing disease in humans.

Virions of influenza virus contain a segmented negative-strand RNA genome. In all, eight RNA segments varying from 0.2 to 0.9 $\times$ 10^6 daltons are present in the virion (Pons and Hirst, 1968; McGeogh *et al.*, 1976; Desselberger and Palese, 1978; Scholtissek *et al.*, 1976). These segments are separately transcribed and polyadenylated in the infected cell to produce mature (plus strand) RNAs. Sequence analysis has revealed that all eight segments contain a common sequence of 12 nucleotides at the 5′ terminus and another common sequence of 13 nucleotides at the 3′ terminus (Skehel and Hay, 1978; Robertson, 1979; Desselberger *et al.*, 1980) (see Fig. 8) which

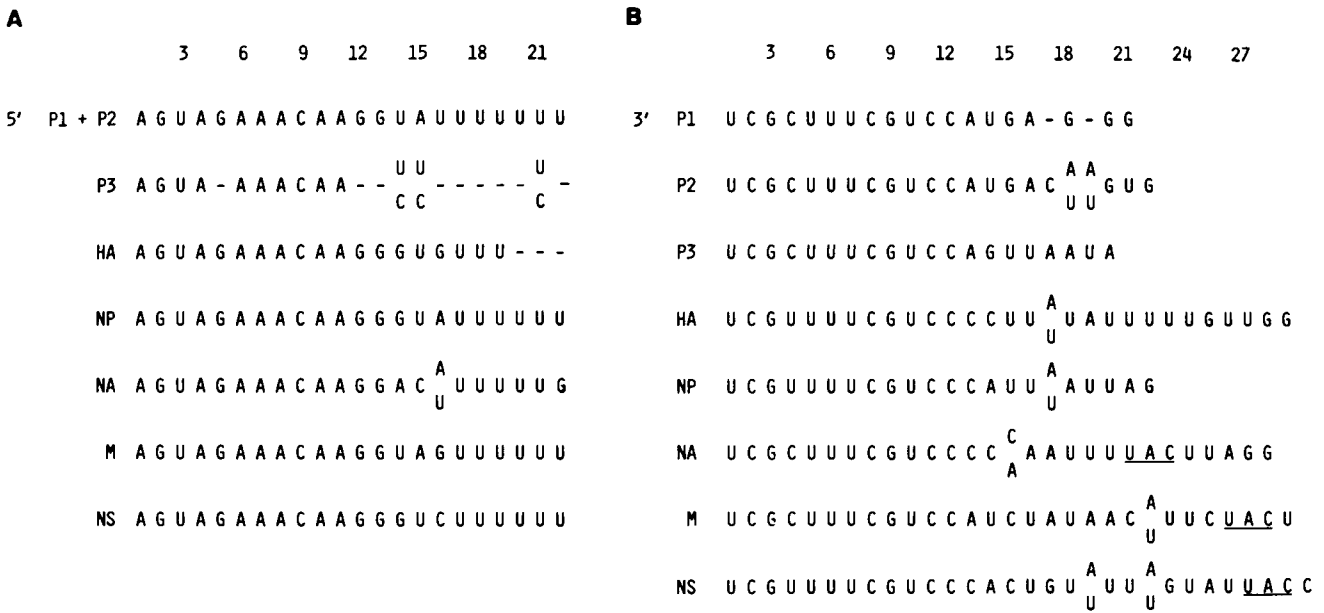

FIG. 8. Homologies between the nucleotide sequences at the 5′ termini (A) and at the 3′ termini (B), of the different influenza RNA segments.

may have roles in transcription and/or replication of the segments. These RNA segments are transcribed and polyadenylated in the infected cell to produce mature (positive-stranded) mRNA species (Etkind *et al.*, 1977; Hay *et al.*, 1977).

Each segment, apart from segment 8, appears to code for a specific viral component (Lamb and Choppin, 1979; Lazarowitz *et al.*, 1971; Inglis *et al.*, 1979); segment 8 seems to code for two nonstructural proteins (NS1 and NS2) (Both *et al.*, 1975; Colonno and Daner-Jee, 1978). The virion contains at least seven virus-coded proteins: the nucleoprotein (NP), the matrix protein (M), three polymerase proteins (P1, P2, P3), and two surface proteins, the hemagglutinin and the neuraminidase (Ritchev *et al.*, 1976; Skehel, 1972). The role that each of these viral proteins plays in the life cycle of the virus is not understood; in particular, it is unclear how the eight RNA segments are correctly assembled into the virion. The mechanism governing the genetic variation that generates the novel viral subtypes responsible for pandemics or epidemics (Lai *et al.*, 1980) is also poorly understood.

Recombinant DNA technology has provoked an explosion of structural data on the genes and gene products of the influenza viruses. It is now clear that antigenic *shift* does not occur by direct mutation of one subtype into another and antigenic *drift* occurs by point mutation in the genes.

A. GROWTH OF INFLUENZA VIRUS

Fields and Winter (1982) injected dilutions of influenza virus A/PR/8/34 (seed virus in allantoic fluid) into 10-day-old chick eggs, which were then incubated at 37°C for 48 hr. The allantoic fluid was harvested and clarified, and the virus was collected by high speed centrifugation. Subsequent centrifugation through a 15–60% sucrose gradient yielded a concentrated viral band.

B. SYNTHESIS AND CLONING OF INFLUENZA cDNA

Purified virus was adjusted to 0.5% SDS and treated with 500 μg of proteinase K per milliliter for 20 min at 56°C prior to phenol extraction and ethanol precipitation (Fields and Winter, 1982).

Two main methods have been used to synthesize cDNA.

1. *Polyadenylation of mRNA and Priming with Oligo(dT)*

Polyadenylation of viral RNA was performed using poly(A) polymerase. Oligo(dT) hybridized to these tails was used to prime reverse transcriptase, and the individual ssDNA segments corresponding to each of the RNA genes were fractionated on a 2.8% acrylamide, 7 *M* urea gel. This gel resolved

seven of the eight viral cDNA segments, which were then eluted (Maxam and Gilbert, 1977) and converted to double-stranded DNA individually (Fields and Winter, 1981).

2. *Priming with Specific Oligonucleotide*

Sequence analyses of the genome segments (Sesselberger *et al.*, 1980; Robertson, 1979) show a high degree of conservation at the 3′ ends as well as at the 5′ ends, as shown for the A/PR/8/34 viruses in Fig. 8.

It is thus possible to design oligonucleotides that will prime specifically the synthesis of influenza cDNA. Davis *et al.* (1980) have, for instance, successfully used the oligonucleotide (5′-AGCAAAAGCAGC-3′), which is complementary to the 3′-terminal sequence of the HA and NP genes for the preparation of hemagglutinin cDNA. The use of identical or closely related oligonucleotides has also been reported by Fields and Winter (1982), Lai *et al.* (1980), Fields *et al.* (1981), and Winter and Fields (1980).

To ensure production of full-length cDNA, Winter *et al.* (1981) used a second oligonucleotide (5′-AGTAGAAACAAGG-3′) complementary to the 3′ end of the sscDNA to prime second-strand synthesis.

VII. Rabies Virus

Rabies has been recognized as a transmissible disease for at least 2000 years, yet successful control of the virus has proved to be extremely difficult. This is due, at least in part, to the extensive reserve of rabies virus in populations of foxes, dogs, skunks, bats, and other animals, where the virus may often persist without producing symptoms of the disease or significant population decline.

Apart from Australia and New Zealand, and certain countries such as Britain and Japan that have succeeded in eradicating the disease, rabies is still present in most parts of the world. Infection of man, often through being bitten by a rabid animal, is usually fatal without treatment. Nevertheless, rabies is unusual in that postexposure vaccination, if administered soon, can prevent development of the disease.

Infection with rabies virus is often followed by a long incubation period. During this time the virus travels through peripheral nerves to the spinal chord and brain, and at this stage the behavioral symptoms become apparent. Subsequent appearance of the virus in other organs, such as the salivary glands, facilitates its transmission.

The virus consists of a rod- or bullet-shaped virion enclosed in a lipid bilayer through which the glycoprotein (G; hemagglutinin) protrudes. Other proteins contained in the virus include the transcriptase (L), the matrix pro-

teins (NS, M2), and the nucleoprotein (N). The viral RNA, about 10 kb of single-stranded (negative-strand) RNA, exists as a helical ribonucleoprotein complex with N protein.

After infection, mRNA transcription mediated by L proceeds directly upon the viral negative strand, and the five mRNAs, corresponding to the five viral proteins, are polyadenylated by a virus-dependent function. The generation, in addition, of full-length plus-strand transcripts permits the subsequent production of viral RNA for encapsidation in the virion.

The glycoprotein spike of rabies virus is the only protein that traverses the envelope, and for this reason it is the sole antigen capable of binding neutralizing antibodies and of eliciting their production (Cox *et al.*, 1977). Existing vaccines (for a review, see Wiktor, 1980), however, rely on the use of whole virus inactivated by treatment with β-propiolactone or a similar agent, but the high price of such vaccines has restricted their use. Several groups have therefore attempted to use recombinant DNA techniques to produce a more cost-efficient rabies vaccine.

A. PREPARATION OF RABIES GLYCOPROTEIN MESSENGER RNA

As mentioned above, the G protein is the only viral antigen capable of eliciting the production of neutralizing antibodies; thus work has concentrated on this protein.

Wunner *et al.* (1980) infected monolayer cultures of rodent cells (BHK21-S3) in roller bottles with rabies virus (ERA strain) at a multiplicity of infection of 25 and recovered the infected cells after 20 hr. Washed cells were treated for 30 min with 2.5% sodium dodecyl sulfate (SDS) and 200 μg of Pronase per milliliter. Total RNA was extracted with phenol and precipitated with ethanol prior to DNase digestion and reextraction with phenol.

This preparation was heat denatured and subjected to oligo(dT)-cellulose chromatography. RNA bound in 300 m*M* salt was eluted with distilled water and concentrated by ethanol precipitation.

Wunner *et al.* (1980) were able to demonstrate that this material, after injection into amphibian oocytes, was capable of programming the synthesis of proteins reacting with antiglycoprotein and antinucleoprotein antisera.

Since G, without its carbohydrate groups (Dietzschold, 1977), has a molecular weight of about 65,000–70,000, the mRNA should be about 1.8 kb in length and sediment at 18 S (Wunner *et al.*, 1980). N is slightly smaller (ca. M_r 60,000), and the corresponding mRNA should sediment slower, at about 16 S. Accordingly, Wunner *et al.* (1980) were able partially to separate the two messenger species. After sucrose gradient centrifugation they injected each fraction into *Xenopus laevis* oocytes and analyzed the newly

synthesized proteins with monoclonal antibodies raised against G or N. By this means they were able to isolate an mRNA fraction enriched for glycoprotein coding sequences.

B. CLONING OF GLYCOPROTEIN CODING SEQUENCES IN *E. coli*

Commencing with this enriched material, Anilionis *et al.* (1981) prepared cDNA by using AMV reverse transcriptase and an oligo(dT) primer hybridized to the polyadenylate tails. A second DNA strand was synthesized using *E. coli* DNA polymerase. After single-strand specific S1 nuclease treatment, the double-stranded cDNA was tailed (3′) with dCMP residues using terminal transferase, and large (≥ 1 kb) tailed cDNA was further purified by sucrose gradient centrifugation. This material was annealed with pBR322 plasmid, which had been previously tailed at its *PstI* site with dGMP residues, then transformed into *E. coli* strain $\chi 1776$.

Tetracycline-resistant colonies emerging from this procedure were examined for the presence of rabies sequences by hybridization with radiolabeled virion RNA. Positive clones were further examined by hybridization to sucrose gradient-fractionated mRNA. As shown in Fig. 9, two hybridization profiles were obtained, one corresponding to an 18 S mRNA, pre-

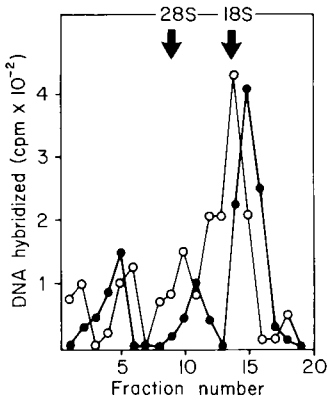

FIG. 9. Detection of mRNAs for glycoprotein and nucleocapsid by hybridization to cloned DNAs. Poly(A) RNA from cells infected with rabies ERA strain was fractionated on a 5 to 23% sucrose density gradient (76,000 *g*, 16 hr, 10°C). Fractions were hybridized in 50% formamide, 750 m*M* NaCl with purified ^{32}P-labeled cDNA fragments with plasmids B333 (nucleocapsid, •) or A344 (glycoprotein, ○). Unhybridized DNA was removed by single-stranded specific nuclease S1 treatment, and acid-insoluble material remaining was determined by precipitation with trichloroacetic acid. Adapted, with permission, from Anilionis *et al.* (1981).

sumably the G protein messanger, and the other to a 16S mRNA, the N protein messenger.

Sequence analysis of the G protein clones revealed a contiguous open reading frame comprising 1572 nucleotides capable of coding for a 524 amino acid protein (Anilionis *et al.*, 1981). Comparison of the deduced amino acid sequence with the partial amino acid sequence of mature G revealed a stretch of 19 additional residues preceding the first amino acid sequence of mature G, probably representing a signal sequence for the partially extracellular protein. Further, a domain of 22 hydrophobic amino acids exists near the C terminus of the encoded polypeptide, and this is likely to represent a transmembrane "anchor" zone for the membrane-bound glycoprotein.

Similarly, the G protein coding sequence from the related CVS strain of rabies virus has been cloned and characterized (Yelverton *et al.*, in preparation; cited in Yelverton *et al.*, 1983).

C. MAPPING OF ANTIGENIC DETERMINANTS

Dietzschold *et al.* (1982) reported that native G protein could be cleaved by cyanogen bromide into seven peptide bands, and they separated these fragments by polyacrylamide gel electrophoresis in the presence of SDS. Only two peptides (see Fig. 10) reacted with antiglycoprotein antiserum,

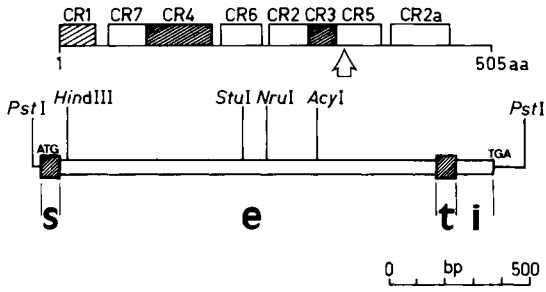

FIG. 10. The rabies glycoprotein cDNA clone and the encoded polypeptide are shown. The *Pst*I sites define the limits of the cDNA fragment inserted into the plasmid vector; additional internal restriction recognition sites are indicated; **s**, **e**, **t**, and **i** indicate the signal, extracellular, transmembrane, and intracellular segments, respectively. Cyanogen bromide fragments CR1, CR3, and CR4 elicit the formation of virus-neutralizing antibodies; CR3 and CR4 fragments were shown to react with antiserum raised against the glycoprotein. aa, Amino acids; bp, base pairs. The arrow indicates the location of the amino acid substitution that eliminates pathogenicity (see text). Data are from Dietzschold *et al.* (1982, 1983) and Anilionis *et al.* (1981).

whereas three peptides induced the formation of virus-neutralizing antibodies after injection into mice. Thus at least three antigenic determinants are present in the glycoprotein polypeptide.

In a subsequent study, Dietzschold *et al.* (1983) examined antigenic variants of rabies virus that had been selected for the ability to grow in the presence of a monoclonal virus-neutralizing antibody. In one case the nonpathogenic mutant virus isolated by this procedure was shown to have an altered amino acid (Arg → Ile) at position 333; this alteration falls outside, but adjacent to, the third region identified by analysis of cyanogen bromide peptides (see Fig. 10).

D. EXPRESSION OF RABIES GLYCOPROTEIN SEQUENCES IN *E. coli*

1. *Expression in a Bacteriophage Vector*

Bacteriophage M13 and its derivatives (Messing and Vieira, 1982; Kieny *et al.*, 1984, submitted for publication) can be usefully employed for the expression of foreign genes in *E. coli* (Slocombe *et al.*, 1982). Although a virus, M13 infection does not kill the host. Instead, the viral genome proliferates in the cytoplasm as a double-stranded circular episomal form. Concomitantly, phage particles containing circular single-stranded DNA are exported from the cell surface.

Derivatives of M13 have been constructed in which the *lac* promoter (*lac*P) has been integrated into a nonessential region of the phage genome (Messing and Vieira, 1982). The inducible (by isopropyl thiogalactoside, IPTG) *lac* promoter is adjacent to the N-terminal section of the *E. coli lac*Z gene encoding β-galactosidase and a region containing multiple restriction recognition sequences. Thus the transcription signals for efficient and controlled gene expression are followed by suitable sites for the integration of exogenous DNA segments.

It should be noted that in a related rhabdovirus, vesicular stomatitis virus (VSV), expression of the glycoprotein N-terminal hydrophobic signal peptide in *E. coli* has been shown to be detrimental to the host (Rose and Shafferman, 1981). In contrast, expression of the C-terminal hydrophobic domain had no deleterious effect. Nevertheless, this result stresses the importance of using an inducible system for the expression of foreign proteins in bacteria.

Subfragments of the rabies glycoprotein coding sequence lacking the N-terminal signal sequence region have been cloned adjacent to the *lac*P-*lac*Z fragment in such a way as to permit the synthesis of a hybrid β-galactosidase-rabies glycoprotein polypeptide (Kieny *et al.*, unpublished observations). Here advantage was taken of a unique *Hin*dIII recognition sequence lying 9 amino acids downstream from the first amino acid of the mature

glycoprotein coding sequence. These 9 amino acids were replaced, in most cases, by 11 amino acids from the N terminus of the *E. coli* β-galactosidase.

Infected bacterial cultures were induced with IPTG, radiolabeled with [^{35}S]methionine, and subjected to immunoprecipitation with polyclonal antiserum raised against the rabies virus glycoprotein. As shown in Fig. 11, the bacterially synthesized glycoprotein, and subfragments thereof, were efficiently recognized by the antiserum. Surprisingly, a number of other proteins of bacterial origin were also precipitated. This is probably due to cross-reaction between *E. coli* proteins and bacterial proteins present in the adjuvant used to prepare the antiglycoprotein antiserum.

2. *Expression in a Plasmid Vector*

Yelverton *et al.* (1983) described a cDNA clone corresponding to the G protein of a different laboratory strain of rabies, the CVS strain. At the amino acid level the two proteins share about 90% homology. In order to express the gene coding for G-CVS in bacteria, a different approach was adopted. Commencing with a cloned full-length cDNA copy of the G messenger, oligonucleotide mutagenesis (see Smith and Gillam, 1981) was used to introduce a bacterial translation initiation signal at a point immediately before the first amino acid of the mature glycoprotein coding sequence, thus "deleting" the hydrophobic signal peptide. At the C terminus, the hydrophobic trans membrane domain was removed by introducing a TAG translation stop codon at a *Bgl*II recognition site 14 amino acids prior to the hydrophobic zone. This coding sequence was placed under the control of an inducible (by tryptophan starvation) promoter derived from the *trp* operon of *E. coli*, and directed the synthesis, in *E. coli*, of a polypeptide cross-reacting with antiserum raised against the authentic rabies-CVS glycoprotein (Yelverton *et al.*, 1983).

We have seen that peptides corresponding to rabies glycoprotein can be produced in a bacterial host. This eliminates the costly production and containment procedures necessary for vaccines based upon inactivated rabies virus.

As mentioned earlier, rabies is unusual in that postinfection vaccination is effective in preventing development of the disease. Nevertheless, it has not yet been demonstrated that purified authentic glycoprotein can protect when administered after exposure, although it does elicit the production of high titers of neutralizing antibodies.

The prospects for a bacterial vaccine for preexposure use are much brighter, for purified authentic glycoprotein gives a level of protection comparable to that obtained with inactivated virus vaccine (Cox *et al.*, 1977, 1980). Levels of antibodies produced, and the protection afforded, never-

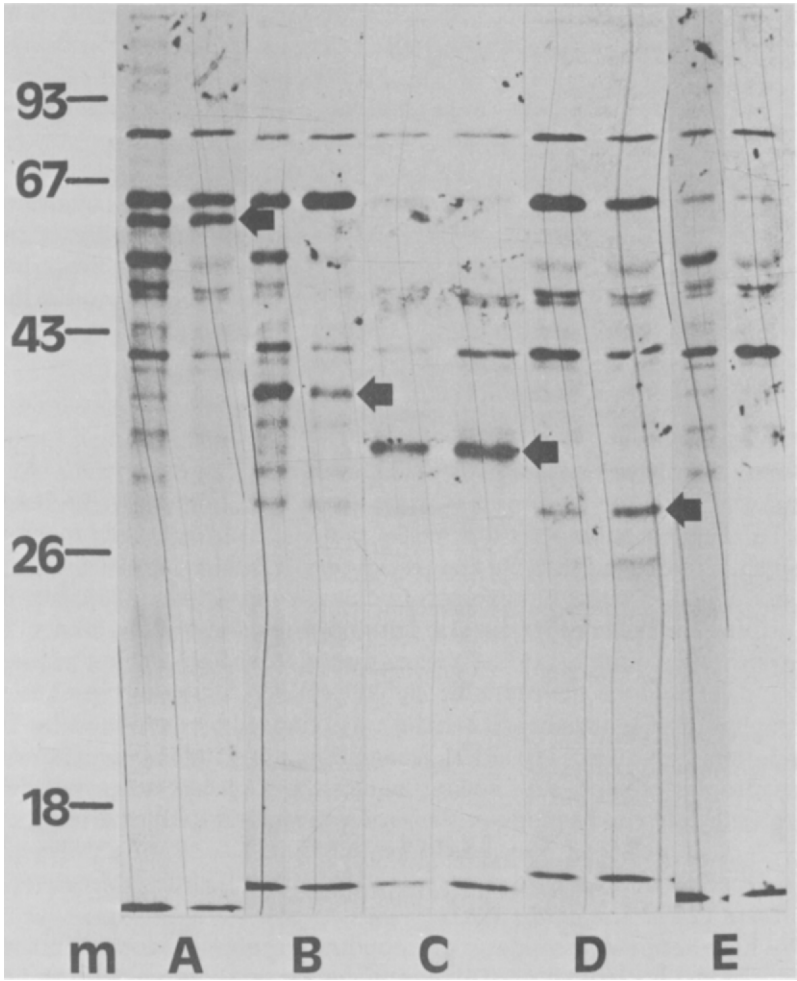

FIG. 11. Expression of rabies glycoprotein polypeptides in *Escherichia coli* (Kieny, Lathe, Lecocq, Curtis, and Wiktor, unpublished observations). Cell cultures in minimal medium were infected with various subclones of the glycoprotein cDNA in vector bacteriophage M13*tg*109, induced with IPTG and radiolabeled with [^{35}S]methionine for 1 hr at 37°C. The labeled cells were lysed by sonication, clarified, and subjected to immunoprecipitation with antiserum raised against the authentic rabies glycoprotein. Radioactive proteins bound were visualized by sodium dodecyl sulfate gel electrophoresis and fluorography. Various subfragments of the glycoprotein cDNA were inserted into the M13 vector bacteriophage: A, *Hin*dIII-PstI, B, *Hin*dIII-*Acy*I; C, *Stu*I-*Pst*I; D, *Hin*dIII-*Nru*I (see Fig. 10). In each case two independent M13 clones were examined. Lane E contains *E. coli* infected with the vector phage alone. Molecular mass standards (m) are given in kilodaltons.

theless depend greatly upon the presentation of the antigenic determinants (Wunner *et al.*, 1983).

VIII. Expression of Viral Antigens in Bacillus subtilis

A. GENE EXPRESSION VECTORS BASED ON PLASMIDS

The ability to express efficiently heterologous genes and the variety of molecular techniques now available to accomplish this have made *E. coli* the organism of choice for cloning and expressing viral genes. A foreign gene product manufactured by *E. coli* must, however, undergo costly purification procedures to free it from contaminating endotoxins, which are known to exert a pyrogenic effect on man. Biologically safe organisms, such as *B. subtilis* and *Saccharomyces cerevisiae*, have become promising alternative hosts for molecular cloning of genes whose products are destined for vaccines, foods, or beverages.

Bacillus subtilis, a nonpathogenic bacterium free of endotoxins, is not a normal resident of the gut, as is *E. coli*. Many of the *Bacillus* spp. are important producers of enzymes and antibiotics purified from highly developed, large-scale fermentation processes. Secretion of many extracellular proteins into the surrounding growth medium is an additional feature that makes *B. subtilis* a suitable candidate as the host microorganism for cloned genes. Furthermore, *B. subtilis* is the most widely studied and thoroughly mapped gram-positive microorganism. It is capable of genetic transformation at relatively high frequencies, sometimes as high as 10% (Bettinger and Young, 1975). Since the discovery that *Staphylococcus aureus* plasmids can replicate and express genetic information in *B. subtilis* (Ehrlich, 1977), recombinant DNA technology on *Bacillus* sp. has advanced rapidly. Several plasmid and bacteriophage cloning vehicles have been successfully employed (for reviews, see Dean and Dooley, 1981; Gryczan, 1982).

To express efficiently foreign genes cloned in *B. subtilis,* transcriptional and/or translational signals that differ from those of *E. coli* are required (McLaughlin *et al.*, 1981). For this reason, so-called "promoter-probe" vectors are needed to detect genetic regulatory signals that allow expression of heterologous genes in the host of interest. Plasmid vectors suitable for cloning in *E. coli* fragments of DNA that carry transcriptional promoter or termination signals are well characterized. Detection in these systems is based on expression of genes that encode β-galactosidase (Casadaban and Cohen, 1980; Casadaban *et al.*, 1980; Gentz *et al.*, 1981) or confer antibiotic resistance to host cells (West *et al.*, 1979; An and Friesen, 1979).

More recently, three promoter-probe systems for *B. subtilis* have been constructed. All are based on activating the expression of genes that are not normally expressed in *B. subtillis*. This "insertional activation" is accomplished by ligating a fragment of DNA that promotes gene expression into a cleavage site upstream from the structural gene. The latter must encode a gene product that is readily detected and its activity easily quantified. Of the three systems for *B. subtilis,* two are similar in that they are based upon expression of chloramphenicol acetyltransferase (CAT) genes originating from either *Bacillus pumilus* (Williams *et al.,* 1981) or the *E. coli* transposable genetic element Tn9 (Goldfarb *et al.,* 1981). The other approach is a method whereby fragments of DNA that promote expression of a foreign gene in *B. subtilis* are detected by a change of color of bacterial colonies (Zukowski *et al.,* 1983). This sensitive chromogenic assay is based upon the expression, in *B. subtilis*, of the *xyl*E gene that encodes for catechol 2,3-oxygenase (C230) of the TOL plasmid from *Pseudomonas putida* mt-2. A brief description of all three systems follows.

1. *Methods for Isolating Fragments of DNA That Promote Expression of CAT Genes in B. Subtilis*

In the system devised by Lovett and co-workers (Williams *et al.,* 1981), a 2.2 kb *Eco*RI-generated fragment of DNA carrying the CAT gene responsible for chloramphenicol resistance (Cm^r) of *B. pumilus* NCIB 8600 is transferred to the neomycin-resistance (Nm^r, 10 μg/ml) plasmid pUB110. The new plasmid, pPL531, confers high-level Cm^r (200 μg/ml) to *B. subtilis* host cells. Sequential digestion with *Pst*I, ligation, digestion with *Bam*HI and *Bgl*II, followed by religation leads the promoter-probe plasmid pPL603 (Fig. 12). Because the CAT gene of pPL603 no longer carries its promoter, host cells demonstrate only low-level Cm^r (5 μg/ml). Insertion of *Eco*RI or *Eco*RI* fragments of DNA into the unique *Eco*RI site upstream from the CAT gene can lead to high-level Cm^r. The authors demonstrated that select fragments of DNA from *B. subtilis, Bacillus licheniformis,* plasmids pUB110 and pPL10, and bacteriophages SP02 and ϕ105 promote expression of the *B. pumilus* CAT gene in *B. subtilis.* It was not determined whether the genetic regulatory signals exerted their control at the transcriptional or translational level.

A similar approach exploits the CAT gene from Tn9 (Goldfarb *et al.,* 1981). The promoter-probe plasmid is the 8.4 kb pGR71, an E. coli/*B. subtilis* cointegrate (Fig. 12). The plasmid confers kanamycin (Km^r)/neomycin (Nm^r) resistance to both *E. coli* and *B. subtilis* and low-level Cm^r (5 μg/ml) in *E. coli*. The CAT structural gene and its putative ribosome binding site are present on pGR71, but *B. subtilis* host cells are Cm^s and demonstrate no CAT activity as determined by spectrophotometric assay

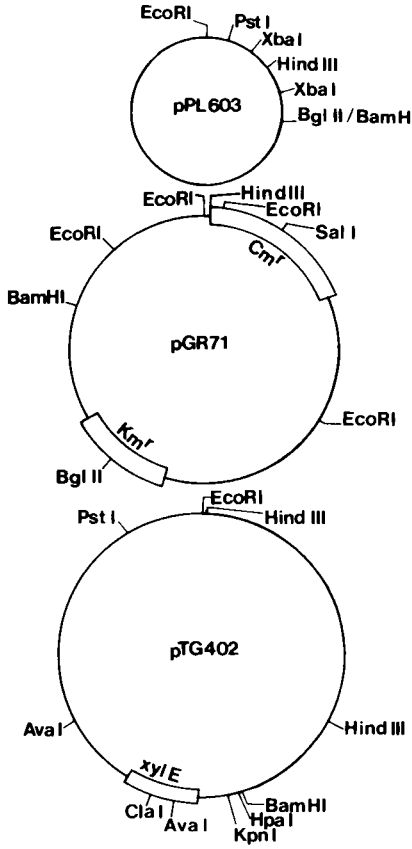

FIG. 12. Promoter-probe plasmid vectors for *Bacillus subtilis*. Top, pPL603 (after Williams *et al.*, 1981); middle, pGR71 (after Goldfarb *et al.*, 1982); bottom pTG402 (Zukowski *et al.*, 1983).

(Shaw, 1975). To activate the CAT gene in *B. subtilis*, *Hin*dIII-generated fragments of *B. subtilis* chromosomal DNA (or any appropriate source) are ligated into the unique *Hin*dIII site of pGR71, which is situated 33–38 base pairs 5′ to the AUG translational initiation site of the CAT structural gene. The ligation mixture is used directly for transformation of *B. subtilis* competent cells. Transformants are selected first on complete medium plates supplemented with Km (10 μg/ml) and are then replica-plated onto medium containing various levels of Cm. CAT activities are estimated by the ability of cells to grow on Cm medium or, more accurately, by the spectrophotometric assay. Since *B. subtilis* is unable to use the *E. coli* ribosome bind-

ing site efficiently for the Tn9-derived CAT gene on pGR71 (Goldfarb *et al.,* 1982), the fragments of DNA that generate Cm^r in *B. subtilis* must necessarily include genetic regulatory signals for transription *and* translation. CAT produced in *B. subtilis* results from a fusion between a native *B. subtilis* gene correctly in reading phase with the Tn9 CAT gene. This system, then, offers the opportunity to select amino-terminal fragments of exogenous proteins that also carry functional translational initiation signals for *B. subtilis*.

2. *Method to Isolate Fragments of DNA That Promote Expression of the xylE Gene in B. subtilis: A Chromogenic Assay*

A novel method to isolate fragments of DNA that promote gene expression in *B. subtilis*, and perhaps many other microorganisms, has been described (Zukowski *et al.,* 1983). The system is based upon production of catechol 2,3-oxygenase [C230; catechol:Oxygen 2,3-oxidoreductase (decyclizing), EC 1.13.11.2] encoded by the *P. putida* TOL plasmid gene *xyl*E. The gene is transferred as a *Bam*HI/*Xho*I fragment of TOL to the *B. subtilis*/*E. coli* cointegrate plasmid pHV33 (Primrose and Ehrlich, 1981). This results in the promoter-probe vector pTG402 (Fig. 12). Although *xyl*E is functionally expressed in *E. coli*, C230 is not detected in *B. subtilis* unless a fragment of DNA capable of promoting gene expression is ligated into a cleavage site (*Bam*HI, *Hpa*I, *Kpn*I) on pTG402 upstream from *xyl*E. The *Bam*HI site is amenable to ligation with DNA fragments generated by digestion with *Bam*HI, *Bcl*I, *Bgl*II, or *Sau*3A, whereas any blunt-ended fragment can be ligated into the *Hpa*I site. A unique feature of this system is the ease of detection: colonies of cells that express *xyl*E become yellow within seconds after Cm selection plates are sprayed with a 0.5 *M* solution of catechol. The latter, a colorless substrate, is converted by C230 to the yellow product, 2-hydroxymuconic semialdehyde. The C230 produced in *B. subtilis* is identical to that produced in *P. putida* and *E. coli* by criteria of molecular weight and enzymatic activity as determined in a simple spectrophotometric assay (Sala-Trepat and Evans, 1971). Strong complementarity between the ribosome binding site for *xyl*E and *B. subtilis* 16 S rRNA suggests that *xyl*E mRNA translation in *B. subtilis* most likely commences at the same initiation codon as that recognized by *P. putida* and *E. coli*. This system, therefore, permits the detection of transcriptional promoters; it does not necessitate the synthesis of fusion polypeptides. The authors have demonstrated that fragments of DNA from *B. subtilis, B. licheniformis, B. pumilus, E. coli,* plasmid pUB110 (unpublished), and bacteriophage ϕ29 (unpublished) all have the capacity to promote *xyl*E gene expression in *B. subtilis*. The assay is rapid and inexpensive, does not require special indicator plates but offers the advantages of a genetic indicator test (Miller,

1972), and can be used for the development of plasmid gene expression vectors.

B. EXPRESSION OF HBV CORE ANTIGEN IN *B. subtilis*

Because the construction of gene expression vectors for *B. subtilis* is a recent development, expression of viral antigens in the microorganism has yet to be fully explored. Success in expressing the genes for HBV core antigen and the major antigen of foot-and-mouth disease virus (FMDV) has, however, been realized (Hardy *et al.*, 1981). To express the HBV core antigen gene, the following method was employed.

The plasmid vector pKH80 (Fig. 13) was made by joining pBD9 (Gryczan and Dubnau, 1978; Gryczan *et al.*, 1980) to pBR322 (Bolivar *et al.*, 1977) by ligating the two plasmids at their respective and unique *Eco*RI sites. Plasmid pKH80 replicates in *B. subtilis* wherein genes to render host cells resistant to Km (10 μg/ml) and erythromycin (Em^r, 10 μg/ml) are expressed. The Em^r gene encodes a protein of 29,000 daltons, the synthesis of which is inducible by subinhibitory concentrations (0.05 μg/ml), of erythromycin. The entire sequence of the Em^r gene has been determined (Horinouchi and Weisblum, 1982). In *E. coli*, cells with pKH80 are resistant to ampicillin (Ap^r, 50 μg/ml), tetracycline (Tc^r, 15 μg/ml), Km (10 μg/ml), and Em (at high concentrations, such as 60 μg/ml, Em partially inhibits *E. coli* unless pKH80 is present).

The HBV core antigen was isolated as a *Pst*I fragment from pHBV139A (Pasek *et al.*, 1979). An average of 100 bp was trimmed from each end of the fragment by using exonuclease *Bal*31, then *Bam*HI and *Hin*dIII linkers were ligated, at random, to the extremities. Digestion with *Bam*HI and purification of the DNA fragment led to the isolation of the HBV core antigen gene flanked by a *Hin*dIII linker 5′ to the gene, a *Bam*HI linker 5′ to the *Hin*dIII linker, and a single *Bam*HI linker 3′ to the HBV core gene. This *Bam*HI fragment was amplified by cloning it in the *Bam*HI site of pBR322. The *Bam*HI-generated fragment was ligated in the unique *Bcl*I site of pKH80; the *Bcl*I cleavage site lies within the Em^r gene. This construct maintains the proper reading frame for expressing the HBV core antigen under the control of genetic regulatory signals for the inducible Em^r gene on the plasmid vector. Ribosomes translating the mRNA of the Em^r gene should arrive in phase at a termination codon (UAG) that lies 4–6 bp 5′ to the AUG initiation codon of the HBV core antigen gene. Translation would then start de novo from the transcript of the viral DNA sequence.

Although *B. subtilis* strains that carry this hybrid plasmid gave positive results when assayed for HBV core antigen by solid-phase radioimmunoassay, the concentration of HBV core protein was determined to be $\leq$

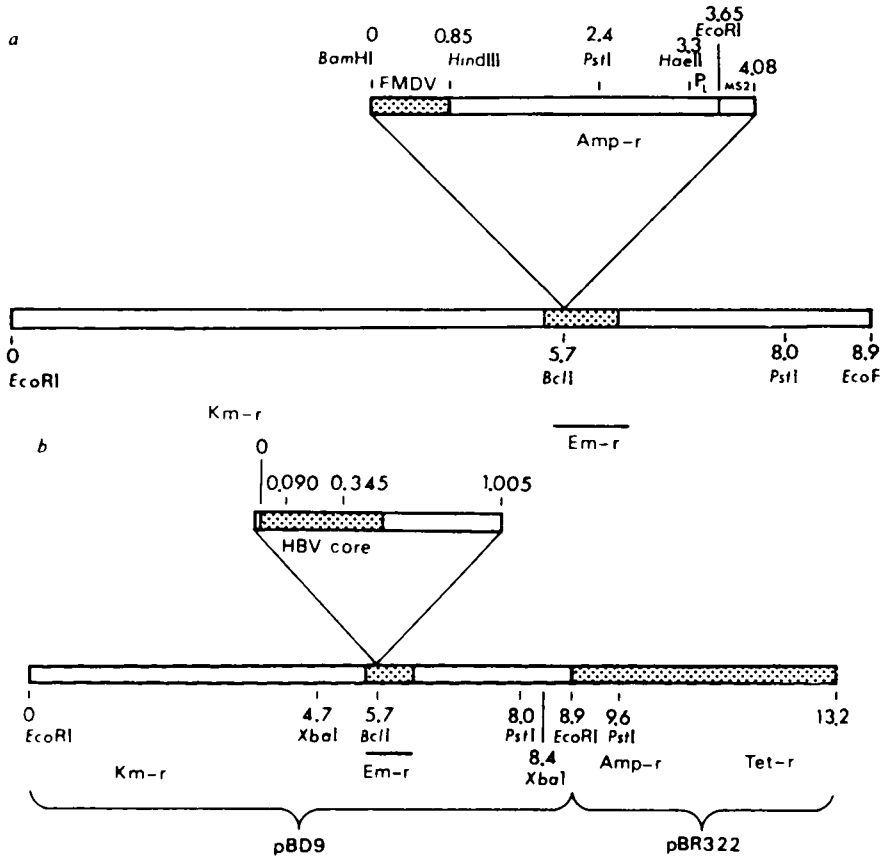

FIG. 13. (a) Restriction endonuclease cleavage map of pEVP1 used to express the major antigen of FMDV in *Bacillus subtilis*. The upper line represents pPLVP1 (Küpper *et al.*, 1981); the lower line represents pBD9 (Gryczan and Dubnau, 1978; Gryczan *et al.*, 1980). (b) Restriction endonuclease cleavage map of pKH80 (lower line) and derivatives containing the HBV core gene (upper line). Reprinted by permission from Hardy *et al.*, *Nature* **293**, 481–483. Copyright © 1981, Macmillan Journals Limited.

0.1% of total cellular protein. The stop-start type of construction is presumably inefficient in this case, but expression might be increased by incorporating an efficient ribosome binding site for *B. subtilis* upstream from the restart site.

C. EXPRESSION OF THE MAJOR FMDV ANTIGEN IN *B. subtilis*

To express the VP1 antigen for FMDV in *B. subtilis*, Hardy *et al.* (1981) utilized the VP1 gene from *E. coli* plasmid pPLVP1 (Küpper *et al.*, 1981).

The latter was digested with *Bam*HI then joined to pBD9 previously digested with *Bcl*I. In one of the two possible orientations, the pPLVP1 and pBD9 hybrid plasmid (pEVP1, Fig. 13) should result in a DNA sequence encoding a fusion polypeptide comprising 73 amino acids of the Em^r gene on pBD9, 284 amino acids of FMDV, and 13 amino acids of the pPL c24 moiety of pPLVP1. The plasmid was introduced into *B. subtilis* by transformation, then cells were treated with subinhibitory concentrations of Em to induce expression of the fused gene. Proteins from an extract of these cells were resolved on a SDS–polyacrylamide (10%) gel. The polypeptides in the gel were transferred to nitrocellulose filters (Bowen *et al.*, 1980), then a radioimmunoassay was used to detect the expected 41,000-dalton fusion polypeptide that binds VP1-specific antibodies. Approximately 1% of the total cellular protein was calculated to be the VP1 protein in its hybrid form.

IX. Expression of Viral Antigens in Saccharomyces cerevisiae

A. PLASMID VECTORS FOR EXPRESSION OF GENES IN YEAST

The yeast *Saccharomyces cerevisiae* has been developed as a host organism for cloned genes. Because it is a eukaryotic organism, yeast is a suitable host for the expression of genes from higher organisms: posttranscriptional and posttranslational processes closely resemble those of higher cells, the organism has complex membrane systems, and the ability to secrete and glycosylate proteins has been observed (Novick *et al.*, 1980). Cloning vectors for yeast are normally constructed as yeast/*E. coli* cointegrate plasmids. They consist of an origin of replication and antibiotic resistance gene(s) that function in *E. coli,* the 2-μm DNA sequence (Cameron *et al.*, 1977) needed for replication of the plasmid in yeast, and a yeast gene that encodes a product that allows selection by complementation in yeast and *E. coli* recipient cells that carry the corresponding mutation. For details on cloning procedures in yeast, Beggs (1981) provides an excellent review.

B. EXPRESSION OF HBV SURFACE ANTIGEN IN YEAST

As previously noted, the HBV surface antigen gene cloned in *E. coli* results in very inefficient production of HBsAg. However, in the yeast systems described below, not only is a substantial level of HBsAg produced, but it is synthesized in the form of particles or aggregates similar in size and shape to those found hepatoma cell lines or in sera from HBV-infected individuals.

In the method of Valenzuela *et al.* (1982), the HBsAg gene was expressed

in yeast by using a vector that utilizes the 5′-flanking region of the yeast alcohol dehydrogenase I (ADH I) gene as a promoter to transcribe HBsAg coding sequences. The HBsAg gene was isolated on a 835 bp *Tac*I–*Hpa*I fragment from pHVB-3200 (Valenzuela, *et al.*, 1979). The fragment, which carries 26 bp preceding the AUG methionine codon of the mature HBsAg, was ligated to *Eco*RI linkers and cloned in the *Eco*RI site of pBR322. The *Eco*RI-generated fragment was then purified by preparative gel electrophoresis and ligated to the yeast/*E. coli* hybrid plasmid, pMA-56, which was previously digested with *Eco*RI and treated with bacterial alkaline phosphatase. The resulting plasmid (Fig. 14) was introduced into yeast cells, and HBsAg was detected by radioimmunoassay from cell extracts of mid-log phase cultures. From the assay data, it was calculated that 10–25 ng of HBsAg were made per milliliter of yeast culture.

An alternative method to express HBsAg in yeast employs the promoter from the gene P-60, which encodes a 60-kilodalton acid phosphatase (Miyanohara *et al.*, 1983). The expression of the P-60 gene is controlled by the level of inorganic phosphate (P_i) in the growth medium; i.e., low levels of P_i (1.5 g/liter in Burkholder minimal medium) induce the synthesis of P-60. Cells are grown to a density of 4×10^6 cells/ml in high P_i (1.5 g KH_2PO_4/liter), then a sample is removed, centrifuged, and suspended in low-P_i minimal medium for induction. Control samples are treated similarly, except that resuspension is in high-P_i minimal medium.

The plasmid vector pAM82, used to express HBsAg, was constructed from pAT77, which carries the sequence of the NH_2-terminal coding region of P-60 and its upstream control region (Rogers *et al.*, 1982). The P-60 polypeptide coding sequence was completely removed by digesting pAT77 with *Sal*I. The plasmid was then treated with exonuclease BAL-31 under conditions adjusted to remove 85–130 bp of DNA, and ligated with *Xho*I linkers.

HBV DNA was recovered from pHBV4, a derivative of pACYC177 that carries the entire HBV (subtype *adr*) genome in double-stranded form as a *Xho*I fragment. pHBV4 was digested with *Xho*I to yield a fragment of DNA with a *Xho*I terminus located 27 bp upstream of the methionine codon for the mature HBsAg. The *Xho*I-generated fragment was ligated in the *Xho*I site of pAM82.

The recombinant plasmid was propagated in *E. coli* χ1776, then transformed into a yeast recipient strain AH22 (a *leu*2 *his*4 *can*1 *cir*$^+$) or its acid phosphatase constitutive derivative, AH22 *pho*80. Cells were grown in liquid medium, then induction was performed in phosphate-free medium. Radioimmunoassays for HBsAg from cell extracts demonstrated that induction was necessary for high-level synthesis of HBsAg. Induced cells were estimated to produce 250 ng of HBsAg per milliliter of yeast culture. Uninduced cells produced 10^3-fold less HBsAg.

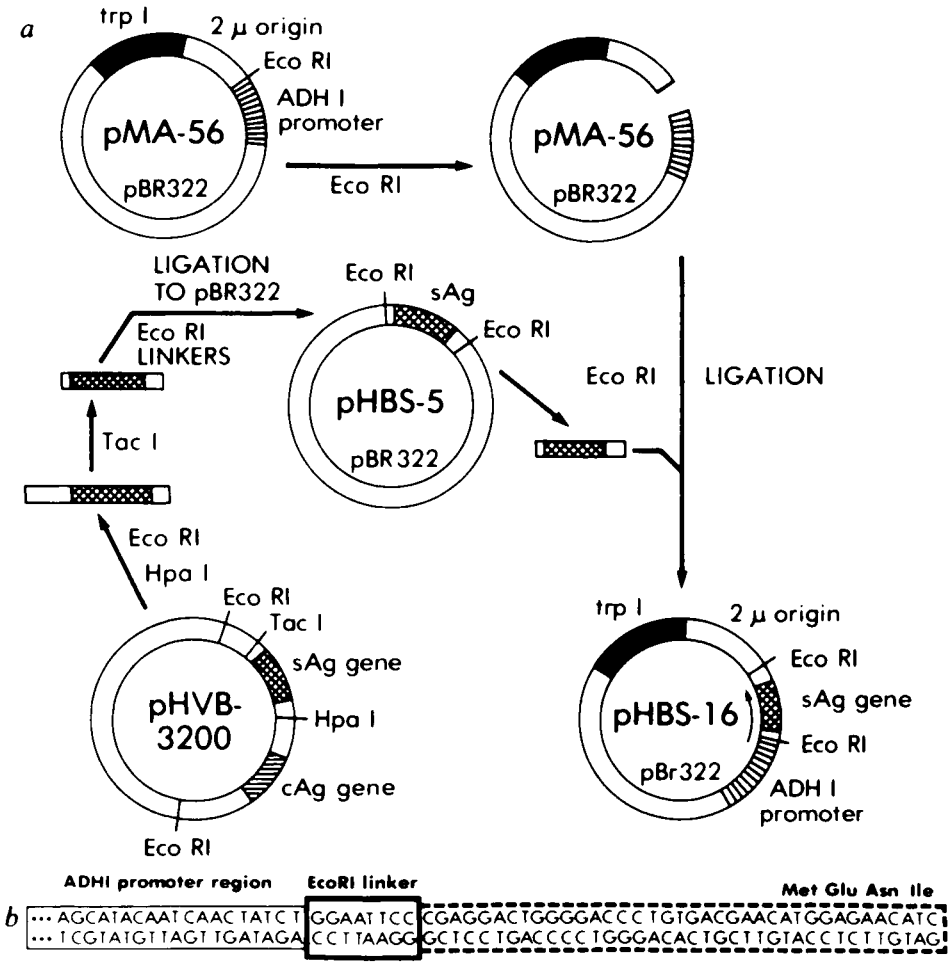

FIG. 14. (a) Construction of plasmids for the expression of HBV surface antigen in yeast. (b) Nucleotide sequence of the ADH1-HBsAg gene junction present in pHBS-16. Reprinted by permission from Valenzuela *et al.*, *Nature* **298**, 347–350. Copyright © 1982, Macmillan Journals Limited.

HBsAg produced in yeast by either cloning method can be purified by equilibrium sedimentation through a discontinuous CsCl gradient, where it bands at 1.2 g/cm^3. Velocity sedimentation in sucrose gradients demonstrates that HBsAg from yeast sediments at ~50S. Both values are in agreement with those of HBsAg from the human hepatoma cell line PLC/PRF/5 (Alexander *et al.*, 1976) and suggest that HBsAg is synthesized in yeast in

the form of particles. Examination of yeast HBsAg purified by sedimentation or by precipitation with anti-HBsAg antibodies established that the yeast particles are more variable in size and smaller than Dane particles. The particles are free of DNA and do not contain significant quantities of higher molecular weight glycoprotein, which suggests that glycosylation is not required for particle formation. Because the HBsAg produced in yeast was demonstrated to be sufficiently immunogenic, this suggests that glycosylation is also not required for immunogenicity. Taken together, the data clearly suggest that the HBsAg particle produced in yeast bears valuable potential for use as a vaccine.

X. Conclusion

In this review we have discussed in some depth how viral antigens can be cloned and expressed in *E. coli* and other microorganisms. For the most part we have not approached in similar depth questions of how suitable such microbially synthesized antigens are likely to be for vaccine purposes.

However, the production of viral proteins via recombinant DNA technology has so far yielded extremely promising results, and we await developments in this field with expectation.

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6 *Spot Hybridization for Detection of Viroids and Viruses*

R. A. Owens and T. O. Diener

I. Introduction

Viroids are low-molecular-weight, covalently circular RNAs present in certain species of higher plants afflicted with specific diseases. They are not detectable in healthy individuals of the same species, but, after introduction into such individuals, they are replicated autonomously and cause the appearance of the characteristic disease syndrome (Diener, 1979). Viroids constitute a novel class of subviral pathogens and, as the smallest known agents of infectious disease, represent a minimal genetic and biological system. Although viroids have been discovered because they cause readily recognizable disease symptoms in certain hosts, they are often replicated in other hosts without causing obvious damage.

Several plant diseases that are now known to be viroid incited are of considerable economic importance (reviewed by Diener, 1979). Although

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at least one viroid, the potato spindle tuber viroid (PSTV), can be eliminated from infected plants by cold treatment and meristem culture (Lizarraga *et al.*, 1980), most control measures are based on prevention rather than cure, thus necessitating reliable and sensitive methods for their detection in plant stock. Because viroids lack the antigenic protein coat that encapsidates viral and "viroid-like" (Randles *et al.*, 1981) RNAs, they pose a problem in diagnosis. Not surprisingly, viroid assays based on immunological principles, such as the sensitive and widely used enzyme-linked immunosorbent assay (ELISA) technique, have not been reported.

Two types of diagnostic test have been used to detect PSTV: bioassay on suitable tomato cultivars (Raymer and O'Brien, 1962; Fernow, 1967) and polyacrylamide gel electrophoresis (PAGE) of extracted nucleic acids (e.g., Morris and Wright, 1975; Pfannenstiel *et al.*, 1980). Although Fernow *et al.* (1969) have demonstrated that a double inoculation technique can be used to detect both mild and severe strains of PSTV from potato seed stocks, bioassays on tomato are slow and unreliable under certain environmental conditions. Detection by PAGE, on the other hand, is laborious and expensive. Neither method is suitable for the rapid screening of thousands of seed potato tubers.

Nucleic acid hybridization in solution is extremely sensitive and has been used to quantitate viroid concentrations in purified RNA preparations (Owens *et al.*, 1978; Palukaitis and Symons, 1979) and to index avocados for the presence of avocado sunblotch viroid (Palukaitis *et al.*, 1979, 1981; Allen and Dale, 1981), but even partial purification of nucleic acids from multiple samples is laborious. One possible alternative is hybridization of highly radioactive DNA complementary to PSTV (PSTV cDNA) with PSTV bound to a solid support, followed by autoradiographic detection of the resulting DNA–RNA hybrids.

Two conditions had to be fulfilled before hybridization with PSTV cDNA could be developed into a practical diagnostic test for the detection of PSTV in numerous samples: (1) PSTV cDNA had to be available in unlimited quantities and at high specific radioactivity, requiring molecular cloning of the cDNA by recombinant DNA technology and *in vitro* labeling; and (2) to expedite and simplify sample preparation, clarified sap rather than purified nucleic acid had to be suitable as the viroid source. Both conditions have been fulfilled, and a practical diagnostic test for the detection of PSTV based on nucleic acid hybridization has been developed (Owens and Diener, 1981). It is also at least 10 times more sensitive than PAGE. In this chapter we describe existing nucleic acid spot hybridization methodology and consider modifications that may be required for its use with other viroids or plant viruses.

II. PSTV Diagnosis by Nucleic Acid Spot Hybridization

Figure 1 summarizes the sample preparation and testing procedure developed by Owens and Diener (1981). Clarified plant sap, rather than purified nucleic acid, serves as the source of PSTV. The relatively high ionic strength and diethyldithiocarbamate concentration of the extraction buffer are required to release PSTV from nuclei (Diener and Raymer, 1969) and inhibit enzymatic polyphenol oxidation (Loomis, 1974). After any PSTV present in the sap samples has been bound to the nitrocellulose membrane, the immobilized PSTV is hybridized with ^{32}P-labeled recombinant DNA containing sequences complementary to PSTV. The resulting ^{32}P-labeled PSTV cDNA–PSTV hybrids are detected by autoradiography. Although the entire testing procedure requires 4 days for completion, no manipulations are required during much of this time. Many samples may be applied to one membrane and processed simultaneously. Automated sample preparation (Gugerli, 1979) may further reduce the labor required.

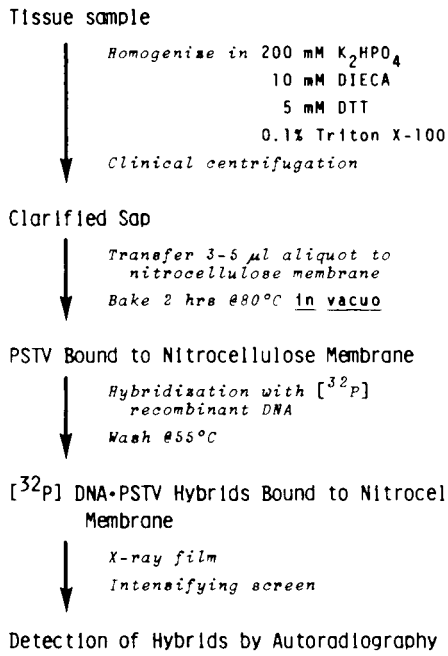

FIG. 1. Outline of nucleic acid hybridization procedure. DIECA, Sodium diethyldithiocarbamate; DTT, dithiothreitol.

A. PREPARATION OF SAMPLE

1. Homogenize a small sample of tissue in an appropriate volume of freshly prepared 200 m*M* K_2HPO_4–10m*M* sodium diethyldithiocarbamate–5 m*M* dithiothreitol–0.1% Triton X-100. Conical ground-glass tissue homogenizers (Bellco Glass Inc.,* Cat. No. 1977) are useful when only a limited number of samples must be homogenized. A buffer:tissue ratio of 1.5 ml/g for leaf or tuber sprout samples or 0.2 ml/16 seeds for true potato seeds has given good results. Seeds are allowed to imbibe water overnight on moist filter paper before homogenization.
2. Centrifuge the homogenate for 2–3 min at top speed in a clinical centrifuge to remove cellular debris.
3. Prepare nitrocellulose membranes (Cat. No. BA85, Schleicher & Schuell) by wetting with water, equilibrating with two or three changes of 3 *M* NaCl–0.3 *M* sodium citrate, blotting on absorbent tissue, and drying at 60°C (Thomas, 1980). Spot small (2–3 μl) aliquots of clarified sap on nitrocellulose membranes and bake for 2 hr at 80°C in a vacuum oven. After baking, the membranes are quite brittle but can be stored indefinitely at room temperature in a desiccator.

B. DNA–RNA HYBRIDIZATION

Articles by Maniatis *et al.* (1982) and Alwine *et al.* (1979) should be consulted for comprehensive discussions of the techniques used in experiments involving recombinant DNA or nucleic acid hybridization where one of the reactants is immobilized. The following conditions have been specifically developed for PSTV–PSTV cDNA hybridization under stringent conditions (Owens and Diener, 1982).

1. Combine 20 ml of deionized formamide, 5 ml of 1.8 *M* NaCl–168 m*M* sodium cacodylate–32 m*M* cacodylic acid–10 m*M* EDTA (pH 7.0), 0.25 ml of 20% SDS, 1 ml of 20 mg/ml yeast tRNA, 2.5 ml of 2% bovine serum albumin–Ficoll–polyvinylpyrrolidone solution, 10 ml of 50% (w/v) dextran sulfate, and 11.25 ml of water. Formamide is deionized by stirring with Bio-Rad AG501–X8 mixed-bed resin (3.5 g/100 ml, 1 hr per change of resin) until the conductivity measures less than 50 μmho, and it is then stored at −20°C. We have found that addition of bovine serum albumin, Ficoll, and polyvinylpyrrolidone to final concentrations of 0.1% is unnecessary, but

*Mention of a commercial company or specific equipment does not constitute its endorsement by the U.S. Department of Agriculture over similar equipment or companies not named.

others (Wahl *et al.*, 1979) have found these components to be essential to prevent nonspecific binding of ^{32}P-labeled probe DNA to the nitrocellulose membrane during hybridization.

2. Seal the nitrocellulose membrane in a plastic pouch, clip off one corner, and add an appropriate volume of the above solution ($\geq$ 1 ml/35 cm^2). Be sure that the membrane is completely wet with the hybridization solution before adding the ^{32}P probe.
3. Combine equal volumes of ^{32}P-labeled probe DNA and deionized formamide, seal in a glass capillary, heat for 2 min at 100°C, and quench in an ice-water bath. Add the denatured [^{32}P]DNA to the contents of the plastic pouch, being careful not to place it directly on the nitrocellulose membrane.
4. Reseal the corner of the pouch after removing as much air as possible and lay the pouch on a smooth, flat surface. Briefly "massage" the pouch with a pipette to distribute the ^{32}P-labeled probe DNA evenly.
5. Incubate overnight ($\geq$ 10 hr) in a water bath at 55°C.
6. Remove the nitrocellulose membrane from the pouch and wash at 55°C with 4–5 changes of 0.36 *M* NaCl–20 m*M* Tris-HCl (pH 7.5)–0.1% SDS and two changes of the same buffer diluted 10-fold. Each washing step lasts 10–15 min. Blot on absorbent paper and allow the membrane to dry at room temperature.
7. Visualize ^{32}P-labeled PSTV cDNA–PSTV hybrids by autoradiography at −70°C using Dupont Cronex Lightning-Plus intensifying screens and either Kodak XAR or XRP X-ray film. A thin sheet of Mylar placed between the film and nitrocellulose membrane will prevent chemical exposure of the film during autoradiography.

C. ISOLATION AND RADIOACTIVE LABELING OF PLASMID DNA

The construction of recombinant plasmid DNAs containing sequences complementary to PSTV has been described by Owens and Cress (1980). Plasmid DNA is isolated by the boiling lysis procedure of Holmes and Quigley (1981) and labeled by *in vitro* "nick translation" (Rigby *et al.*, 1977) in the presence of [α-^{32}P]dCTP according to a protocol supplied by the Amersham Corporation.

1. *Plasmid Isolation*

1. Prepare a 20-ml overnight culture of *Escherichia coli* C600 carrying the desired recombinant pBR322 plasmid. The culture medium is LB broth containing the appropriate antibiotic—20 μg of tetracycline per milliliter if the viroid DNA has been inserted in the ampicillin resistance gene or 50 μg of ampicillin per milliliter if the viroid DNA

has been inserted in the tetracycline resistance gene. LB broth contains 10 g of Bactotryptone, 5 g of Bacto yeast extract, and 10 g of NaCl per liter; the pH is adjusted to 7.4 with 1 *M* NaOH before sterilization.

2. Eighteen hours later, inoculate 1 liter of LB broth containing antibiotic with the 20-ml overnight culture. Incubate with shaking at 37°C for 3–4 hr until the cell density reaches 120–150 Klett units (No. 54 filter). Add 200 mg of chloramphenicol and continue incubation with shaking overnight.
3. Collect the bacteria by centrifugation (10 min at 10,000 rpm in a Sorvall GSA rotor). Resuspend the pellets in a total of 70 ml of 8% sucrose–5% Triton X-100–50 m*M* Tris-HCl–50 m*M* EDTA (pH 8.0), add 6 ml of 1% lysozyme solution, and distribute between two 125-ml Erlenmeyer flasks.
4. Immerse in a boiling water bath for 10 min with intermittent stirring. The resulting lysate should turn milky.
5. Centrifuge for 45 min at 19,000 rpm in a Sorvall SS-34 rotor. Measure the supernatant volume, dispense into centrifuge tubes, and add an equal volume of isopropanol to each tube. Mix and allow precipitation to proceed for at least 15 hr at −20°C.
6. Collect the nucleic acid precipitate by a 10-min centrifugation in a Sorvall SS-34 rotor at 11,000 rpm. Rinse the pellets with 70% ethanol and invert the tubes to dry the pellets. A gentle stream of N_2 can be used to hasten the drying.
7. Resuspend the pellets in 25.6 ml of 10 m*M* Tris-HCl–1 m*M* EDTA (pH 8.0) and add 600 μl of pancreatic RNase (2.5 mg/ml) and 120 μl of T1 RNase (5000 units/ml) and incubate for 15 min at 37°C. Pancreatic and T1 ribonuclease stock solutions contain 50 m*M* Tris-HCl (pH 7.5)–1 m*M* EDTA–50 m*M* NaCl–5% glycerol. The pancreatic RNase stock solution is heated at 90°C for 15 min to inactivate any contaminating DNase activity.
8. Add 29.6 g of CsCl and mix very gently. Add 6.4 ml of a 5 mg/ml ethidium bromide solution and centrifuge for 20 hr at 48,000 rpm in a Beckman VTi80 rotor at 20°C.
9. Visualize the band containing supercoiled plasmid DNA with long-wavelength UV illumination, and collect the DNA via a 16-gauge syringe needle inserted through the tube wall. Add additional CsCl solution ($\rho = 1.57$ g/ml) containing 800 μg of ethidium bromide per milliliter as necessary and recentrifuge as above.
10. Collect the supercoiled plasmid DNA and extract 5–10 times with equal volumes of isopropanol equilibrated with a saturated solution of CsCl. Dialyze at 4°C against four 2-liter volumes of 10 m*M* Tris-

HCl–1 m*M* EDTA (pH 8.0) and store at 4°C. A yield of >1 mg of DNA per liter of culture is typical.

2. *Nick Translation of Plasmid DNA*

The procedure described below is essentially that supplied by the Amersham Corporation as part of their Nick Translation Kit (Cat. No. N.5000). Specific activities of 1 to 2 × 10^8cpm/μg are routinely obtained, and the ^{32}P-labeled probe DNA remains usable for 3–4 weeks after *in vitro* labeling.

1. Combine on ice 5 μl (1 μg) of plasmid DNA, 10 μl (100 μCi) of stabilized aqueous [α-^{32}P]dCTP (specific activity = 400 Ci/mmol), 4 μl of Amersham nick translation buffer, and 2 μl of Amersham DNA polymerase I–pancreatic DNase solution. Incubate for 2–3 hr at 15°C before adding 80 μl of 2 m*M* EDTA (pH 8.0) and 1 μl (20 μg) of yeast tRNA.
2. Extract with an equal volume of buffered phenol–$CHCl_3$ mixture (1:1) and chromatograph on a small column of Sephadex G-50 fine to remove unincorporated [α-^{32}P]dCTP as described by Maniatis *et al.* (1982). Store at −20°C.

Figure 2 illustrates the type of data obtained when nucleic acid spot hybridization was used to examine several types of tissue for the presence of PSTV. Inspection of rows A and C shows that PSTV is still detectable after 625-fold dilution of infected tomato leaf sap with sap prepared from uninfected leaves. No hybridization is observed with sap from uninfected leaf tissue. PSTV may constitute as much as 1–2% of the 2 *M* LiCl-soluble RNA extracted from systemically infected leaf tissue, a concentration equivalent to 1–2 mg/kg fresh weight (Niblett *et al.*, 1980). A 2 to 3-μl aliquot of infected leaf sap should, therefore, contain ≤10 pg of PSTV.

PSTV can be detected also in extracts prepared from true seed harvested from PSTV-infected potatoes (rows B and D) and tuber sprouts (row E). The dilution series suggests that seed transmission of PSTV at rates approaching 1% can be detected. Three of the four tubers tested were infected with PSTV.

Comparison of spot intensities from clarified and deproteinized samples (compare row A with row C and row B with row D) shows that the presence of protein reduces the binding of PSTV to the nitrocellulose membrane approximately 3-fold. In other experiments binding was reduced as much as 10-fold (Owens and Diener, 1981).

Three relatively minor modifications of our routine protocol could significantly increase its sensitivity: use of higher specific activity ^{32}P-labeled probe DNA, increasing the duration of autoradiography (typically 24–48 hr in our experiments), and deproteinization of the clarified sap samples

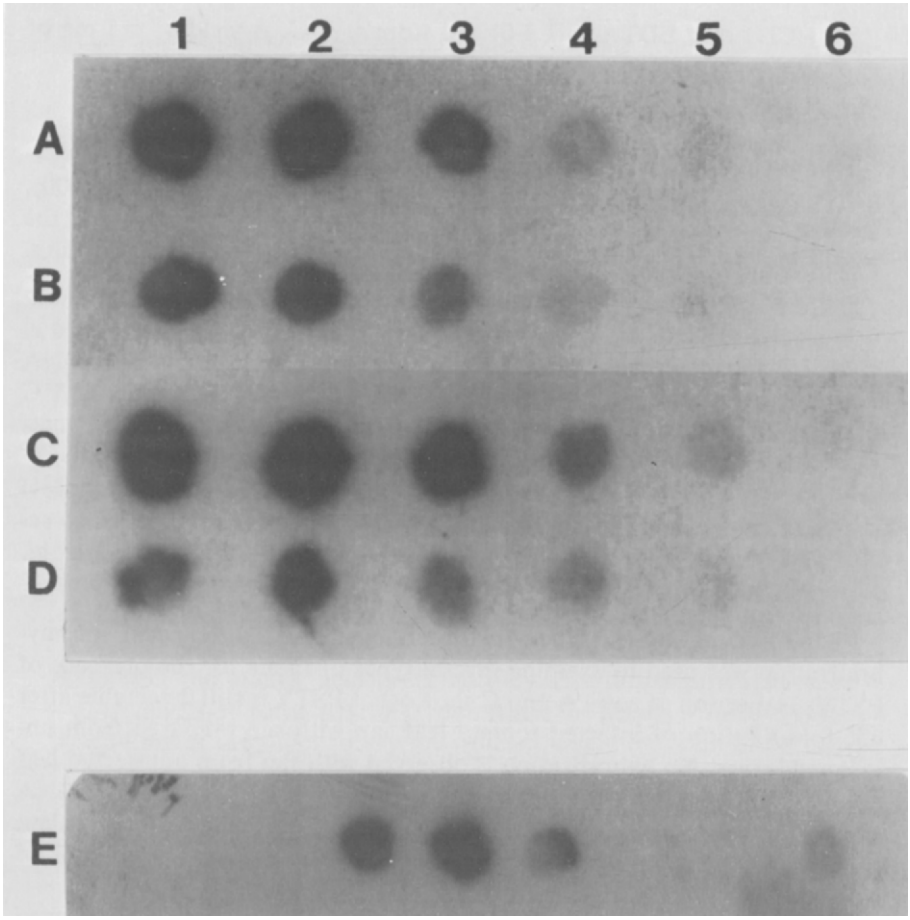

FIG. 2. Detection of PSTV in three different tissues by nucleic acid spot hybridization. Rows A and C: extract from PSTV-infected tomato leaves, undiluted and diluted 1:5, 1:25, 1:125, and 1:625 with extract from uninfected tomato leaves (columns 1–5, left to right). Rows B and D: extracts of seeds from PSTV-infected potato plants, undiluted and diluted 1:3, 1:9, 1:27, and 1:81 with extracts of seeds from uninfected plants (columns 1–5, left to right). Samples in column 6, rows A–D, are undiluted extract from uninfected tissue. Samples in rows C and D were extracted with phenol and chloroform before application to the nitrocellulose membrane. Row E contains samples prepared from potato tuber sprouts (columns 1–4), uninfected tomato leaves (column 5), and extract from PSTV-infected tomato leaves diluted 1:125 with extract from uninfected tomato leaves (column 6).

by phenol-$CHCl_3$ extraction. The specific radioactivity of the probe DNA can be increased either by raising the concentration of [α-^{32}P]dCTP during nick translation according to the Amersham Corporation protocol or by nick translation in the presence of more than one labeled deoxynucleotide triphosphate. The desirability of any of these modifications depends upon the requirements of an individual experiment.

III. Application to Other Viroid Diseases

Diagnostic tests paralleling the one developed for the detection of PSTV evidently may be initiated for the diagnosis of economically important diseases caused by other viroids. Indeed, Murphy *et al.* (1982) have shown that concentrations of avocado sunblotch viroid as low as 40–100 pg per gram of leaf fresh weight can be detected by nucleic acid spot hybridization when concentrated nucleic acid extracts are applied to the nitrocellulose membrane. The extensive sequence homologies between PSTV and certain other viroids (Visvader *et al.*, 1982; Gross *et al.*, 1982) may allow the use of specific fragments of cloned PSTV cDNA as probes for their detection, but synthesis and molecular cloning of the respective viroid cDNAs will probably give more reliable results.

Diagnostic tests are most urgently needed with vegetatively propagated crops that are susceptible to viroid-induced diseases, crops such as chrysanthemum (stunt and chlorotic mottle viroids), hops (stunt viroid), citrus (exocortis viroid), and avocado (sunblotch viroid). Specific spot hybridization tests for these viroids would replace presently used, less satisfactory diagnostic procedures based on bioassay in suitable indicator plants or PAGE analysis. Bioassays for avocado sunblotch and cadang-cadang viroids may require as long as 2 years to complete.

Nucleic acid spot hybridization tests may also become useful for the early detection of viroid infection in young seedlings. A case in point is the coconut cadang-cadang disease (Haseloff *et al.*, 1982, and references cited therein). Because of the great sensitivity of the hybridization test, routine testing of seedlings in new plantations could identify infected palm trees long before symptoms appear or before the cadang-cadang viroid is detectable by PAGE analysis. Early roguing of infected palm trees might materially diminish tree-to-tree spread of the viroid in these plantations. Similar considerations may apply to avocado plantations and their protection from the avocado sunblotch viroid (Palukaitis *et al.*, 1979, 1981; Allen and Dale, 1981; Murphy *et al.*, 1982).

Each viroid–host combination to be tested will present unique difficulties. Problems encountered in the design of a rapid indexing procedure for

avocado sunblotch viroid illustrate two important problems in viroid diagnosis: low and extremely variable viroid titers and the presence of substances in crude extracts that interfere with the hybridization reaction. Palukaitis *et al.* (1981) have measured the concentration of avocado sunblotch viroid by RNA-cDNA hybridization in solution and found that it varied 10,000-fold—from 0.2% (comparable to PSTV-infected tomato) to as low as 2×10^{-5}%. Where such a wide range of concentrations is present, the limit of detectability can often be lowered by increasing the concentration of the nucleic acid to be tested. When the concentration of avocado nucleic acid was increased, however, impurities (possibly polysaccharides) in the partially purified nucleic acid extracts led to increased viscosity and spurious hybridization results. Allen and Dale (1981) found that CF-11 cellulose chromatography of low-molecular-weight avocado RNA preparations could increase subsequent hybridization sensitivity 3- to 10-fold. Successful indexing for some viroids will, therefore, require the use of concentrated, partially purified nucleic acid rather than clarified sap as the source of viroid (Murphy *et al.*, 1982).

IV. Application to Viral Diseases

A quantitative immunochemical technique known as enzyme-linked immunosorbent assay (ELISA) has been widely adopted for the diagnosis of plant virus diseases and the measurement of low virus concentrations (reviewed by van Regenmortel, 1982; pp. 112–120). Numerous variations of ELISA have been developed, including an indirect form using antiserum to a single virus strain and an antiglobulin conjugate that can detect even distantly related viral serotypes. Antigen concentrations as low as 1–10 ng/ml can be detected using sample volumes of 300 μl (van Regenmortel, 1982). The sensitivity and simplicity of ELISA, as well as the low cost and long shelf-life of the necessary reagents, make it well suited for mass indexing.

Preparation of antisera requires the ability to purify virus for use as antigen, but host constituents often make virus isolation difficult. Because nucleic acid isolation using phenol-detergent extraction avoids many of these difficulties, Morris and Dodds (1979) have proposed PAGE analysis of virus-specific double-stranded RNAs as a diagnostic tool for RNA plant virus identification. Disease diagnosis via analysis of virus-specific double-stranded RNAs, in contrast to ELISA, is completely independent of virus purification. Nucleic acid spot hybridization, as well as PAGE, could be used to detect virus-specific double-stranded RNA.

Application of nucleic acid spot hybridization techniques to plant virus detection in crude extracts will require either a sufficient concentration of

unencapsidated virus-specific RNA or a simple method to release the viral genome by disrupting the viral capsid. Potential sources of unencapsidated virus-specific RNA include subgenomic messenger RNAs and double-stranded RNA replicative intermediates. Double-stranded RNAs would have to be denatured before the sample was spotted on the nitrocellulose membrane. Brandsma and Miller (1980) have shown that nucleic acid spot hybridization can be used to screen cultured mammalian cells for the presence of Epstein-Barr virus DNA. Cell suspensions are spotted on a nitrocellulose membrane and treated with NaOH to lyse the cells and denature the viral DNA simultaneously. Careful comparative studies will be required to discover whether ELISA, PAGE analysis of virus-specific double-stranded RNA, or nucleic acid spot hybridization is best suited for routine diagnosis of a specific virus disease.

V. Supplementary Methodologies

Although specific spot hybridization tests for PSTV and other viroid diseases will probably supersede procedures based on bioassay in suitable indicator hosts or PAGE analysis for routine indexing, these older procedures remain quite useful. Bioassays can be used to confirm initial results obtained by nucleic acid spot hybridization (Salazar *et al.*, 1983). Despite their drawbacks they can provide information that cannot presently be obtained by nucleic acid spot hybridization.

A. BIOASSAY OF PSTV

A number of independently isolated PSTV strains have been described and can be classified as either severe or mild strains on the basis of the symptoms produced in *Lycopersicon esculentum* L. cv. Rutgers. Tomato has been used as a diagnostic host for PSTV because symptoms in some potato cultivars are indistinct. Severe strains cause extreme shortening of the internodes, severe epinasty, shortening of petioles and midribs, and necrosis of stems, petioles, and midribs in tomato. Symptoms of mild strains, on the other hand, are slow to develop and often so mild that they are easily overlooked (Fernow, 1967).

Gross *et al.* (1981) have compared the complete nucleotide sequences of a severe and a mild strain of PSTV and found that they differ in only three nucleotide exchanges. RNA-cDNA hybridization techniques that use cDNAs complementary to a large portion of PSTV cannot detect such small sequence differences, but it may be important to know whether an infected plant contains a severe or a mild strain of PSTV. Figure 3 shows that bioas-

FIG. 3. Symptoms of PSTV in Rutgers tomato plants inoculated with sprout extracts prepared from 12 potato tuber clones (pots 1–3 and 6–8 from the left in each row) or inoculated with purified PSTV (severe strain) (pot 5). The fourth pot in each row is an uninoculated control. From Salazar *et al.* (1983).

say on Rutgers tomato can provide this information. In all cases, the presence of PSTV in plants with symptoms was confirmed by nucleic acid spot hybridization of the tomato foliage. Important considerations in the use of systemic bioassays for viroids have been discussed by Diener *et al.* (1977) and Diener (1979).

B. EXTRACTION AND PURIFICATION OF LOW-MOLECULAR-WEIGHT RNA

Several protocols for the detection of PSTV by PAGE have been published (see, for example, Morris and Wright, 1975; Pfannenstiel *et al.*, 1980). Each contains a simple and rapid procedure for extraction of 2 *M* LiCl-soluble nucleic acid from the plant tissue to be tested. We have added poly-

saccharide extraction and DNA digestion steps to the detergent-phenol extraction and 2 *M* LiCl precipitation steps of these protocols; the result is a relatively rapid procedure for the purification of RNA from small (<1 g) to intermediate (100 g) quantities of tomato leaf tissue. Because 2 *M* LiCl-soluble RNA is the starting material for a number of different types of viroid studies (including PSTV detection by PAGE), this procedure is presented below. All centrifugations are performed at 10,000 rpm for 10 min in Sorvall GSA or SS-34 rotors.

1. Powder frozen leaf tissue in liquid N_2 using a chilled mortar and pestle before homogenizing for 2 min in a Polytron homogenizer (Brinkmann) with 1 ml of 0.5 *M* K_2HPO_4–1% bentonite–0.1% sodium diethyldithiocarbamate–0.1% ascorbic acid, 0.1 ml of 20% SDS, 1 ml of buffer-equilibrated phenol containing 0.1% 8-hydroxyquinoline, and 1 ml of $CHCl_3$ per gram of tissue.
2. Separate the aqueous and phenol–$CHCl_3$ phases by centrifugation, reextract the aqueous phase with 0.8 ml/g each of phenol and $CHCl_3$, and serially reextract the phenol–$CHCl_3$ phases with 0.6 ml/g of 0.5 *M* K_2HPO_4–1% bentonite–0.1% ascorbic acid–0.1% sodium diethyldithiocarbamate.
3. Remove polysaccharides by adding equal volumes of 2.15 *M* K_2HPO_4–0.35 *M* KH_2PO_4, and 2-methoxyethanol to the pooled aqueous phases and shaking the resulting mixture for 5 min at 4°C. Recover the upper (2-methoxyethanol) phase by centrifugation and reextract the lower phase with an equal volume of "upper phase" from a parallel mock extraction. Add a quarter volume of 1% hexadecyltrimethylammonium bromide (CTAB) to the pooled upper phases and allow the nucleic acids to precipitate overnight at −20°C.
4. Collect the nucleic acid precipitate by centrifugation, and wash three times by resuspension in 70% ethanol–0.1 *M* sodium acetate. Dissolve the washed precipitate in sterile water, add 0.1 volume of 2.2 *M* potassium acetate–10 m*M* EDTA (pH 6.0) and 2.5 volumes of ethanol, and store overnight at −20°C.
5. Collect the nucleic acid precipitate by centrifugation, dry briefly *in vacuo,* and dissolve in 10 m*M* Tris-HCl–2 m*M* $MgCl_2$ (pH 7.5) at a final concentration of ca. 5 mg/ml. Add pancreatic DNase (Worthington, electrophoretically purified) to 20 μg/ml, incubate for 60 min at 37°C, and add SDS and EDTA to respective final concentrations of 0.1% and 10 m*M*.
6. Extract with one-half volume of buffered phenol containing 0.1% 8-hydroxyquinoline and one-half volume of $CHCl_3$. Recover the aqueous phase by centrifugation, extract three times with 2–3 volumes of diethyl ether, and remove the residual ether with an N_2 stream. Recover

total RNA by addition of 0.1 volume of 2.2 *M* potassium acetate, 10 m*M* EDTA (pH 6.0), and 2.5 volumes of ethanol; storage at −20°C.

7. Collect the total RNA by centrifugation, dry briefly *in vacuo,* dissolve in sterile water to a concentration of 2 mg/ml. Add an equal volume of 4 *M* LiCl and allow the high-molecular-weight RNA to precipitate overnight at 4°C.
8. Centrifuge and recover the low-molecular-weight RNA from the 2 *M* LiCl supernatant by addition of 2.5 volumes of ethanol; storage at −20°C. Collect the precipitate by centrifugation, dissolve in sterile water, and reprecipitate by addition of 0.1 volume of 2.2 *M* potassium acetate, 10 m*M* EDTA (pH 6.0), and 2.5 volumes of ethanol.

RNA yields are ca. 750 μg of tomato leaf tissue per gram (ca. 200 μg of LiCl-soluble and 550 μg of LiCl-insoluble RNA per gram). Although RNA preparations from tomato have proved to be quite satisfactory for PAGE and hybridization analyses (Owens and Diener, 1982), chromatography on CF-11 cellulose (Niblett *et al.,* 1980; Allen and Dale, 1981) may be required to remove high levels of contaminating polysaccharides when RNA is extracted from hosts such as chrysanthemum or avocado.

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NOTE ADDED IN PROOF

Maule *et al.* (*J. Virol. Methods* **6,** 215–224, 1983.) have described the application of spot hybridization to the detection of DNA and RNA viruses in crude plant homogenates.

7 *Detection of Viral Nucleic Acids by in Situ Hybridization*

Ashley Haase, Michel Brahic, Linda Stowring, and Hubert Blum

I. Introduction

Methods to adapt hybridization techniques to preparations of cells and chromosomes were devised and introduced in 1969 (Gall and Pardue, 1969a,b; Pardue and Gall, 1970; John *et al.*, 1969; Buongiorno-Nardelli and Amaldi, 1970) and used successfully to localize highly reiterated or amplified genes (Chandler *et al.*, 1979; Grigliatti *et al.*, 1974; Wimber *et al.*, 1974; Jones, 1970; Henderson, 1972). Improvements in both hybridization techniques (Brahic and Haase, 1978) and labeling of specific probes have extended the compass of *in situ* hybridization to detection of single genomes of viruses in cells (Haase *et al.*, 1982) and single genes in chromosomes (Tereba *et al.*, 1979; Trent *et al.*, 1982; Neel *et al.*, 1982; Gerhard *et al.*, 1981; Harper *et al.*, 1981). We undertook this chapter with the view that these developments herald a new era in the analysis of viral infections and

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the role of viruses in chronic diseases, and present some exceptional opportunities to examine the expression of genes in individual cells in other disciplines, such as developmental biology (Harrison *et al.*, 1973, 1974; Conkie *et al.*, 1974).

In this chapter we have tried to achieve a balance between techniques and applications, but we make no claim to have been exhaustive in the latter case. Rather we have chosen to provide unabashedly detailed descriptions of methods drawn from personal experience, to cite examples we think particularly telling or interesting of the use of *in situ* hybridization in virology, and to indicate some of the important issues whose solution we believe will be greatly influenced by *in situ* hybridization.

II. Materials and Methods

A. Overview

In *in situ* hybridization a nucleic acid probe, labeled radioactively or with a "reporter" molecule, is annealed to suitably prepared cells or chromosomes, and the formation of hybrids is assessed by autoradiography, by fluorescence, or by histochemical means. The great increase in sensitivity achieved in current techniques is the result of the increased signal generated by contemporary probes and the increased efficiency of hybridization. This section is structured to reflect these improvements, first in the preparation of probes, and second, in the treatment of cells and conditions for hybridization. Figure 1 summarizes the principal steps in the procedures in a flow diagram.

B. Viral Probes

In general we prepare virus-specific probes by reverse-transcribing purified viral RNAs, or by nick-translating viral DNAs, usually cloned to obviate problems with contaminating cellular sequences. Although in principle there may be theoretical advantages to the use of RNA probes (Casey and Davidson, 1977) transcribed with RNA polymerase, in practice we prefer DNA probes for their stability. The examples that follow illustrate how to make probes for two plus-strand RNA viruses, a minus-strand RNA virus, and DNA viruses cloned in plasmids.

1. *Isolation of Viral RNA Templates*

a. Visna. Visna virus is a plus-strand RNA virus in the family of retroviruses that causes a slow infection in sheep and a rapid lytic infection in

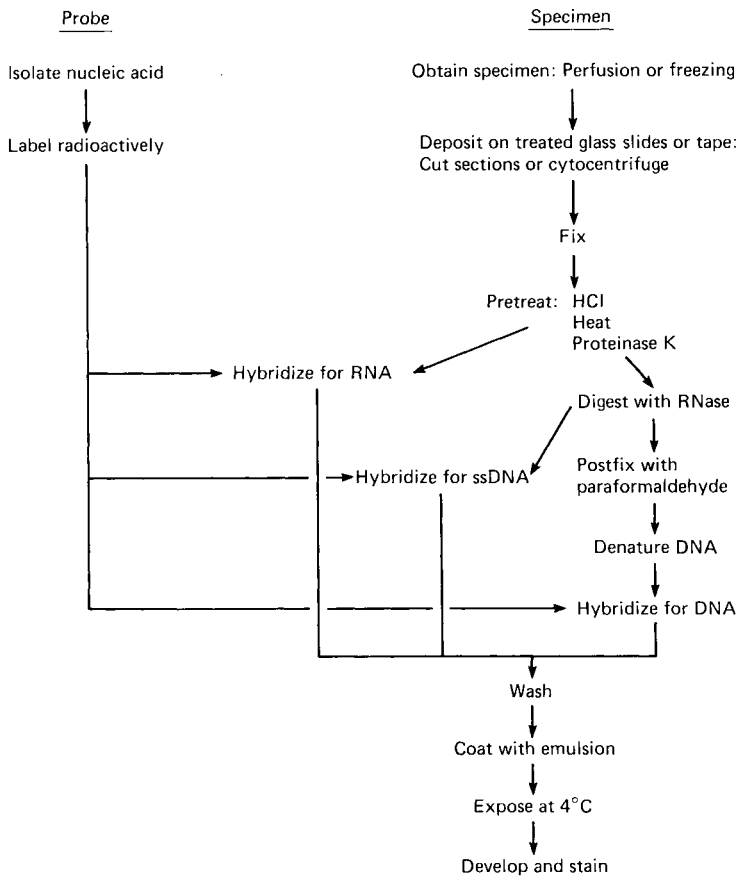


FIG. 1. Overview of *in situ* hybridization procedures. See text for explanation.

tissue culture. To isolate RNA (Haase *et al.*, 1974) infect 2 to 5 × 10^8 (about a hundred 75-ml flasks) sheep choroid plexus (SCP) cells with 3 PFU of visna virus per cell. Adsorb for 2 hr at 37°C. Add L-15 medium with 2% lamb serum and incubate at 37°C. Discard medium at 24 hr and replenish with 10 ml of fresh medium per 75-ml flask. Collect and pool the medium at 48 and 72 hr, clarify, and store at −70 to −80°C. Concentrate the virus by slowly adding solid ammonium sulfate to a final concentration of 50% (w/v); maintain the pH at 7.4 with Tris base as the ammonium sulfate dissolves. Stir for 1 hr at 4°C. Centrifuge at 13,200 *g*. Resuspend the pellet in 1/20th of the original volume with 0.1 *M* NaCl, 0.01 *M* Tris-HCl (pH 7.4), 0.001 *M* EDTA (STE). Prepare discontinuous gradients in

SW27 cellulose nitrate tubes consisting of 4 ml of 40% (w/w) potassium tartrate as a cushion and 10 ml of 17% (w/v) sucrose in STE buffer. Overlay concentrated virus, 24 ml per tube. Centrifuge at 22,500 rpm in SW27 rotor for 2 hr at 4°C. Collect the visible virus band at the sucrose–potassium tartrate interface. Dilute and pellet the virus for 1 hr in an SW27 rotor at 25,000 rpm. Resuspend the pellet in 400 μl of 1% lithium dodecyl sulfate, 100 μg/ml proteinase K in LTE buffer (0.01 *M* LiCl, TE). Incubate at 37°C for 30 min. Layer the lysate on a 12-ml 15–30% sucrose density gradient in LTE buffer with 0.5% lithium dodecyl sulfate. Centrifuge in an SW41 rotor at 40,000 rpm for 4 hr at 4°C. Collect fractions of about 400 μl from the bottom. The 70 S viral complex of RNA subunits will be in fractions 10–12; add 0.1 volume of 20% sodium acetate (pH 4.5) and 2 volumes of ethanol and chill (15 min in dry ice–ethanol or 30 min at −80°C, or overnight at −20°C). Collect the precipitate by centrifuging at 20,000 *g* for 25 min at 4°C. Drain, dry *in vacuo,* resuspend in 0.4 ml of STE. Determine the concentration and the $OD_{260/280}$ ratio in cuvettes that have been soaked in 0.1% diethyl pyrocarbonate (DEP) for 1 hr and then washed several times in autoclaved distilled water. The $OD_{260/280}$ ratio should be ≥ 1.8; if less than 1.8, extract with redistilled phenol saturated with STE for 2 min at room temperature. Centrifuge at 1000 *g* for 30 sec, remove the aqueous layer, and ethanol precipitate again. Wash the pellet once with 70% ethanol containing 0.2 *M* sodium acetate and chilled to −20°C. Dry the pellet, redissolve in 400 μl of STE, and check the $OD_{260/280}$. Aliquot the RNA in amounts convenient for preparation of probes, e.g., 1 μg. Add sodium acetate and ethanol, and store at −20°C.

b. Theiler's Virus. Theiler's virus is a murine picornavirus responsible for a persistent infection of the central nervous system of mice and a primary demyelinating disease. The procedure for purifying Theiler's virus is derived from the method described by Phillips *et al.* (1968) for poliovirus. It takes into account the fact that the majority of virus particles are associated with cell debris:

Infect BHK cells grown on thirty 75-ml plastic flasks with a tissue culture-adapted strain of Theiler's virus (GD VII, DA, or WW) at a multiplicity of infection of 10 PFU/cell. After the complete cytopathic effect develops (10–12 hr after infection), shake the flasks to suspend the cell debris. Harvest the culture medium (450 ml); centrifuge at 1200 *g* for 15 min. Resuspend the pellets in 20 ml of ice-cold distilled water, homogenize in a tight-fitting Dounce, 10–20 strokes. Centrifuge the lysate at 1200 *g* for 15 min, and add the supernatant to the clarified culture medium. Add SDS to a final concentration of 1%; centrifuge the mixture at 35,000 rpm for 2 hr at 20°C in a 35 Ti rotor to pellet the virus. Resuspend the pellets in 3 ml of 10 m*M* Tris-HCl, pH 7.4. Cool to 0°C; centrifuge at 1200 *g* for 10 min;

discard the SDS pellet. Add 6.6 ml of cesium chloride in 10 m*M* Tris-HCl, pH 7.4 (density 1.51 g/ml). Cool the mixture to 0°C for 20 min, centrifuge at 1200 *g* for 10 min. Dilute the supernatant to 6 ml with cesium chloride in 10 m*M* Tris-HCl, pH 7.4 (density 1.35 g/ml). If necessary adjust the final density to 1.35 g/ml, centrifuge the solution at 40,000 rpm for 20 hr at 4°C in two tubes of the SW50.1 rotor. Recover visible virus bands at a density of 1.35 g/ml, dilute to 13 ml with 10 m*M* Tris HCl (pH 7.4), and centrifuge at 40,000 rpm for 2 hr at 4°C in an SW41 rotor. Resuspend the virus pellet in 150 μl of STE buffer, digest with SDS and proteinase K as described for visna virus. Extract twice with phenol, ethanol precipitate twice, and assess the purity of the viral RNA by electrophoresis in 1% horizontal slab agarose gel.

c. Measles Virus. Measles virus is a negative-strand RNA virus in the family of paramyxoviruses that causes both acute and chronic infections of man and animals. We isolate RNA from intracellular nucleocapsids (Haase *et al.,* 1981b); we generally have not obtained useful quantities of undegraded RNA from virus. Select for stable and rapid growing virus by picking plaques of wild-type virus that are 3 mm or greater in size after 3 days of growth in Vero cells at 39°C. Infect one 75-ml flask of Vero cells with one plaque. Replaque purified virus from the clarified tissue culture fluid of this culture obtained 3 days after infection. Use this isolate to prepare a stock virus pool. Infect 75-ml flasks with individual plaques of virus from the virus stock. Discard the supernatant fluid after the first day and replenish with medium containing 1 mCi of [^{3}H]uridine per flask. By the third day of infection the cytopathic effect (CPE) (formation of giant cells) should involve nearly the entire monolayer. Wash the cell sheet and scrape into 5 ml of cold PBS. Centrifuge at 1000 *g* at 4°C for 10 min. Discard the supernatant. Without disturbing the pellet of cells, use a few drops of distilled water to remove buffer left on the sides of the tube. Resuspend the infected cells in distilled water at a concentration of about 10^7 cells per milliliter. Transfer to a Dounce homogenizer, and chill for 10 min. Disrupt the cells by 10–20 strokes. Separate the nuclei from the cytoplasm by centrifuging at 10,000 *g* for 5 min.

It is important to prepare cytoplasmic extracts by this method, as RNA degradation occurs in cytoplasmic extracts prepared with detergents. Layer the cytoplasmic extract over a discontinuous gradient of renografin: 3 ml of 30%, 3 ml of 40%, 1 ml of 50%, and 3 ml of 65%. Centrifuge in an SW41 rotor at 30,000 rpm for 17 hr at 4°C. The virus nucleocapsids appear as a visible lower band, separated from cellular nucleoprotein species by 1.5 cm. Collect 20 fractions of 0.2 ml from the bottom. Measure the radioactivity in an aliquot of each fraction. The viral nucleocapsids should be in fractions 10–12. Pool these, dilute at least 5-fold with STE, and fill

up tubes with STE. Pellet at 40,000 rpm in the SW41 rotor for 17 hr at 4°C. Resuspend the pellet in 1 ml of STE with 1% SDS and 100 μg of proteinase K per milliliter. Incubate at 37°C for 60 min, phenol extract, and precipitate in ethanol. Take the pellet up in 0.4 ml of 1% SDS, and layer over a gradient of 15–30% sucrose in STE with 0.5% SDS. Centrifuge in the SW27.1 rotor at 20,000 rpm for 4 hr at 20°C. Collect fractions of 0.5 ml. Measure the radioactivity in aliquot of each fraction. The peak of 50 S viral RNA should be in fractions 10–12. Pool the peak fractions, precipitate in ethanol, redissolve in 400 μl of STE, check the purity and concentration of RNA, and store as described above.

2. *Comments on Purification of DNA Templates*

For DNA viruses we use DNA cloned in a variety of vectors. The propagation of vectors and isolation of insert DNA is beyond the scope of this chapter, but is well described in recently published manuals (Maniatis *et al.*, 1982).

3. *Labeling Viral Probes*

a. General Considerations. The binding of virus-specific probes to viral nucleotide sequences in cells can be detected by autoradiography for radioactively labeled probes or by fluorescent or enzymatic methods. Examples of the latter approach include detection of hybrids by immunofluorescence with antisera to RNA–DNA hybrids (Rudkin and Stollar, 1977; Stuart *et al.*, 1981; van Prooijen-Knegt *et al.*, 1982), detection of hybrids by fluorescence generated by RNA probes labeled at their 3′ ends with thiosemicarbazide derivatives of fluorescein or rhodamine (Bauman *et al.*, 1980, 1981a,b), and detection of hybrids by immunofluorescence or immunoperoxidase methods (Langer *et al.*, 1981; Langer-Safer, *et al.*, 1982; Singer and Ward, 1982). In the latter method the probe is synthesized with biotinylated nucleotides, and hybrids are detected with antibodies to biotin. The sensitivity of these methods appear to be adequate to detect reiterated genes, but none of the published work indicates that they are comparable in sensitivity to methods using radioactive probes. For this reason, we will focus here on the synthesis of radioactive probes and autoradiographic means of detection.

b. Choice of Radioisotopic Precursor. For most applications ^{3}H-labeled probes remain the standard for several reasons: the probes have both a long half-life and a long shelf life; localization of the signal by autoradiography is excellent at both the electron microscopic and light microscopic level; and sufficiently high specific activities can be achieved to detect confidently a few copies of genomes in the 10-kb size range. However, to detect nonreiterated genes or single copies of smaller viral genomes, the limited specific

activities and lower efficiency of grain development (about 0.1 grain per disintegration) (Rogers, 1979) with ^{3}H make it necessary or advantageous to turn to precursors containing ^{125}I or ^{35}S. High specific activities and efficient grain development can be achieved with ^{32}P-labeled probes, but localization is poor (Rogers, 1979). More recently introduced thiophosphate deoxynucleotide triphosphates (Vincent *et al.*, 1982) have much to recommend their use in *in situ* hybridization: high efficiency of grain development (0.5 grain per disintegration) (Rogers, 1979), adequate localization at the light microscopic level, and specific activities of greater than 10^9 disintegrations per microgram for reverse-transcribed probes. Probes of comparable specific activity can also be prepared by nick translation with [^{125}I]dCTP, and although the efficiency of grain development is less (0.2 grain per disintegration) (Rogers, 1979), localization is sufficient for electron microscopy. Probes made with either isotope have a useful shelf life of 1–2 months (the shelf life is less than the half-life, presumably because of radiochemical damage to the probe).

c. Reverse Transcription with Random Primers

i. Introduction. Probes of high specific activity can be prepared from any purified RNA template of plus or minus polarity by reverse-transcribing the RNA. The necessary primers for reverse transcription are provided by DNA fragments generated by digestion of DNA from a convenient source, e.g., calf thymus or *Escherichia coli,* with deoxyribonuclease, followed by chromatographic separation of fragments of about 10–15 nucleotides (Maniatis *et al.*, 1982). These fragments of DNA will anneal at random along the RNA template and can be extended by reverse transcriptase (Fig. 2). Radioactive deoxynucleotide triphosphate (dNTP) precursors are incorporated into complementary DNA (cDNA) pieces that are about 400 nucleotides long (unpublished observation) and collectively represent all the sequences in the RNA template. If actinomycin D is omitted, the cDNA is copied to some extent such that the probe is comprised of partially duplex DNA that can form networks in the presence of dextran sulfate (Wahl *et al.*, 1979). As a consequence, a single hybridization event results in an amplified signal in proportion to the extent of network formation, with a corresponding increase in the sensitivity of hybridization. Thus, these probes are particularly well suited for *in situ* hybridization in secondary structure, size, sequence representation, and specific activity.

ii. Primers. We prepare primers of an optimal size by digesting calf thymus DNA with deoxyribonuclease and separating fragments by chromatography on DEAE-Sephadex. A detailed protocol has been published recently (Maniatis *et al.*, 1982).

iii. Reverse transcription. We generally reverse-transcribe about 1 μg of RNA by the following procedure.

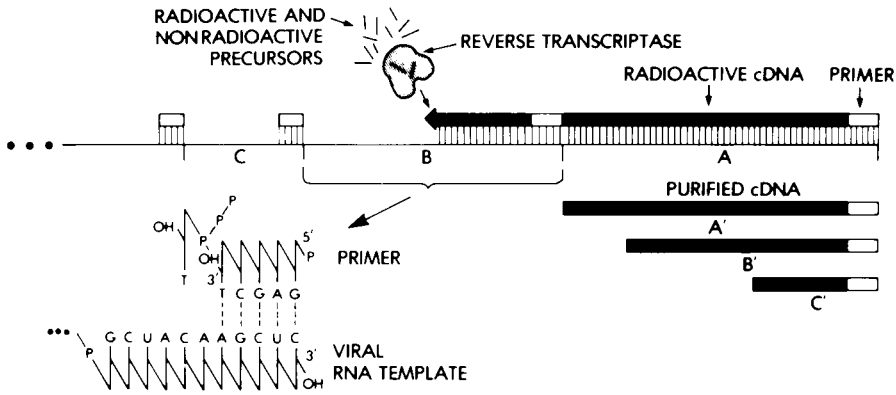


FIG. 2. Artist's depiction of reverse transcription by random priming. Fragments of DNA produced by digestion with deoxyribonuclease in the size range of 8–15 nucleotides will anneal to complementary sequences in viral RNA at random loci. The fragments provide the necessary free 3′-OH to prime DNA synthesis. The extension of the random primers by reverse transcriptase with radioactive precursors results in a collection of radioactive DNA products of varying lengths representative of all the sequences in viral RNA.

Siliconize and heat a lyophilization flask to 108° for 2 hr or more to destroy any ribonuclease. Cool, and add radioactive dNTPs in sufficient quantity so that their concentration in a 50–100-μl reaction will be 100–300 μM; e.g., for a ^{3}H-labeled probe of specific activity of 4 to 5 $\times$ 10^8 dpm/μg, use 2 mCi each of [^{3}H]dATP, -dCTP, and -dTTP at 50–100 Ci/mmol. Freeze at $-80°$; lyophilize to dryness. Make up a solution on ice that will contain as final concentrations in 50 μl: 50 mM Tris HCl (pH 8.1–8.3), 2 mM dithiothreitol (DTT), 250 μM to 1 mM dNTP not used for labeling (in this case dGTP), 8–10 mM $MgCl_2$, and 40 mM KCl. The latter improves transcription for some RNAs such as visna virus, but not others (Theiler's, measles virus) and should be omitted. Add calf thymus primers in 1000-fold excess (by weight) of template. Use this solution to recover carefully the dried radioactive precursors. Transfer to a 1.5-ml microfuge tube on ice. Centrifuge an aliquot of 1 μg of RNA in ethanol, e.g., 10 min in a microfuge in the cold. Drain, then dry *in vacuo*. Use the solution on ice to dissolve the RNA. Add AMV reverse transcriptase to 1500–2000 units/ml, mix. Remove a small sample (0.5–1 μl) to precipitate with perchloric acid and carrier. Incubate the reaction at 42°C. The reaction is usually complete by 45–60 min. Take a second point to determine the extent of incorporation. Expect to synthesize 250–1000 ng or more of cDNA per microgram of RNA template. Place the reaction mixture on ice and add Na_4 EDTA to 20 mM and SDS to 1.0%, or Sarkosyl to 0.5% if the reaction mixture con-

tains KCl. Add proteinase K to 100 μg/ml, and incubate for 30 min at 37°C. Add an equal volume of phenol saturated with STE, vortex for 2 min, centrifuge, and remove the aqueous phase. Precipitate the nucleic acids with ethanol and carrier RNA in a dry ice–ethanol bath (cf. Section II,B,1). Centrifuge, drain, dry *in vacuo*. Digest the residual RNA template to nucleotides by dissolving the pellet in TE buffer, 0.3 *N* NaOH. Incubate at 37°C for 6 hr or overnight at room temperature. Place the reaction on ice and make it 100 m*M* in Tris-HCl (pH 7.4). While vortexing, slowly neutralize with 0.3 *N* HCl. Use pH indicator paper to check the pH on a small aliquot. Separate the labeled DNA product from unincorporated precursors and primers by chromatography on a 50 × 1 cm column of Sephadex G-50 in 0.3 *M* NaCl-TE buffer containing 0.5% SDS. Concentrate the [^{3}H]cDNA in the excluded volume by addition of 50–100 μg of carrier RNA and 2 volumes of ethanol.

Probes with specific activities greater than 10^9 dpm/μg can be synthesized with one or more [^{35}S]dNTPs. The reaction volume should be reduced to 25 μl to decrease the amount of isotopic precursor needed. Moreover, at present these precursors are supplied in 10 m*M* dithiothreitol (DTT), to decrease the rate of radioactive decomposition. The DTT must be removed prior to lyophilization, as concentrated DTT markedly inhibits reverse transcription. Directions for removing DTT by chromatography are supplied by the manufacturer. Once the DTT has been removed, the [^{35}S]dNTPs decompose at the rate of 3 to 10% per day (K. O'Brien, personal communication). Because incorporation of [^{35}S]dNTPs is slower, the extent of incorporation should be followed at intervals of 30–60 min for 3 hr. The reaction with visna RNA is complete by this time, but the kinetics of transcription may be different with other RNA templates. In the determination of acid-precipitable radioactivity with [^{35}S]dNTPs, include 10 m*M* DTT in the solutions used to precipitate the reaction products, to reduce nonspecific binding of ^{35}S to the filters.

In principle it should be possible to synthesize probes with specific activities of 4×10^9 dpm/μg with carrier-free [^{125}I]dCTP (2200 Ci/mmol). However, the K_m for some of the dNTPs in reverse transcription is about 75 μ*M*, (Hizi *et al.*, 1977) and although these concentrations of [^{125}I]dCTP can be achieved for small reactions, we have found in practice that incorporation is very poor (cDNA synthesized per input RNA = 0.01–0.05). Radiochemical damage to the reverse transcriptase may be responsible, but maneuvers to limit such damage such as inclusion of bovine serum albumin (BSA) in the reaction or addition of fresh enzyme did not increase transcription (R. Peluso, unpublished work).

d. M13 Probes. Region and polarity specific probes can be synthesized by extension of the commercially available sequencing primer. Dry down

100–200 ng of M13 DNA containing the inserted fragment of either plus or minus polarity. Add 1 μl of 10× buffer [100 m*M* Tris-HCl (pH 7.4), 600 m*M* NaCl, 66 m*M* $MaCl_2$, 10 m*M* DTT], 3 μl of primer (also 100–200 ng), and 6 μl of water. Denature by boiling for 2 min. Cool slowly to room temperature. Add in a volume of 10 μl each unlabeled nucleotide to 500 μM and the labeled precursors to 1 μM. Add 2 μl (1 unit) of Klenow fragment of DNA polymerase. Incubate for 2 hr at room temperature. Extract with phenol, separate labeled DNA from unincorporated isotope on Sephadex G-50, and probe from M13 DNA by denaturation followed by sedimentation through an alkaline sucrose density gradient (Ricca *et al.*, 1982).

e. Nick Translation. For cloned DNA, nick translation with ^{3}H-, ^{35}S-, or ^{125}I-labeled precursors provides probes suitable for *in situ* hybridization. The same general considerations apply to the choice of isotope, but specific activities will reflect not only the specific activity of the precursor, but also the extent of replacement of nucleotides in the reaction. With three [^{3}H]dNTPs of the highest specific activities available commercially, the DNA will be labeled to 1 to 2 × 10^8 dpm/μg; for ^{35}S and ^{125}I, the specific activities are 3 to 4 × 10^8 dpm/μg and 0.8 to 1.0 × 10^9 dpm/μg, respectively. For some applications, such as detection of genes on both a macroscopic and microscopic scale, it is advantageous to dual-label probes with ^{125}I and ^{35}S. In all instances the probes have the same desirable characteristics for *in situ* hybridization described for probes synthesized with random primers and reverse transcriptions: small size for diffusion into the cell (about 400 nucleotides), duplex structure to form networks with dextran sulfate, and faithful sequence representation.

Our reaction conditions, slightly modified from those described by Rigby *et al.* (1977), are as follows:

Concentrate radioactive precursors by lyophilization; aim for a concentration of 10 μM dNTP in a 25- to 50-μl reaction. Since the starting volume of isotope is small, the lyophilization can be done in a microfuge tube to be used for the reaction, covered at the top with Parafilm with holes punctured with a 26-gauge needle to limit contamination of the lyophilizer with isotope. Place the tube on ice. Add 500 ng of DNA template, dNTPs except for the labeled species to 10 μM 1/10th volume of 10× buffer consisting of 500 m*M* Tris-HCl (pH 7.4), 100 m*M* $MgCl_2$, 10 m*M* DTT, 500 μg of acetylated BSA per milliliter. Add deoxyribonuclease I (DNase) to 0.1 unit per milliliter. In order to assure reproducible "nicking" with DNase, dissolve DNase I at 1 mg/ml in 50 m*M* Tris-HCl (pH 7.4), 10 m*M* $MgSO_4$, 1 m*M* DTT, 50% glycerol. DNase is sensitive to denaturation; do not vortex or shake; aliquot in small quantities, store at −20°C, and use a fresh aliquot for each reaction. Dilute the enzyme in 50 m*M* Tris-HCl (pH 7.4), 10 m*M* $MgSO_4$, 50 μg of BSA per milliliter, 1 m*M* DTT, and keep on ice for

2 hr before initiating the reaction. Allow the DNase to nick the DNA for 3–4 min at room temperature. Add *E. coli* polymerase I to 175 units/ml. Incubate at 14°C, and follow the extent of incorporation. With ^{3}H and ^{125}I, levels of incorporation plateau at 1.5–3 hr; the incorporation of ^{35}S is variable, but usually slower—6–8 hr or more to plateau. Stop the reaction by chilling and adding SDS to 1% and EDTA to 20 m*M*. Extract with phenol, and separate products from isotope on Sephadex G-50 (Section B,3,c,iii). Add carrier, concentrate by precipitation with ethanol, and store at −20°C in small aliquots.

f. Tailed Probes. Probes bearing a long "tail" of radioactive DNA can be prepared as described by Tereba *et al.* (1979). The signal generated in hybridization will be amplified by the radioactivity in the "tail." Although these methods greatly increase the sensitivity of hybridization to paper and chromosomes, they do not offer major advantages when hybridizing cells or tissues. Indeed, single genes can be detected with conventionally prepared probes (Gerhard *et al.*, 1981; Harper *et al.*, 1981; Neel *et al.*, 1982; Trent *et al.*, 1982), and the large size of the tailed probes interferes with efficient *in situ* hybridization in cells (Brahic and Haase, 1978).

C. PREPARATION OF CELLS AND TISSUES FOR *in Situ* HYBRIDIZATION

In this section we discuss techniques for obtaining and storing specimens for *in situ* hybridization and transferring them to glass or other materials for the hybridization procedures. These methods satisfy two important requirements: morphological and architectural features of cells and tissues are retained at the level of the light microscope, and nucleic acids are fixed in the cell under optimal conditions for hybridization.

1. *Fixatives*

Fixatives fall into two general classes, precipitants and cross-linking agents. In *in situ* hybridization the optimal conditions for fixation reflect a compromise between preservation of morphological detail and efficient hybridization. We summarize in Table I our assessment of the effect on hybridization efficiency (HE) of a number of fixatives from each class.

We and others (Moar and Klein, 1978) find that precipitating fixatives such as ethanol, ethanol–acetic acid, methanol, and methanol–acetone afford the highest HE compatible with good preservation of morphology at the level of the light microscope (Table I). The cross-linking fixatives, on the other hand, are required for ultrastructural studies with *in situ* hybridization (Croissant *et al.*, 1972; Geuskens and May, 1974) and are superior for retention of morphological detail for the light microscope; however,

TABLE I
FIXATIVES FOR *in SITU* HYBRIDIZATION

Fixative	Reference	Conditions	Efficiency of hybridization[a]
Precipitants			
Ethanol-acetic acid	Haase *et al.* (1982)	Ethanol-acetic acid, 3:1 (v/v) 15 min; ethanol 5 min, room temperature	10
Ethanol	Unpublished	Ethanol, 20 mins, 4°C	9
Methanol-acetone	Moar and Klein (1978)	Methanol-acetone 1:2 (v/v) 4 min, −20°C	8
Methanol	Unpublished	Methanol 20 min, −4°C	8
Acetone	Unpublished	Acetone, 4 min, −20°C; acetone 20 min, 4°C	7
Chromic acid	Langenberg and Sharpee (1978)	Chromic acid, 0.1%, 4°C, 20 min	5
Picric acid	Luna (1968)	75% solution, 2 hr, room temperature	5
Mercuric cholride	Luna (1968)	5%, 2 hr, room temperature	5
Cross-linking agents			
Formaldehyde	Luna (1968)	0.1–4%, room temperature, 20 min	2
Glutaraldehyde	Godard and Jones (1979)	0.1–2%, room temperature, 20 min	1
Paraformaldehyde-lysine-periodate (PLP)	McLean and Nakane (1974)	PLP, room temperature, 15 min	4
Ethyldimethylaminopropylcarbodiimide	Willingham *et al.* (1978)	1%, room temperature, 20 min	3
Dimethylsilserimidate	Hand and Hassell (1976)	20 mg/ml, 4°C, 30 min	4

[a]Ranking of fixatives for *in situ* hybridization: Hybridization efficiencies are ranked on a scale of 1–10; a score of 10 indicates maximum level of hybridization. Efficiency was determined from a comparison of average number of grain counts per cell infected with visna virus, hybridized independently *in situ* for viral DNA and for viral RNA.

they may greatly reduce HE. We have not been able to confirm reports (Godard and Jones, 1979) of higher HE with dilute solutions of glutaraldehyde, and therefore recommend ethanol-acetic acid as the standard primary fixative for *in situ* hybridization and light microscopy.

This recommendation, however, must be considered provisional. We know of situations where other fixatives are better, and we can envision new protocols that will make it possible to employ cross-linking fixatives without impairing hybridization. The choice of fixative in individual situ-

ations must be determined empirically guided by an admittedly limited understanding of the fixation process.

1. Fixation of cells varies with accessibility and permeation. Thin sections or monolayers favor rapid fixation. Fixation is much slower in perfused animals or intact tissues.
2. Fixation with formaldehyde and glutaraldehyde cross-links proteins and alkylates amino groups of bases in single-stranded nucleic acids. The anticipated effect is a decrease of HE as a consequence of formation of a proteinaceous network that will both hinder diffusion of probes and impair annealing of bases after alkylation and cross-linking of nucleotides (Langenberg, 1980; Chaw *et al.*, 1980; Grossman *et al.*, 1961). The extent to which hybridization is decreased will depend on the secondary structure of the nucleic acid and will be influenced greatly by the conditions and timing of fixation and by posttreatments. For example, a brief fixation in formaldehyde followed by a more extensive digestion with proteinase K has been used successfully by Singer and Ward (1982) to localize actin mRNA in muscle cells, and we found (Haase *et al.*, 1982) that formaldehyde fixation after removal of RNA improved *in situ* hybridization for DNA.

These considerations, examples, and appreciation of the enigmatic aspects of fixation warrant an individualized approach to fixation.

2. *Preparation of Tissues for in Situ Hybridization*

At present we are largely limited to *in situ* hybridization to sections cut from frozen tissues and fixed with precipitants or to sections of tissues embedded in paraffin after perfusion of animals with fixatives.

a. Frozen Sections

i. Freezing methods; autolysis. Tissues from infected animals or human tissues obtained postmortem should be frozen as quickly as possible after death to minimize autolysis and degradation of nucleic acids. This is especially true for single-stranded RNA viruses. We find, for example, that more than half of measles virus RNA is degraded in infected hamster brain if the brains are held at room temperature for 6–18 hr prior to freezing (L. Stowring and A. Haase, unpublished).

In our experience the simplest and most effective way to freeze tissue is to section the tissue into pieces 0.5–1.0 cm thick, and to freeze them between aluminum sheets sandwiched between two blocks of dry ice; a more elaborate device can be constructed of two aluminum plates cooled with liquid N_2. For studies of whole brains or small animals, there are three possibilities: freeze the intact animal or skull directly in liquid N_2; freeze

the animal or skull in carboxymethyl cellulose (CMC) in a metal frame, in a bath of dry ice–pentane (Ullberg, 1977); or freeze the brain in Freon 12 in a vessel suspended over liquid N_2 (B. Moyer, unpublished work). The temperature should be lowered to the point where only the Freon in the bottom of the vessel is frozen. After freezing, store specimens at −70 to −80°C.

ii. Sectioning and transfer to treated glass slides or tape. The microscope glass slides must be treated to minimize nonspecific adsorption of probe. Clean the slides for 30 min in 1 *M* HCl. Wash in distilled water, dip in 95% ethanol for 30 min, and wipe dry with gauze. Soak the slides for 3 hr in Denhardt's medium (DM) (Denhardt, 1966) [DM is 0.02% BSA, 0.02% polyvinylpyrrolidone, 0.02% Ficoll, 3 × SSC] at 65°C. Dip briefly in water. Transfer to ethanol–acetic acid (3:1, v/v) for 20 min. Air dry. Acetylate (Hayashi *et al.*, 1978) as follows: Dip the slides in 0.1 *M* triethanolamine, pH 8.0. Add acetic anhydride to 0.25% (v/v) and mix vigorously for 10 min. Wash twice in water and once in 95% ethanol for 5 min, air dry, and store at room temperature. Alternatively, the slides can simply be acetylated or be washed with Extran and treated with aminopropyltriethoxysilane (van Prooijen-Knegt *et al.*, 1982).

Cut frozen sections of 4–10 μm with a conventional cyrostat. Air dry briefly. Fix in ethanol acetic acid (3:1) at room temperature for 15 min, rinse in ethanol for 5 min, air dry. The fixed sections can be stored at room temperature for as long as 6 months before hybridization.

Larger pieces of tissue or whole animals, brains, and other organs require a special cryostat (Ullberg, 1977). For this apparatus, embed the tissues in a block of CMC frozen in a metal frame. The bottom of the frame is a plate that attaches to the cryostat. Remove the frame, then pass the block of CMC under a large knife and transfer the section (Fig. 3) to tape. Fix the sections in ethanol–acetic acid and ethanol and store dry at room temperature.

b. Perfusion, Paraffin Embedding. Tissues also can be embedded in paraffin, sectioned, and hybridized *in situ*. These procedures offer excellent preservation of morphology and are particularly suitable for small fragments of tissue, e.g., mouse spinal cord. In this procedure we perfuse animals with fixative, dissect the organs of interest, embed them in paraffin, and prepare histological sections. These are hybridized *in situ* after removal of the paraffin. The procedure applied to mouse central nervous system follows.

Anesthetize the animal with ether and mount it ventral side up on a Styrofoam board on top of gauze pads. Open the thorax and abdomen and make an incision in the right atrium of the heart. Perfuse the left ventricle with a 20-gauge needle mounted on a two-way stop cock connected with two

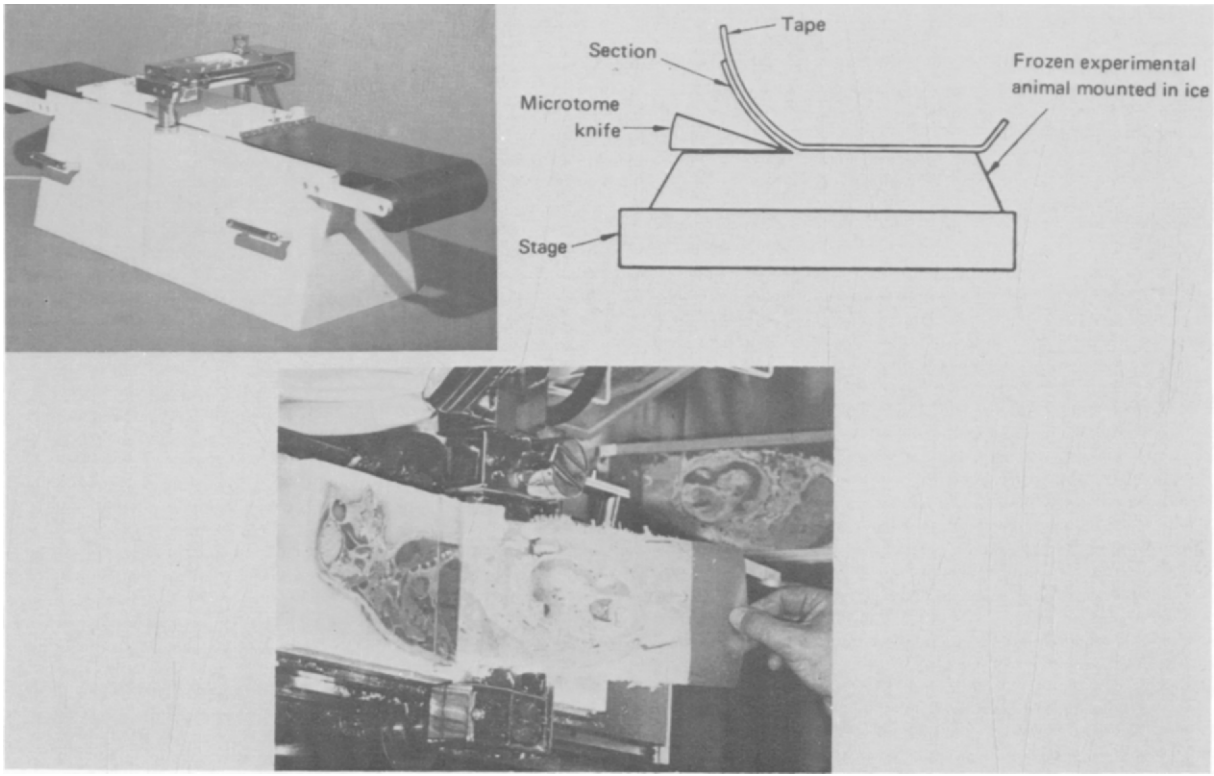


FIG. 3. Transfer of large sections to tape for hybridization of whole organisms, organs, or large pieces of tissue. *Top left:* LKB microtome outside cryostat. *Top right:* Principle of operation. A piece of clear tape is applied to the top of the block of tissue frozen in carboxymethyl cellulose. As the knife cuts the section, it is transferred to the tape. *Bottom:* An example of sectioning of an animal. Adapted from Fig. 6-8 of Ullberg (1977).

syringes containing normal saline and fixative. Perfuse first with about 30 ml of saline in order to eliminate blood, then with the same volume of fixative, either ethanol–acetic acid (3:1, v/v) or PLP cooled to 4°C. Ethanol–acetic acid is optimal for hybridization of cells in gray matter but dissolves most of the white matter of the central nervous system. PLP preserves the morphology of both gray and white matter; it diminishes hybridization levels of cells in gray matter approximately 5-fold compared with ethanol–acetic acid, but improves hybridization of cells in white matter (M. Brahic, unpublished).

After perfusion dissect out the brain and spinal cord, immerse in fixative for 2 hrs, at room temperature for ethanol–acetic acid, or at 4°C for PLP. When using PLP, rinse for 30 min in PBS at 4°C, and transfer to 70% ethanol. Tissue blocks can be kept in 70% ethanol for several days without affecting the hybridization level.

Process the tissues for paraffin embedding: 1 hr in 80% ethanol, 1 hr in 95% ethanol, two steps of 1 hr each in absolute ethanol, two steps of 1 hr each in xylene, two steps of 1 hr each in melted paraffin (Paraplast or its equivalent at 58°C). RNA is stable in paraffin blocks held at room temperature. Cut sections of 10 μm with a histology microtome and float them at 45°C on distilled water containing 0.1% Elmer's white glue. Pick up the sections on microscope slides treated for *in situ* hybridization as described above. The concentration of glue can be raised to 2% when sectioning small pieces of tissue (e.g., mouse spinal cord) in order to increase their adherence to the slide. Even higher concentrations of glue are without effect on *in situ* hybridization. After overnight incubation at 37°C, the slides can be stored for several months at 4°C.

For *in situ* hybridization deparaffinize the slides by dipping twice, 5 min each time, in xylene followed by two washes, 5 min each, in ethanol. After drying, process the slides for *in situ* hybridization as described below.

3. *Preparation of Cells for in Situ Hybridization*

In many cases hybridization *in situ* will involve single cells, e.g., infected cells suspension, cells in monolayers removed with trypsin–EDTA, and peripheral blood leukocytes. For *in situ* hybridization we deposit these cells on treated glass slides with the Shandon cytocentrifuge. For most situations that we have examined, cytocentrifugation is superior to options such as smears, squashes, and filtration of cells onto transparent filters.

Remove cultured cells with trypsin–EDTA (5 min, 37°C), centrifuge at 400 *g* for 5 min. Discard the trypsin, resuspend the cells in PBS lacking Ca^{2+} and Mg^{2+} (PBS-CMF). Collect by centrifugation and resuspend cells at 8×10^5 cells/ml of PBS-CMF. Use 0.3 ml of the cell suspension per slide. Cytocentrifuge at 350–500 rpm for 5 min. Dry the slides and fix in

ethanol–acetic acid and ethanol. Generally with this method about 10^5 cells are deposited in a circle with a diameter of 6 mm. Optimal conditions vary with cell type, and, for smaller cells such as leukocytes or macrophages, the initial density should be 10^6 cells per milliliter.

Alternatively cells can be grown on acetylated glass coverslips placed at the bottom of tissue culture petri dishes. The coverslips are acetylated as described above. The cells are fixed on the coverslip in the petri dish. After drying, the coverslips are mounted (cell side up) on glass slides using a toluene-base mounting medium. After drying overnight the slides are processed for *in situ* hybridization.

D. HYBRIDIZATION

1. *Treatments to Increase Diffusion*

One of the factors that limit the efficiency of *in situ* hybridization is diffusion of the probe through the section. The rate of diffusion is increased approximately 2-fold by treatments that remove some protein from the section without altering the morphology. For this purpose we place the slides containing material of interest for 20 min in 0.2 *N* HCl. We have not been able to confirm the report (Godard and Jones, 1979) of the loss of RNA from cells with HCl treatment. Wash briefly in distilled water, incubate for 30 min at 70°C in 2× SSC, wash briefly in water, digest for 15 min at 37°C with 1 μg of proteinase K per milliliter in 20 m*M* Tris-HCl (pH 7.4), 2 m*M* $CaCl_2$, wash twice in water for 5 min, and dehydrate in graded ethanols (70% twice, 95% once, 5 min each time).

2. *Hybridization for RNA*

a. Length of Probe. The length of the probe affects both its diffusion rate and the extent of network formation. Probes synthesized either by nick translation of cloned genes, or by reverse transcription of RNA using random primers, with an average length of about 400 nucleotides, appear to be optimal for hybridizing *in situ* in the presence of dextran sulfate.

b. Concentration of Probe. The concentration of the probe affects several parameters of *in situ* hybridization. High concentrations are desirable to increase diffusion, to enhance the rate of hybridization, and to saturate available viral sequences as a prerequisite for quantitative work. These gains must be weighed against the increases in background that are proportional to the amount of probe used and against practical considerations of the expense of preparing large quantities of probe. As guidelines we recommend, for genomes in the range of 3–10 kb, 0.06 ng/μl for 1–10 copies per cell; 0.14 ng/μl for 20–100 copies per cell; and 2–4 ng/μl for 1000 copies per cell or more. For hybridizations involving sequences of greater or lesser

complexity, the amounts of probe added should be proportionally increased or decreased for the relevant sequences. These concentrations are in 10-fold excess or more for preparations of 10^5 cells with these copy numbers and yield acceptable backgrounds of 5–10 grains per cell for the lowest concentration of ^3H probes of 1 to 5 $\times$ 10^8 dpm/μg with autoradiographic exposures of 1–2 months. We use a volume of 0.05 μl of hybridization solution per square millimeter of the coverslip used to cover the cells.

c. Hybridization Solution and Temperature of Hybridization. The hybridization solution has three components, which can be prepared in advance and stored at −20°C. (a) 20% dextran sulfate dissolved in deionized formamide; deionize the formamide by stirring for 30 min at room temperature with 5 g of mixed-bed resin (Bio-Rad AG501 $\times$ 8) per 100 ml of formamide; filter; (b) 20 m*M* HEPES (pH 2), 1.2 *M* NaCl, 2 m*M* EDTA, 0.04% (w/v) Ficoll, 0.04% (w/v) polyvinylpyrrolidone, 0.04% (w/v) bovine serum albumin, 200 μg of sonicated denature calf thymus per DNA per milliliter, 1 mg of total RNA extracted from uninfected tissues of the species under study, 100 μg/ml of poly(A); (c) radioactive probe dissolved in 3 m*M* EDTA or TE at a concentration of approximately 10–15 ng/μl.

Prepare the hybridization solution by mixing equal volumes of components a and b and then add the amount of component c required to obtain the desired final concentration of probe; or, add 10 μg of RNA carrier to the requisite amount of probe, 0.1 volume of 2 *M* sodium or ammonium acetate and 2 volumes of ethanol, chill for 5 min in a dry ice–ethanol bath, centrifuge, and dry the pellet *in vacuo*. Add the hybridization solution. In both cases, after vigorous mixing, heat the solution to 100°C for 30 sec and quickly cool to 0°C. Add dithiothreitol to a final concentration of 10 m*M* if the probe is labeled with ^{35}S to reduce background.

Place the appropriate volume of solution on the specimen; cover with a glass coverslip that has been cleaned in HCl and ethanol (cf. Section C,2,a,ii), siliconized, and heated to 180°C for 2 hr. Seal the edges of the coverslip with rubber cement or trace a circle around the coverslip with a wax pencil and cover this area with a few drops of mineral oil.

Hybridize at a temperature of 20–25°C. This is the optimal temperature for *in situ* hybridization in this medium (Alonso *et al.*, 1974; Brahic and Haase, 1978; Haase *et al.*, 1982; Simon *et al.*, 1982); increasing the temperature to 40°C reduces the hybridization level approximately 5-fold. Hybridize in the dark to minimize decomposition of formamide.

d. Hybridization Kinetics. When *in situ* hybridization is performed in probe excess, the hybridization kinetic becomes pseudo-first order with respect to the probe concentration (Szabo *et al.*, 1977). Consequently the time required to achieve saturation is only a function of the probe concentration. For 0.06 ng/μl of probe and the hybridization conditions described, this is

achieved in 60–72 hr. The rate of hybridization *in situ* is about one-tenth that of liquid hybridization performed in probe excess (Szabo *et al.*, 1977; Brahic and Haase, 1978).

3. *Hybridization for DNA*

a. Introduction. To measure viral DNA in cells that also contain viral RNA, often as the predominant species, the RNA must be digested with ribonucleases. This step and the subsequent denaturation of DNA partially solubilize the DNA so that during hybridization more than half of it is lost. We found that these losses could be prevented by refixing the cells with paraformaldehyde after the ribonuclease digestion (Haase *et al.*, 1982), presumably by cross-linking single-stranded regions of DNA to proteins. This second fixation must be introduced after removal of RNA, since it will also fix the RNA in the cell and prevent complete digestion, and before the denaturation step since formylation of bases will impair hybridization. The conditions for denaturation have been investigated extensively, and have been optimized to fully denature even GC-rich DNA (Hubbell *et al.*, 1976) under conditions that minimize loss of DNA and morphological detail.

b. Pretreatments and Ribonuclease Digestion. For DNA hybridization we employ the same treatments to increase diffusion of probes as for RNA hybridization. Following the proteinase K step and dehydration, cover the cells or tissue sections with a solution of 2× SSC containing, per milliliter, 100 μg of DNase-free ribonuclease A and 10 u of ribonuclease T1 (a convenient and economical way to do this is to use 15–20 μl of ribonucleases under a 18-mm coverslip), and incubate in a humidified chamber in a water bath for 30 min at 37°C. Wash the slides for 5 min twice in 2× SSC, and postfix in paraformaldehyde. We have monitored the ribonuclease step with cells where RNA has been labeled with [^{3}H]uridine. The digestion conditions described remove virtually all the labeled RNA. This step also obviously contaminates glassware with ribonuclease; we therefore treat all glassware with diethyl pyrocarbonate before washing it, and we dry the glassware at 180°C for 2 hr.

c. Postfixation in Paraformaldehyde. Prepare a fresh solution of paraformaldehyde each time by dissolving 5% paraformaldehyde in 0.3 *N* NaOH in PBS. As soon as the solution clears on stirring, neutralize with HCl to pH 7–8. Fix the slides for 2 hr at room temperature in this solution in the dark, then wash twice in 2× SSC, 5 min each time, and dip briefly in water. Denature.

d. Denaturation. Make a solution of 95% deionized formamide in 0.1 × SSC. Allow the solution and the vessel to be used for denaturation (e.g., a large, covered staining dish) to come to 65°C. This will take approximately 2 hr and usually requires a higher temperature in the water bath

(about 70°C). Transfer the slides from the postfixation step to the formamide solution. After 15 min at 65°C, quickly transfer the slides in a mixture of ice and 0.1 × SSC for 2 min. Wash for 2 min in water, then dehydrate in graded ethanols and dry.

e. Hybridization. Similar considerations of probe size, concentration, temperature, and components of the hybridization solution apply to DNA. As a rule the probe should be small, from 50 to 500 nucleotides; and the reaction should be driven by excess probe in solution. The T_{opt} for in *situ* hybridization for DNA in 50% formamide is quite broad over the range of 22–33°C; we hybridize for convenience at room temperature. As for RNA, the kinetics of *in situ* hybridization for DNA are slow. Even with dextran sulfate, the reaction with 0.6 ng of cDNA per microliter requires 60–70 hr to go to completion, and the overall hybridization efficiency is only about 20–25% of that expected theoretically. This is estimated as follows: If the cell population has 30 copies per cell of visna DNA, there are 30×10^{-5} pg of viral DNA per cell. If all the DNA hybridized to a ^3H probe with a specific activity of 5×10^8 dpm/μg, and if 0.1 grain develops per ^3H disintegration, there should be 150 grains per cell with an autoradiographic exposure of 1 week. The average count per cell is closer to 30–40 grains per cell, suggesting that the efficiency of hybridization is only 20–30%. Since hybridization *in situ* to RNA occurs at 100% efficiency, this problem is peculiar to DNA and may reflect rapid renaturation of spatially contiguous strands of DNA in the cell (Alonso *et al.*, 1974).

f. Tissue Blot Hybridization. Sections of whole animals, organs, or large fragments of tissue can be cut with the LKB 2258 or 2250 cyromicrotome and transferred to tape or glass. The fixed and treated tissue on tape is placed section side down on coated and acetylated glass slides 8.2 × 10.1 cm. Probe (20 ng) in 250 μl of hybridization solution is introduced between the section and tape by puncturing the tape with a 30-gauge needle. The sides of the tape are pressed down firmly and the hole is sealed with rubber cement to form the hybridization chamber.

E. TREATMENTS AFTER HYBRIDIZATION; AUTORADIOGRAPHY

1. *Washing*

To measure single copies of viral genomes requires long exposure times and very low backgrounds. The washing procedures therefore are critical. We describe the most stringent procedures for long exposures, compatible with backgrounds of less than 10 grains per cell after 2 months of exposure with ^3H probes (specific activity 5×10^8 dpm/μg, 0.06 ng/μl). For short exposures, the first four steps suffice.

1. If oil has been used to cover the section, remove it by two 5-min washes in chloroform; do not dislodge the coverslip or allow the chloroform to evaporate, as this irreversibly precipitates probe. If the hybridization was conducted under a sealed coverslip, simply break the rubber seal with the tip of a scalpel. For tissue blots, simply remove the tape and secure it to metal frames.
2. Wash the slides twice for 5 min, in hybridization wash medium (HWM) consisting of 50% formamide, 0.6 *M* NaCl, 10 m*M* phosphate buffer (pH 6), 1 m*M* EDTA.
3. Transfer the slides to 2× SSC at 55°C for 1 hr (Rein *et al.*, 1982). This should be omitted for paraffin sections, as they tend to detach from the slide during this step.
4. For short exposures wash in HWM for 1 hr at room temperature, 1 liter, two changes. Dehydrate in ethanol solutions containing 0.3 *M* ammonium acetate (to stabilize hybrids): 70% ethanol, 5 min, twice; 95% ethanol, 5 min, once. Air dry. For long exposures wash for 3 days in 1500 ml of HWM with scraps of nitrocellulose paper. Dehydrate and air dry as above.

2. *Autoradiography*

After washing the slides, we coat the hybridized slide with nuclear track emulsion and place them at 4°C. The volume by Rogers (1979) is an invaluable source of information on the theoretical and practical aspects of autoradiography. Our current practice is to dip the slides in melted nuclear track emulsion (Kodak NTB-2) to which a volatile salt has been added (0.3 *M* NH_4 acetate) to stabilize hybrids during dipping.

Work with emulsion in total darkness. Put a histology water bath in the dark room, several racks to hold microscope slides in a vertical position (test tube racks made of plastic-coated wire are convenient), and lightproof slide boxes. Equilibrate the water bath to 42–45°C. Melt one bottle (118 ml) of emulsion by immersing it in the water bath for 30 min. Place a beaker containing 118 ml of 0.6 *M* NH_4 acetate in the water bath at the same time. Pour the melted emulsion into the beaker; mix it with the salt solution by gently stirring with a clean microscope slide. The size of the beaker should be such that 240 ml fills it to a level corresponding to approximately three-fourths of the length of a microscope slide. Dip a blank slide in the beaker for a few seconds and observe it outside the dark room. If a large number of air bubbles are present on the slide, deaerate the emulsion by repeatedly dipping a slide in the beaker. Dip hybridized slides in the emulsion for a few seconds, and place them vertically in racks for drying. Pour the excess emulsion back into a bottle and store it at 4°C in a light-tight box. The same bottle of emulsion can be melted several times over a period of 1 or

2 months without significant increases in background. After drying for 1 hr at room temperature, place the slides in lightproof slide boxes containing a small amount of drying agent. This is an important precaution, since humidity greatly increases fading of latent images in Kodak emulsions. Seal the boxes with tape, cover with aluminum foil, and put the boxes in a refrigerator (4°C) for the appropriate amount of time.

Before developing the slides, warm the boxes to room temperature for 1 hr to prevent condensation on the slides. Develop the slides in the dark: 3 min in Kodak D-19 developer at room temperature, a brief dip in water, 3 min in Kodak fixer. At this point the light can be turned on. Wash for approximately 1 min in two changes of distilled water. Stain the slides.

The choice of stains depends on the nature of the specimen. Giemsa is a general purpose stain, well suited to cells grown in culture and deposited on microscope slides (Brahic and Haase, 1978). Animal tissues, especially nerve tissue, are usually stained with hematoxylin-eosin (Luna, 1968). In this case the ammonium hydroxide solution in which the slides are dipped to obtain a blue coloration of nuclei must be made in 80% ethanol in order to avoid swelling and loss of the emulsion. The subsequent washes should also be done in ethanol. After drying, mount the slides under coverslips. Photographic emulsion sometimes interacts with toluene-base mounting medium to render observation of the specimen impossible. This can be avoided by using water-base mounting medium (e.g., Dako-Glycergel).

3. *Fluorography*

There have been claims that exposure times can be dramatically reduced by incorporation of liquid scintillators in nuclear track emulsions (Durie and Salmon, 1975). We and others (Rogers, 1981) have been unable to verify these claims, and we agree with Rogers that the reported apparent decrease in exposure times may have been the result of adequate drying of the emulsion exposed to liquid scintillators and inadequate drying in the controls. Certainly little increase in grain development would be expected with scintillants, as the emulsions used in autoradiography, in contrast to X-ray films, are quite insensitive to photons. To underscore this distinction we incorporated 0.1–10% PPO in the toluene–methacrylate solutions used to mount coverslips and used this solution to cover cells labeled with thymidine. After drying, the coating with PPO remained clear and readily darkened X-ray film, but no grains formed in NTB 2 or NTB 3 emulsions applied over the layer of PPO.

4. *Color Microradioautography and Double-Label in Situ Hybridization*

With ^{3}H- and ^{35}S-labeled probes to two different viruses or genes it is possible to identify cells containing different genes by a color autoradi-

ographic process (Haase *et al.*, in preparation). After hybridization and development, the silver grains are converted to magenta colored grains in the first layer of emulsion with color developer (Kodak CD2) and a magenta dye coupler (Eastman Kodak M-38). This first layer is then coated with a thin film of Krylon polymer (Borden Co.) and a second layer of emulsion. After a second exposure and development, grains in the second layer are converted to cyan colored grains with CD2 and C-16 (Eastman Kodak) a cyan dye coupler. Cells that have bound ^{35}S will contain magenta grains in the first layer, cyan grains in the second. Cells that bound ^{3}H probe will contain magenta grains in the first layer; because of the thin barrier film and lower energy of ^{3}H, insignificant numbers of cyan grains will be produced in the second layer.

5. *Detection of Genes and Gene Products in the Same Cell*

It is now possible to detect simultaneously genes and gene products in the same cell (Brahic *et al.*, 1984). Cells are reacted first with specific antibody and then developed with avidin–biotin peroxidase to deposit an insoluble polymer of diaminobenzidine at sites in the cell where antigen has accumulated. The diaminobenzidine resists the subsequent treatments involved in *in situ* hybridization. The autoradiograph therefore will have cells in which the large numbers of silver grains indicate specific genes, and the brown polymer will mark antigen deposits in the same cell.

F. ANALYSIS OF DATA

1. *Controls*

The expected outcome of the hybridization is nucleotide sequence-specific binding of radioactivity, recorded through formation of silver grains in the emulsion. Since grains form in response to other forms of energy, and probes may bind to cells by interactions not involving base pairing, it is essential to include a number of controls.

a. Background. During autoradiography some silver grains will result from the development process or from spurious causes such as pressure, exposure to light, chemography, and environmental radiation (Rogers, 1979). These grains constitute the background in the emulsion itself. With each batch of emulsion we ascertain the level of background from a slide dipped in emulsion. Cells and glass also bind probes nonspecifically. In most instances most of the background can be attributed to such nonspecific binding. We estimate background levels of binding from the developed autoradiographs of uninfected cells.

b. Nuclease Controls. Nucleic acid probes may bind to proteins and other cellular constituents as well as nucleic acids, and on occasion this binding may give rise to very convincing artifacts. We found, for example,

that the granules of eosinophiles contain a very basic protein (Gleich *et al.*, 1974) that will interact strongly with probes to give the impression that viral genomes are present in eosinophiles (R. Peluso, unpublished). Although this problem is readily circumvented with dextran sulfate in the hybridization medium, it illustrates how misleading *in situ* hybridization can be without appropriate controls.

One such control is nuclease digestion of the infected cells before hybridization. For RNA, digestion described in Section II,D,3 should decrease the grain count to the level of uninfected cells. For DNA we digest cells or sections before the postfixation step with 200 μg of DNase per milliliter in 20 m*M* Tris (pH 7.4), 10 m*M* $MgCl_2$ for 60 min at 37°C. The DNase should be freed of contaminating ribonuclease (Zimmerman and Sandeen, 1966).

c. Nucleotide Sequence Specificity. The results of tests described in subsections *a* and *b* above should provide assurance that the probe is bound through base pairing. The sequence specificity of this interaction should be determined by comparing the binding of a probe of comparable specific activity but unrelated sequence to the cells or sections. This heterologous probe can be the DNA of the plasmid vector of a cloned viral DNA, or a probe for a virus with a host range confined to tissues of species other than those being tested. Competition experiments with unlabeled nucleic acid corresponding in sequence to probe offer an additional or alternative control, particularly in this era when the requisite large quantity of unlabeled DNA is readily obtained by cloning.

d. Reproducibility, Limitations of the Controls. It is both necessary and easy to demonstrate specific hybridization of probe to preparations of infected cells or to sequential sections in which the foci of infection are quite extensive. This criterion of reproducibility should be satisfied in time and space, i.e., independent hybridizations of subjacent sections should continue to be positive, throughout the depth of an infected focus. Because lesions are not precisely aligned in every section, it is unrealistic to expect that the positive foci will be superimposable in every case, but the positive areas should be within a microscopic field or two. If only an occasional cell is infected it may not be possible to reproduce a positive focus. This situation unfortunately also invalidates the other controls and therefore limits the analysis to infections that are positive at several foci and depths. The recent development of *in situ* double-label hybridization should make it possible to control internally for specificity within a single section.

2. *Quantitation*

a. Theoretical Considerations. In principle, the number of copies of viral genetic material per cell can be determined from the number of grains formed over that cell. Consider a hypothetical cell with 30 copies of visna

virus RNA. If the probe is perfectly representative, does not self-anneal, and is labeled with ^3H at a specific activity of 4×10^8 dpm/μg, and if 100% hybridization efficiency is achieved, the amount of radioactivity in the cell will be $30 \times (5 \times 10^{-12}) \times (4 \times 10^8) = 0.06$ dpm. (The molecular weight of one strand of visna RNA is 3×10^6, which is equivalent to 5×10^{-12} μg). If we assume that the emulsion responds with a linear increase of grain number with time of exposure, we expect 60 grains per cell after 1 week of exposure. Or, to put it another way, if we find 60 grains per cell after 1 week of exposure we conclude that the cell contains 30 copies of visna virus DNA.

In practice, the conditions listed above are rarely met because (1) probes synthesized by nick translation of cloned DNA, or by reverse transcription of RNA using random primers, do self-anneal and in dextran sulfate form networks by hybridization to tails of partially hybridized probes; (2) the hybridization efficiency reaction for DNA is not 100%, probably because of competition from reassociation of contiguous DNA in the cell; (3) the actual efficiency of grain formation, which varies with isotopes, also depends on the thickness of the section and the distribution of viral nucleic acids within it. As a result a direct computation of viral genome numbers, as described above, can serve only as a guide.

b. Calibration Curves. In practice we determine copy numbers by determining the relationship between grain counts and copy numbers in productively infected cells hybridized *in situ* under conditions where the hybridization reaction is driven to completion (probe excess, hybridization for 72 hr). We determine the relationship of grain count to copy numbers at different times of infection to show that the relationship holds over a wide range of copy numbers.

We will illustrate the construction of such a calibration curve for the quantitation of visna virus RNA in sheep tissue culture cells. Infect sheep choroid plexus cells at 3 PFU/cell. Harvest the cells by trypsinization at various times in the replication cycle. Deposit one aliquot on a slide and hybridize *in situ* for viral RNA. Expose the slide for a period of time compatible with accurate counting of grains (12–50 per cell). Determine the average number of grains per cell for 50–100 cells selected randomly. Extract total cellular RNA from another aliquot of cells. Measure the average number of copies of viral RNA per cell by solution hybridization (Brahic and Haase, 1978) or by a "dot blot" technique (Thomas, 1980). Plot the average number of grains per cell per unit time of autographic exposure against the average number of viral RNA genomes per cell. The relationship of grain count to copy number is linear between a few and several hundred viral RNA genomes per cell, and nonlinear for higher copy numbers (Fig. 4). The curve can be used to estimate the number of viral RNA genomes

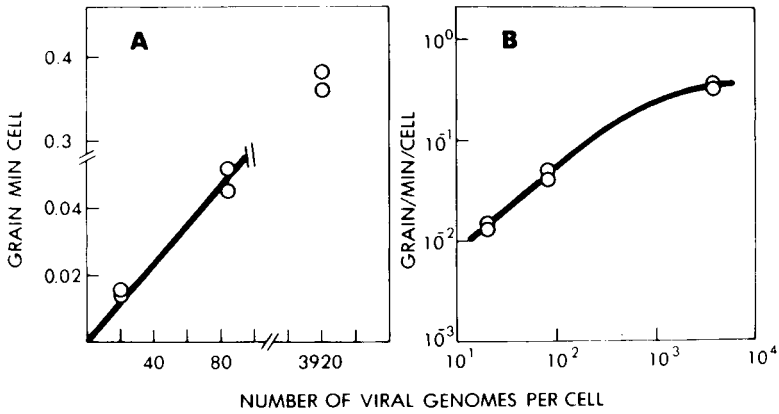


FIG. 4. Quantitation of copy numbers by *in situ* hybridization. Linear (A) and logarithmic (B) plots of the relationship between grain counts and viral RNA copy number determined by *in situ* hybridization and solution hybridization, respectively, in cells infected with visna virus and harvested at different times after infection. Reprinted from Brahic and Haase (1978) with permission.

present in infected cells detected in sections of animal tissues with *in situ* hybridization performed under the same conditions (Brahic *et al.*, 1981b).

c. Definition of the Labeled Cell. In tissue sections of infected animals, the cells with viral genetic material usually occur in foci. We base our recognition of the focus of infected cells on criteria derived from the Poisson distribution of grains. Silver grains are randomly distributed over cells of equal size such that the probability that x grains are found over a cell is $Px = m^x e^- m(x!)^{-1}$ where m is the average number of grains per cell in the uninfected controls (Arnold, 1981). We usually choose exposures that result in 5–10 grains per uninfected cell. The probability that a cell is infected is >0.99 for cells that contain 12 grains (background 5 grains). We use the 0.99 criteria to identify infected cells in sections from infected animals and determine the average grain count and copy numbers in this population.

III. Applications

Shortly after the introduction of *in situ* hybridization to localize reiterated genes in cells, virologists utilized the technique to localize viral genes in productively infected cells (McDougall *et al.*, 1972; Orth *et al.*, 1970, 1971), and subsequently in cells transformed by DNA and RNA tumor viruses (Loni and Green, 1973, 1974; Rein *et al.*, 1982). In the ensuing decade improvements in probes and techniques have brought the sensitivity of *in*

situ hybridization to the point that single copies of viral genes can be mapped on chromosomes (Gerhard *et al.*, 1981; Harper *et al.*, 1981; Neel *et al.*, 1982; Tereba *et al.*, 1979; Trent *et al.*, 1982), and single copies of viral genomes can be detected in cells (Haase *et al.*, 1982). *In situ* hybridization is therefore a powerful complement to other techniques for gene mapping (Ruddle, 1981), and is virtually indispensable in situations in which replication is restricted within individual cells or within a population of cells, or in which the limited amount of tissue available in a biopsy precludes the use of other hybridization techniques.

We have elected to organize this section on applications around the general problem of virus infection and pathogenesis, with the view that the heterogeneity of virus replication presents just the situation in which the contributions of *in situ* hybridization have been or are expected to be unique. Although the appreciation of heterogeneity of virus replication *in vitro* achieved with *in situ* hybridization can be enlightening (Fig. 5), we will devote our discussion to examples of applications to issues of viral pathogenesis *in vivo,* to show how quantitative *in situ* hybridization can be used to study virus life cycles *in vivo*. This information provides the foundation for such fundamental reconstructions of virus infection as restricted gene expression as a unifying theme in slow infections and impetus for a search for virus genes in chronic diseases of man.

A. CELL TYPE AND PATHOGENESIS

Identifying viral genes in a particular cell type by *in situ* hybridization can immediately shed light on pathogenesis and suggest appropriate lines of investigation.

1. *Demyelinating Diseases*

a. Theiler's Virus Infection. Theiler's agent is a picornavirus of mice that, in natural infections, replicates initially in the gut and subsequently may disseminate to the central nervous system, where it causes a biphasic disease. The first phase is an encephalitis in which neurons, particularly those in the hippocampus, are infected and destroyed. Animals that survive the acute disease may develop later a curious paralytic condition characterized by inflammation and primary demyelination in the white matter of spinal cord. This white matter disease, which strikingly resembles multiple sclerosis (MS) in man, has been attributed to an immunopathological process (Lipton and Dal Canto, 1976). The demonstration (Brahic *et al.*, 1981a) by *in situ* hybridization that viral RNA persists in glial cells raises the possibility that viral gene products may invoke and sustain the inflammatory and demyelinating response.

b. Marek's Disease. A different mechanism of demyelination likely op-

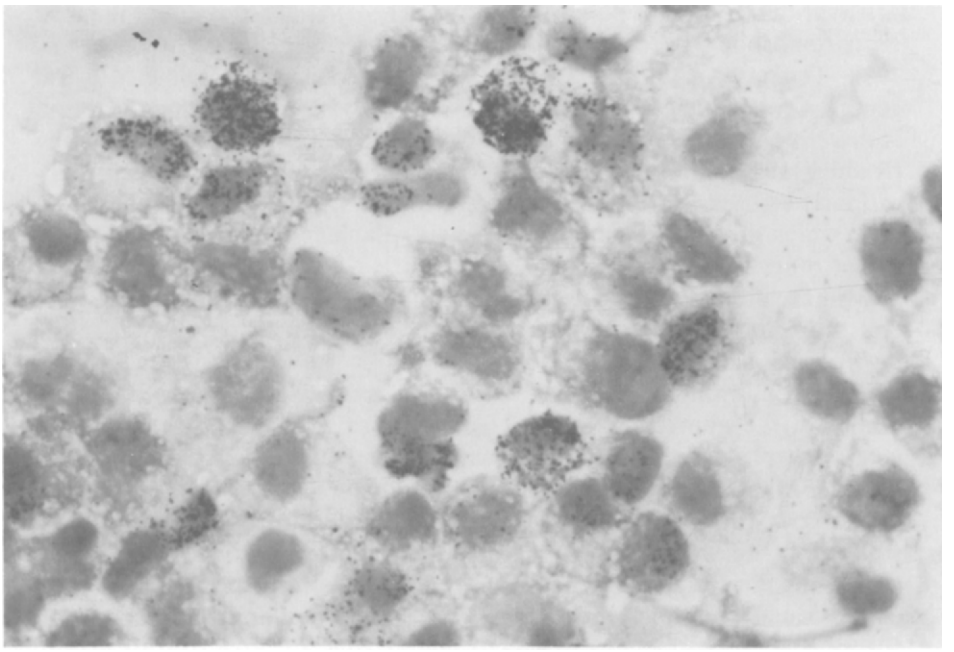


FIG. 5. Heterogeneity of visna virus replication *in vitro* revealed by *in situ* hybridization. Sheep tissue culture cells uniformly and synchronously infected with visna virus were hybridized *in situ* for viral RNA 20 hr after infection. The heterogeneity in transcription is evident, ranging from negligible accumulation of RNA in the cells, to the onset of transcription in nuclei of cells and RNA in both nucleus and cytoplasm. Investigations of this heterogeneity lead to the hypothesis that gene dosage may regulate gene expression and the tempo of replication in infections by this prototype of conventional agents of slow infections (Haase *et al.*, 1982). Hybridization conditions were as described by Haase *et al.* (1982).

erates in Marek's disease of fowl, an infection by a member of the herpes virus family. The causative agent transforms lymphocytes, and these cells infiltrate viscera and peripheral nerve. The demyelination in nerve that ensues is mediated in some way by the transformed lymphoblasts, not by an immune reaction to viral products in the Schwann cells, since Ross *et al.* (1981) by *in situ* hybridization readily documented viral nucleic acid in lymphocytes in the nerve, but not in Schwann cells.

2. *Virus Dissemination*

In situ hybridization has played an essential part in clarifying the basic mechanisms involved in the horizontal and vertical transmission of viruses, both in individuals and in populations. Epstein-Barr virus (EBV) is har-

bored in B lymphocytes, but replicates in the epithelial cells of the oropharynx. Identification by *in situ* hybridization of the EBV genome in epithelial cells provided an explanation for transmission of virus in infectious mononucleosis (Lemon *et al.*, 1977) and a further link between EBV and nasopharyngeal carcinoma, another situation in which *in situ* hybridization revealed EB virus genomes in epithelial cells (Wolf *et al.*, 1973). Cytomegalovirus (CMV) genomes have been demonstrated by *in situ* hybridization in peripheral blood leukocytes (Joncas *et al.*, 1975), in immature and mature germ line cells such as spermatozoa (Dutko and Oldstone, 1979), and in cultured embryo cells derived from mice infected with murine CMV (Chantler *et al.*, 1979). These findings suggest a mechanism for the vertical and horizontal transmission of CMV. *In situ* hybridization has also been enlightening in hepatitis B virus infections, in which viral genomes have been discovered in endothelial and biliary epithelial cells (Fig. 6), raising the possibility that virus may traffic in and out of the liver in the blood and bile by replicating in these cells (Blum *et al.*, 1983).

B. VIRUS REPLICATION AND PATHOGENESIS

We regard as particularly important applications of quantitative *in situ* hybridization to assessment of the biochemistry of virus replication in single cells in tissues (Brahic *et al.*, 1981a; Simon *et al.*, 1982). This approach is likely to be even more rewarding in the future, allied with recombinant DNA technology to provide probes specific for individual genes and of defined polarity, a prerequisite to distinguish between synthesis of negative-strand genomes and plus-strand mRNAs. By developing a detailed picture of the anatomy of the virus genome in individual cells, and the expression of specific regions of the genome, we may hope to penetrate the enigmas of virus latency and carcinogenesis (Galloway *et al.*, 1979; McDougall *et al.*, 1982), persistence, and slowness. The examples that follow are drawn from our investigations of the slow and persistent infections caused by visna virus and by measles virus.

1. *Visna*

Visna virus is the prototype of the subfamily of lentiviruses responsible for slow infections of sheep (Brahic and Haase, 1981). The virus life cycle of this and other retroviruses involves the transfer of genetic information from the RNA genome of the virus to a DNA intermediate or provirus in the infected cell. In productive infections in tissue culture the DNA serves as a template for the synthesis of viral mRNA and genomic RNA, and about 50–100 infectious progeny are produced per cell.

In order to explain the slow evolution of the disease in nature, and the

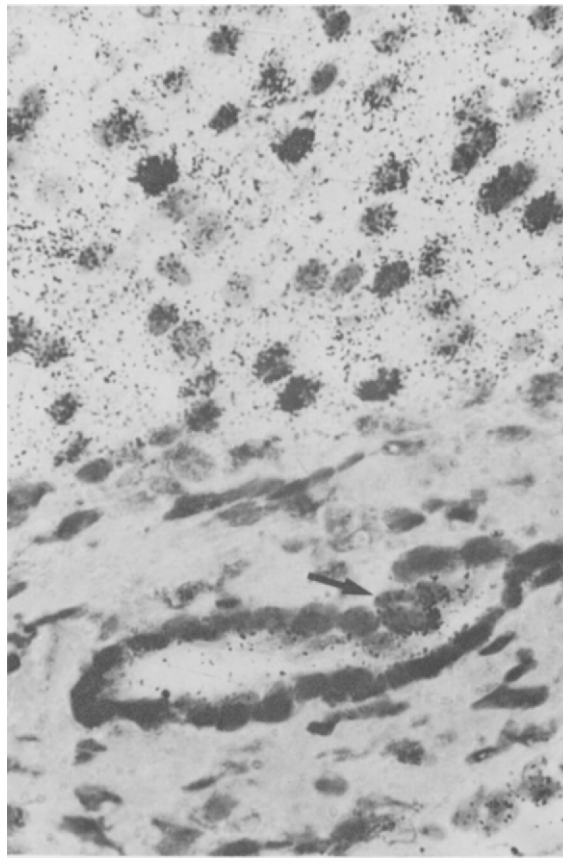


FIG. 6. Demonstration by *in situ* hybridization of hepatitis B virus DNA in hepatocytes and biliary tract epithelium. Sections from a liver biopsy of a patient with chronic peristent hepatitis were hybridized *in situ* and exposed for 48 days before developing and staining. The arrow points to grains over the nucleus of an epithelial cell in a bile duct cut in cross section. There are larger numbers of grains evident in the nuclei and cytoplasm of hepatocytes in the adjacent half of the section.

ability of the virus to withstand the immune attack mounted by its host, we postulated that the visna *in vivo* is analogous to lysogeny in bacteriophage λ. The expression of proviral DNA was held to be so reduced that the cell had insufficient antigen to be detected and destroyed by immune surveillance or to sustain a rapid rate of tissue destruction. To test this hypothesis we employed *in situ* hybridization to detect viral genomes in tissue sections, and we measured viral antigen production by immunofluorescence in subjacent sections. The predictions of the lysogeny model were

satisfied: visna DNA was demonstrated in foci of cells that had little if any detectable antigen (Haase *et al.*, 1977). This molecular analysis has been extended to establish that the block occurs at the level of transcription (Brahic *et al.*, 1981b). This result is consistent with the lysogeny model, or with a gene dosage model of regulation of expression, since *in vitro* we found a correlation between the extent of early DNA synthesis and transcription (Haase *et al.*, 1982).

2. *Pseudolysogeny and Restricted Gene Expression as a Unifying Theme in Slow Infections in Vivo*

Two axiomatic conditions that must be fulfilled for a virus to cause a slow and persistent infection *in vivo* are that (1) the synthesis of virus antigen and particles must be sufficiently limited for the virus to survive immune surveillance; and (2) the destructive aspects of the virus host interaction characteristic of productive infections *in vitro* must be sufficiently mitigated for the host to survive, and thus continue to provide the cellular substrates to perpetuate infection (Braude, 1981). Analysis of infections by other than visna by quantitative *in situ* hybridization suggests that restricted synthesis and expression of viral genes may be a general theme in slow infections and provide explanation of how viruses satisfy these logical conditions (Haase *et al.*, 1981b; Brahic and Haase, 1981). Figure 7 and Table II trace the decline in RNA copy number and production of antigen from acute to chronic infectious states with measles virus and with visna virus.

TABLE II
RESTRICTED SYNTHESIS AND EXPRESSION OF VIRAL GENES IN TWO SLOW INFECTIONS[a]

	Frequency of cells with RNA (%)		Frequency of cells with DNA (%)	Average number of copies of RNA		Cells with viral antigen detectible by immunofluorescence (%)	
Type of Infection	Measles	Visna	Visna	Measles	Visna	Measles	Visna
Permissive infection of tissue culture cells	>90	>90	>90	2500	10,000	>90	>90
Acute infection of animals	0.9	—	—	1500	—	>90	—
Chronic infection in animals	0.3	1.9	1.6	3	140	0–0.1	0–0.025
SSPE	0.3	—	—	7	—	0–0.01	—

[a]The synthesis and expression of viral genomes in two slow infections was qunatitated by *in situ* hybridization; viral antigen production was measured by immunofluorescence with antisera to major virus polypeptides. The table was modified from Brahic *et al.* (1981b) and Haase *et al.* (1981b), by permission of the publishers.

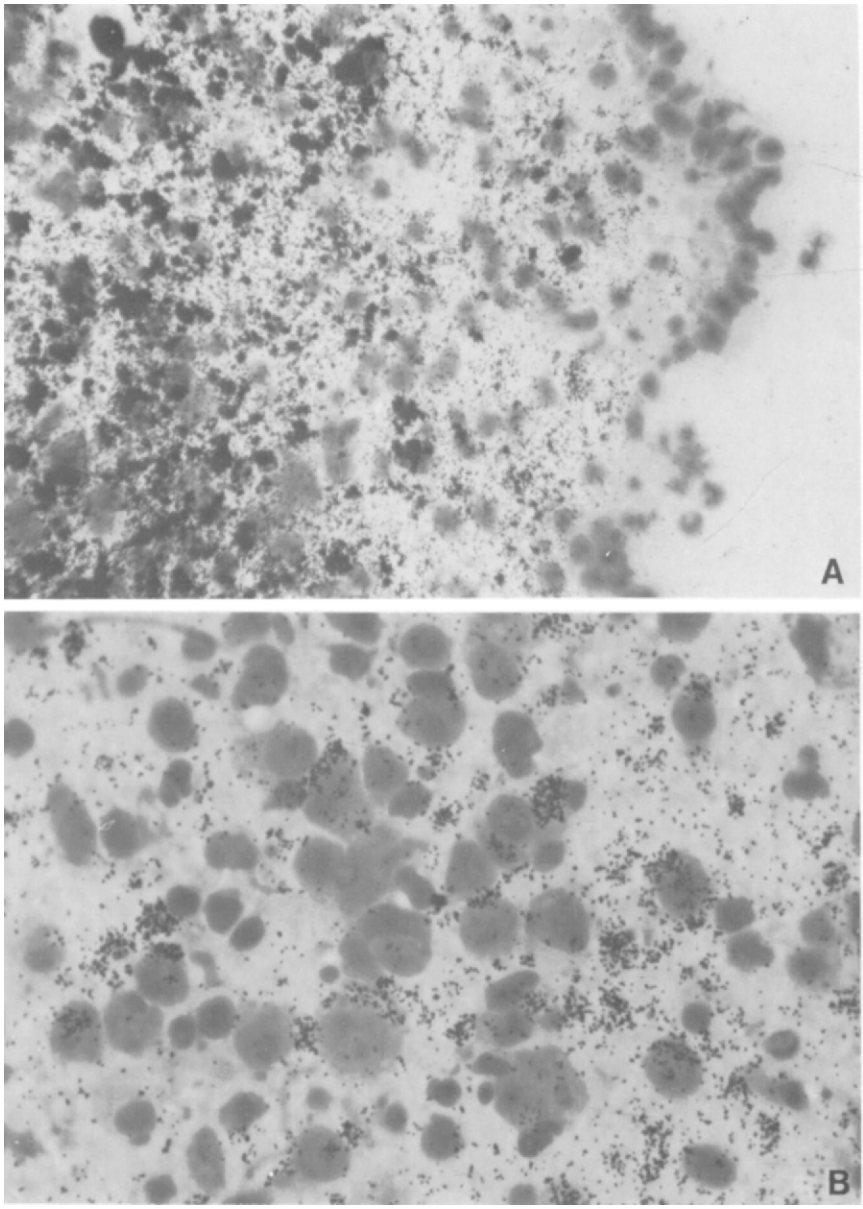
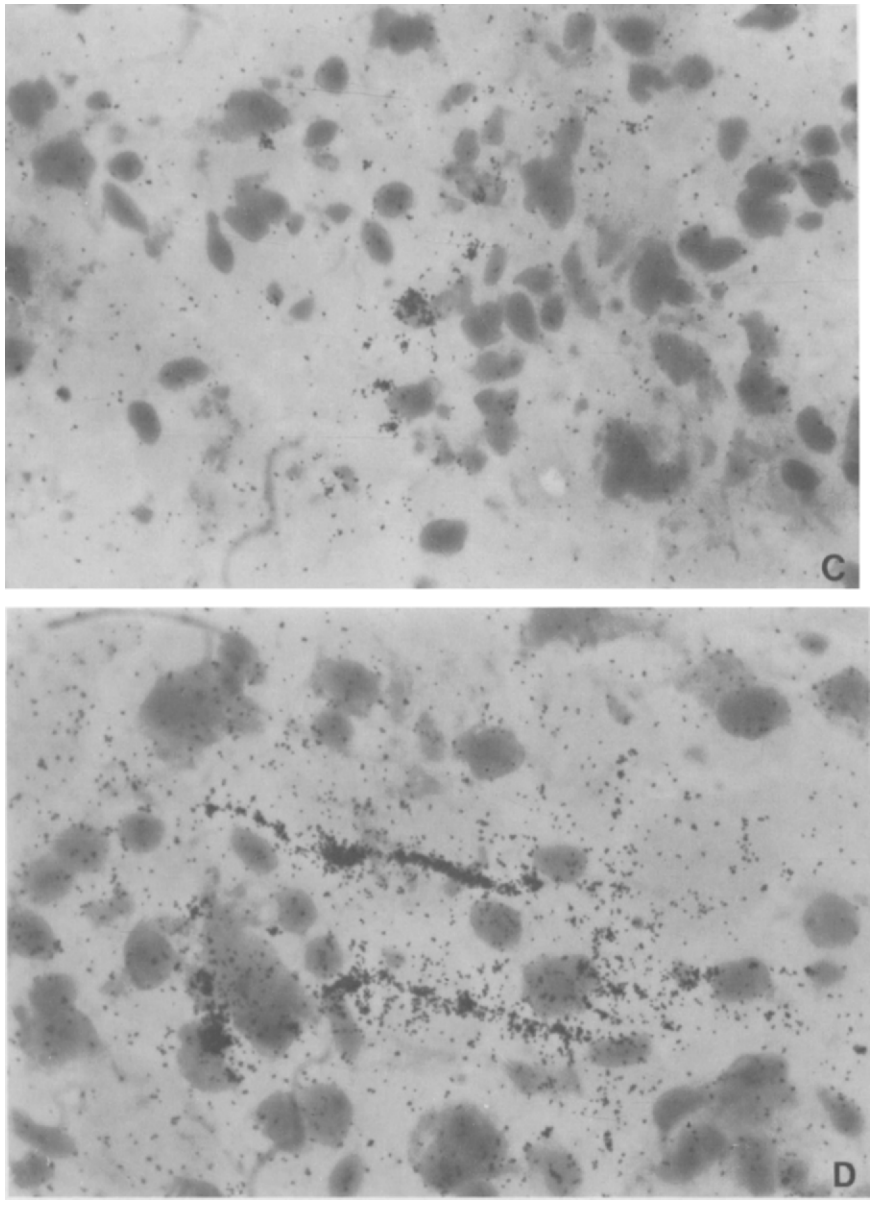


FIG. 7. Restricted synthesis and expression of measles virus genes in chronic infections. Measles virus RNA was detected by *in situ* hybridization as described by Haase *et al.* (1981a,b). (A and B) Sections from an acutely infected hamster; (A) overexposed (1 week) to show accumulation of silver grains over cells in a periaqueductal focus, average of 1000 copies per cell of viral RNA; (B) short exposure (6 hr) of cells in focus shown in (A). (C–F) Chronic



infections with fewer than 10 copies of viral RNA per cell; decrease in copy number can be appreciated by comparing number of grains in cells (B) with a 6-hr exposure to the number of grains per cell with a 3-week exposure (C–F). (C and D) Sections from case of SSPE; (D) shows axonal distribution of viral RNA. (E and F) Sections from two cases of multiple sclerosis.

(*cont.*)

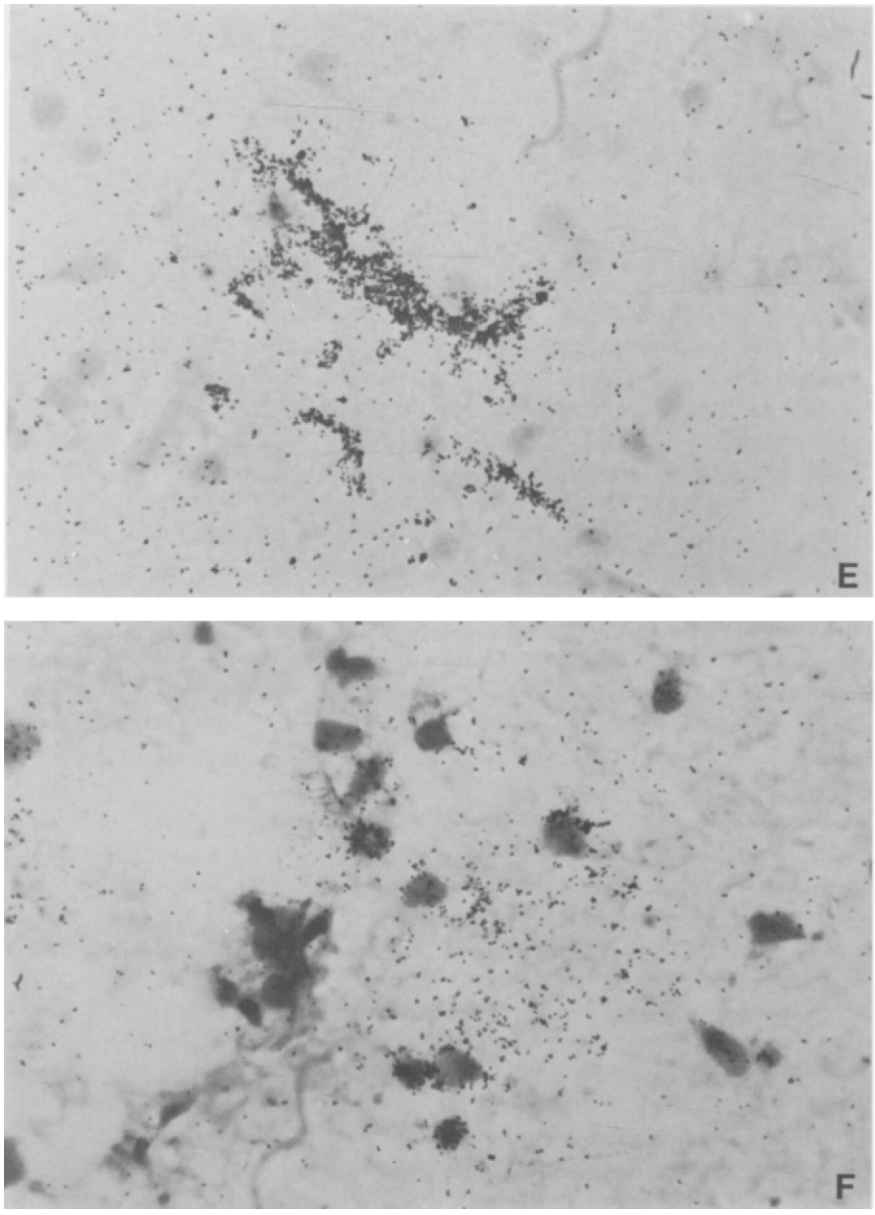


Fig. 7. (*cont.*)

C. THE SEARCH FOR VIRAL GENES IN CHRONIC HUMAN DISEASES

Viruses have been advanced as the cause of diseases as diverse as cancer and multiple sclerosis (MS), but with few exceptions the evidence is hardly compelling, as it has not been possible consistently and reproducibly to demonstrate infectious virus, particles, antigens, or nucleic acids in the tissues. However, all these analyses have relied on assays that predictably would have been negative if viral gene expression is curtailed *in vivo,* and viral genomes focally distributed such that extraction of nucleic acid from the tissue would dilute the virus sequences beyond detectibility by solution or blot hybridization methods. This line of reasoning provides the rationale for a reevaluation by *in situ* hybridization of the role of viruses in chronic diseases of man. This approach has been taken in analyses of measles virus in MS (Haase *et al.,* 1981a) and in cervical carcinoma (McDougall *et al.,* 1982) and will likely be profitable in the search for hepatitis B virus in hepatocellular carcinoma (Gowans *et al.,* 1981) and other cancers in which viruses have been implicated.

D. PROSPECTUS

In our view the full promise of *in situ* hybridization will be realized in the next few years, based on developments that make it possible to define the spatial and temporal distribution of specific genes in tissues of whole organisms, and to define gene expression at the single cell level: (1) The use of probes dual labeled with ^{125}I and ^{35}I in the tissue hybridization method described in this chapter represents a new approach in hybridization that combines macroscopic localization of genes in tissue detectable on X-ray film with microscopic localization in cells in the autoradiographs. In this way large areas of tissues can be sampled to locate the position of cells that warrant closer examination with the light microscope. (2) The assays for genes and their products in the same cell and the color autoradiographic-facilitated analysis of double-label *in situ* hybridization provide unprecedented opportunities to examine gene expression in individual cells. We envision numerous applications of these new strategies in virology, and in many other disciplines such as developmental biology for which the genetic programs of particular types of cells is of importance.

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8 *Exploring the Gene Organization of Baculoviruses*

Lois K. Miller

I. Introduction

Molecular biological research on insect baculoviruses was originally stimulated by interest in the commercial employment of these viruses as pesticides (Summers *et al.*, 1975; Burges, 1981). Occluded baculoviruses are, however, also intrinsically fascinating from both molecular biological and virological perspectives. These viruses have two distinct infectious forms

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that differ functionally and biochemically. Synthesis of these two forms is temporally controlled by the virus; the formation of extracellular nonoccluded virus (NOV) occurs relatively early in infection (e.g., 12 hr postinfection) and the formation of occluded virus (OV) occurs late in infection (e.g., 18–72 hr postinfection) in the nucleus. The synthesis of occluded virus also appears to be influenced by the host cell type (e.g., midgut epithelial cell versus fat body cell). Several recent reviews on the nature of baculoviruses and their mechanisms of infection *in vivo* and *in vitro* are available (Carstens, 1980; Faulkner, 1981; Harrap and Payne, 1979; Summers, 1978; Tweeten *et al.*, 1981). Exploration into the nature of baculovirus genes, their organization of genes, and the molecular mechanisms controlling their expression is fundamental to understanding the strategy of the baculovirus infection process. In pursuing this goal, baculovirologists are bound to uncover some very important principles regarding gene organization and expression in invertebrate cells.

Several unique features of baculoviruses make them highly attractive as potential recombinant DNA vectors for passenger gene expression in eukaryotes also (Miller, 1981a). These features include (1) a large (100–250 kb) double-stranded, circular, supercoiled DNA genome that replicates in the nucleus; (2) an extendable rod-shaped nucleocapsid and thus a potentially large capacity (e.g., 100 kb) for passenger DNA; (3) a very strong promoter controlling the synthesis of a dispensable gene product (the occlusion matrix protein); and (4) the temporal control of expression from this promoter, thus permitting the expression of passenger genes after the synthesis of infectious virus but well before cytolysis. In fact, evidence exists that in nature the virus can act as a vector for cellular sequences (Miller and Miller, 1982). The development of these viruses as recombinant DNA vectors has thus been recommended for cases where a large vector capacity is needed and where the gene product or metabolic pathway product may be cytotoxic. This provides an extra impetus to explore the gene organization of these complex viruses.

The availability of permissive cell lines and plaque assay methods has made several baculoviruses highly amenable to molecular biological and genetic analysis. Rapid progress has been made in the analysis of one virus particularly, *Autographa californica* nuclear polyhedrosis virus (AcNPV). Ten laboratories in North America and Europe are now focusing on AcNPV and its closely related variants (*Trichoplusia ni* mNPV, *Rachiplusia ou* NPV, and *Galleria melonella* NPV) as the basic model system for molecular biological analysis of occluded baculoviruses. This review will concentrate on the recent advances made in the molecular biology of AcNPV with special attention to the methodology used to determine the gene organization of this virus. Studies of the gene organization of other baculoviruses will be considered in relation to AcNPV work.

Most of the methodology used in mapping the gene organization of AcNPV was originally developed in the study of animal tumor viruses. The rapid progress made with AcNPV stems in large part from the availability of these techniques and the ease with which they may be applied to baculovirus systems. Rather than reviewing details of the methods developed with other virus systems, this review instead focuses on their specific application to baculovirus research and the type of data obtained to date. A few suggestions for future research are also offered. The volume "Molecular Cloning" (Maniatis *et al.*, 1982), which provides complete details for gel electrophoresis of nucleic acids, cloning, blotting, hybridization, hybrid selection, and so forth, is highly recommended. This monograph also provides strategies and many helpful practical suggestions.

The most notable progress in determining baculovirus gene organization has been made in (1) constructing physical restriction endonuclease maps; (2) constructing a translation (or gene product) map of AcNPV by hybrid selection and *in vitro* translation of specific RNAs; and (3) the development of marker rescue (or gene replacement) procedures for creating and/or mapping AcNPV mutants. The research described herein provides a very strong framework on which some exquisite molecular biology and genetic engineering can be performed.

II. Establishing the Physical Map of a Baculovirus

A. Basic Considerations in Mapping Restriction Endonuclease Sites

Obtaining a pure, preferably plaque-purified, baculovirus isolate is an important first step in preparing to map the virus physically. Genotypic heterogeneity of a virus stock results in the presence of restriction fragments in submolar quantities (Knell and Summers, 1981; Lee and Miller, 1978; Smith and Summers, 1978) that could be misleading in determining linkage relationships. If plaque purification is not possible, owing to the lack of a permissive cell culture system, then attempts to obtain as pure a virus preparation as possible should be made. It is interesting that even plaque purification of baculoviruses does not necessarily result in genetic purity. There are now three reports of genetic heterogeneity remaining in baculovirus preparations after several successive plaque-purification steps (Carstens, 1982; Huang *et al.*, 1982; Miller and Miller, 1982). This genetic heterogeneity may be due to recombination between repeated, inserted, sequences in the viral DNA (see discussion in Section III, A on transposable element effects on genotype). It is therefore recommended that several different plaque isolates or purified virus preparations be compared by restriction

endonuclease analysis before choosing a virus preparation for physical mapping and further characterization.

The large size of the baculovirus DNA genome makes physical mapping of restriction endonuclease sites a somewhat difficult and tedious process. Several of the techniques used to map restriction sites on small viral DNAs, such as papovavirus DNAs, are not easily applied to baculovirus DNAs. For example, the probability of finding a restriction endonuclease that recognizes only one site in a 125 kb DNA is extremely low. Instead, a restriction enzyme that recognizes a hexanucleotide sequence will probably recognize 10–20 sites on a given baculovirus DNA. An enzyme that produces fewer than 10 fragments, which are easily separable on agarose gels, is a good candidate for initial mapping.

In mapping smaller DNAs, advantage has also been taken in isolating "partial" digestion intermediates and redigestion of these intermediates to obtain fragment linkage information. This approach has limited utility in mapping a large baculovirus genome because restriction endonucleases tend to produce either (1) too many fragments, so that it is difficult to isolate pure partial digestion products, or (2) a few large fragments that cannot be purified on agarose gels from the partial digestion products.

The methods that have been successfully employed for mapping restriction endonuclease sites of baculoviruses include (1) a double-digestion technique in which a large restriction fragment is isolated from a gel and redigested with a second restriction endonuclease; and (2) a hybridization technique in which linkage information is derived by determining sequence homology relationships among fragments by a Southern blot technique. The inherent advantages and disadvantages of these techniques are discussed in turn below.

B. Double Digestion for Mapping Restriction Sites

The double-digestion technique, in which a large restriction fragment is separated by gel electrophoresis, excised from the gel, and redigested with a second enzyme and the digestion products are analyzed by gel electrophoresis, was used to establish the basic physicl map of AcNPV (Miller and Dawes, 1979; Smith and Summers, 1979). Smith and Summers (1979) relied solely on this technique to establish their physical map. Miller and Dawes (1979) used this technique primarily to confirm the map they established by sequence homology–Southern blotting techniques. The double-digestion technique is a tedious one, and, in order to be successful, the sites of a number of different restriction endonucleases must be mapped simultaneously. Linkage of fragments and alignment of the maps are accomplished by careful deduction. With this approach, there is a total reliance on cor-

relating the fragments on the basis of size. The advantage of the technique is that the relative positions of the sites within a restriction fragment can be determined from the sizes of the secondary cleavage products.

C. DNA SEQUENCE HOMOLOGY TO MAP RESTRICTION SITES

In the sequence homology techniques, a fragment produced by one restriction enzyme is hybridized to a Southern blot of viral DNA fragments produced by a second restriction enzyme (Miller and Dawes, 1979). One, two, or several restriction fragments may be homologous to the fragment used to probe the blot, and this provides information on the relationship between the two restriction fragment maps as well as linkage information for the fragments tested on the blot. The technique can often establish linkage information for large fragments more definitively than the double-digestion technique. The cross-blot hybridization, employed by Loh *et al.* (1981) to map the *Bam*HI and *Hin*dIII sites of *Spodoptera frugiperda* MNPV, eliminates the need to isolate separately each fragment used to probe the Southern blots.

If the baculovirus DNA contains repeated sequences, then spurious linkages may be deduced. This was not a problem in mapping AcNPV or SfNPV because both genomes contain very little repeated DNA (Cochran *et al.*, 1982; Loh *et al.*, 1981; Miller and Dawes, 1979; Summers *et al.*, 1980). Cochran *et al.* (1982), however, have detected homologous regions of AcNPV DNA between *Hin*dIII L and Q located at 18.4–20.4 and 87.2–88.8 physical map units, respectively. NPVs from four different *Spodoptera* species contained a readily reannealing fraction representing 2–3% of the DNA, suggesting a small amount of repeated sequence (Kelly, 1977). To minimize the effect of small regions of partial homology, two precautions can be taken: (1) the use of stringent hybridization conditions (Section VI) that favor only completely homologous DNA reassociations; and (2) reliance only on strong hybridization signals for linkage information. In most cases, reassociation kinetics indicate the lack of repeated sequences in NPV DNA (Jurkovicova *et al.*, 1979; Rohrmann *et al.*, 1977; Scharnhorst *et al.*, 1977), although Huang *et al.* (1982) reported that HZ-IV DNA may contain up to 13% repeated sequences. The hybridization technique may not be useful for a DNA with dispersed, totally homologous, repeated sequences.

D. MOLECULAR CLONING TO FACILITATE MAPPING

When the early physical maps of AcNPV were constructed, the NIH Guidelines for recombinant DNA research restricted the cloning of baculovirus DNA in *Escherichia coli*. More recently, these restrictions have been

lifted and baculovirus DNA fragments may be cloned in *E. coli* K12 using P1 containment in the United States. Two groups have employed cloned AcNPV fragments to facilitate mapping of their isolates (Cochran *et al.*, 1982; Lübbert *et al.*, 1981). Cloning facilitates both the double-digestion technique and the hybridization technique because it eliminates the need to purify the fragments on gels before redigestion or probing. It is particularly useful because cloned fragments are not contaminated by other fragments. Cloned fragments allowed Cochran *et al.* (1982) to establish that the *Hin*dIII L and Q fragment have some sequence homology. Cloning of baculovirus DNA fragments is recommended as an early step in mapping a baculovirus genome, since it facilitates detailed physical mapping and is very valuable for further work such as translational mapping, transcriptional mapping, and marker rescue (see below).

E. The Physical Map of AcNPV and Its Variants

Four different groups have established physical maps of AcNPV (Cochran *et al.*, 1982; Lübbert *et al.*, 1981; Miller and Dawes, 1979; Smith and Summers, 1979), and there is now mutual agreement on a common orientation and basic fragment nomenclature for the AcNPV genome (Vlak and Smith, 1982). Although map positions are frequently referred to with first decimal point accuracy, it should be remembered that there still is considerable doubt about the precise positions of some sites. Until finer mapping is done, some ambiguity will remain.

The genotypes of the viruses characterized by the different groups vary slightly from each of the others. AcNPV L-1 (Miller and Dawes, 1979) was originally chosen as the predominant variant found among 12 different plaque-purified isolates from a virus stock passaged once or twice in *A. californica* after the original isolation by Vail (Lee and Miller, 1978). The AcNPV L-1 physical map is presented in Fig. 1 and is thus far identical to the physical map of AcNPV E-2 (Smith and Summers, 1979) with the exception that AcNPV L-1 has an additional *Hin*dIII site in *Hin*dIII-B resulting in *Hin*dIII-B1 and *Hin*dIII-B2 fragments. The HR-3 strain of Cochran *et al.* (1982) is identical to the AcNPV L-9 variant and is similar to the AcNPV E-2 variant in all respects except for the presence of a small addition of approximately 0.2 kb in the 18.4–20.4 region of the genome. The AcNPV-E isolate of Lübbert *et al.* (1981) differs from AcNPV E-2 in at least two respects: there appears to be an additional *Eco*RI site as well as additional DNA (0.25 kb) in the *Eco*RI-H region and an insertion of about 0.15 kb in the *Eco*RI-L fragment.

There are a number of viruses that are closely related to AcNPV, but, because they were isolated from insects other than *A. californica,* they are known by other names. Thus *T. ni* NPV, *G. melonella* NPV, and *R. ou*

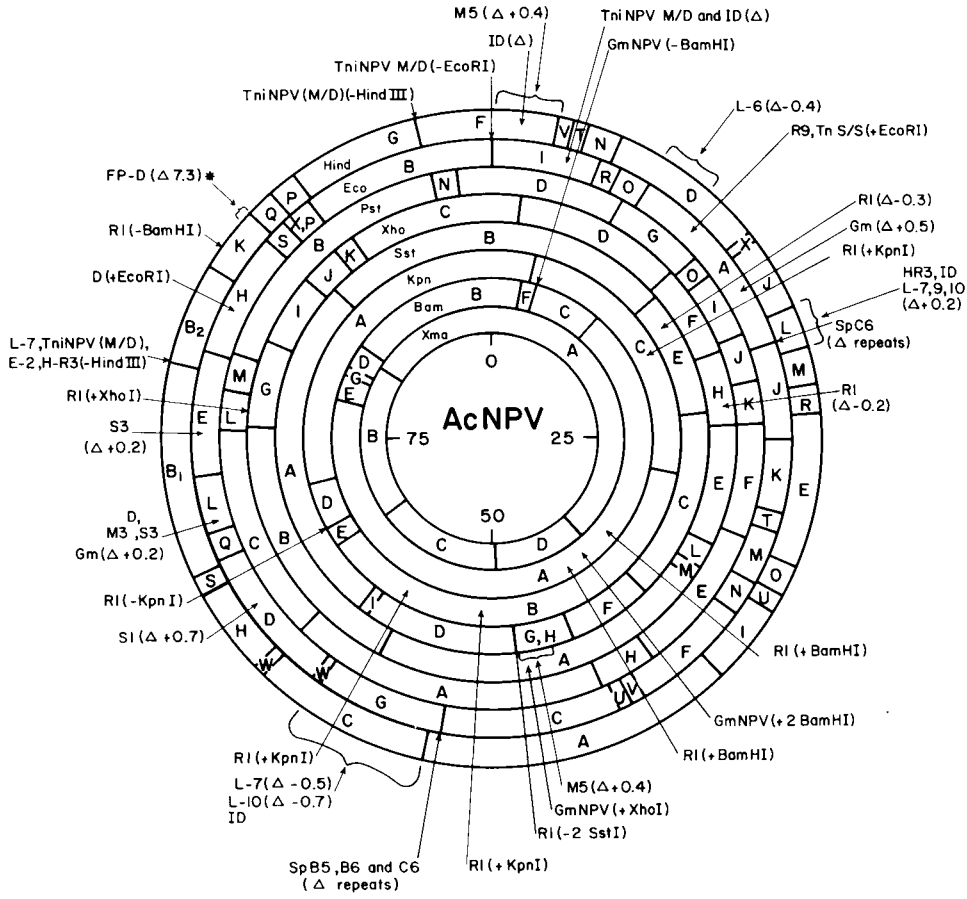

FIG. 1 A physical map of *Autographa californica* NPV (AcNPV) L-1 and the location of variations found in closely related virus strains, independent isolates, and virus mutants. Variants of AcNPV cloned from virus stocks derived from the original AcNPV isolate of Vail include L-1, L-6, L-7, L-9, and L-10 (Lee and Miller, 1978); E-2, M3, S1, and S3 (Smith and Summers, 1978); H-R3 (Cochran *et al.*, 1982); and D (isolate of E of Lübbert *et al.*, 1981). One AcNPV variant, ID, was independently isolated in Idaho (Miller *et al.*, 1980). Mutants of AcNPV (or clones obtained after serial passage in cell culture) having altered restriction enzyme patterns are M5 (Carstens, 1982), FP-D (Miller and Miller, 1982), and SpB5, SpB6, and SpC6 (Burand and Summers, 1982). Closely related variants of AcNPV, but isolated from other (related) insects, are *T. ni* NPV (M/D refers to Miller and Dawes, 1978a), *T. ni* NPV (S/S refers to Smith and Summers, 1979), *Galleria melonella* NPV (GmNPV, Smith and Summers, 1979), *Rachiplusia ou* NPV (R; Smith and Summers, 1980a). Variations observed include the absence of a restriction site (e.g., $-$*Hin*dIII), an extra site (e.g., $+$*Bam*HI), deletion of DNA (e.g., $\Delta - 0.4$ refers to a deletion of 0.4 kb of DNA) or the addition of DNA (e.g., $\Delta + 0.5$ means an addition of 0.5 kb). The asterisk next to FP-D emphasizes the unusual genetic instability of the 7.3-kb transposable element inserted at this site. Variations of the AcNPV DNA genome appear to be dispersed throughout the DNA genome.

NPV can be considered to be related variants or strains of the same virus (Miller and Dawes, 1978b; Smith and Summers, 1979). The differences in the restriction maps of these viruses have been determined (Smith and Summers, 1979, 1980a). The *R. ou* NPV is the most divergent virus in this group, having 35 out of 60 restriction sites in common with AcNPV. This amount of site variation reflects an average of approximately 0.03–0.04 base substitutions per nucleotide as calculated by Upholt (1977), or roughly 96% sequence homology (Jewell and Miller, 1980). The *R. ou* NPV/AcNPV relationship and the Upholt equation are illustrative of an important feature to remember in considering the relatedness of different baculoviruses; two baculovirus DNAs may have restriction endonuclease patterns that appear to be totally dissimilar and yet share considerable sequence homology (see Section VI).

A summary of a number of the variations observed in the genotype of AcNPV and its closely related variants is presented in Fig. 1. The summary is relatively comprehensive for variations reported in the literature with the exception that many of the *Eco*RI and *Xho*I variations in the physical map of *R. ou* NPV are not included because they are numerous. The variations are distributed throughout the AcNPV genome. The common variations are small (0.1–1 kb) insertions or deletions of DNA and the loss or gain of restriction endonuclease sites. One is left with the impression that the AcNPV genome is plastic. However, the general genotype of cloned virus variants is maintained in at least several passages through insect larvae (Lee and Miller, 1978). The results of Knell and Summers (1981) indicate that long-term passage of *S. frugiperda* NPV in insect larvae may result in the generation and/or selection of different genotypic variants. The fact that the variations characteristic of AcNPV E-2 and HR-3 were observed in very early stocks of the Vail isolate (Lee and Miller, 1978; Fig. 1) supports a selection mechanism. It would not be surprising to find that the insect host as well as passage conditions (multiplicity of infection, age of host upon infection, etc.) may affect the selection of a particular variant. Once a physical map of a baculovirus is established, some attention should be paid to the retention of that pattern in virus preparations.

III. Mapping Mutuations of Baculoviruses

A. Physical Mapping of Insertions and Deletions and the Effects of a Transposable Element on Genotype

With the construction of a sufficiently detailed physical map as shown in Fig. 1, it is relatively easy to map insertions and deletions in baculovirus DNAs by comparing mobilities of restriction endonuclease fragments of the

mutant DNA with wild-type DNA. In this way, a number of insertions and deletions have been localized with respect to the physical map. To date, none of these insertion or deletions has been correlated with a phenotypic characteristic of the virus. In many cases when an insertion or deletion has been observed, there is no obvious phenotypic difference between the "wild-type" and altered virus strains, and indeed it is difficult to determine which is the "wild-type" strain. For example, Andrews *et al.* (1980) were unable to detect any difference in virulence between five different AcNPV genotypic variants, three of which contained significant deletions ranging from 0.4 to 0.7 kb. Burand and Summers (1982) selected wild-type (MP) viruses after 30 serial passages of AcNPV in cell culture. Of the 20 plaque-purified virus isolates contained, 7 reiterated viral sequences appearing as insertions at two different regions of the map (between *Eco*RI-G and C and *Eco*RI-A and J). Although Burand and Summers (1982) suggested that these sequences may be reiterations of DNA replication origins (and thus confer a replication advantage), a correlation between these sequences and more rapid replication has not been directly demonstrated.

Two interesting insertions described in the literature suggest a very novel effect that certain types of insertions may have on baculovirus genotype (Carstens, 1982; Miller and Miller, 1982). Carstens (1982) studied the genotype of a polyhedron morphology mutant, AcNPV-M5 (Brown *et al.*, 1980), and found that M5 contained two 0.4-kb insertions: one was located between 0.0 and 3.3 and the other between 45.6 and 48.6 on the physical map (Fig. 1). The DNA of M5 was of two distinct lengths: one was approximately full length whereas the other was 58% the length of the larger DNA. Careful observation of the restriction patterns indicated that certain fragments (including those from 0.0 to 48.6 map units) were in submolar quantities despite several successive plaque-purification steps.

Miller and Miller (1982) detected the insertion of a *copia*-like transposable element in the genome of an FP mutant, FP-D, of AcNPV (Potter and Miller, 1980b). The 7.3-kb element, TE-D, is of host (*T. ni*) origin. At the ends of the element are 0.27-kb-long terminal repeat (LTR) sequences. The FP-D mutant is actually composed of two different DNA size classes despite successive plaque purification; one DNA, FP-DS, contains only a single 0.27-kb LTR; the other DNA, FP-DL, contains the entire 7.3-kb TE-D insertion. A virus containing only FP-DS can be isolated by plaque purification from FP-D stocks, but FP-DL cannot be plaque purified free of FP-DS. The mechanism by which FP-DL is converted to FP-DS is currently being studied. Preliminary results indicate that recombination can occur between LTRs of TE-D and generate FP-DS as well as tandem duplications of the element (Miller and Miller, unpublished results). In addition, TE-D may move by mechanisms characteristic of transposable elements.

It is tempting to make an analogy between M5 and FP-D. If the two 0.4-

kb inserts of M5 are identical to each other (and are thus analogous to the LTRs of TE-D), recombination between the 0.4-kb inserts would result in two smaller circles (42 and 58% of the genome), only one of which (58%) can be replicated and/or packaged into virions. It will be interesting to determine whether the 0.4-kb inserts in M5 are of host origin and are transposable elements or single LTRs left behind by two larger transposable elements. An important consequence of the ability of baculoviruses to accommodate additional DNA sequences within their rod-shaped nucleocapsids may be that the evolution of baculoviruses is more strongly influenced by the movement of host transposable elements than are other eukaryotic viruses that cannot stably accommodate additional DNA sequences. Clearly reiterated insertion sequences can result in very unusual baculovirus genotypes.

In both the M5 and FP-D cases, the insertions have not been correlated yet with any specific phenotype although both viruses are mutants. The TE-D insertion does affect transcription of viral sequences (Miller and Miller, unpublished results), but a direct correlation between this insertion and the FP phenotype has not been demonstrated to date (see Section III,C).

B. Mapping Point Mutations

It is extremely rare to detect a specific point mututation by restriction endonuclease analysis. There are approximately 125,000 base pairs in AcNPV DNA, and fewer than 1000 of these base pairs constitute the approximately 150 restriction sites physically mapped on the AcNPV genome. Surprisingly then, Carstens (1982) has made a tentative correlation between the *Bam*HI-B/F junction and the site of the M5 mutation affecting polyhedron morphology. This *Bam*HI-B/F alteration is distinct from the 0.4-kb insertions described above. The usual approach to correlating an altered restriction site with a particular phenotype is to isolate a number of revertants and to demonstrate restriction site reversion in a substantial proportion of the revertants. Carstens analyzed a single revertant and observed restriction site reversion. His correlation of this site with the polyhedrin gene is much strengthened by the independent observation that the gene for the polyhedrin protein (the size of which is affected in the M5 mutant) maps in the *Bam*HI-B/F region of the AcNPV genome (see Section IV,B).

Other approaches have been utilized in mapping temperature-sensitive (*ts*) mutations of baculoviruses. These approaches are more general in their utility than the restriction site reversion technique. Of the two discussed, the marker rescue technique is preferable to recombination frequency determination because marker rescue permits the direct correlation of a genetic map with a restriction map.

1. *Recombination Frequency and Complementation Analyses*

Brown and Faulkner (1980) described a partial genetic map of AcNPV *ts* mutants based on the recombination frequency (RF) between pairs of *ts* mutants. Their "two-factor" genetic crosses and the more powerful "three-factor" genetic cross represent the classical genetic approach to mapping virus mutants. They found RFs ranging from 0.4 to 48%; three mutants belonging to the same complementation group had RFs between 0.4 and 1.2% ± 0.6%. A genetic map encompassing nine *ts* mutants was constructed based on the RFs (Brown and Faulkner, 1980; Faulkner *et al.*, 1980). The problem with a map of this nature is that it is difficult to correlate RFs with physical distances and there is no direct correlation of genetic map position with physical map location. If two or three mutations can be correlated with the physical map (see marker rescue below), then there may be some utility in first determining the relative map position by RF analysis and then determining the exact physical location by marker rescue.

Complementation analysis to determine whether two closely linked mutations are located in the same gene or different genes can be used advantageously with baculovirus mutants. Complementation analysis of AcNPV *ts* mutants has been based on polyhedrin production or polyhedra formation (Brown *et al.*, 1979; Lee and Miller, 1979). The basic concept of complementation analysis is that coinfection of cells with two mutants will result in a productive infection if the two mutations are in two different genes. With AcNPV, a productive infection results in the formation of polyhedra that can be visually observed in the light microscope. Thus Lee and Miller (1979) compared the percentage of cells containing polyhedra following infection with one virus and with both viruses. Brown *et al.* (1979) compared the levels of polyhedrin synthesis in singly and doubly infected cells by radioimmunoassay.

Complementation analyses can be misleading and must be carefully interpreted. Some mutants fail to complement other mutants in other genes, and cases of intragenic complementation (two mutants defective in the same gene complement each other) are known to exist in other systems.

2. *Marker Rescue to Map Point Mutations*

The ability to map *ts* mutations of AcNPV by marker rescue was described by Potter and Miller (1980a) and then extended (Miller, 1981b) to map six *ts* mutations with respect to the physical map. The marker rescue procedure involves the transfection of host cells with intact mutant DNA and individual restriction fragments from wild-type virus. In $CaCl_2$ transfection procedures, both types of DNA usually enter the same cell. A region of the wild-type fragment can replace homologous sequences in the mutant DNA by either a double recombination or a gene conversion event within

the cell. If the wild-type fragment being tested is homologous to the mutated region, the mutant can be "rescued" (i.e., converted to wild type) by the fragment. Thus scoring for plaques at the restrictive temperature allows one to determine which wild-type restriction fragment can rescue the *ts* mutant. Because the location of fragment on the physical map is generally known, the *ts* mutation can be directly correlated to the physical map.

There are four $CaCl_2$-based DNA transfection procedures described for AcNPV DNA (Bud and Kelly, 1980; Burand *et al.*, 1980; Carstens *et al.*, 1980; Potter and Miller, 1980b). All these procedures are based on the original work of Graham on adenovirus and herpesvirus DNA transfections (Graham and van der Eb, 1973; Graham *et al.*, 1977), and the reader is referred to these papers for factors involved in DNA infectivity using $CaPO_4$ precipitation. The $CaCl_2$-transfection procedure is more efficient than a DEAE-dextran procedure on *S. frugiperda* cells (Potter and Miller, 1980b). The relative levels of infectivity observed varied from approximately 1 to 4×10^3 PFU/μg (Burand *et al.*, 1980; Kelly and Wang, 1981) to as high as 3 to 6×10^4 PFU/μg (Carstens *et al.*, 1980; Potter and Miller, 1980b). Glycerol or DMSO "boosts" gave only 2.5- to 4-fold increases in infectivity and are not routinely used. DNA transfection does not appear to expand the host range of baculoviruses (Burand *et al.*, 1980; Kelly and Wang, 1981).

In developing a baculovirus marker rescue technique, two basic techniques were considered. The marker rescue techniques first developed for mapping the small papovavirus DNAs (Lai and Nathans, 1975; Miller and Fried, 1976) involve the formation of a heteroduplex DNA containing mutant information in one strand and wild-type information in the other. The heteroduplexes were formed by denaturation of full-length mutant DNA and excess wild-type DNA fragments and reannealing of the two DNAs. After transfection of cells with the heteroduplex DNA, gaps, breaks and mismatched nucleotides in the DNA are apparently repaired by cellular enzymes (Miller *et al.*, 1976). The second "recombination" technique, a procedure in which mutant and wild-type fragment DNA are simply mixed together, and the one used for our baculovirus mapping work, was more simplistic in its form and avoided the denaturation step involved in heteroduplex formation. We chose first to use the DNA mixture procedure, and, since it worked successfully, no further assessments of the techniques in terms of their relative rescue capabilities have been explored. It may be worthwhile to do so.

Knipe *et al.* (1979) compared the two marker rescue techniques for herpesvirus mutants. They took advantage of the phosphonoacetic acid resistance mutants (PAA^r), which provided an easily scorable phenotype. After transfections with either heteroduplexes or DNA mixtures, they incubated the cells in liquid culture and then screened for percentage of PAA^r progeny. When the DNA mixture technique was used, an increase in percentage

of PAA^r virus was observed from 0.1 to 1 μg of fragment DNA (in the presence of 0.1 μg of herpesvirus DNA). The percentage of PAA^r virus varied depending on the set of restriction fragments used; the highest reported rescue was 10% PAA^r using this technique. Using the heteroduplex technique, a considerably higher proportion of the progeny obtained resistance (over 30%); approximately an 8-fold increase in percentage of PAA^r progeny was observed with the heteroduplex procedure using the *Xba*I fragment series. Rescue using the DNA mixture approach was dramatically affected by the size of the DNA fragment used, larger fragments rescuing more efficiently than smaller ones. The effect of fragment size on the heteroduplex technique was not explored. The total progeny virus obtained from the transfections with either DNA mixtures or heteroduplexes was not reported and would be useful data to have in comparing the utility of the two techniques. It might be assumed that the denaturation–renaturation steps involved in heteroduplex formation would significantly decrease the specific infectivity of the 128-kb AcNPV DNA compared to the more gentle DNA mixture approach. Assuming that the specific infectivities of the heteroduplex technique are high enough to be feasible, this heteroduplex technique might be very valuable for baculovirus marker rescue, particularly in cases where a high percentage of rescued progeny is critical to successful scoring of rescue (see below).

In the DNA mixture (recombination) procedure used to map *ts* mutants of AcNPV (Potter and Miller, 1980a; Miller, 1981b), a molar ratio of 10 fragment DNAs to one circular mutant DNA was used. This ratio is consistent with the ratio determined to give maximum rescue of the PAA locus (Knipe *et al.*, 1979). Parris *et al.* (1980), studying rescue of herpesvirus *ts* mutants, found that varying the molar ratio of intact DNA-to-DNA fragment from 1:0.7 to 1:333 had no effect on the efficiency of rescue. More critical in their study was the absolute amount of DNA fragment; they routinely used 0.1 μg or more of DNA fragment per 10^6 cells in 35-mm plates. When feasible, we also maintained DNA fragment levels at 0.1 μg or more, although, in the absence of cloned fragments, this is difficult when very small fragments are tested.

The marker rescue technique should be greatly facilitated by the availability of cloned segments of AcNPV genome. One of the most tedious and tenuous steps in the current procedure is the isolation of large quantities of pure fragments from agarose gels; fragment contamination results in ambiguity concerning which fragment rescues the mutant.

The relative success of the "DNA mixture" marker rescue technique in mapping a given mutation depends on the nature of the mutant itself. We have been most successful in the marker rescue of conditional lethal (*ts*) mutants having very poor plaquing efficiency at the nonpermissive condition (e.g., high temperature). We estimate that the frequency of rescue in

most cases is approximately 1%. Thus, if the plaquing efficiency of a given mutant at the restrictive temperature is 0.1% of that observed at the nonrestrictive temperature, one may expect approximately a 10-fold increase (above background) in the number of plaques observed at the restrictive temperature. This is a definitive result. However, if the mutant is leaky and one observes a high plaquing efficiency (1% or more) at the restrictive vs nonrestrictive temperature, the rescue will be difficult to observe owing to background plaques.

Related to this aspect of marker rescue is the mapping of mutants that are defective only in occlusion (e.g., NOV^+OV^- mutants). Rescue of these mutants must be monitored by observing the presence or the absence of polyhedra in the nuclei of infected cells within a plaque. As long as the OV^- mutant is not leaky (0.1% or less of the infected cells produce OV), the mutation should be easily mapped by simply scoring the number of plaques with OV. However, it is relatively more difficult to map an FP mutant; these mutants produce only a few polyhedra per nucleus compared to many polyhedra produced in wild-type (MP) infected cells. In the case of FPs, scoring for rescue becomes much more tedious and, to some extent, subjective, since a 5- to 10-fold difference in the level of polyhedra per cell is being scored. Hundreds of plaques must be carefully screened, and this requires considerable patience. The use of the heteroduplex technique described above might increase the percentage of rescued progeny, which would facilitate mapping of these mutants and is therefore worth investigating.

The relative frequency of marker rescue can vary from experiment to experiment. Therefore, all fragments in a series should be scored simultaneously. Otherwise careful monitoring of "background" plaques arising from reversion and/or leakiness of the mutant should be made in all experiments in the series. Monitoring for only background plaques is recommended only if cloned (pure) fragments are used for rescue. The relative frequency of rescue also appears to depend on the distance of the mutation from the end of the fragment; the closer the mutation is to the end, the less efficiently the fragment rescues the mutation (Miller, 1981b). The size of the fragment may also influence the relative efficiency of rescue (Knipe *et al.*, 1979), although we have observed efficient rescue with a 2.2-kb AcNPV DNA fragment (Miller, 1981b). Many other factors may also influence rescue; a comprehensive survey of factors involved and quantitation of effects has not been undertaken.

Marker rescue is able also to provide information concerning whether a mutant contains two or more mutations if those mutations are located at a distance from each other (Parris *et al.*, 1980; Miller, 1981b). In these cases, the mutant might be rescued by a very large fragment (e.g., *Xma*-A, which represents 60% of the genome) or by the full complement of restric-

tion fragments of a given series (e.g., all the *Hin*dIII fragments), but fail to be rescued by small single fragments of several different restriction fragment series (e.g., individual *Hin*dIII or *Eco*RI fragments). Using mutagenesis conditions in which greater than 1% of the viruses contain *ts* mutations, the probability of isolating double mutants is significant.

C. CORRELATING GENOTYPE AND PHENOTYPE

In a survey of the genotypes and phenotypes of our *ts* mutants, we observed one mutant that had two different genotypic alterations (observed by restriction endonuclease analysis) and phenotypically differed in the mobility of a virus-induced polypeptide (Miller *et al.*, 1983b). It would not be surprising to find that none of these observed genotypic or phenotypic alterations corresponded with the *ts* mutation in this mutant, generated by bromdeoxyuridine mutagenesis. Marker rescue is the only reliable way to begin mapping the *ts* mutation. Once marker rescue information is obtained, it can be compared to any restriction endonuclease-detectable changes observed. Revertants or marker-rescued viruses can be screened phenotypically for the alteration of protein mobilities to correlate these phenotypic properties with a specific region of the genome. In this way phenotypes and genotypes can be correlated.

In some cases, a reverse type of marker rescue may be more convenient for correlating a specific genotype with a given phenotype. For example, a deletion or insertion may be observed by restriction endonuclease analysis. To correlate that alteration with a specific observed phenotype (e.g., FP or OV^- phenotype), one could isolate the mutant fragment, transfect with intact wild-type DNA, and attempt to isolate a mutant virus (FP or OV^-). Approximately 1% of the progeny should have mutant phenotype, and these mutants could be selected and screened for the deletion or insertion by restriction endonuclease analysis. Several mutants should be screened with this approach. This is a preferred procedure for mapping FP mutations generated by serial passage in cell culture, since each FP mutant probably contains multiple mutations responsible for the FP phenotype and would be difficult to map by the normal marker rescue approach. The construction of mutants by site-directed mutagenesis will also provide a correlation of genotype and phenotype (see Section VII).

IV. Correlating Proteins with the Physical Map

A. INTERTYPIC RECOMBINATION FOR GENE MAPPING

Summers *et al.* (1980) have introduced the use of AcNPV and *R. ou* NVP (RoNPV) recombinants to map virus structural proteins. These two viruses

share sequence homology throughout their genome (Jewell and Miller, 1980; Summers *et al.,* 1980) so that recombination between these viruses may occur at all regions of the genomes. Smith and Summers (1980a) constructed a restriction map of RoNPV and compared the map to its relative AcNPV. Of the 60 RoNPV restriction sites mapped, 35 mapped in positions similar to those found in AcNPV.

Summers *et al.* (1980) phenotypically and genotypically analyzed seven AcNPV/RoNPV recombinants isolated after coinfection of AcNPV and RoNPV in *T. ni* cells. Restriction endonuclease analysis determined the portions of recombinant genomes that were derived from each parent. Thus, the crossover points would be determined with respect to the physical map. RoNPV and AcNPV vary phenotypically with respect to the electrophoretic mobilities of three enveloped nucleocapsid structural proteins of 37,000, 56,000, and 90,000 daltons. The polyhedrins of AcNPV and RoNPV can be distinguished by two-dimensional tryptic peptide mapping. By phenotypically analyzing the recombinants and comparing this information with the crossover points of the recombinants, Summers *et al.* determined approximately where these proteins map with respect to the physical map. The 37,000-dalton protein mapped within the 89–100% or the 53–80% region of the current AcNPV physical map, the 55,000-dalton protein mapped from 53 to 100% on the AcNPV map, and the 90,000-dalton protein mapped from approximately 25 to 60% (see Fig. 2). Polyhedrin was located between approximately 90% through 0–9% of the physical map.

Although a similar intertypic recombinant technique has been successfully employed for mapping structural proteins and *ts* mutants of adenoviruses and herpesviruses (Mautner *et al.,* 1975; Williams *et al.,* 1975; Morse *et al.,* 1977, 1978), the AcNPV/RoNPV recombination technique cannot be strongly recommended for use as a mapping technique because of several technical difficulties. The technique is extremely tedious in its current form because only a few recombinants are obtained in an AcNPV/RoNPV cross. Of the hundred plaques genotypically analyzed, only seven proved to be recombinants (Summers *et al.,* 1980). In order to increase the frequency of recombinants, two technical changes in the original protocol are suggested: (1) reduce the multiplicity of infection from 500 PFU per cell to approximately 5–10 PFU per cell; and (2) visually select for *small* plaques in picking potential recombinants. The first strategy is aimed at allowing time for recombination between parental strains to occur. At a multiplicity of infection of 500, the parental genomes may simply be packaged without replicating and recombining extensively. The second proposed modification is based on our observations that RoNPV forms small plaques relative to AcNPV. Random picking would favor AcNPV and, indeed, Summers *et al.* (1980) observed 88 parental AcNPV genotypes versus 5 parental RoNPV

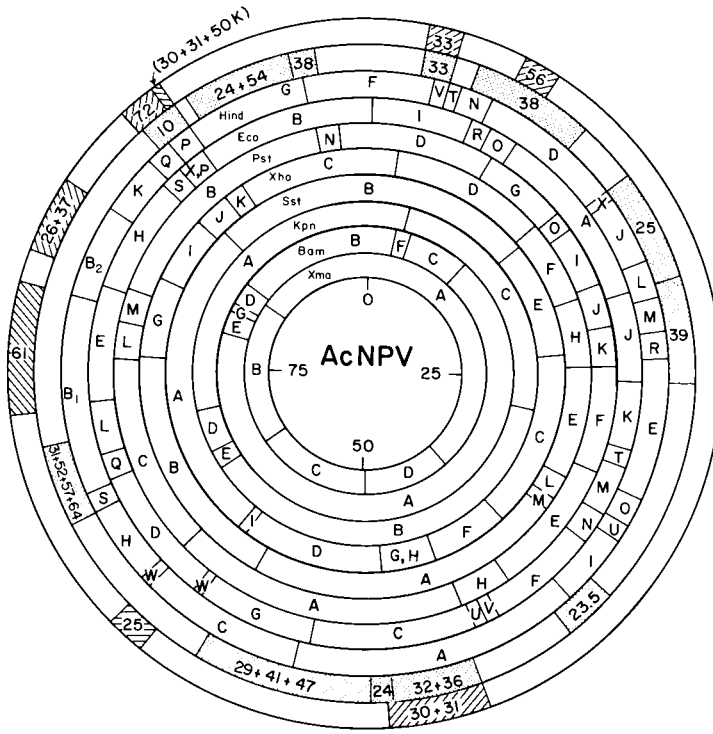

FIG. 2 A translational map of the AcNPV DNA genome. Regions of the DNA have been correlated with specific proteins by *in vitro* translation of late mRNAs selected by hybridization either to genomic fragments (Vlak *et al.*, 1981; Smith *et al.*, 1982) or by cDNA (Adang and Miller, 1982). The size of the *in vitro*-synthesized protein is given in kilodaltons. The outermost concentric circle contains those proteins mapped with the aid of cDNA clones; the general location of cDNA clone determined by Southern blotting is shown by the hatched lines. The second concentric circle shows the proteins located using genomic fragments for hybridization selection of mRNAs; the stippling represents the regions of the DNA that select for mRNAs encoding the specified proteins.

genotypes in a random selection of 100 plaques from a recombination experiment.

The second technical difficulty may also be related to preferential replication of AcNPV in culture. Six of the seven recombinants screened by Summers *et al.* (1980) revealed a crossover site between 78 and 89% of the genome. The reason for this is not known, but it does interfere with the isolation of recombinants with a variety of crossover points. Thus, Summers *et al.* (1980) were limited in their ability to map more closely the positions of the viral proteins; the most closely mapped protein was mapped only to within a 19% region of the physical map.

Marker rescue is currently the preferred technique for mapping *ts* mutations of adenoviruses, herpesviruses, and baculoviruses. Intertypic recombination mapping can provide information on the location of structural polypeptides that marker rescue cannot provide unless it is known that a particular *ts* mutation affects a specific structural protein. However, the more recent *in vitro* translation techniques are perhaps more direct and precise methods than intertypic recombination for mapping structural virus proteins (see Section IV,B).

B. Hybridization Selection and *in Vitro* Translation

The most powerful technique for determining the specific regions of a baculovirus DNA genome encoding specific proteins involves the isolation of mRNAs encoded by a given DNA region, *in vitro* translation of the purified mRNAs, and the subsequent analysis of the protein products. Since such homologous mRNAs are isolated by hybridization to specific single-stranded DNA which has been immobilized on nitrocellulose, the technique is referred to as hybridization selection. The original technique was primarily developed in mapping polypeptides with respect to tumor virus DNA genomes, and application of the technique to mapping baculovirus proteins was first reported by Vlak *et al.* (1981).

1. *Use of Virus Genomic Fragments for RNA Selection*

Restriction fragments of the AcNPV DNA genome were used to select homologous RNAs for subsequent *in vitro* translation. Vlak *et al.* (1981) and Smith *et al.* (1982) obtained specific DNA fragments either by eluting electrophoretically separated fragments from gels or by cloning fragments in *E. coli.* Cloning is much preferred to gel purification because of the possibility of fragment contamination, which is particularly problematic in this technique if an mRNA of a neighboring fragment is highly abundant compared to the mRNA of the fragment of interest. This problem can be assessed by determining also the translation products of the neighboring fragments.

In the first report, Vlak *et al.* (1981) isolated *Eco*RI-I and -J fragments from agarose gels and hybrid-selected homologous RNA by a modification of the McGrogan *et al.* (1979) procedure developed for adenovirus mapping. This method calls for specific washing procedures to remove non-homologous or weakly homologous RNAs from the DNA-containing nitrocellulose filters. The temperature and nature of the buffers used in the washing steps used to remove RNAs with limited sequence homology but retain specific (rigorously homologous) RNAs must be empirically determined. These conditions depend primarily on the G + C content of the

viral DNA being characterized. If there are widely divergent regions of G + C within a single DNA, the appropriate washing temperature should be empirically established for each region.

AcNPV *Eco*RI-I and *Eco*RI-J fragments select RNAs that direct the synthesis of a 33,000- and a 39,000-dalton protein, respectively (Vlak *et al.*, 1981). The 33,000-dalton protein was postulated to be polyhedrin, based on its comigration with polyhedrin in SDS–PAGE gels and by prior knowledge (from intertypic mixing experiments) that polyhedrin mapped in that general region of the AcNPV genome (see Section IV,A). They also demonstrated that polyhedrin is synthesized *in vitro* from *total* RNA isolated late in infection by immune precipitation of a 33,000-dalton protein with antiserum raised against polyhedrin. To confirm rigorously that the 33,000-dalton protein synthesized from *Eco*RI-I-selected RNA is indeed polyhedrin, it is necessary to demonstrate that the 33,000-dalton protein is also immune precipitable with polyhedrin antibody. Adang and Miller (1982) have demonstrated by immune precipitation that the 3′ end of polyhedrin mRNA is homologous to *Hin*dIII-V, thus confirming the location of the polyhedrin gene within *Eco*RI-I.

Smith *et al.* (1982) used 18 restriction fragments, representative of almost the entire AcNPV DNA genome, to hybrid-select RNA, *in vitro*-translate the mRNA, and thus establish a more extensive translation map. The approximate locations of genes encoding 19 AcNPV polypeptides were determined in this way and are shown in Fig. 2. More precise positions for these genes could be established by subcloning the fragments and testing smaller regions of the genome for their ability to select mRNAs encoding a given protein. There are surely many more proteins encoded by AcNPV that have not been detected in this initial genomic screen. Late RNA, isolated 21 hr after infection, was used in the hybridization selection step. In other experiments, Smith *et al.* (1982) translated total early mRNA *in vitro* and found that early mRNA directs the synthesis of at least six polypeptides (47,000, 39,000, 32,000, 31,000, 29,000, and 25,000 daltons). Polypeptides of these sizes have also been found as *in vitro* translation products of late RNA (Smith and Summers, 1982, and Fig. 2), but the correspondence of these similar-sized early and late proteins has not been rigorously established. Since AcNPV induces more than 19 polypeptides, more work, using subclones of fragments and different RNA sampling times, will be needed to map more fully the AcNPV DNA genomes by this method.

2. *Use of cDNA Clones for RNA Selection*

Adang and Miller (1982) took a different approach to translational mapping of AcNPV by employing cDNA clones to hybrid-select specific mRNAs for *in vitro* translation. The cDNAs were synthesized from RNA isolated

late (27 hr postinfection) in the AcNPV infection process. The cDNAs were then cloned in *E. coli* using pBR322 as a vector. Forty-five cDNAs containing viral DNA were mapped with respect to the AcNPV physical map by hybridization to *Hin*dIII and *Eco*RI blots of the viral DNA genome. Eleven different cDNA clones were identified and used to hybrid-select mRNA. Ten different proteins were mapped with respect to the physical map; the locations of these proteins are illustrated in the outermost concentric circle of Fig. 2. It is likely that many more late mRNA transcripts and proteins could be identified by screening more cDNA clones to late viral mRNA. Also the cloning of cDNAs synthesized from RNAs isolated at earlier times after infection would probably reveal many other transcripts.

Comparison of the translational maps established using genomic fragments and cDNAs of AcNPV indicates that the proteins encoded by the most abundant cDNAs, the 7200- and 33,000-dalton proteins in the *Hin*dIII-P and -V regions, respectively, were identified by both groups. The 7200- and 10,000-dalton proteins in the *Hin*dIII-P region are probably the same protein; the accurate size of small proteins (less than 14,000) must be determined using alternative SDS–polyacrylamide gel systems (Adang and Miller, 1982). The 31,000-dalton protein of Adang and Miller (1982) may also correlate with the 32,000-dalton protein of Smith *et al.* (1982) located in the *Sst*-G/H region at 45 map units (Fig. 2). Otherwise, the two groups have mapped apparently different proteins with respect to the map. The results are compatible and illustrate the fact that different sources of DNAs used to select mRNAs will provide different sets of information. Smith *et al.* (1982) seem to have detected the majority of proteins that are synthesized in large quantities, whereas Adang and Miller (1982) have detected several very abundant proteins as well as some proteins synthesized in small quantity. Why some relatively abundant proteins were not detected in the initial cDNA screen remains to be determined. Precautions that must be taken in interpreting the data and resulting map positions derived by the two techniques are discussed below.

3. *Interpretation of Hybrid Selection Data*

The use of cDNAs as "selectors" for specific mRNAs differs in several very important respects from the use of genomic fragments as "selectors." The cDNAs are synthesized from a single mRNA and thus must represent a single mRNA or a family of sequence-related transcripts. In contrast, genomic fragments may be homologous to several RNAs that have no sequence relationship to each other but merely lie adjacent to each other on the genome. Second, cDNAs are synthesized from the 3′ end of the mRNA if oligo(dT) is used to prime cDNA synthesis. In the work of Adang and

Miller (1982), the cDNAs were generally quite small since no size fractionation technique was used to select for large cDNAs, and thus these cDNAs primarily represent 3′ ends of viral mRNAs.

Adang and Miller (1982) observed that the *in vitro* products of some cDNA-selected mRNAs were difficult to detect in the autoradiographic analysis. We found that the 7200- and 33,000-dalton proteins were easily detected with a relatively short autoradiographic exposure time. Proteins encoded by infrequently found cDNAs, probably correlating with infrequently found mRNAs, were detected only after long autoradiographic exposures of gels containing the *in vitro* translation products. In a few cases, we were unable to detect an *in vitro* translation product for a given cDNA. There are several possible reasons for this: (1) the mRNA is not readily translated by the *in vitro* translation system used; (2) the mRNA is not abundant; (3) the product is a protein of very high molecular weight, and the mRNA was not isolated intact or the *in vitro* translation system did not translate the total protein; and (4) the DNA region was a relatively low G + C content and the mRNA was "washed off" in the selection process. Some of these factors may also account for the fact that only 19 proteins were mapped during the genomic fragment screen of the AcNPV proteins (Smith *et al.*, 1982).

The main advantage of the cDNA is that the probe is small and selects for at least one mRNA, and one can look very hard by long autoradiographic exposure for the expected product. Genomic cloning apparently misses low-abundance mRNA products. Both techniques may provide misleading information concerning the location of the gene if the gene is spliced. This may be more of a problem with the genomic fragment selection than the cDNA selection if baculoviruses utilize 5′ leader sequences as adenovirus does (see below).

If more than one protein is made using cDNA-selected mRNA for *in vitro* translation, then three alternative possibilities that can be investigated are (1) more than one mRNA is hybrid-selected by the cDNA; (2) more than one protein is synthesized from a single mRNA owing to different initiation sites; or (3) the smaller protein is an artifact of premature chain termination in the *in vitro* protein-synthesizing system. More than one RNA might be selected for a variety of reasons including the obvious possibilities that (1) the RNA is spliced in different ways; and (2) there is symmetric transcription (both DNA strands serve as templates for transcription) in that region of the genome. By using the cDNA as a probe of Northern blots (blots of gels containing RNA), one can determine whether more than one size of RNA is homologous to the cDNA probe.

In this manner, it has been possible to show that the 30,000- and 31,000-dalton proteins encoded, at least in part, by the *Hin*dIII-A/*Eco*RI-C/*Sst*I-

G region of the genome are related at the DNA level. A cDNA from this region is homologous to at least two different mRNAs as determined by Northern blots (D. W. Miller and L. K. Miller, unpublished results) and mRNA homologous to the cDNA directs the synthesis of both the 30,000- and 31,000-dalton proteins (Adang and Miller, 1982). The data are consistent with splicing, overlapping mRNAs, and/or symmetrical transcription. The cDNA of polyhedrin mRNA hybridizes to a 1.2-kb mRNA as well as at least one larger but less abundant mRNA (Miller *et al.*, 1983). The RNA directs the *in vitro* synthesis of a 33,000-dalton polypeptide corresponding in size and antigenicity to polyhedrin as well as an 18,000-dalton polypeptide that is antigenically related to polyhedrin. Adang and Miller (1982) suggested that this 18,000-dalton polypeptide might be an artifact of the *in vitro* translation system; there are many precedents in the literature for such *in vitro* artifacts. However, at this point we cannot eliminate the possibilities that the 18,000-dalton protein is (1) synthesized from a minor mRNA that is related in sequence by a splicing event to the 1.2-kb polyhedrin RNA; (2) a proteolytic product of polyhedrin; or (3) synthesized from an internal AUG of the 1.2-kb polyhedrin mRNA.

These experiments highlight some of the factors that must be taken into account with *in vitro* translation systems. Such factors become even more difficult to resolve if genomic fragments are used for hybridization selection. For instance, Smith *et al.* (1982) showed that *Kpn*I-D-selected mRNA directs the synthesis of 31,000-, 52,000-, 57,000-, and 62,000-dalton polypeptides. Whether the DNA sequences encoding any of the proteins are common or related and whether any of the smaller polypeptides are artifacts of the translation system cannot be easily investigated. Extensive subcloning of the genomic fragments or gel electrophoresis of the selected mRNAs followed by *in vitro* translation is necessary. The latter techniques can be powerful methods for both transcriptional and translational mapping and will probably be useful in fine-structure mapping of the baculovirus genome.

Another factor that must be considered, particularly in using genomic fragments or full-length cDNAs as RNA selectors, is the possibility that a common 5′ end may be found on many mRNAs as a result of splicing; this situation is encountered in adenovirus late mRNA transcripts where a common 5′ leader sequence is found on a variety of mRNAs (Ziff, 1980; Flint, 1981). A DNA containing this 5′ end region would select for a variety of mRNAs, and, although the gene products would appear to be encoded by this region from the *in vitro* translation studies, the sequences actually encoding the polypeptides observed may be located at a considerable distance downstream. Hybrid-arrested translation (Paterson *et al.*, 1977) is one method that can be used to resolve whether a sequence is merely a leader

sequence or actually encodes a given protein. The question may also be addressed by observing whether neighboring sequences, when used as RNA selectors, also direct the synthesis of the same proteins. Smith *et al.* (1982) screened the products selected by DNAs encompassing nearly the entire AcNPV genome, and a protein of 24,000 daltons was found in two different locations. This may merely reflect the existence of two proteins of 24,000 daltons or it may reflect a splicing phenomenon. It is quite probable that different proteins of the same size will be found on the AcNPV map. A similar situation is found for a 38,000-dalton protein (Smith *et al.*, 1982). It is of critical importance that a criterion other than size be used to correlate *in vitro* translation products with viral structural proteins or other proteins of known function. Immune precipitation or peptide analysis techniques are needed for such functional assignments.

In summary, the translation map presented in Fig. 2 must be used with some caution until more is known concerning the nature of the mRNA transcripts and the mechanisms of RNA processing in baculovirus-infected cells. At the current time, the maps provide a valuable starting point in the vast task of translational mapping of a baculovirus genome.

V. Transcriptional Mapping of Baculovirus Genomes

Studies on the control of transcription and the location of transcripts on the baculovirus genome are just beginning. Late AcNPV and *Heliothis zea* NPV (HzNPV) transcripts appear to be transcribed by an α-amanitin-insensitive RNA polymerase (Grula *et al.*, 1981). The polymerase II of *H. zea* is sensitive to α-amanitin and remains sensitive after infection (Grula *et al.*, 1981). These results suggest that HzNPV DNA is transcribed by host RNA polymerase I or III or by a new viral RNA polymerase II, which is insensitive to α-amanitin. The latter possibility seems more likely considering the compartmentalization of polymerase I in the nucleolus and the unique promoter control of polymerase III. In either case, the α-amanitin insensitivity of viral transcripts is novel and should be further pursued. Late polyadenylated RNAs are capped (Jun-Chuan and Weaver, 1982). Both a cap 0 structure ($m^7GpppXp$) and cap 1 structure ($m^7GpppXp^m$) were observed.

Vlak *et al.* (1981) initiated studies on the mapping of AcNPV transcripts with respect to the physical map. Late polyadenylated RNA (21 hr postinfection) was radiolabeled and hybridized to Southern blots of AcNPV DNA *Eco*RI and *Bam*HI fragments. Most, if not all, of the fragments contained sequences homologous to the late mRNA. However, some fragments were more abundantly represented in the RNA population than others; there were

more polyadenylated RNAs homologous to *Bam*HI-F, *Eco* RI-I, and *Eco*RI-P than to other fragments. These regions correspond to regions now known to encode 33,000-dalton polyhedrin (*Hin*dIII-V, *Eco*RI-I) and the abundant 7200 (10,000)-dalton polypeptide synthesized late in infection (Adang and Miller, 1982; Smith *et al.*, 1982; Vlak *et al.*, 1981). This general approach of determining the location of the relatively abundant late AcNPV RNA transcripts was further explored by Vlak and van der Krol (1982). They also noted that a region between 20 and 25% physical map units was abundantly expressed late in infection. One precaution that must be taken with this technique is to test each set of blots with uniformly labeled total AcNPV DNA as a probe to determine whether all the fragments are transferred during blotting in a proportionate fashion.

The majority of polyadenylated RNA found at 21 hr postinfection appears to be transcribed from viral DNA. *In vitro* translation products of total polyadenylated RNA were compared to *in vitro* translation products of AcNPV-selected RNA (Vlak *et al.*, 1981). The patterns were virtually identical indicating that essentially all translatable mRNAs in the cell at 21 hr postinfection are of viral origin. A comparison of proteins synthesized *in vivo* at 18–21 hr postinfection have been compared with those synthesized *in vitro* (Smith *et al.*, 1982). The patterns are generally similar, indicating that what is observed in *in vitro* translation systems accurately reflects the *in vivo* situation. However, very large polypeptides (greater than 65,000 daltons) are poorly synthesized in the *in vitro* translation system, and a greater abundance of small molecular weight polypeptides (10,000–30,000 daltons), which may not all correspond to *in vivo* proteins, are observed. Two polypeptides (20,000 and 30,000 daltons) found *in vivo* were not found *in vitro*, and at least one polypeptide (e.g., 31,000 daltons) was found *in vitro* but not *in vivo*.

Most of the early mRNA appears to be of host origin by similar analysis (Smith *et al.*, 1982). Six early proteins (47,000, 39,000, 32,000, 31,000, 29,000, and 25,000 daltons) were detected by *in vitro* translation of viral DNA-selected mRNA. Information on the physical location of the transcripts for these proteins is not yet available. Although it is possible that these proteins correspond with those proteins of similar size found late in infection, size alone is not a sufficient criterion for equating proteins.

The cDNA approach of Adang and Miller (1982) provides information on the physical location of the 3′ ends of the transcripts, the relative abundance of transcripts, the protein products of these transcripts, and, through Northern blotting (Miller *et al.*, 1983a), it can provide information on the temporal control of transcription. The physical map positions were determined for 45 cDNAs that were synthesized from late (27 hr postinfection)

mRNA, cloned in *E. coli,* and found to be homologous to viral mRNA. Seventeen of the 45 cDNAs were homologous to *Hin*dIII-P/*Eco*RI-P, and 12 of the cDNAs were homologous to *Hin*dIII-V/*Eco*RI-I as determined by Southern blotting. Thus almost two-thirds of the AcNPV-homologous cDNAs found at 27 hr postinfection correspond to regions of the DNA genome that Vlak *et al.* (1981) reported to be abundantly expressed late in infection. Furthermore, these two cDNAs select mRNAs that are translated *in vitro* into polyhedrin and 7200-dalton proteins (Adang and Miller, 1982). Smith *et al.* (1982) showed that polypeptides of 33,000 and approximately 10,000 daltons are the predominant proteins synthesized very late (48–51 hr) in infection. There is thus a correlation between the abundance of these mRNAs, the frequency of cloning their cDNAs, and the level of protein synthesis from these mRNAs. A cDNA to the 39,000-dalton protein was not detected; a single cDNA homologous to *Hin*dIII-R was isolated, but the protein encoded by this cDNA could not be identified.

Using a polyhedrin cDNA clone to probe Northern blots of RNA isolated at 0, 6, 12, 18, and 24 hr postinfection, Miller *et al.* (1983a) showed that the polyhedrin transcript is approximately 1.2 kb and its synthesis is temporally controlled. Although some viral mRNAs are expressed by 6 hr postinfection, polyhedrin mRNA is not observed until 12 hr postinfection and increases in concentration through 24 hr postinfection. It will be interesting and useful to isolate full-length cDNAs and compare their DNA sequence with the genomic sequence. We can anticipate that much more information will be available shortly concerning the precise map locations of these predominant messages and the presence or the absence of intervening sequences. The techniques used in the transcriptional mapping of tumor viruses (Ziff, 1981; Flint, 1981, and references therein) can be readily adapted to baculovirus mapping.

Rohrmann *et al.* (1982b) took advantage of the prevalence of polyhedrin mRNA to identify and clone the polyhedrin gene of *Orgyia pseudotsugata* NPV (OpNPV). By *in vitro* translation of RNAs purified by sucrose gradient centrifugation, fractions of the gradient were identified that directed the synthesis of a protein identical in size to OpNPV polyhedrin and immune precipitable with antipolyhedrin antibodies. Complementary DNA was synthesized to the RNA fraction directing polyhedrin synthesis and was used to detect the homologous genomic fragment by probing Southern blots. A genomic fragment (*Xho*I-J) was cloned, and then a 2.5-kb *Sal*I fragment, homologous to polyhedrin mRNAs, was subcloned. Caution must be taken in assuming that this fragment encodes only polyhedrin; hybrid selection and *in vitro* protein synthesis would be expected to give predominantly polyhedrin products owing to the prevalence of polyhedrin mRNA. A more

precise localization of the polyhedrin gene within the 2.5-kb fragment was determined by hybridization to 3′-end labeled polyhedrin mRNA and R-loop mapping.

The polyhedrin mRNA was further characterized by R-loop mapping and partial sequencing of corresponding DNA sequences (Rohrmann *et al.*, 1982b). There is no indication of intervening sequences, although small intervening sequences might not be detected by this procedure. The R loops are 980 ± 75 nucleotides long. The DNA sequence corresponding to the N terminus of polyhedrin was determined directly by sequencing the DNA and correlating these sequences with the known amino acid sequence through the genetic code. Comparison of DNA sequence and R-loop information suggest that the OpNPV mRNA has a very small 5′ noncoding sequence and a relatively long (250 nucleotide) noncoding region at the 3′ end.

VI. DNA Sequence Homology to Facilitate Mapping of Baculoviruses Other than AcNPV

Different baculoviruses exhibit varying degrees of DNA sequence homology; the regions of homology may be located with respect to the AcNPV map by Southern blotting (Jewell and Miller, 1980; Rohrmann *et al.*, 1982a; Smith and Summers, 1982). In general, AcNPV shares more detectable sequence homology with multiply embedded NPVs (often abbreviated MNPVs; AcNPV is an MNPV) than it shares with singly embedded NPVs (the SNPVs). Sequence homology between AcNPV and several granulosis viruses has also been detected. A small but detectable amount of homology is observed between AcNPV and the nonoccluded HZ-1 viral DNA (Smith and Summers, 1982).

In functionally mapping a baculovirus other than AcNPV, it might be helpful to determine first whether the virus shares any sequence homology with AcNPV. Nonstringent hybridization conditions should be used to favor hybridization of regions containing some mismatch (Rohrmann *et al.*, 1982a; Smith and Summers, 1982). Adjusting the stringency of hybridization involves changing the formamide concentration and/or temperature at which the hybridizations are carried out. The higher the formamide concentration, the more stringent are the hybridization conditions. The work of Howley *et al.* (1979) on papovirus DNA sequence homology should be consulted for theoretical and empirical considerations. As noted by Rohrmann *et al.* (1982a), even the least stringent conditions of hybridization may not be sufficient to detect homologous sequences containing more than 33% mismatched nucleotides.

If DNA sequence homology is detected between AcNPV and the homology is distributed in a variety of areas as determined by Southern blot

hybridization, then a variety of cloned regions of AcNPV DNA could be used to probe Southern blots of the partially homologous baculovirus DNA. The use of cDNAs or carefully subcloned genomic DNAs, known to contain only a single gene, are strongly recommended for this purpose. If the physical map of the second baculovirus has been established, then this approach would rapidly establish an "analogy" map with AcNPV. It will be of considerable interest to determine if different baculoviruses have common gene organizations.

VII. Site-Directed Mutagenesis and Allelic Replacement

The successful marker rescue of AcNPV mutants (Miller, 1981b) demonstrates the ability to transfer genetic information from homologous DNA fragments to intact circular AcNPV DNA. This allelic (or gene) replacement technology may be utilized to mutate specific regions of AcNPV DNA as desired or to introduce new DNA sequences into AcNPV for genetic engineering purposes (Miller, 1981a; Miller *et al.*, 1983a). The basic approach is to clone a segment of AcNPV DNA in a plasmid in *E. coli,* mutate a specific region (Shortle *et al.*, 1981), or insert a new DNA sequence (e.g., passenger DNA) into the segment, isolate and propagate the altered plasmid in *E. coli,* and then cotransfect insect cells with the plasmid DNA and intact viral DNA. The mutant could be selected either by altered phenotype (e.g., defective occlusion body synthesis) or by altered genotype (using an appropriate probe).

The replacement of the polyhedrin gene is a particularly attractive approach to using AcNPV as a recombinant DNA vector system (Miller, 1981a; Miller *et al.*, 1983a). The location of the polyhedrin gene with respect to the physical map has been firmly established (see above). The gene is actually transcribed late in infection, and the gene product, polyhedrin, constitutes a high proportion of the total infected cell protein by 72 hr postinfection. Furthermore, polyhedrin is dispensable for virus replication in cell culture, and a simple selection method (visual observation of plaques) is available to detect mutants defective in polyhedrin production. Finally, the synthesis of polyhedrin mRNA is delayed until infectious extracellular virus synthesis has begun (10–12 hr postinfection) so that even passenger genes encloding cytolytic gene products may be propagated. Since AcNPV has a rod-shaped nucleocapsid that can apparently vary considerably in length, a potentially large amount of passenger DNA may be inserted into the DNA without affecting packaging capabilities.

Clearly, site-directed mutagenesis via allelic replacement will play an important role in exploring the organization and function of baculovirus genes. Baculoviruses may also become extremely valuable tools to future genetic

engineers attempting to express eukaryotic genes in a eukaryotic environment.

VIII. Summary

Rapid and remarkable progress has been made in determining the gene organization of the baculovirus AcNPV. Among the more recent advances are (1) the construction of a physical restriction map of the viral DNA; (2) the isolation of variants and mutants by plaque purification; (3) the development of transfection and marker rescue methods for mapping mutants or for site-specific mutagenesis of AcNPV genes; (4) the construction of translational maps of AcNPV gene products by hybridization selection of mRNA, using genomic DNA fragments or cDNAs of late mRNA, followed by *in vitro* translation; and (5) the initial characterization and mapping of AcNPV transcripts.

The first glimpses of baculovirus gene organization revealed by the AcNPV work suggest that the organization may be very complex. Genes present late in infection are distributed throughout the genome (Fig. 2). Genes that are known to be turned on late in infection, those encoding polyhedrin and the 7200-dalton protein, are located at approximately 3.5 and 89 map units, respectively (Fig. 2). A very early gene affected by the *ts821* mutation (Miller *et al.*, 1983b) is located between 90 and 2% of the genome (Fig. 1). Thus a very early gene lies between two very late genes. How extensive this intermixing of late and early genes is remains to be determined. With the exception of the polyhedrin gene, the *in vitro* translation products of hybrid-selected mRNA have been identified only by size; the 33,000-dalton polypeptide encoded by *Hin*dIII-V-homologous mRNA has been identified as polyhedrin by immune precipitation. Although the 39,000-dalton protein is similar in size to an early 39,000-dalton protein and the 41,000- and 64,000-dalton proteins are similar in size to extracellular virus structural proteins, rigorous structural or immunological correlations have not yet been made, so that a "functional" map of AcNPV is still in the early construction phase.

The research on AcNPV gene organization may be readily applied to determine the gene organization of other baculoviruses that share at least partial (detectable) sequence homology with AcNPV. Physical maps of essentially any baculovirus can be developed following initial selection for a single or predominant variant; a physical map of *S. frugiperda* NPV (SfNPV) has been established. Location of regions analogous to AcNPV might be quickly identified using appropriate AcNPV clones as probes of Southern blots. The polyhedrin gene of OpNPV was isolated by indepen-

dent methods. R-loop mapping of the OpNPV polyhedrin mRNA and DNA sequencing of the gene are beginning to reveal information on the nature of polyhedrin transcripts and their promoter regions. A vast amount of information on the gene organization of a variety of baculoviruses can be expected shortly.

AcNPV is extremely amenable to genetic and molecular biological analysis owing to the availability of several excellent permissive host cell lines and simple plaque assays. The technology developed for exploring the gene organization of tumor viruses is readily applied to the study of baculoviruses with their circular DNA genome containing primarily single-copy sequences. It will be fascinating to continue the exploration of baculovirus gene organization and structure. Understanding these features will be a prerequisite for the intelligent genetic manipulation of baculoviruses as biological insecticides as well as recombinant DNA vectors. The field of baculovirology has clearly emerged as one of the most fascinating new areas of molecular virology.

IX. Addendum

A relevant article by Esche *et al.* (1982) was not covered in the original writing of this review. Using cloned genomic fragments of AcNPV DNA for hybridization selection of homologous early and late mRNAs, Esche *et al.* physically mapped at least four early (6 hr postinfection) viral polypeptides and 20–24 late (24 hr postinfection) polypeptides. The basic approach was similar to that of Smith *et al.* (1982) except that cloned *Eco*RI fragments were used. The results of the two laboratories are difficult to compare precisely owing to the variations in molecular weights of the polypeptides determined in the different laboratories or, for that matter, in different gels. For instance, Smith *et al.* (1982) report 36K, 32K, and 24K proteins in the *Eco*RI-C region and Esche *et al.* report 38K, 35K, and 21K proteins in this same region. Notable differences between the results of the laboratories are the observation of a prominent 39K protein in the *Eco*RI-J region by Smith *et al.* (1982); Esche *et al.*, however, observe only a 22K protein in this same region but observe a 36K protein gene located in the neighboring *Eco*RI-K fragment. The region from 43 to 60 map units also reflects considerable differences between the results of the two groups. Further work is needed to resolve these differences, but the *in vitro* translations of Esche *et al.* (1982) appear to be more sensitive and the use of cloned fragments should provide more pure mRNAs.

The difficulty of comparing results of different experiments based on the molecular weights of proteins emphasizes the need to develop alternative

methods of identifying proteins. In addition, the new challenge to the baculovirus field is to determine the function of the proteins. This will require a more rigorous genetic and biochemical analysis of the baculovirus infection process.

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9 *Detection and Characterization of Subgenomic RNA in Plant Viruses*

Peter Palukaitis

I. Introduction

Most plant viruses contain single-stranded RNA genomes and many also give rise to positive-strand subgenomic RNAs [see Davies and Hull (1982) for a review of the genome organization of plant positive-strand RNA viruses]. Subgenomic RNAs are defined as RNAs related by sequence to a viral genome, which are generated during the course of infection. These subgenomic RNAs are often found encapsidated, the presence of some being more

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obvious than that of others (see below). In many cases, however, certain subgenomic RNAs are found only in infected plant cells, not in virions.

Subgenomic RNAs function as mRNAs *in vitro,* and are believed to fulfill this role as well *in vivo* (Atabekov and Morozov, 1979; Davies, 1979; Lane, 1979; Zaitlin, 1979). Thus, the generation of certain viral proteins is temporally controlled by the synthesis of the antecedent mRNA—the subgenomic RNA. Hence, an analysis of the subgenomic RNAs generated by a virus produces information on the genome organization and the control of the expression of the genes of that virus.

Over the last 10 years, a number of new biochemical techniques have been developed or modified that enable subgenomic RNAs to be detected, isolated, purified, and characterized. These new procedures are never static. They are forever being modified; naturally, always for the better. Thus, although details of many of the procedures as used in this laboratory and several others to detect and characterize subgenomic RNAs are given here, there exist many modifications of these procedures as used by other laboratories.

II. Detection and Characterization of Encapsidated Viral Subgenomic RNAs

If an RNA is found in virions, is isolated (Section II,A), purified (Section II,B), and shown to be neither infectious on its own nor an essential requirement for infectivity, then it could be a subgenomic RNA. On the other hand, the RNA could also be a satellite RNA, a satellite virus, or a viral negative-strand RNA. For positive-strand viruses, the criteria for determining the subgenomic nature of an RNA are (1) *in vitro* translation to produce a protein characteristic of the virus (e.g., coat protein) or one required for infectivity (Sections II,C and II,D); and (2) a relationship by sequence to (one of) the genomic RNA(s). This can be shown either by molecular hybridization analysis (Section II,E) or by RNA fingerprinting (Section II,F).

A. RNA ISOLATION

A number of procedures exist to extract RNAs from virions. Most of these are based on direct disruption of virions with buffer, detergent, metal chelators, and organic denaturants. Both the virion proteins and any contaminating nucleases must be denatured. Standard procedures involve slightly alkaline buffers (50–100 m*M* Tris-HCl or Tris-acetate at pH 7.5–

9.0), 0.1–0.5% (w/v) sodium dodecyl sulfate (SDS), 1 m*M* ethylenediaminetetraacetic acid (EDTA), and water-saturated phenol containing 0.1% 8-hydroxyquinoline (Loening, 1969; Peden and Symons, 1973; Lot *et al.*, 1974; Bruening *et al.*, 1976). In some cases, chloroform is combined with the organic phase to aid in denaturation (Ingle and Burns, 1968; Zimmern, 1975; Hari *et al.*, 1979). In the case of poly(A)-containing viruses, the above solution with chloroform (Perry *et al.*, 1972) or a high-pH buffer (Brawerman, 1974), or both (Otal and Hari, 1983), is used to avoid loss of poly(A)-RNA into the phenol phase or interface. Bentonite, 0.02–1% (w/v), is sometimes added as a nuclease inhibitor (Fraenkel-Conrat *et al.*, 1961; Bockstahler and Kaesberg, 1965; Brakke and van Pelt, 1969). The mixture is shaken or stirred for 5–10 min and is usually separated by low-speed centrifugation. The upper aqueous phase is reextracted once or twice more with phenol, and the RNA is recovered from the aqueous phase by the addition of salt (NaCl or preferably sodium acetate) to 0.1–0.2 *M* and 2.5–3 volumes of cold ethanol. After incubation (−20°C, 3–16 hr; −80°C, 15 min), the RNA precipitate is collected by centrifugation at 5000–20,000 *g* for 10–20 min at 0–4°C and either washed several times with ethanol containing 0.1 *M* sodium acetate, or dried (*in vacuo* to remove the ethanol), resuspended, and reprecipitated as above. The final RNA pellet is dissolved in either sterile, deionized water or, better, in sterile 0.1 − 1 m*M* EDTA. (Whenever possible, all glassware and solutions coming into contact with RNA should be sterile.) If the removal of all salt and buffer ions is important (depending on further use), dialysis or gel filtration is often employed.

In some cases, phenol treatment of detergent-treated virions is neither the only nor the best way to extract RNA. For example, digestion of virions with 0.1% (w/v) Pronase in 0.5% (w/v) SDS and 0.1 *M* sodium acetate followed by extraction with 90% phenol:10% cresol (v:v) has been used on nepoviruses (Murant *et al.*, 1972) and potyviruses (Abu-Samah and Randles, 1981) [*m*-cresol is reported by Kirby (1965) to improve phenol extraction of proteins]. Another technique used with potyviruses and luteoviruses is extraction by dissociation of virions in 100 m*M* ammonium carbonate (pH 9.0), 1 m*M* EDTA with or without 0.2 mg of bentonite per milliliter (Brakke and van Pelt, 1970; Brakke and Rochow, 1974; Dougherty and Hiebert, 1980a). Other extraction procedures include the LiCl denaturation method of Francki and McLean (1968), the sodium perchlorate extraction method of Wilcockson and Hull (1974), and the phenol–cetyltrimethylammonium bromide (CTAB) method of Ralph and Bellamy (1964).

Countless variations of these techniques exist. The best one is that consistently yielding the most RNA in an intact state *in one's own hands.*

B. FRACTIONATION AND PURIFICATION

The discovery of subgenomic RNAs was a by-product of the analyses of RNAs inside virions and determinations of the minimum number of components required for infection (Lane and Kaesberg, 1971). When it was recognized that the nonrequired components were generated from the RNAs required for infectivity and that the nonrequired components contained genes of the virus, then the concept of subgenomic RNAs became established.

The technology of fractionating and visualizing virion RNAs has improved markedly since subgenomic RNAs were first discovered. Early, and still useful, fractionation methods were based on rate-zonal (sucrose) density gradient ultracentrifugation (Diener *et al.*, 1964; Bockstahler and Kaesberg, 1965; Gillaspie and Bancroft, 1965; Hull *et al.*, 1969; van Kammen and van Griensven, 1970). This was useful in providing a crude fractionation of RNA. However, RNAs with molecular weight differences of only about 10–30% are inseparable by this procedure. Furthermore, several cycles of sucrose gradient ultracentrifugation are required to "purify" RNAs with about 50% or more difference in molecular weight (personal observation).

A far superior fractionation of nucleic acids is possible on polyacrylamide gels (Loening, 1967; Peacock and Dingman, 1968) or agarose gels (Hayward and Smith, 1972; Sharp *et al.*, 1973). The drawbacks of gel electrophoresis are 2-fold: (1) extraction of RNA from the gels after electrophoresis; and (2) removal of contaminating macromolecules from the gel. A large number of elution-by-soaking and electroelution procedures have been published testifying to the difficulty of the problem. Reviews of various elution methods and procedures for the removal of gel contaminants have covered most of the different approaches available (Southern, 1979; Yang *et al.*, 1979; Smith, 1980). Electroelution from acrylamide gels (Symons, 1978; Ho, 1983) and the melting of low-temperature-setting agarose gels (Wieslander, 1979) appear to be the simplest and most reliable methods for general applicability. Sucrose density-gradient centrifugation of the eluted RNA also appears to be the simplest method for removing contaminating agarose and polyacrylamide macromolecules and gel pieces. These macromolecules often bind enzymes and interfere with or inhibit a number of enzymatic reactions involving RNA (or DNA).

When RNAs are fractionated by gel electrophoresis and visualized by staining, care must also be taken. Some visible stains (e.g., methylene blue, toluidine blue O, acridine orange, and pyronin Y) have been reported to react with RNAs in strong (visible) light, but not under subdued lighting (Schuerch *et al.*, 1975), especially when the staining and destaining process is brief (Symons, 1978). The effect of the reaction of RNA with visible

stains may result in strand cleavage or RNA modification resulting in a reduction in infectivity. Presumably, such modifications may also affect the *in vitro* translation, template activity, hybridization ability, and RNase digestibility of RNAs.

Although a stain such as ethidium bromide does not appear to cause such problems (Schuerch *et al.*, 1975), it has other drawbacks: (1) ethidium bromide is a carcinogen and must be handled with care; (2) scission of DNA has been reported by treatment with ethidium bromide and strong (visible) light (Tai *et al.*, 1972); and (3) exposure of RNA to UV light (used to view ethidium bromide-stained RNA) can result in the cross-linking of RNA molecules.

Electrophoretic fractionation of RNA on polyacrylamide gels was previously covered in this series (Adesnik, 1971); fractionation on agarose gels is covered in Section III,B.

C. *In Vitro* TRANSLATION, GEL ANALYSIS, AND DETECTION OF PROTEIN PRODUCTS

The classification of an RNA as a subgenomic mRNA of a virus is established by showing that (a) the unidentified RNA contains sequences present in the viral genomic RNA (Sections II,E, II,F, and III,E), and (b) the subgenomic RNA can be translated *in vitro* into protein products (this section), some of which may be identified as well-characterized virus-associated proteins (Section II,D), e.g., virus coat protein. The first point differentiates subgenomic RNAs from satellite RNAs (Gould *et al.*, 1978; Mossop and Francki, 1978), and the second point aids in differentiating subgenomic mRNAs from random viral breakdown products.

In this section five *in vitro* systems used to translate RNAs into proteins will be briefly described, as well as a number of polyacrylamide gel and detection methods. Detailed procedures for the preparation of the various *in vitro* translation systems and the reaction protocols have been adequately covered elsewhere (Marcus *et al.*, 1974; Woodward, *et al.*, 1974; Ranu and London, 1979; Schleif and Wensink, 1981; Maniatis *et al.*, 1982) and will not be covered here per se; however, the components of the wheat and the rabbit reticulocyte cell-free lysate systems are listed in Table I.

1. *In Vitro Translation*

a. Wheat Germ Cell-Free Lysate (Roberts and Paterson, 1973; Efron and Marcus, 1973; Marcu and Dudock, 1974). The preparation and use of the wheat germ cell-free lysate for *in vitro* translation have been described in detail by Schleif and Wensink (1981). This system has been used for the translation of RNAs of tobacco mosaic virus (Bruening *et al.*, 1976; Beachy

TABLE I
COMPONENTS OF TWO *In Vitro* TRANSLATION SYSTEMS[a]

Component	Cell-free lysate derived from	
	Wheat germ[b]	Rabbit reticulocyte[c]
RNA	0.1–2 μg	0.1–2 μg
Lysate	5–10 μl[d]	10–16 μl[e]
HEPES·KOH, pH 7.2–7.6	12–37 m*M*	20–25 m*M*
Creatine phosphate	6–8 m*M*	8–17 m*M*
Creatine phosphokinase	0.5–1.0 μg	0.25–2.5 μg
19 unlabeled amino acids	25–30 μ*M*	25–100 μ*M*
Spermidine·HCl	40–400 μ*M*	100–600 μ*M*
ATP	1 m*M*	1 m*M*[f]
GTP	20–80 μ*M*	400 m*M*[f]
Magnesium acetate	2.0–3.5 m*M*	1–2 m*M*
Potassium acetate	80–130 m*M*	70–120 m*M*[g]
Potassium chloride	10–38 m*M*[h]	—[g]
Dithiothreitol	2 m*M*	1–2 m*M*
tRNA[i]	—[j,k]	2.5 μg
[^{3}H]-Leu (60–140 Ci/mmol), or	10–100 μCi	10–100 μCi
[^{35}S]-Met (500–1300 Ci/mmol)	10–100 μCi	10–100 μCi
Duration of incubation	1–3 hr	1–1.25 hr
Temperature of incubation	22–25°C	30–37°C

[a]Composite concentrations derived from the sources listed in Section II,C; amounts are given per 25 μl of reaction volume.

[b]Roberts and Paterson (1973).

[c]Pelham and Jackson (1976); Paterson *et al.* (1977).

[d]The volume of cell-free lysate used after passage through a Sephadex G-25 column.

[e]The volume of cell-free lysate used after treatment with micrococcal nuclease, hemin, and EGTA.

[f] These components are not included in most descriptions of the components used, but Paterson *et al.* (1977) do include them.

[g]Maniatis *et al.* (1982) use 80 m*M* KCl rather than KOAc; other workers report only KOAc.

[h]Since the wheat germ lysate contains KCl, the amount of KCl in the final mixture will depend on how much lysate is used. Chloride ions are inhibitory to the wheat germ cell-free translation system at concentrations above 50 m*M* (Davies *et al.*, 1977).

[i]Rat, mouse, or calf liver tRNAs are added to micrococcal nuclease-treated lysates.

[j]The wheat germ cell-free lysate is not usually treated with micrococcal nuclease; however, it may be so treated if high levels of endogenous RNAs are present. Under such circumstances, tRNA is added as a supplement to the wheat germ system as well.

[k]Bruening *et al.* (1976) add several additional components to their wheat germ translation mixture: 50 μ*M* $CaCl_2$, 10 μ*M* EDTA, and 80 μ*M* CTP.

and Zaitlin, 1977), cowpea mosaic virus (Davies *et al.*, 1977), brome mosaic virus (Davies and Samuel, 1975), cucumber mosaic virus (Schwinghamer and Symons, 1977), alfalfa mosaic virus (Thang *et al.*, 1976), tobacco rattle virus (Mayo *et al.*, 1976), and turnip yellow mosaic virus (Klein *et al.*, 1976) among others.

b. Wheat Embryo Cell-Free System (Marcus *et al.*, 1968; Marcus, 1970; Klein *et al.*, 1972). The preparation and use of this system have been described in detail by Marcus *et al.* (1974). The system has been used for the translation of RNAs of satellite tobacco necrosis virus (Klein *et al.*, 1972), brome mosaic virus (Shih and Kaesberg, 1973), cucumber mosaic virus (Schwinghamer and Symons, 1975, 1977), southern bean mosaic virus (Salerno-Rife *et al.*, 1980), and turnip crinkle virus (Dougherty and Kaesberg, 1981) among others.

c. Frog Oocyte System (Gurdon *et al.*, 1971). The preparation, method of injection, and translation *in vivo* in frog (Gurdon *et al.*, 1971) or toad (May and Glenn, 1974) oocytes have been described previously. This system has not been as extensively used as the above systems, presumably because of the complexities of the system and the high endogenous synthesis levels. Nevertheless, it has been used with tobacco mosaic virus (Knowland, 1974), cucumber mosaic virus (Schwinghamer and Symons, 1977), and brome mosaic virus (Semancik *et al.*, 1977).

d. Rabbit Reticulocyte Lysate. Prior to 1976, the rabbit reticulocyte cell-free lysate (Gilbert and Anderson, 1970) had been used to translate a few plant viral mRNAs (Knowland *et al.*, 1975; Mohier *et al.*, 1975; Schwinghamer and Symons, 1977); however, it was not the system of choice because of the high level of endogenous mRNA that produced large amounts of globin upon *in vitro* translation. In 1976, Pelham and Jackson developed a method of making the rabbit reticulocyte lysate mRNA dependent; they digested the mRNA in the lysate with the Ca^{2+}-dependent, micrococcal nuclease, which could then be conveniently inactivated by treatment with ethylene glycol tetraacetic acid (EGTA). The micrococcal nuclease-treated, rabbit reticulocyte cell-free system has since been used to translate the RNAs of cowpea mosaic virus (Pelham and Jackson, 1976; Pelham, 1979a), eggplant mosaic virus (Ricard *et al.*, 1978), tobacco rattle virus (Pelham, 1979b), alfalfa mosaic virus (van Tol and van Vloten-Doting, 1979), tobacco etch virus and pepper mottle virus (Dougherty and Hiebert, 1980a,b), tomato black ring virus (Fritsch *et al.*, 1980), southern bean mosaic virus (Salerno-Rife *et al.*, 1980), turnip crinkle virus (Dougherty and Kaesberg, 1981), turnip rosette virus (Morris-Krsinich and Hull, 1981), barley stripe mosaic virus (Gustafson *et al.*, 1981), and cucumber mosaic virus (Gordon *et al.*, 1982) among others.

e. Escherichia coli Cell-Free Lysate (Glover and Wilson, 1982). With the possible exception of tobacco necrosis virus (Salvato and Fraenkel-Conrat, 1977), carnation mottle virus (Salomon *et al.*, 1978), and potyviruses (Dougherty and Hiebert, 1980b), internal genes on polycistronic plant viral RNAs are not separately initiated during *in vitro* translation with eukaryotic systems; they are translated either from the subgenomic RNAs or as part of a polycistronic protein. The *E. coli* cell-free lysate, however, seems to

be capable of efficient translation of the internal genes on plant viral RNAs, although the system has been tested only with tobacco mosaic virus thus far (Glover and Wilson, 1982).

f. Comparing Systems. Several of the more recent translational analyses of plant viral RNAs or subgenomic RNAs have involved translation in both the wheat embryo (or wheat germ) and the rabbit reticulocyte cell-free systems (Ricard *et al.*, 1978; Salerno-Rife *et al.*, 1980; Dougherty and Kaesberg, 1981; Gustafson *et al.*, 1981). The results obtained suggest that translation in both systems is the wisest course. The wheat embryo system is not very efficient at synthesizing high-molecular-weight proteins and often produces a number of premature termination products. The rabbit reticulocyte system is capable of synthesizing much larger amounts of protein and full-length, high-molecular-weight polypeptides with fewer premature termination products. On the other hand, the rabbit reticulocyte system is also more selective about which RNAs it will recognize and translate; the wheat embryo system is more promiscuous in this regard.

The two major elements that are varied in *in vitro* translation systems are the Mg^{2+} concentration and the K^+ concentration; the concentrations of both affect the initiation of protein synthesis, while the K^+ concentration affects also elongation in protein synthesis. Hence, with high-molecular-weight proteins, a delicate balance between the K^+ optimum for initiation and for elongation must be established. Unfortunately, because of the way the rabbit reticulocyte system is normally prepared, it is more difficult to control the concentration of ions and amino acids in different preparations; an unknown concentration of ions and amino acids coming from the reticulocytes themselves would vary with different preparations. Passage of the lysate over G-25 Sephadex columns usually resulted in a very considerable loss of translation activity. However, these problems have been overcome: Salerno-Rife *et al.* (1980) were able to adjust the ionic strength of the lysate by dialysis, and Jackson *et al.* (1983) have determined the cause of the previous inactivation and have developed corrective procedures. Concentration and quality of RNA can also have a marked effect on the actual translation products obtained.

2. *Gel Analyses*

The identification of the translation product and its characterization have usually involved polyacrylamide gel electrophoresis under denaturing conditions. A number of gel systems are available for analyzing proteins, but the three most used types of gels are: (1) discontinuous SDS–polyacrylamide gels (Laemmli, 1970; Maizel, 1971); (2) gradient-polyacrylamide gels containing SDS (Margolis and Kenrick, 1968; O'Farrell, 1975; Lambin *et al.*, 1976); and (3) highly cross-linked, polyacrylamide gels containing SDS and urea (Swank and Munkres, 1971).

a. Discontinuous SDS-Polyacrylamide Gels (Laemmli, 1970; Maizel, 1971). This is the system most widely used to analyze proteins. The components of the two versions of the system are listed in Table II. The translation products and marker proteins are boiled in SDS containing 2-mercaptoethanol, and the protein samples are electrophoresed on an 8–15% polyacrylamide gel overlaid with a 3–5% polyacrylamide "stacking" gel. The use of buffers with different ionic strengths and counterions results in the stacking of proteins into sharp bands. Thus, the system is superior to continuous SDS-polyacrylamide gels that had previously been used (e.g., Weber and Osborn, 1969). A modification of the above gel system was reported by Conejero and Semancik (1977); this gel contains in addition a spacer gel between the stacking gel and the separating gel.

b. Gradient Polyacrylamide Gels Containing SDS (Margolis and Kenrick, 1968; O'Farrell, 1975; Lambin *et al.*, 1976). Since a number of proteins of widely varying molecular weights are often analyzed together and many of

TABLE II
ELECTROPHORESIS SYSTEMS FOR PROTEINS[a]

Components	Laemmli (1970) version	Maizel (1971) version
Separating gel	8–15% Acrylamide, acrylamide:bisacrylamide 37.5:1[b] or 125:1[c]	8–15% Acrylamide, acrylamide: bisacrylamide 37.5:1
Stacking gel	5% Acrylamide, acrylamide:bisacrylamide 37.5:1	3% Acrylamide,[d] acrylamide:bisacrylamide, 37.5:1
Separating gel buffer	375 m*M* Tris-HCl (pH 8.8), 0.1% SDS	375 m*M* Tris-HCl (pH 8.9), 0.1% SDS
Stacking gel buffer	125 m*M* Tris-HCl, (pH 6.8), 0.1% SDS	62.5 m*M* Tris-HCl (pH 6.7),[e] 0.1% SDS
Electrode buffer	25 m*M* Tris, 192 m*M* glycine (pH 8.3), 0.1% SDS	50 m*M* Tris,[e] 384 m*M* glycine (pH 8.3), 0.1% SDS
Sample loading buffer	62.5 m*M* Tris-HCl (pH 6.8) 2% SDS, 10% glycerol, 5% 2-mercaptoethanol, 0.001% bromphenol blue	62.5 m*M* Tris-HCl, (pH 6.7)[f] 1% SDS, 10% glycerol, 0.1% 2-mercaptoethanol, 0.002% bromphenol blue

[a]Although originally designed for tube gels, virtually all analysis is now done using these systems in polyacrylamide slab gels; the length and thickness of the stacking gel and the separating gel vary with the user. For a review of the effect of such variations, see Dunn and Burghes (1983).

[b]The acrylamide:bisacrylamide stock is usually 30% acrylamide:0.8% bisacrylamide.

[c]To prevent cracking of the gel during the "drying down" step (Maizel, 1971), the acrylamide:bisacrylamide ratio may be changed to 125:1 (Schwinghamer and Symons, 1977). The acrylamide:bisacrylamide stock is 40% acrylamide:0.32% bisacrylamide.

[d]Subsequently, 5% acrylamide stacking gels were used with the Maizel system.

[e]Note that the Tris concentration of the stacking gel is twice as high in the Laemmli system as in the Maizel system, and the Tris-glycine concentration in the Maizel system is twice as high as in the Laemmli system.

[f] The Laemmli sample loading buffer is now used for both gel systems, since it contains higher SDS and 2-mercaptoethanol concentrations, which may be required with some proteins.

these are best resolved or characterized on different percentage polyacrylamide gels, the use of a gradient gel containing polyacrylamide concentrations varying from 7.5 to 20% (or percentages within this range) enables virtually all the proteins to be analyzed on the one gel. Problems may arise, however, in the quantitative and qualitative detection of proteins electrophoresed on gradient gels (Dunn and Burghes, 1983; Harding and Scott, 1983). Furthermore, elution of proteins (for further analysis) from gradient gels is often more difficult (Harding and Scott, 1983).

c. Highly Cross-Linked, Polyacrylamide Gels Containing SDS and Urea (Swank and Munkres, 1971). Low-molecular-weight proteins and peptides (2000–12,000) generated by cyanogen bromide cleavage are best analyzed on a highly cross-linked, polyacrylamide gel system containing both SDS and urea (Swank and Munkres, 1971). Although the original method was developed for tube gels, slab gels containing low ratios of acrylamide:bisacrylamide can be prepared by lowering the bisacrylamide concentration to 0.3% (w/v) and increasing the acrylamide concentration from 10 to 12% (w/v) (unpublished observation). Under these conditions, excessive shrinkage of the slab gel during polymerization does not occur. Furthermore, the spots observed on the gels can be sharpened to bands by including a stacking gel (Ghosh *et al.*, 1979).

Unfortunately, not all proteins show a linear relationship between mobility and log-molecular weight on such gels (Swank and Munkres, 1971). There are, however, alternative analytical gels for analyzing low-molecular-weight polypeptides; e.g., 20 or 25% SDS-discontinuous polyacrylamide gels or 15–30% gradient polyacrylamide gels containing SDS.

3. *Detection of Translation Products in Polyacrylamide Gels*

The detection of *in vitro* translation products is usually accomplished by either autoradiography (for ^{14}C- or ^{35}S-labeled proteins) or fluorography (for ^{3}H-, ^{14}C-, or ^{35}S-labeled proteins). Autoradiography involves covering a gel, which often has been "fixed" (i.e., incubated in an acid or acid–alcohol mixture to precipitate proteins and prevent diffusion of the proteins in the gel), with a sheet of X-ray film and detecting the position of the radiolabeled proteins in the gel by the appearance of dark spots on the X-ray film after development. Fluorography, on the other hand, is a more sensitive procedure that involves further processing of the gel (Bonner and Laskey, 1974; Laskey and Mills, 1975).

The original procedure of Bonner and Laskey (1974) involves dehydrating the fixed gel with dimethyl sulfoxide (DMSO) and saturating the gel with DMSO containing 2,5-diphenyloxazole (PPO). The PPO is precipitated in the gel, and the DMSO is removed by washing the gel in water. The gel is dried down onto Whatman 3MM paper, covered with presensi-

tized X-ray film, and stored at −80°C to expose the film. As electrons decay from the radioactive source, their energy is absorbed by the PPO (a fluor), which in turn emits light of the far-blue wavelength. Many brands of X-ray film are particularly sensitive to blue light. A detailed explanation of the process of fluorography has already been given (Laskey and Mills, 1975; Laskey, 1980).

Because the original method involves a large number of steps with expensive reagents, alternative methods of introducing fluors into the gel have been developed. Three such methods are (1) the use of 20–25% (w/v) naphthalene containing 1% (w/v) PPO in DMSO instead of 22% (w/v) DMSO (my unpublished results reported by Gill *et al.*, 1981); (2) the use of 1 *M* sodium salicylate in water (pH 5–7 with NaOH; Chamberlain, 1979); and (3) the use of 20% (w/v) PPO in glacial acetic acid (Pulleyblank and Booth, 1981). The last two procedures are considerably shorter than the original method or method 1. All these procedures seem to be equally efficient and comparable to the use of commercial fluorography cocktails (e.g., Enhance or Enlightning, New England Nuclear). Naphthalene–PPO mixtures cannot be used with glacial acetic acid alone because of poor solubility (unpublished observation).

In order to ascertain the molecular weight(s) of the translation product(s), protein size markers are used. These can be detected by staining the gel with Coomassie blue prior to fluorography, or by using radiolabeled proteins that are then detected by fluorography. The latter can most easily be prepared by reductive methylation of lysine residues with either [^{14}C]formaldehyde and sodium borohydride (Rice and Means, 1971), or formaldehyde and sodium boro[^{3}H]hydride (Kumarasamy and Symons, 1979). Alternatively, proteins containing tyrosine residues can be iodinated by either the chloramine-T procedure of Greenwood *et al.* (1963), reviewed by McConahey and Dixon (1980); the lactoperoxidase-catalyzed procedure of Morrison (1980); the Bolton–Hunter reagent (Bolton and Hunter, 1973), reviewed by Langone (1980); and the chloroglycoluril-catalyzed method of Fraker and Speck (1978).

As a stain for proteins, "silver staining" is much more sensitive than Coomassie blue (Okley *et al.*, 1980). Ochs *et al.* (1981), compared six silver staining procedures and judged their own protocol to be the most satisfactory.

D. CHARACTERIZATION OF TRANSLATION PRODUCTS

The two most useful methods for establishing the relationship between a subgenomic viral RNA *in vitro* translation product and a bona fide virus-encoded protein [usually the viral coat protein or virus-induced inclusion body protein(s)] are peptide mapping and immune precipitation.

1. *Peptide Mapping*

a. Tryptic Peptides: Two-Dimensional Separation. Viral translation products are cut out of dried, fluorographed gels (Section II,C,3) and the gel slices are soaked in either 10% methanol or DMSO followed by 10% methanol to remove salicylate or PPO–naphthalene, respectively. The gel slices are dried at 60°C and incubated in 50 m*M* NH_4HCO_3 (pH 8.0) containing 50 μg of trypsin per milliliter (DPCC-treated) at 37°C for 18 hr. The tryptic peptides elute out of the gel and are lyophilized to remove the NH_4HCO_3. Peptides are solubilized in electrophoresis buffer I (formic acid:acetic acid:water, 5:15:85) and stored frozen until use.

Peptides (1 to 2 × 10^5 cpm) are applied to a 250-μm Avicel cellulose thin-layer plate (20 × 20 cm; Analtech). Up to 50 μl can be applied to a 2.5-cm linear origin. Electrophoresis is carried out in electrophoresis buffer I for 1.5 hr at 500 V on a cooling plate at 5°C. After electrophoresis, the protein spots are compressed by chromatography in 1% (v/v) acetic acid (Bieleski and Turner, 1966). Separation in the second dimension is accomplished by ascending chromatography in butanol:pyridine:acetic acid:water (32.5:25:5:20). The plate is air dried and either autoradiographed to detect ^{35}S-labeled peptides (Section II,C,3), indirectly autoradiographed to detect ^{125}I-labeled peptides (Section III,E,2), or fluorographed to detect ^{3}H-labeled proteins. Fluorography of thin-layer plates can be accomplished by including 7% (w/v) PPO in the chromatography buffer (Leonard and Zaitlin, 1982), by a second chromatography in 7% PPO (w/v) in acetone (Laskey and Mills, 1975), by dipping the thin-layer plate in 30% PPO (w/v) in ether or other solvents (Bonner and Stedman, 1978), or by spraying 0.4% (w/v) PPO–10% toluene in 2-methylnaphthalene onto the thin-layer plate (Bonner and Stedman, 1978). The last two methods were judged to be up to 15-fold more efficient than the second method (Bonner and Stedman, 1978). After impregnation, the sample is covered with presensitized X-ray film and exposed at −70° to −80°C (Laskey and Mills, 1975).

The two-dimensional tryptic peptide map enables minor differences between subgenomic RNA translation products of viral strains (Leonard and Zaitlin, 1982) as well as precursors and products to be identified.

b. Limited Proteolysis: One-Dimensional Mapping. One-dimensional peptide mapping by limited proteolysis of proteins is carried out as described by Cleveland *et al.* (1977). A polyacrylamide gel slice containing the radiolabeled translation product is either pretreated to remove fluors and/or fixers as described above (Section II,D,1,a) or, preferably, only stained and destained briefly to locate the protein to be digested. The gel slice is soaked for 30 min in equilibration buffer (125 m*M* Tris-HCl, 0.1% SDS, 1 m*M* EDTA, pH 6.8) and placed in a well of a 15–20% polyacrylamide (acry-

lamide:bisacrylamide, 15:1) Laemmli (1970) or Maizel (1971) gel. The gel slice in the well is overlaid with either 0.5–1.0 μg of *Staphylococcus aureus* V8 protease, 2–5 μg of elastase, 0.5–2 μg of papain, or 2–5 μg of chymotrypsin in equilibration buffer containing 10% glycerol and 0.01% bromphenol blue. The protein and dye are electrophoresed into the stacking gel and the current is turned off for 30 min to allow partial digestion to take place. Electrophoresis is then continued until the dye reaches the bottom of the gel. The gel is fixed (and stained and destained in some instances) and fluorographed as described above (Section II,C,3).

c. Chemical Cleavage. A problem often encountered with the limited-proteolysis method of peptide mapping is determining the ratio of enzyme to substrate either when two substrates (e.g., viral coat protein and coat protein mRNA translation product) are to be compared and one of these is present in very low amounts, or when one is present in a gel slice and the other is in solution. Therefore, as an alternative to limited proteolysis catalyzed by enzymes, limited proteolysis by chemical cleavage is sometimes used. Three such chemical cleavage procedures are cyanogen bromide cleavage of methionine residues, formic acid cleavage of aspartyl-prolyl bonds, and *n*-chlorosuccinimide cleavage of tryptophan residues.

i. Cyanogen bromide cleavage: Methionine (Gross, 1967). Protein dissolved in 0.1 *N* HCl is combined with excess cyanogen bromide (at least a 30-fold molar excess of reagent over methionine residues, or as much as a 1:1 ratio by dry weight of cyanogen bromide to protein) and incubated at room temperature for 24 hr. The cyanogen bromide is then removed by lyophilization, and the peptides are analyzed on polyacrylamide gels (Section II,C,2).

Stained and destained gel slices are first rinsed in water for 15–30 min, dried, and incubated at room temperature in 0.5 *M* 2-mercaptoethanol for 1.5 hr [to reduce methionine sulfoxide to methionine; the former is readily formed from methionine and is not cleavable by cyanogen bromide (Caldwell *et al.*, 1978)] before being incubated in 450 μg of cyanogen bromide per milliliter in 88% formic acid for 2 hr at 37°C and 2.5 hr at room temperature (Collmer *et al.*, 1983). The cyanogen bromide-treated gel slices are then rinsed several times in water, once in 1.5 *M* Tris-HCl (pH 8.8), and once in gel loading buffer (10 min at 37°C) prior to loading onto a 15% or 20% polyacrylamide gel (Section II,C,2). The peptides in the gel can be localized by staining, autoradiography, or fluorography.

ii. Formic acid cleavage: Asp-Pro (Sonderegger *et al.*, 1982). The gel containing the proteins to be examined is briefly stained and destained. Gel slices containing protein bands are equilibrated with 5 ml of 75% formic acid at room temperature for 4 hr. The excess formic acid is removed, and the gel slice is covered with 2 ml of paraffin oil and incubated at 37°C for

18–24 hr. The paraffin oil is removed, and the gel slice is lyophilized three times (and reswollen in water between lyophilizations) before finally being reswollen for 15 min in 125 m*M* Tris-HCl (pH 6.8), 0.5% SDS, 20% glycerol. The gel slice is then loaded onto a 12% or 15% polyacrylamide gel (Section II,C,2).

iii. N-Chlorosuccinimide cleavage: Tryptophan (Lischwe and Ochs, 1982). Gel slices containing the protein bands to be examined, are washed at room temperature; twice for 20 min in 25 ml of water and twice for 20 min in 10 ml of urea:water:acetic acid (1 g:1 ml:1 ml). The gel slices are then incubated in 5 ml of 15 m*M* *N*-chlorosuccinimide in urea:water:acetic acid (as above) for 30 min at room temperature. The gel slices are subsequently washed twice more with water and four times for 1.5 hr in 10 ml of 62.5 m*M* Tris-HCl, pH 6.8, 3% SDS, 15% 2-mercaptoethanol, 10% glycerol. The gel slices are then loaded onto a 15% polyacrylamide gel (Section II,C,2).

The three chemical cleavage procedures described above cleave amino acids that are found infrequently in proteins, resulting in large peptides being produced that can be analyzed on polyacrylamide gels. Unfortunately, some proteins to be examined or compared may not contain either methionyl, trytophanyl or aspartylprolyl residues (or bonds). However, rarely will a protein not contain all three of these groups.

2. *Immune Precipitation* (Kessler, 1975, 1981)

The following is a modification of the procedure of Kessler (1975) as used by Dougherty and Hiebert (1980a). After translation of viral subgenomic RNAs, the mRNAs are digested with RNase A (0.7 mg/ml), and the proteins are dissociated by the addition of 2 volumes of 62.5 m*M* Tris-HCl, pH 6.8, 2% SDS, 5% 2-mercaptoethanol, and 10% glycerol, followed by heating at 90°C for 3–5 min. Four parts of dissociated translation mixture are incubated with one part of antiserum and eight parts of immune precipitation buffer [150 m*M* NaCl, 5 m*M* EDTA, 50 m*M* Tris-HCl (pH 7.4), 0.05% Nonidet P-40 (NP-40), 1 mg of ovalbumin per milliliter, and 2 m*M* unlabeled amino acid corresponding to the labeled amino acid used during the translation reaction] at room temperature for 1 hr, followed by the addition of 2 volumes of (10% w/v) *Staphylococcus aureus* (Cowan strain) protein A solution, and a further incubation of 30 min at room temperature. The mixture is centrifuged at 2000 *g* for 5 min, and the immunoprecipitate pellet is resuspended in 250 μl of 50 m*M* Tris-HCl (pH 7.4), 5 m*M* EDTA, 150 m*M* NaCl, 0.05% NP-40. The material is centrifuged and resuspended thrice more in this manner (as above). The final pellet is resuspended in 50 μl of 62.5 m*M* Tris-HCl (pH 6.8), 2% SDS, 5% 2-mercaptoethanol, and 10% glycerol, heated at 90°C for 5 min, and centrifuged

at 2000 *g* for 5 min. The final supernatant is loaded onto a polyacrylamide gel for analysis (Section II,C,2).

Figure 1 shows such a gel with antisera prepared to tobacco etch virus coat protein (lane 2), nuclear inclusion protein (lane 3), and cylindrical inclusion protein (lane 6) being used to immunoprecipitate the respective viral translation products from an *in vitro* translation of tobacco etch virus RNA. As described here, not only can immune precipitation be specific (Fig. 1, lanes 8–10), but it is also capable of identifying precursor molecules as well as premature termination products. This is in contrast to some earlier procedures where nonspecific precipitation (i.e., trapping) of proteins was observed.

E. MOLECULAR HYBRIDIZATION ANALYSIS

One of the most useful techniques for determining the sequence relationships of two RNAs is molecular hybridization analysis. Two classes of such hybridization reactions will be considered here, both involving the hybridization of complementary DNA (cDNA) transcribed *in vitro* from an RNA template to the template RNA, or to other RNA species, where (1) both the RNA and the cDNA are in solution, the so-called liquid–liquid hybridization (Gillespie *et al.*, 1975); and (2) one component is immobilized on a solid support (e.g., nitrocellulose, or chemically activated paper, or cellulose powder) and the other is free in solution, the so-called solid–liquid hybridization (Section III) (Gillespie *et al.*, 1975; Alwine *et al.*, 1977; Thomas, 1980; Seed, 1982). In the case of liquid–liquid hybridizations, the extent of hybrid formation can be estimated by the use of the single-stranded specific nuclease S1 (Ando, 1966; Sutton, 1971), by hydroxyapatite chromatography, or by binding to nitrocellulose. Only the enzymatic assay procedure will be considered here.

Although it is beyond the scope of this treatise to discuss the theory of hybridization or an analysis of the kinetics of hybridization, it is important to consider briefly some of the factors affecting the rate of the hybridization reaction.

The rate of the association of cDNA and RNA is governed by a number of physical parameters that were deduced from earlier analyses of the rates of reassociation of denatured double-stranded DNA (Wetmur and Davidson, 1968; Bishop, 1972; Gillespie *et al.*, 1975; Wetmur, 1976). The parameters affecting the rates of reassociation (or "hybridization") are (1) the RNA concentration; (2) the time of incubation; (3) the temperature of incubation; (4) the monovalent cation concentration; (5) the pH; and (6) the viscosity of the solution. Most of the conditions used for hybridization have been optimized with respect to the last four points. The first two points are

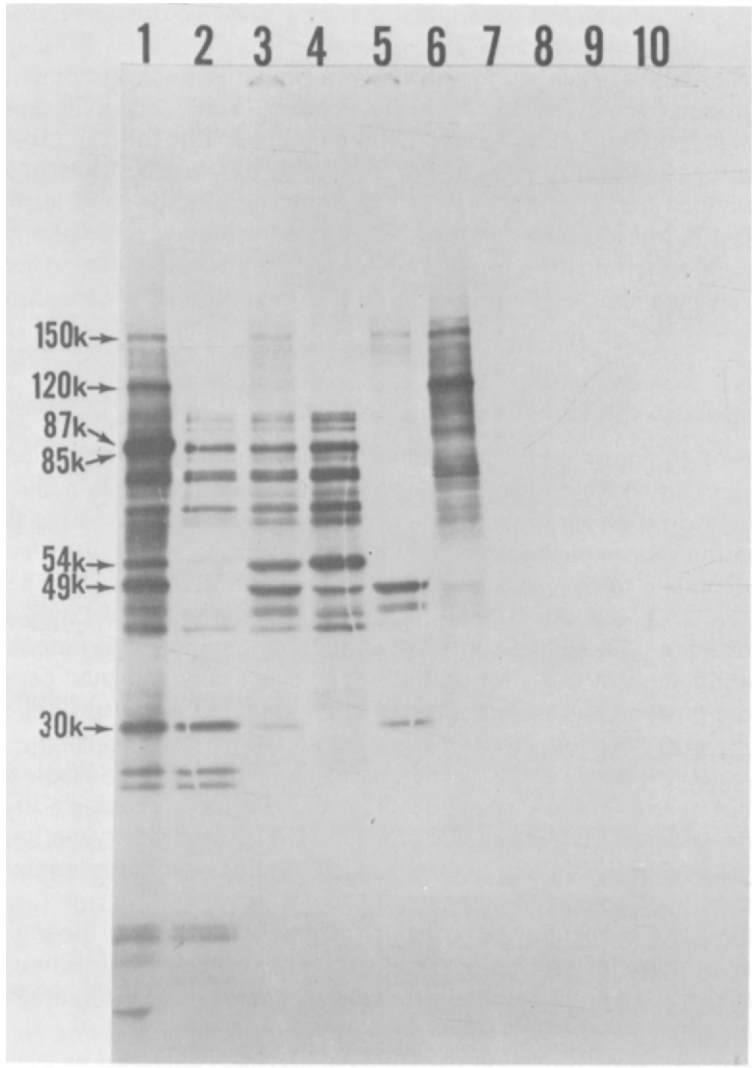


FIG. 1. Fluorogram of *in vitro* translation products electrophoresed on a 7.5–15% linear gradient polyacrylamide gel. [^{35}S]Methionine-labeled translation products of tobacco etch virus (TEV) RNA were electrophoresed on an SDS–discontinuous polyacrylamide gel (Laemmli, 1970) before (lane 1) or after (lanes 2–10) immunoprecipitation with antiserum to the following: TEV capsid protein (lane 2); total nuclear inclusion protein (lane 3); gel-purified nuclear inclusion protein (54K) (lane 4); gel-purified nuclear inclusion protein (49K) (lane 5); TEV cylindrical inclusion protein (lane 6); watermelon mosaic virus 1 cylindrical inclusion protein (lane 7); potato virus X capsid protein (lane 8); proteins of uninoculated tobacco (lane 9); and nonimmunized rabbits (lane 10). This figure is Fig. 7 of Dougherty and Hiebert (1980b) and was generously provided by the senior author.

then varied to provide information on the "analytical complexity" of an RNA, or the "percentage of sequence homology" between RNAs.

An estimation of the analytical complexity of an RNA [i.e., the minimum number of nucleotides (or their molecular weight) that comprises all the nonidentical nucleotide sequences of the nucleic acid species] can be achieved by studying the rate of association of known concentrations of an RNA species to its cDNA, since the rate of hybridization is inversely proportional to the analytical complexity of the reacting species and directly proportional to the (molar) concentration of the reacting species. Therefore, if the rate of hybridization is determined by plotting the percentage of cDNA being converted to a cDNA:RNA duplex as a function of the log of the product of the RNA concentration (measured in moles of ribonucleotide per liter) and the time of incubation (measured in seconds), then a sigmoidal curve will be produced (Fig. 2). The curve is referred to as an "R_0t (or C_rot) curve" and the point at which half of the cDNA has hybridized to the RNA is referred to as the "R_0t 1/2."

By comparing the R_0t 1/2 of a "homologous" hybridization reaction (cDNA hybridized to its template RNA) with the R_0t 1/2 of a "heterologous" hybridization reaction, it is possible to determine whether two RNAs are related by sequence; i.e., whether one RNA is a subgenomic RNA of the other, or if one RNA is merely contaminated by sequences of the other. Comparisons of this sort have been used to characterize subgenomic RNAs of cucumber mosaic virus (Gould and Symons, 1977), alfalfa mosaic virus (Gould and Symons, 1978), and barley stripe mosaic virus (Taliansky *et al.*, 1979).

1. *Hybridization Procedure*

Complementary DNA to RNA is prepared by one of the procedures described in Section III,D.

The R_0t curve-hybridization reaction is carried out by setting up a series of dilutions of the RNA in hybridization buffer [10–50 m*M* Tris-HCl (pH 7.0), 0.18 *M* NaCl, 0.05% (w/v) SDS, 1 m*M* EDTA] and adding 2000–4000 cpm of either [^{3}H]cDNA or [^{32}P]cDNA to each sample dilution. The samples (10–15 μl) are either prepared in siliconized glass tubes or Eppendorf tubes and are covered with mineral oil; alternatively, they are drawn up into siliconized glass capillaries, both ends of which are then sealed to prevent evaporation. The RNA and cDNA are boiled for 3–5 min and then incubated at 60°C for the various times required to obtain particular R_0t values. (Either the RNA concentration or the time of incubation, or both, can be varied to obtain a given R_0t value.) After incubation, the sample is rapidly cooled to inhibit any further hybridization and is either stored at −20°C or assayed immediately. The temperature of hybridization (60°C) is optimal for the formation of most cDNA:RNA hybrids in 0.18 *M* Na^+.

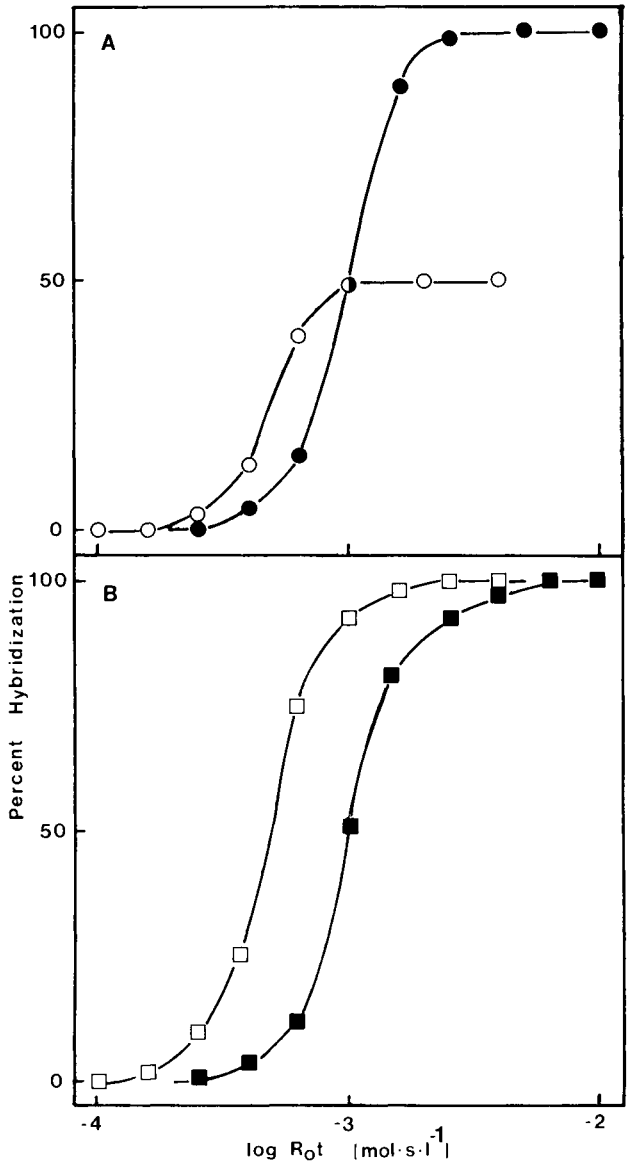


FIG. 2. R_0t curves of the hybridization of (A) $cDNA_I$ against RNA_I (●) or against RNA_{II} (○) and (B) $cDNA_{II}$ against RNA_I (■) or against RNA_{II} (□). Hybrids were formed and assayed as described in Section II,E.

Others have used higher Na^+ concentrations to increase the rate of hybridization, and incubated at higher temperatures to compensate for the increased temperature optima. Unfortunately, prolonged incubation at higher temperatures tends to degrade RNA. Therefore, many hybridization reactions are carried out in solutions containing formamide (Friedrich and Feix, 1972; Hutton, 1977). This has the effect of lowering the T_m [the midpoint of the thermal denaturation of the hybrid; T_m minus 15–25°C is generally the optimal temperature for hybridization (Wetmur and Davidson, 1968; Hutton, 1977)] of the hybrid, permitting the incubation to be carried out at lower temperatures. Hutton (1977) has conducted a thorough analysis of the effect of denaturants (formamide and urea) on the rate of hybridization.

2. *Determination of Percentage of Hybridization*

As stated above, the percentage of the cDNA that is in the form of a cDNA:RNA hybrid can best be determined by an assay involving the digestion and elimination of the unhybridized, single-stranded cDNA with the single-strand specific nuclease S1.

The solution containing the hybrids is added to 400 μl of nuclease S1 digestion buffer [30 m*M* sodium acetate (pH 4.6), 50 m*M* NaCl, 1 m*M* $ZnSO_4$, 5% (v/v) glycerol, containing 40 μg of denatured and sonicated salmon sperm or calf thymus DNA per milliliter]. Two samples, each of 200 μl, are taken; to one sample is added 2–10 units of nuclease S1 (see below). Both samples ("+" and "−" nuclease S1) are incubated at 45°C for 30 min, and the digestion is terminated by the addition of 1.0 ml of 10% (w/v) cold trichloroacetic acid (TCA) and bovine serum albumin (100 μg) or yeast RNA (50 μg) as a carrier. After 15–30 min at 0°C, the TCA precipitates are collected onto glass-fiber filters, washed 3–4 times with 4–5 ml of 5% (w/v) TCA (cold) and twice with 4–5 ml of cold ethanol or ether. The filters are dried and counted by liquid scintillation spectrometry in a toluene-based liquid scintillation fluid (made from 3.5 g of PPO and 0.35 g of POPOP per liter of toluene). The percentage of hybridization is given by the counts per minute in the "+ nuclease S1" sample divided by the counts per minute in "– nuclease S1" sample × 100%. When this is done for all the $R_o t$ points, curves such as those in Fig. 2 are generated.

The curves in Fig. 2 show the following: (1) $cDNA_I$:RNA_I hybridizes at the same rate as $cDNA_{II}$:RNA_I, indicating that RNA_{II} contains sequences present in RNA_I; (2) $cDNA_I$:RNA_{II} and $cDNA_{II}$:RNA_{II} hybridize at the same rate, but only half of $cDNA_I$ is capable of hybridizing to RNA_{II}. This observation and the increased analytical complexity of RNA_I, viz. RNA_{II}, indicate that RNA_I is more complex than RNA_{II}, and that RNA_{II} is contained within RNA_I; i.e., RNA_{II} could be a subgenomic RNA of RNA_I.

A detailed R_0t analysis enables one to distinguish contamination from sequence homology. This is not possible if only hybridization to a single, high R_0t point is carried out.

Although nuclease S1 is available commercially, there is no universally accepted definition of a unit. Furthermore, whereas one "unit" of nuclease S1 may digest the single-stranded cDNA prepared to one type of RNA, it may require 5 or 10 units of nuclease S1 to digest completely other cDNAs. Different preparations of nuclease S1 also have different amounts of contaminating double-stranded nuclease activity (Vogt, 1973). This can lead to variable or low maximum percentage of hybridization values. Moreover, some cDNAs either do not seem to hybridize to their homologous RNAs as well as others do or are more susceptible to digestion by the contaminating double-stranded nuclease activity. Therefore, the amount of nuclease S1 that should be used in a given hybridization analysis needs to be empirically determined.

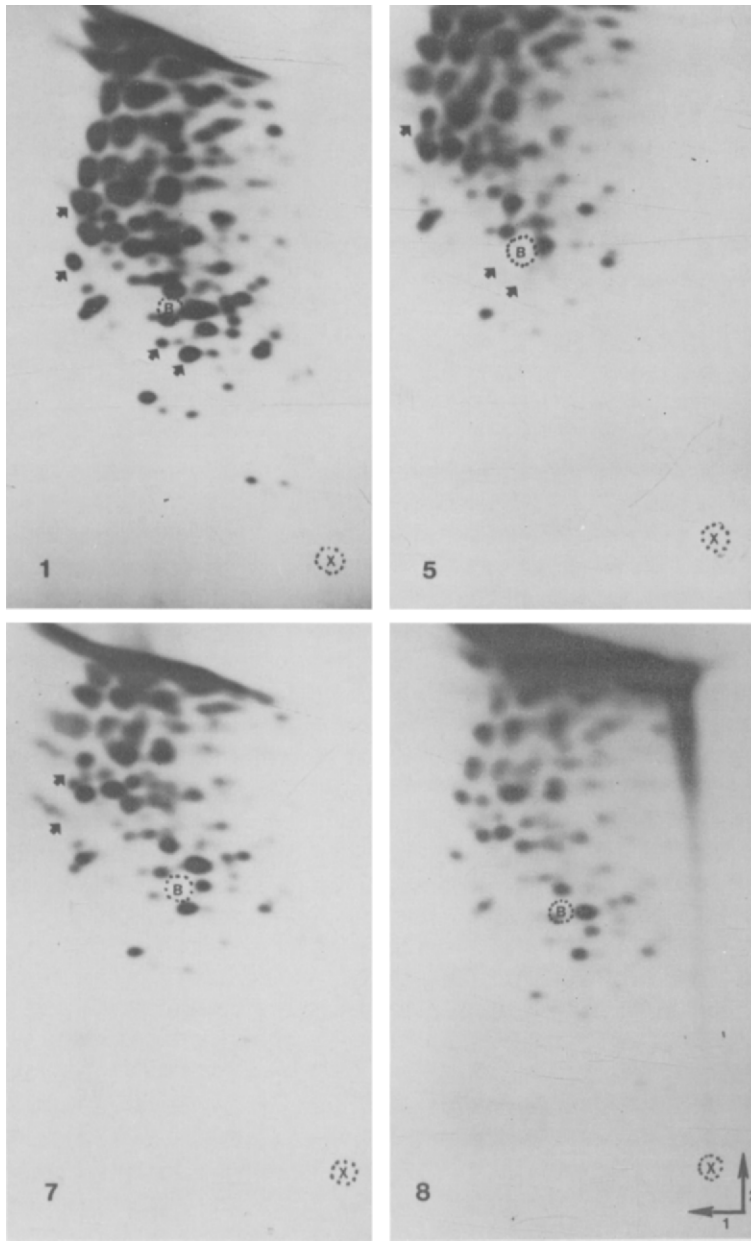
In my experience, homemade nuclease S1, prepared as described by Vogt (1973), has given lower backgrounds and higher percentage of hybridization values and was a more consistently stable enzyme than many commercial preparations.

F. RNA FINGERPRINTING

The relationships between RNAs can also be analyzed by a technique called "RNA fingerprinting," in which an RNA is fragmented by complete digestion with a ribonuclease (either RNase T1 or bovine pancreatic RNase A) and the fragments are separated from each other on some two-dimensional system, usually on the basis of charge in the first dimension and size and base composition in the second dimension. The RNA itself either is labeled *in vivo* or *in vitro* before the digestion or is labeled *in vitro* after the digestion. Thus, after the two-dimensional separation, the fragments of the RNA are visualized as spots (see Fig. 3) by autoradiography, and RNAs differing in sequence will produce RNase digestion fragments of different sizes and charges.

As initially employed (Sanger *et al.*, 1965; Brownlee and Sanger, 1969), the two-dimensional fractionation procedure used electrophoresis on cel-

FIG. 3. Autoradiograms of 5′-^{32}P-labeled oligonucleotides separated by two-dimensional gel electrophoresis. Electrophoresis was at pH 3.5 in the first (horizontal) dimension and at pH 8.3 in the second (vertical) dimension. The RNA samples, from four strains of tobacco mosaic virus designated 1, 5, 7, and 8, were digested with RNase T1 prior to 5′-end labeling with polynucleotide kinase and [γ-^{32}P]ATP. The arrow markers indicate spots present in some strains, but absent in other strains. The positions of the marker dyes bromphenol blue and xylene cyanol blue are indicated.


1
5
7
8
2
1

lulose acetate at pH 3.5 in the first dimension and "homochromatography" on DEAE-cellulose thin-layer plates in the second dimension. This approach, with modification, is still in use (Robertson *et al.*, 1973; Gross *et al.*, 1977; Jonard *et al.*, 1978).

Another approach, developed by De Wachter and Fiers (1972), uses a two-dimensional gel separation of the RNA fragments. The RNAs are electrophoresed at pH 3.5 in an 8–10% polyacrylamide gel in the first dimension and at pH 8.3 in an 18–25% polyacrylamide gel in the second dimension. This approach has been used more often with both plant and animal viruses (Sawyer and Dahlberg, 1973; Frisby *et al.*, 1976; Harris and Brown, 1977; Clewley *et al.*, 1977; Lot *et al.*, 1977; Rowlands *et al.*, 1978; Mohamed *et al.*, 1982) than the cellulose acetate–DEAE-cellulose method (referred to henceforth as the "cellulose method"), perhaps for the following reasons.

1. Many plant viral and most animal viral RNA genomes are quite large and contain a number of large RNase T1-resistant fragments; i.e, greater than 30 nucleotides long. Such large fragments are difficult to separate from each other by the cellulose method, but can readily be separated on polyacrylamide gels.
2. The cellulose method requires exact timing of various steps and appreciable skill in handling cellulose acetate and DEAE-cellulose, whereas the two-dimensional polyacrylamide gel procedure has no such requirements.
3. The cellulose method requires conditions and high-voltage equipment for electrophoresis not found in most laboratories.
4. Different batches of cellulose acetate show considerable variation in their effective uses. Therefore, a description only of the two-dimensional polyacrylamide gel separation procedure will be given here.

1. *Ribonuclease Digestion*

Ribonucleic acid (0.3–2.0 μg), in 5–10 m*M* Tris-HCl (pH 7.5), 1 m*M* EDTA, is denatured by heating at 100°C for 1 min and rapidly cooling on ice. Then RNase T1 (Sankyo, Calbiochem; 2.5–5 units) or RNase A (1–2 μg) is added, and the samples (10 μl) are incubated at 37°C for 1 hr (for RNase T1) or 2 hr (for RNase A). Digestion cannot be terminated by boiling, since neither RNase is inactivated by heating at 100°C. On the other hand, digestion may be terminated by extraction with phenol and precipitation of the RNA fragments by ethanol; however, this is an unnecessary step that can lead to loss of material. It would, however, be necessary if only partial RNase digestion was desired. Many laboratories use shorter or longer times of incubation, different buffers, pH, or concentrations as well as much lower ratios of RNase T1:RNA than used above: ratios of 1:5, 1:10, 1:20,

or 1:50 (Dickson *et al.*, 1979; Domdey *et al.*, 1978; Frisby *et al.*, 1976; Lot *et al.*, 1977). When dealing with RNAs containing appreciable secondary structure, we have used alternative RNase T1 digestion conditions; i.e., after addition of 2.5 units of RNase T1, the RNA is incubated at 37°C for 25 min, followed by boiling for 1 min, cooling on ice, and the addition of a further 2.5 units of RNase T1. The sample is then incubated at 56°C for 25 min. This ensures complete cleavage of the RNA and of the cyclic phosphate intermediate to an (oligo)nucleotide 3′-phosphate. Incubation at 56°C alone for 1 hr is not recommended, because at this temperature the rate of cleavage of 2′,3′-cyclic phosphates by RNase T1 is decreased, and consequently both types of digestion products (those with 2′,3′-cyclic phosphates and those with 3′-phosphates) are observed on two-dimensional gels.

When RNAs labeled *in vivo* with ortho[^{32}P]phosphate or labeled *in vitro* with ^{125}I (see Section III,D,2) are digested, carrier RNA (1–10 μg) is usually added.

2. *In Vitro Labeling of RNA Fragments with ^{32}P*

Of the three types of labeled RNAs that can be used for RNA fingerprinting, *in vivo* labeling with ^{32}P produces RNA fingerprints with the most information. This is because *in vivo* labeling usually results in all RNA fragments being uniformly labeled, whereas radioiodination, which radiolabels only cytidine residues, will result in RNase T1 fragments devoid of C residues and about half of the RNase A fragments being undetected by autoradiography. Furthermore, *in vitro* labeling with ^{32}P is not quantitative or uniform, even though virtually all the RNA fragments are labeled (Frisby, 1977); i.e., some fragments are better substrates than others. Nonetheless, because of the difficulty and hazards of *in vivo* labeling of plant viruses with ^{32}P, the *in vitro* labeling procedure is the method of choice.

In this procedure, the RNase-resistant fragments are 5′-end labeled with [γ-^{32}P]ATP and T4-polynucleotide kinase as follows: The RNase (T1 or A) digest is freeze-dried and resuspended in 5 μl of 2 m*M* spermidine (HCl) in water. The sample is boiled for 0.5–1.0 min and rapidly cooled on ice. Tris-HCl (pH 8.3), $MgCl_2$, dithiothreitol (DTT), and glycerol are added to final concentrations of 50 m*M*, 10 m*M*, 10 m*M* and 10% (v/v), respectively. This solution is then transferred to dried down [γ-^{32}P]ATP (30–50 μCi ~ 3000 Ci/mmol; Amersham) and 2.5 units of T4 polynucleotide kinase [PL Biochemicals; or 4 units of the same enzyme from Bethesda Research Laboratories (BRL)] is added, and the 10 μl of solution is incubated at 37°C for 30 min. After 15 min ATP is sometimes added to 5 μM to drive the reaction; the ratio of ATP: 5′ ends should ideally be 5:1 (Chaconas and van de Sande, 1980). The reaction is stopped by the addition of 10 μl of 98% formamide, 1 m*M* EDTA, and the tracker dyes bromphenol blue and xylene cyanol

blue. The ^{32}P-labeled RNA digest is stored at −20°C until ready for electrophoretic separation.

3. *Two-Dimensional Gel Electrophoresis*

This laboratory routinely uses gels with dimensions of 20 cm (width) × 40 cm (height) × 0.08 cm (thickness). However, wider gels (40 cm; Frisby *et al.*, 1976) as well as thinner (Mohamed *et al.*, 1982) or thicker (Richards *et al.*, 1977; Harris and Brown, 1977) gels have also been used.

The gels are made according to a slight modification of the method of De Wachter and Fiers (1972). The first dimension of electrophoresis is in a gel made from 60 ml of 10% (w/v) acrylamide, 0.3% (w/v) bisacrylamide, 7 *M* urea, 25 m*M* sodium citrate-citric acid (pH 3.5), and polymerization is catalyzed by the addition of 200 μl of 0.5% (w/v) $FeSO_4 \cdot 12H_2O$ (freshly prepared), 200 μl of 10% (w/v) ascorbic acid, and 20 μl of 30% (v/v) H_2O_2. A comb is inserted, and the gel is allowed to set (10–20 min). The gel is preelectrophoresed for 0.5–1.0 hr at 20 mA (constant current). Half of the RNase digest (i.e., 10 μl) is loaded into a 1-cm-wide well of the gel, and the sample is electrophoresed at 18–20 mA (constant current) until the two tracker dyes have migrated 16 cm (xylene cyanol blue) and 22.5 cm (bromphenol blue, green at this pH), respectively (approximately 4 hr of electrophoresis). One glass plate covering the gel is removed. The gel, adhering to the second glass plate, is covered with Saran Wrap (Dow Chemicals) and autoradiographed at room temperature for 0.5–1 min to locate the position of the 5′-end-labeled fragments. A gel strip 1 cm wide and up to 16.5 cm long, containing all the radioactive spots except for the unincorporated [γ-^{32}P]ATP, is cut out with a razor blade, and this strip (still covered by Saran Wrap for ease of handling) is transferred to the bottom of a glass plate for the second-dimension electrophoresis. There should be a 0.5- to 1-cm gap between the gel (side) spacers and the first-dimension gel strip, as well as a 2.5-cm gap between the bottom of the glass plate and the gel strip. The Saran Wrap is removed from the gel strip, the upper glass plate is positioned, and the plates are taped together along the edges with Scotch electrical tape (56, 3M). The second-dimensional gel (60 ml) consists of 20% (w/v) acrylamide, 0.06% (w/v) bisacrylamide, 90 m*M* Tris-borate, 1 m*M* EDTA (pH 8.3), and polymerization is catalyzed by the addition of 60 μl of TEMED and 600 μl of 10% (w/v) ammonium persulfate. The gel solution is carefully poured to avoid trapping air bubbles below, to the sides of, and especially on the upper edge of the first-dimension gel strip. The second-dimension gel is electrophoresed at 600 V (constant voltage) until the bromphenol blue and xylene cyanol blue dyes have migrated 20 and 10 cm, respectively, from the upper edge of the first-dimension gel strip (usu-

ally after 15–18 hr of electrophoresis). One glass plate is removed from the gel, which is then covered with Saran Wrap and autoradiographed at room temperature for 2–30 min.

With RNAs of low molecular weight (e.g., $1–2 \times 10^5$), it is preferable to use a second-dimension gel containing 25% acrylamide and 0.83% bisacrylamide, giving a better separation of the smaller RNA fragments (5–30 nucleotides).

It is unnecessary to deproteinize the nucleic acid at the various steps. Thus, the solution of [^{32}P]RNA fragments contains both RNase and T4 polynucleotide kinase as well as their protein contaminants. In most cases, it is also unnecessary to dephosphorylate the 3′ ends of the RNase fragments prior to 5′-end labeling. Moreover, the introduction of phosphatase at this point requires treatment with phenol and ethanol precipitation to effect its removal, since neither treatment with nitriloacetic acid (Chaconas and van de Sande, 1980) nor 5′-end labeling in the presence of 10–30 m*M* sodium phosphate (personal observations) completely inhibits the calf intestinal alkaline phosphatase.

Figure 3 shows RNA fingerprints of four closely related tobamoviruses. The RNA fragments are sufficiently labeled to be cut out, eluted, and have their RNA sequences determined (Palukaitis and Zaitlin, 1984a,b).

This method has been used to confirm that cucumber mosaic virus (CMV) RNA_4 sequences are present in CMV RNA_3 (Lot *et al.*, 1977) and that an mRNA coding for the coat protein of turnip yellow mosaic virus (TYMV) contains sequences present in TYMV (Richards *et al.*, 1977); i.e., both smaller RNAs are subgenomic RNAs.

III. Detection and Characterization of Subgenomic Viral RNAs in Extracts of Infected Tissues

Although many subgenomic RNAs generated during infection are encapsidated into virions, other subgenomic RNAs are not encapsidated and thus are found only by extraction of RNA from infected tissues normally followed by analysis by gel electrophoresis. Furthermore, (−) strand copies of subgenomic RNAs are also not usually encapsidated and can be found only by analyzing RNAs from infected tissues. Unfortunately, plant tissues themselves contain a large number of RNA species, mostly cytoplasmic and chloroplastic ribosomal RNAs as well as specific breakdown products of these rRNAs. Moreover, the concentration of individual subgenomic RNAs is usually much lower than the level of any of the rRNA species. Therefore, direct visualization of subgenomic RNAs on gels is not usually possible. In

cases where direct visualization of extra (subgenomic?) RNA bands is possible, it is necessary to confirm that the putative subgenomic RNAs are in fact related to the plant virus being analyzed.

Earlier procedures applied to address these problems centered around experiments in which plants, leaf strips, leaf disks, cells, or protoplasts were radiolabeled with either [^{3}H]uridine, [^{3}H]uracil, or ^{32}P-labeled inorganic phosphate in the presence of actinomycin D (Sänger and Knight, 1963) to inhibit host RNA synthesis; the RNAs were extracted, fractionated on polyacrylamide tube gels, which are then sliced, digested, and counted (Jackson *et al.*, 1972; Pinck and Hirth, 1972; Siegel *et al.*, 1973; Mohier *et al.*, 1974; Philipps *et al.*, 1974; Aoki and Takebe, 1975; Bancroft *et al.*, 1975; Takanami *et al.*, 1977).

These analyses did not, however, address the issue of proving that the "new RNA species" were viral encoded RNAs, complementary viral RNAs, or actinomycin D-resistant or stimulated host RNAs. These problems were sometimes pursued, but the methods were quite laborious (Siegel *et al.*, 1973).

In the last 5 years, the techniques for analyzing RNAs extracted from tissues have greatly increased in sophistication and sensitivity. Thus, it is now possible to fractionate nucleic acids by gel electrophoresis under totally denaturing conditions (Section III,B), transfer the RNA from the gels to binding or support media (Section III,C), and detect the presence of viral specific RNAs by (the so-called Northern) hybridization (Section III,E), with radiolabeled probes prepared to various segments of the viral RNA genome (Section III,D).

Using such approaches, it is possible to construct a genetic map of a virus, on the basis of the location of subgenomic RNAs within the genomic RNA (Section III,E).

A. Extraction of RNA from Tissues

1. *Total RNA*

Generally, the smaller the amount of plant material extracted, the higher the yield of RNA and the greater the proportion of intact RNA. Although a large number of different extraction procedures have been used, most of them are variations on the ones given below.

Procedure a. Washed and deribbed leaves (10 g) are ground with a pestle in a prechilled (0°C) mortar in the presence of sterile acid-washed sand, 5 ml of 0.1 *M* Tris-HCl (pH 7.8), 0.2 ml of 2-mercaptoethanol, 0.2 ml of 20% SDS, and 5 ml of phenol:chloroform:isoamyl alcohol (25:24:1). The slurry is centrifuged at 12,000 *g* for 10 min at 0–4°C, and the aqueous phase is reextracted twice with 1 volume of phenol:chloroform:isoamyl alcohol

as described above, followed by a final extraction with 1 volume of chloroform. The RNA is precipitated from the aqueous phase by the addition of sodium acetate to 0.2 *M* and 2 volumes of 2-propanol. The RNA precipitate is collected by centrifugation as described above, dried, and resuspended in water (Otal and Hari, 1983).

Procedure b. Deribbed leaves are frozen with liquid nitrogen in a chilled mortar and ground to a powder. The frozen powder is combined with 0.1 *M* Tris-HCl (pH 7.0), 0.1 *M* NaCl, 0.01 *M* Na_2EDTA, 1% SDS, 5 m*M* 2′(3′)AMP, and 1 volume of phenol and is ground further. The slurry is centrifuged at 12,000 *g* for 10 min at 4°C, and the aqueous phase is twice more extracted with phenol. The RNA is precipitated from the final aqueous phase by the addition of 2–2.5 volumes of 95% ethanol. After overnight incubation at −20°C, the RNA precipitate is collected by centrifugation as described above, dried, and resuspended in water (Bisaro and Siegel, 1980, 1982).

Procedure c. Leaves (10g) are frozen and ground to a powder in liquid nitrogen in a mortar. The powder is extracted with 40 ml of 0.2 *M* Tris-HCl (pH 9.0), 0.4 *M* LiCl, 25 m*M* EDTA, 1% SDS, and 1% diethyl pyrocarbonate (DEP). The slurry is filtered through cheesecloth and the filtrate is extracted three times with 1 volume of phenol:chloroform (1:1), the phases being separated by centrifugation after each extraction. Pectin is removed by the addition of 1 volume of 95% ethanol, incubation at 0°C for 10 min, and centrifugation at 6000 rpm for 5 min at 0°C. The RNA, in the supernatant, is precipitated by the addition of 1 (original aqueous) volume of 95% ethanol, incubation overnight at −20°C, and centrifugation at 8000 rpm for 10 min at 0°C. The dried RNA pellet is resuspended in 2 *M* LiCl for further fractionation (see below; Odell and Howell, 1980).

Procedure d. Deribbed washed leaves (2–3 g) are rapidly ground, with the aid of a chilled mortar and pestle, in 5 ml of extraction buffer [10 m*M* Tris-HCl (pH 7.5), 50 m*M* NaCl, 1% triisopropylnaphthalene sulfonate, 6% 4-aminosalicylate], 1 g of acid washed sand, and 0.05 ml of DEP. The slurry is combined with an additional 5 ml of extraction buffer and emulsified with 10 ml of water-saturated phenol, containing 1% *m*-cresol and 0.1% 8-hydroxyquinoline. The emulsion is broken by centrifugation (8000–12,000 *g* for 10 min at 0–4°C), the aqueous phase is extracted 2 or 3 more times with phenol, and the RNA is precipitated by the addition of 2–2.5 volumes of ethanol (Howell and Hull, 1978).

Procedure e. Deribbed leaf tissue (10–30 g) is frozen and ground in a chilled mortar in the presence of liquid nitrogen with 1 volume of 0.2 *M* glycine, 0.1 *M* Na_2HPO_4, 0.6 *M* NaCl (pH 9.6), 0.2 volume of 10% SDS, 1% 2-mercaptoethanol, 1 volume of water-saturated phenol containing 0.1% 8-hydroxyquinoline, and 1 volume of chloroform:1-butanol (25:1).

The resulting slurry is allowed to thaw while grinding and is then stirred at room temperature for 30 min. After centrifugation at 12,000 *g* for 10 min at 4°C to separate the phases, the RNA is precipitated from the aqueous phase by the addition of 2 volumes of cold 95% ethanol and stored at −20°C for 2–3 hr. The precipitate is collected by centrifugation at 12,000 *g* for 10 min at 4°C, dried, resuspended in 50 m*M* Tris-HCl (pH 7.5), centrifuged briefly to remove any insoluble material, and dialyzed overnight at 4°C against 50 m*M* Tris-HCl (pH 7.5), to remove the sodium phosphate. The nucleic acid solution is then clarified by a brief centrifugation, made 10 m*M* with respect to $MgCl_2$, and incubated with 50 μg of DNase I (Worthington, DPRF; RNase-free) per milliliter for 40 min at room temperature. The solution is reextracted with 1 volume of water-saturated phenol and 20 m*M* Na_2 EDTA and centrifuged; the RNA in the aqueous phase is precipitated by the addition of sodium acetate to 0.1 *M* and 2.5 volumes of 95% ethanol. The RNA is collected by centrifugation as described above, dried, and resuspended in 2–5 ml of 2 *M* LiCl for further fractionation (Zelcer *et al.*, 1981; see below).

Most variations of the above procedures involve the use of additional components in the extraction buffer (e.g., bentonite to bind and inhibit ribonuclease) or different buffers and pH.

After the initial extraction and collection of the nucleic acid by ethanol precipitation, further fractionation is usually carried out. This involves removal of the DNA with DNase I [the purity of which is very important; RNase contaminating DNase I can be removed as described by Maniatis *et al.* (1982) or RNase-free DNase I (DPRF) can be purchased from Worthington] and fractionation of the nucleic acid by 2 *M* LiCl into the LiCl-soluble portion (double-stranded RNA and low molecular weight single-stranded RNA; RNA of molecular weight 1×10^5 is partitioned equally into the 2 *M* LiCl-soluble and -insoluble fractions) and the LiCl-insoluble portion, containing mostly rRNAs and the single-stranded viral RNAs. If only the 2 *M* LiCl-insoluble RNA is to be analyzed, then the DNase I digestion can be omitted; DNA is soluble in 2 *M* LiCl.

The 2 *M* LiCl fractionation can be achieved in two ways. In the first, the initial RNA pellet is thoroughly dried and resuspended directly in 2 *M* LiCl (in procedures c and e above) by vortexing vigorously for several minutes. The slurry is centrifuged at 12,000 *g* for 20 min, and the LiCl-soluble fraction is removed. The LiCl-insoluble fraction is reextracted with 2 *M* LiCl, as just described, and centrifuged; the two LiCl-soluble fractions are combined. The LiCl-insoluble fraction is resuspended in water and made 0.1 *M* with respect to sodium acetate. RNA is recovered from both the LiCl-soluble fraction and the LiCl-insoluble fraction by the addition of 2.5–3.0 volumes of 95% ethanol, stored at −20°C overnight or at −70°C for 30 min,

and centrifuged at 12,000 *g* for 10 min at 0–4°C. The final RNA pellet is redissolved in sterile 0.1 m*M* EDTA, pH 7. If the RNAs are to be used for *in vitro* translation, then a second ethanol precipitation is recommended even though LiCl is very ethanol soluble; translation is inhibited by lithium ions.

In the second way to achieve 2 *M* LiCl fractionation, the initial RNA pellet is resuspended in water and LiCl is added to 2 *M* (usually by the addition of either 1 volume of 4 *M* LiCl or 0.25 volume of 10 *M* LiCl). The mixture is incubated at 0–4°C for 12–18 hr and centrifuged at 12,000 *g* for 10–20 min at 0–4°C; the 2 *M* LiCl-soluble and insoluble RNAs are recovered by ethanol precipitation as described above.

The LiCl-insoluble RNA is analyzed for the presence of viral RNAs and the LiCl-soluble RNA is analyzed for the presence of viral complementary (−) RNA species (see Sections III,C–E).

If the viral RNA contains poly(A) sequences, then it can be further fractionated away from host rRNA species by chromatography on oligo(dT)-cellulose columns (Aviv and Leder, 1972; Nakazato and Edmons, 1974; Hari, 1980). Two cycles of oligo(dT)-cellulose chromatography are recommended to remove virtually all the rRNA (Alwine *et al.*, 1979).

2. *Polyribosomal RNA*

The question of which "subgenomic RNAs" are really mRNAs, not some sort of artifact or breakdown product, is best answered by analyzing which RNAs are found on polyribosomes, on the presumption that they are thus mRNAs.

A simple method for the isolation of polyribosomes containing up to 13 ribosomes (M. A. Sulzinski and M. Zaitlin, personal communication) is via a modification of the method of Jackson and Larkins (1976; also personal communication).

Deribbed, unexpanded apical leaves (0.6 g; 1–5 cm long) are ground in a chilled mortar in 6.0 ml of cold extraction buffer [200 m*M* Tris-HCl (pH 8.5 at room temperature), 400 m*M* KCl, 200 m*M* sucrose, 35 m*M* $MgCl_2$, 25 m*M* EGTA, and 1% 2-mercaptoethanol].

The slurry is centrifuged at 12,000 *g* for 24 min at 4°C, and the supernatant is decanted and made 1% with respect to Triton X-100. The polyribosomes are pelleted from the supernatant by centrifugation at 225,000 *g* for 90 min at 4°C through a 4-ml "cushion" of 1.75 *M* sucrose, 40 m*M* Tris-HCl (pH 8.5), 200 m*M* KCl, 30 m*M* $MgCl_2$, and 5 m*M* EGTA. The upper phase is removed by aspiration, and the top of the sucrose cushion is washed with 1 ml of water and removed by aspiration. The sucrose is poured off, and the pellets are washed once by adding 1 ml of water and quickly decanting. (These washes appear to be important for the isolation

of intact polyribosomal RNA.) The pellets are drained and either stored at −20°C or, if the polyribosomes are to be analyzed, resuspended in 0.2 ml of 40 m*M* Tris-HCl (pH 8.5), 200 m*M* KCl, 30 m*M* $MgCl_2$, and 5 m*M* EGTA by vortexing. The resuspended polyribosomes are layered onto a 5–35% (w/v) sucrose gradient in 40 m*M* Tris-HCl (pH 8.5), 20 m*M* KCl, and 10 m*M* $MgCl_2$, centrifuged for 1.5 hr at 34,000 rpm in a Beckman SW-41 rotor at 4°C, and analyzed on an ISCO density-gradient fractionator.

To release mRNA from the polyribosomes, the frozen polyribosomal pellets are resuspended in 0.2 ml of water, combined with 0.25 ml of 0.1 *M* Tris-HCl (pH 7.5), 1 *M* KCl, 10 m*M* $MgCl_2$, and 0.05 ml of 10 m*M* puromycin, and incubated first at 0°C for 15 min and then at 37°C for 10 min (Blobel, 1971). The reaction mixture is then layered onto a 5–20% (w/v) sucrose gradient in 50 m*M* Tris-HCl (pH 7.5), 0.5 *M* KCl and 5 m*M* $MgCl_2$, centrifuged at 4°C for 6.5 hr at 34,000 rpm in a Beckman SW-41 rotor, and fractionated on an ISCO density gradient fractionator. The UV absorbing material at the top of the gradient consists of puromycin and tRNA; the middle peak is mRNA; and the lower peak is the ribosomal subunits (in the case of tobacco mosaic virus, the virions are pelleted during this centrifugation step; unpublished observation). The mRNA peak is phenol extracted and ethanol precipitated. The final mRNA preparation still contains some rRNA; however, the virion-encapsidated RNA, most of the degraded virion RNA, and most of the host RNA have been removed (Palukaitis *et al.*, 1983).

If the plant viral RNA is polyadenylated, it can be separated from the remaining rRNA at this stage by chromatography on oligo(dT)-cellulose columns (Aviv and Leder, 1972). The RNA can then be analyzed by gel electrophoresis, blotting, and hybridization (Section III,B–E).

B. AGAROSE GEL ELECTROPHORESIS

Subgenomic RNAs can be detected in nucleic acid extracts from infected plants by first fractionating the plant nucleic acid by gel electrophoresis, transferring the RNA from the gel to a binding medium, and incubating with radiolabeled cDNA probes. Although polyacrylamide gels are better at resolving RNAs of similar molecular weights than are agarose gels, transfer of RNA from polyacrylamide gels is more difficult and less efficient than from agarose gels. Hence, agarose gels are generally used for fractionation prior to blotting and hybridization.

1. *Nondenaturing Gel Electrophoresis*

Ribonucleic acids can be analyzed by agarose gel electrophoresis on 1.1–1.8% agarose gels in the electrophoresis buffer of Loening (1967); i.e., 40 m*M* Tris-HCl (pH 7.2–8.2), 20 m*M* sodium acetate, 2 m*M* EDTA (TAE).

In this laboratory, a 12.5 × 14.5 × 0.3 cm gel in a commercial horizontal electrophoresis apparatus (Aquebogue Machine Shop, Aquebogue, NY) is used to fractionate both double-stranded RNAs (1.1% gel; Fig. 4A) or single-stranded RNAs (1.5% or 1.8% gels; Fig. 4B). The gels are run at 75 mA (constant current and about 60 V) for 6.5 hr (1.1% gels) or 4 hr (1.5–1.8% gels). The gels and electrode buffer also contain 0.5 μg of ethidium bromide per milliliter for ready visualization. Alternatively, the gels can be stained with ethidium bromide (20 μg/ml) after electrophoresis and destained with water. The gels are photographed using transmitted UV illumination and Polaroid type 55 negative–positive film. Often, faint bands can be seen on the film negative that cannot be seen by eye or on the positive print.

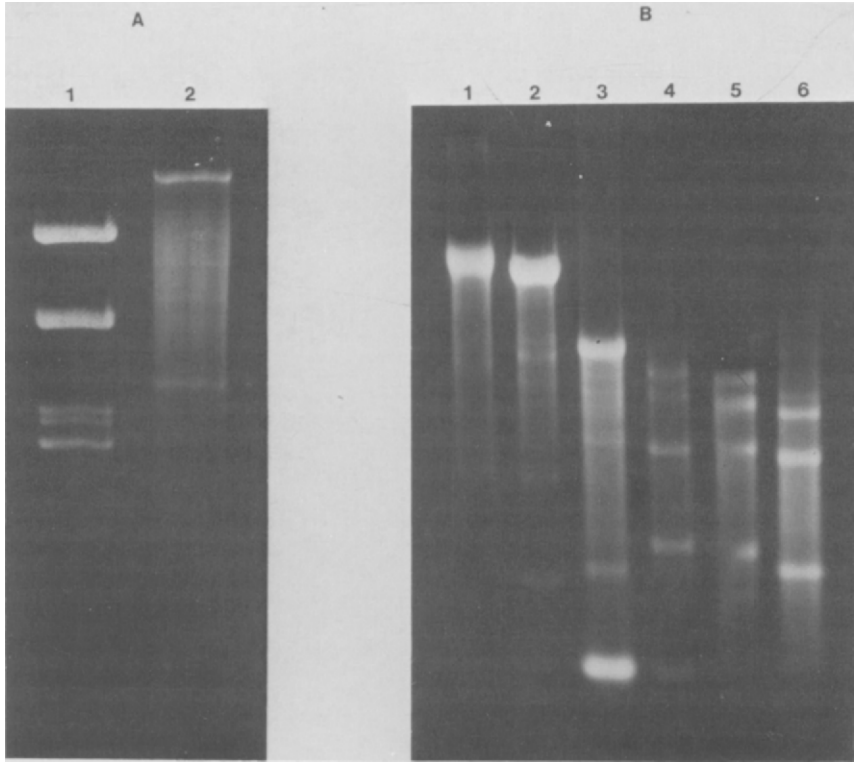


FIG. 4. Agarose gels containing RNAs. (A) Agarose gel (1.1%) containing reovirus double-stranded RNAs (lane 1) and double-stranded RNAs extracted from tobacco mosaic (TMV)-infected tobacco (lane 2), purified by two cycles of CF-11 column chromatography (Franklin, 1966; Zelcer *et al.*, 1981). (B) Agarose gel (1.8%) containing U1-TMV RNA (lane 1), C_c-TMV RNA (lane 2), velvet tobacco mottle virus RNA (lane 3), B-strain cucumber mosaic virus (CMV) RNA (lane 4); L_SS-CMV RNA (lane 5); and brome mosaic virus RNA (lane 6).

2. *Denaturing Gel Electrophoresis*

In order to ensure that the single-stranded RNAs detectable by "blotting" from gels are not aggregates, RNA samples are analyzed by gel electrophoresis under "denaturing" conditions; i.e., the RNAs are covalently modified to disrupt and prevent hydrogen bonding. This method is used also to denature double-stranded RNA and directly compare them to their single-stranded counterparts.

Three types of gels are considered to be denaturing.

a. Methylmercuric Hydroxide (MeHgOH) (Bailey and Davidson, 1976). All work involving MeHgOH must be done in a fume hood. The RNA is dissolved in 2.5 m*M* sodium borate–25 m*M* boric acid (pH 8.2), 5 m*M* sodium sulfate, 0.5 m*M* EDTA, 5% glycerol, and 10 m*M* MeHgOH, mixed, and then analyzed by electrophoresis on 1.0–1.8% agarose gels containing the same buffer as the sample (without glycerol) and 5–10 m*M* MeHgOH, at 15–25 V for 16–20 hr (or 80 V for 4–5 hr) with recirculating buffer. The drawback of this procedure is the extremely toxic nature of MeHgOH. The gel, electrode buffer, and all material coming in contact with MeHgOH must be treated with 10 m*M* 2-mercaptoethanol to inactivate the MeHgOH and convert it to MeHgSEt. The gel may be stained with ethidium bromide during the mercaptoethanol treatment (Alwine *et al.*, 1979).

b. Glyoxal (McMaster and Carmichael, 1977). RNA samples are heated for 1 hr at 50°C in 1 *M* glyoxal (deionized with a mixed-bed resin), 50% (v/v) dimethyl sulfoxide, 10 m*M* sodium phosphate (pH 6.5–6.8). The glyoxalated RNA is electrophoresed on a 1.0–1.8% agarose gel in 10 m*M* sodium phosphate (pH 6.5–6.8), at 25–30 V for 16–20 hr or 90 V for 4–5 hr, with recirculating buffer. The RNA bands are visualized by staining with 33 μg of acridine orange per milliliter in 10 m*M* sodium phosphate (pH 6.5–6.8) for 10 min and destaining three times for 20 min in the above buffer. It is important to circulate the buffer during electrophoresis, otherwise the pH will change drastically; glyoxalation of the RNAs is reversible above pH 8 (Thomas, 1980).

The above recipe has been modified such that DMSO is no longer included in the denaturation buffer. Apparently, most single-stranded RNAs are sufficiently denatured by heat alone to ensure covalent interaction with glyoxal (Alwine *et al.*, 1979).

c. Formaldehyde (Rave *et al.*, 1979; Seed, 1982). The original procedure of Rave *et al.* (1979) contained 20 m*M* sodium phosphate, pH 7. A more recent variation by Seed (1982), uses a MOPS-acetate buffer; the rest of the procedure is the same. That is, RNA in 1× MAE [20 m*M* *N*-(3-morpholino)propanesulfonic acid (MOPS), 5 m*M* sodium acetate, 1 m*M* EDTA (pH 7)], 50% (v/v) formamide (deionized with a mixed-bed resin), 6% (v/v)

formaldehyde, is incubated at 60°C for 10–15 min. The RNA is then electrophoresed in a 1.0–1.8% gel containing 1× MAE and 6% (v/v) formaldehyde. The electrode buffer is 1 × MAE with no formaldehyde. Electrophoresis is carried out at 150–170 V for 2–3 hr or longer at a lower voltage to prevent heating. Care should be taken to avoid inhaling formaldehyde fumes during the preparation of the gel. The agarose is first dissolved in water by boiling, then cooled to 60°C before the buffer and the formaldehyde are added. MOPS is also somewhat sensitive to light.

Formaldehyde gels are stained as described for glyoxal gels or by soaking the gel for 1–24 hr in electrode buffer followed by staining for 30 min in 0.5–20 μg of ethidium bromide per milliliter in 0.1 *M* NH_4 acetate and destaining in water for 1–4 hr.

C. TRANSFER OF RNA FROM GELS TO BINDING MEDIA

1. *Transfer to DMB Paper*

Diazobenzyloxymethyl (DBM) paper (Alwine *et al.*, 1977, 1979) is a "chemically activated" paper capable of binding molecules, first by ionic interaction and then slowly by covalent linkage. Using buffers of low pH to maximize the half-life of the reactive diazonium groups, RNA has been transferred from gels to the environment around the positively charged diazonium groups. A slow reaction between the RNA and the DBM group ensues, resulting in the covalent attachment of the RNA to the DBM paper. The synthesis of DBM paper has been described by Alwine *et al.* (1977, 1979). DBM paper is also commercially available in the more stable nondiazotized form, aminobenzyloxymethyl (ABM) paper.

Prior to transfer, gels must be treated to remove denaturing agents or any components of the buffer that can react with the diazonium groups. Removal of methyl mercuric hydroxide or glyoxal is done as described by Alwine *et al.* (1979). Removal of formaldehyde from the gels is done as described by Rave *et al.* (1979).

The preparatory steps include cleavage of the RNA with 50 m*M* NaOH to increase the rate of diffusion of the RNA, and neutralization with 200 m*M* sodium acetate, pH 4.0.

Transfer is carried out at this low pH (see above and Alwine *et al.*, 1979) by the following procedure. Two sheets of Whatman 3MM are saturated with 200 m*M* sodium acetate (pH 4.0), and the ends of the paper are placed in contact with the above buffer (Fig. 5A). The gel is placed onto the wet 3MM paper, and freshly prepared DBM paper is placed directly on top of the gel. The DBM paper should not overlap the gel and touch the 3MM paper. Saran Wrap, plastic strips, or aluminum foil may be used to prevent this by placing them around the edges of the gel. Three layers of dry 3MM

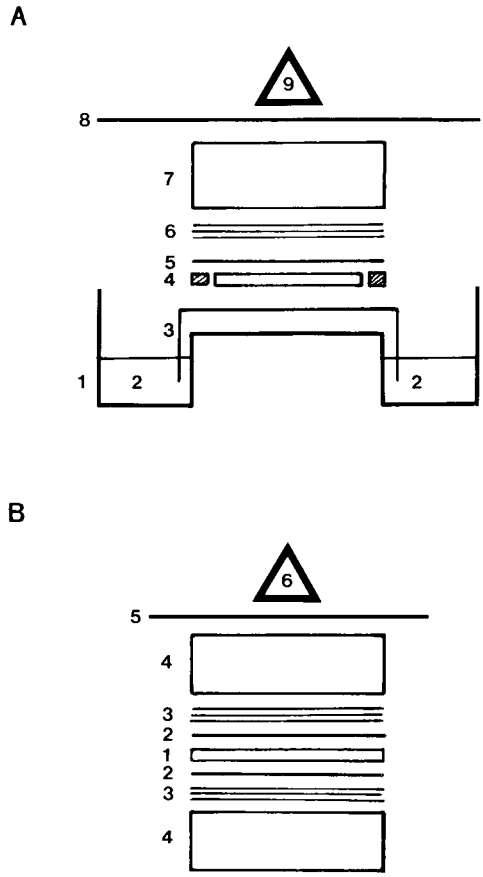


FIG. 5. (A) Northern and Southern blotting. A horizontal gel tank (1), is partially filled with the appropriate blotting buffer (2) (Section III,C). Whatman 3MM paper (one or two layers) (3) is placed over the bridge of the horizontal gel apparatus, with the ends of the 3 MM paper in the gel tank. The 3MM paper is soaked in blotting buffer, covered with the gel to be blotted (4), one layer of either prewetted DBM paper, APT paper, or nitrocellulose (5), three layers of dry 3MM paper (6), a 5–8 cm layer of paper towels (7), a glass plate, or the top of the horizontal gel apparatus (8), and a weight (9). The entire assembly is then covered with Saran Wrap. To prevent the flow of buffer from the tanks (2) around the gel to the binding medium (4) and the dry paper above, either Saran Wrap, aluminum foil, or thin plastic spacers are placed around the gel (crosshatched blocks in 4). (B) Bidirectional blotting. A gel (1) is placed between two sheets of prewetted binding medium (2). These in turn are placed between two sets of three layers of prewetted 3MM paper (3). The entire "sandwich" is placed between two stacks of paper towels (4) and covered with a glass plate (5) and a weight (6). The entire assembly is then covered with Saran Wrap. Since the length and width dimensions of the gel, binding media, 3MM paper, and paper towels are all the same, no spacers are required around the gel.

paper and about an approximately 5- to 8-cm layer of paper towels are placed on top of the DBM paper, as well as a glass plate and a weight. The entire assembly is then covered with Saran Wrap, and the buffer is allowed to blot through the gel, the DBM paper, the 3MM paper, and into the paper towels, either at room temperature or at 4°C. Some laboratories prefer to change the paper towels several times during the blotting, to increase the rate of transfer. The entire blotting procedure can also be conveniently set up inside a horizontal gel electrophoresis apparatus.

After the blotting, the DBM paper is treated with prehybridization buffer to inactivate the diazonium groups and prevent any nonspecific binding of the probe (see below). The prehybridization buffer contains 1% (w/v) glycine (to inactivate any unreacted diazonium groups), 50% (v/v) formamide, 50 m*M* sodium phosphate (pH 7.0), 0.9 *M* sodium chloride, 5 m*M* EDTA, 0.1% (w/v) SDS, 0.1% (w/v) each of bovine serum albumin (BSA), Ficoll, and polyvinylpyrrolidone, and 250–500 μg/ml of either yeast RNA or sonicated and denatured salmon sperm DNA. The DBM paper is placed in a boilable plastic bag with the prehybridization buffer (100–200 μl/cm^2 of paper) and the bag is sealed (e.g., with Seal-N-Save, Sears; or Seal-A-Meal II, Dazy), care being taken to exclude all air bubbles. The bag is incubated at 42°C for 4–24 hr. The transfer blots can then either be used immediately or stored at 4°C (possibly indefinitely).

2. *Transfer to DPT Paper*

Diazophenylthioether (DPT) paper is another aryldiazonium-derivatized paper. Its advantage over DBM is that it is easier to synthesize and is more stable. In fact, DPT paper can be prepared, dried, and kept at −20°C for months (R. Goldberg, personal communication). The synthesis of DPT paper was described in detail by Seed (1982). The blotting procedure is essentially the same as that described above for DBM paper, except that different buffer solutions are utilized (Seed, 1982).

3. *Transfer to Nitrocellulose*

The transfer of DNA fragments to nitrocellulose from agarose gels was developed by Southern (1975). It was the failure of RNA to bind to nitrocellulose that prompted the development of diazotized paper for RNA transfer from agarose gels (Alwine *et al.*, 1977; Seed, 1982). However, Thomas (1980) showed that RNA could be bound to nitrocellulose by slight modifications of the procedure of Southern (1975).

The method of transfer is as described above (and below), except that the transfer buffer was 20× SSC (1× SSC = 0.15 *M* NaCl, 0.015 *M* Na_3 citrate, pH 7.0) and the nitrocellulose membrane was presoaked in water and then in 20× SSC before application to the gel. Furthermore, pretreat-

ment of the gel to remove denaturants is not necessary if glyoxalated or formylated RNAs are used; MeHgOH must still be removed as described by Alwine *et al.* (1977). Transfer is complete in 12–15 hr. If RNAs are not denatured prior to electrophoresis, then transfer from the gel is much less efficient unless the gel is first pretreated with 50 m*M* NaOH (3–4 gel volumes) for 30–40 min and neutralized with four 5-min washes in (3–4 volumes) of 50 m*M* sodium borate–boric acid (pH 8.0). Under these conditions, adequate transfer of the RNA to nitrocellulose is achieved (Zelcer *et al.*, 1981; Bar-Joseph *et al.*, 1983; our unpublished observations), although the efficiency of transfer by this method, cf. RNAs from denaturing gels, is not known. Thomas (1980) claims that alkali cleavage and neutralization or staining with ethidium bromide reduces the efficiency of transfer of RNAs from denaturing gels. On the other hand, I have found that alkali treatment and neutralization improves the transfer of RNAs over 1×10^6 in molecular weight by up to a factor of 5 (unpublished observation). In the case of double-stranded RNAs, electrophoresed on nondenaturing gels and blotted to nitrocellulose, it is necessary to treat the gel with alkali to separate the strands. Double-stranded RNAs will bind to nitrocellulose; however, they will not hybridize to probes unless first denatured. We find that 40–45 min of treatment with 50 m*M* NaOH followed by neutralization is required to denature (and cleave) double-stranded RNAs blotted from a nondenaturing 1.1% agarose gel (Zelcer *et al.*, 1981; Palukaitis *et al.*, 1983). On the other hand, Bar-Joseph *et al.* (1983) can detect both strands of double-stranded RNAs on nitrocellulose blots after only 15 min of treatment with 50 m*M* NaOH; longer treatments lead to less probe hybridizing (R. Hull, personal communication).

After the transfer, the nitrocellulose membrane is either air dried or dried under a heat lamp. The nitrocellulose is then baked at 80°C *in vacuo* for 2–3 hr. At this stage, the nitrocellulose is very brittle and must be handled with care. The membrane is placed in a sealable plastic bag along with prehybridization buffer [50 m*M* sodium phosphate (pH 6.5), 0.75 *M* NaCl, 0.075 *M* Na_3 citrate, 250 μg/ml of either sonicated, denatured salmon sperm DNA or yeast RNA, 0.1% (w/v) SDS, 50% (v/v) formamide, and 0.02% (w/v) each of BSA, Ficoll, polyvinylpyrrolidone], and incubated at 42°C for 8–20 hr. The nitrocellulose membranes can be stored at 4°C in this fashion for months.

Owens and Diener (1981) published a modified protocol in which the BSA, Ficoll, and polyvinylpyrrolidone were replaced by 1% glycine.

A modification of the blotting procedure of Southern (1975) (see Fig. 5A) has been described (Smith and Summers, 1980) that permits RNAs to be bidirectionally transferred from agarose gels to DBM paper (and probably DPT paper as well). The time of transfer is shorter (79% transfer in 3 hr); however, since the rate of covalent bond formation between RNA

and the diazonium groups is relatively slow (Alwine *et al.*, 1977; Stellwag and Dahlberg, 1980), the DBM paper has to be incubated for ca. 2.5 hr to ensure covalent attachment. Nevertheless, this method provides two blots from each gel, which can then be analyzed with different probes. We have used this bidirectional transfer procedure with RNAs blotted from nondenaturing gels, as well as denaturing gels, to nitrocellulose membranes (Palukaitis *et al.*, 1983). We find that bidirectional transfer of RNA to nitrocellulose is essentially complete in 4 hr. The bidirectional blots are prepared by a modification of the method of Smith and Summers (1980). The gel is either blotted directly after electrophoresis (Fig. 5B) or treated as follows: the gel is soaked for 30–45 min in 50 m*M* NaOH (4 gel volumes) and washed four times for 5 min in 50 m*M* sodium borate–boric acid, pH 8.0. Meanwhile, three layers of Whatman 3MM paper, soaked in 20× SSC, are placed on a glass plate. On top of the 3MM paper is placed one layer of nitrocellulose, prewetted by slow immersion into water to avoid trapping air bubbles, then transferred to 20× SSC for a few minutes. The gel is placed onto the nitrocellulose membrane, care being taken to avoid trapping air bubbles between the gel and the nitrocellulose. The gel is in turn covered with a second sheet of nitrocellulose (wetted in 20× SSC) and three layers of 3MM paper wetted in 20× SSC. This "sandwich" is then placed between two 2.5–5 cm layers of paper towels and covered with a glass plate and Saran Wrap. A lead weight (1.5–4.5 kg) is placed on top of the glass plate, and bidirectional transfer is carried out for 4–16 hr.

After transfer, the blots are baked either at 80°C *in vacuo* for 2–3 hr or at 100°C with no vacuum for 3–4 hr. Alternatively, if the filters are properly air dried, they can be baked at 80°C without a vacuum for 2–18 hr (Bar-Joseph *et al.*, 1983). Formaldehyde is removed from the RNAs on the nitrocellulose by the 2-hr baking; however, glyoxal may require 3–4 hr of baking to ensure removal (Bruening *et al.*, 1982). The blots are placed in sealable bags and prehybridized in 50 m*M* sodium phosphate (pH 7.0), 0.75 *M* NaCl, 0.075 *M* Na_3 citrate, 50% (v/v) deionized formamide, 250 μg of yeast RNA per milliliter, 0.1% (w/v) SDS, and 0.1% (w/v) each of BSA, Ficoll, and polyvinylpyrrolidone. Incubation is carried out at 42–45°C for 12–24 hr. The blots can be stored at 4°C for several months with no appreciable loss of hybridizable RNA from the membranes. Alternatively, the blots can be kept dry indefinitely (after baking and prior to prehybridization).

D. PREPARATION OF RADIOACTIVE PROBES

Radioactive probes used to detect subgenomic RNAs can be of two polarities: (1) DNA probes complementary to the subgenomic RNA; (2) RNA probes of the same polarity as the subgenomic RNA that are used to detect

RNA complementary to the subgenomic RNA, usually in the form of double-stranded RNAs extracted from tissues.

1. *cDNA Probes*

Complementary DNA to an RNA can be prepared by one of three methods.

a. Oligo(dT)-Primed cDNA (Green and Gerard, 1974). Ribonucleic acids that contain 3′-polyadenylate residues may be used as templates for cDNA synthesis, with oligo(dT)$_{8-10}$ as a primer (Green and Gerard, 1974), by the following method. The reaction mixture (50 μl) contains 0.5–2 μg of poly(A)-containing RNAs, 50 μM Tris-HCl (pH 8.3), 8–13 mM $MgCl_2$, 100 mM KCl, 20 mM dithiothreitol (DTT), 5 μg of actinomycin D, 0.5–0.7 mM of each of three dNTPs, 20–100 uM of the fourth dNTP (25 Ci/mmol [^{3}H]dNTP, or 50–200 Ci/mmol [α-^{32}P]dNTP), 1 μg of oligo(dT)$_{8-10}$, and 8–16 units of avian myeloblastosis virus reverse transcriptase (National Cancer Institute. Note: unit definitions vary considerably with the supply source). The mixture is incubated at 37–45°C for 1–3 hr, and the reaction is stopped by the addition of 5 μl of 5% SDS, 125 μl of water, and 20 μl of 3 N NaOH. The RNA is then hydrolyzed either at 37°C for 3 hr or at room temperature overnight. The cDNA is separated from the low-molecular-weight components by gel filtration on a Sephadex G-50 column (5.5–6.0 ml), made in a 5-ml glass pipette. The column is sterilized with 0.1 N NaOH, washed, equilibrated, and eluted with either freshly prepared 0.1 M NH_4HCO_3 or 10 mM Tris-HCl (pH 8.3), 1 mM EDTA, and 0.1% SDS; 0.5-ml fractions are collected, and 10-μl aliquots are counted. The cDNA elutes in the void volume. The peak fractions are combined and either stored at −20°C, ethanol-precipitated with yeast carrier RNA (100–200 μg/ml final concentration), or, in the case of cDNA, eluted in 0.1 M NH_4HCO_3 combined with a 0.1 volume of redistilled triethylamine (7 M, neat) and lyophilized. The ethanol-precipitated or dried cDNA is then resuspended in either 1 mM EDTA or a hybridization buffer.

The optimal temperature (37–45°C) and maximum time of incubation (1–3 hr) vary with the template RNA used. Similarly, the optimal dNTP concentration, Mg^{2+} concentration, KCl (or NaCl) concentration, or even whether salt is required, vary with the RNA template. If higher dNTP concentrations are used, then higher Mg^{2+} concentrations should also be used. Actinomycin D reduces the counts per minute incorporated, but prevents second-strand DNA synthesis, which may occur with some RNAs. Alternatively, inorganic pyrophosphate (4 mM) can be added to inhibit "anti-complementary DNA synthesis" without reducing the yield of complementary DNA (Kacian and Myers, 1976). Conditions that optimize counts per minute incorporated are not necessarily optimal for the length of the cDNA.

If an RNA does not contain a 3′-end "poly(A) tail" or internal poly(A) sequences, then a 3′-end poly(A) tail may be synthesized *in vitro* (Devos *et al.*, 1976; Gould *et al.*, 1978). This reaction mixture (50 μl) contains 0.5–2 μg of RNA, 50 m*M* Tris-HCl (pH 7.9), 10 m*M* magnesium acetate, 2.5 m*M* $MnCl_2$, 1 m*M* EDTA, 1 m*M* DTT, 0.1 m*M* [^{3}H]ATP (0.1 Ci/mmol), 0.25 *M* NaCl, and poly(A) polymerase [either 0.5 unit of the BRL enzyme or 10–15 μl of enzyme prepared as described by Sippel (1973)]. After incubation at 37°C for 30–45 min, the reaction is stopped by extraction with phenol:chloroform (1:1), the organic phase is back-extracted with 100 μl of water, and the combined aqueous phases are passed through a sterile Sephadex G-50 column (as described above) and eluted with water. Fractions (0.5 ml) are collected, and aliquots are counted to localize the RNA, which should be in the void volume. The peak fractions are pooled and lyophilized. The RNA can be stored frozen in 0.1 m*M* EDTA or used directly for cDNA synthesis.

Once again, my experience has been that homemade poly(A) polymerase is more active and more stable than commercial preparations. The enzyme may be stored for 6 months to 1 year at 4°C, or it can be stored in aliquots in liquid nitrogen indefinitely.

Because the poly (A) polymerase activity has a very narrow salt optimum (Sippel, 1973) and the enzyme itself is usually in about 0.7 *M* NaCl, it is necessary to adjust the added NaCl to compensate for the NaCl present with the enzyme. Bovine serum albumin is not usually added to the reaction, since it appears to be unnecessary for enzyme stability over the course of a 30–45 min reaction. However, should it be necessary to add BSA to this or any enzyme reaction, then ribonuclease-free BSA must be used. This can be purchased commercially or can be easily prepared by the reaction of BSA with acetic anhydride followed by dialysis (Gonzalez *et al.*, 1977).

b. Specific-Sequence-Primed cDNA. If an RNA does not contain a poly(A) tail or if cDNA to only a specific sequence of the viral RNA genome is desired, then a specific-sequence primer should be used. In this case, some working knowledge of the RNA sequence is required in order to synthesize a specific oligodeoxyribonucleotide. Two examples of specific-sequence-primer cDNA follow: (1) cDNA to the 5′ half of brome mosaic virus RNA_3 could be primed with p(dT_8dA), which binds to an internal poly(A) sequence, bordered by a 5′-U (Ahlquist *et al.*, 1981). (2) A cDNA probe specific to the 3′ end of tobacco mosaic virus (TMV) was prepared using a synthetic primer complementary to the 3′-terminal 13 nucleotides of TMV RNA (Goelet and Karn, 1982; Goelet *et al.*, 1982).

cDNA prepared to an RNA by this approach is synthesized as described above for oligo$(dT)_{8-10}$-primed cDNA (which is itself a sequence-specific primer). With short oligonucleotide primers, 15-min preincubations at 30°C

have been used to ensure binding of the primer to the template (Goelet *et al.,* 1982).

c. Random-Primed cDNA (Taylor *et al.,* 1976). Primers specific for 3′ termini of plant viral RNAs often result in the synthesis of cDNAs that do not contain sequences complementary to the 5′ end or even the 5′ half of large RNA molecules.

This situation varies with the RNA templates, component concentrations of the cDNA synthesis reaction mixture, and the source of reverse transcriptase (usually due to low levels of RNase). If commercial or homemade specific-sequence primers are not available, then a ready alternative is to use random primers prepared as described by Taylor *et al.* (1976). These primers are prepared by the DNase I digestion of high-molecular-weight eukaryotic DNA (usually commercial salmon sperm DNA is used) in the presence of 25 m*M* Tris-HCl (pH 7.4), 10 m*M* $MgCl_2$ for 2 hr at 37°C (5 mg of DNA and 70 μg of DNase I per milliliter). The DNase I can be inactivated by boiling for 30 min or autoclaving for 10 min. Unfortunately, pancreatic RNase, contaminating most DNase I preparations, is not so readily inactivated. Therefore, phenol extraction to remove the proteins and ethanol precipitation is recommended. The DNA fragments are resuspended in the Tris-Mg buffer used for digestion. These DNA fragments, 10–20 nucleotides in length (Taylor *et al.,* unpublished data), are of random sequence, and by random chance some will bind to complementary sequences in an RNA. The cDNA is then synthesized by a reaction essentially identical to the one described above for oligo$(dT)_{8-10}$-primed cDNA, except that the oligo$(dT)_{8-10}$-primer is replaced with 2.5 mg/ml of DNase I-digested salmon sperm DNA. Such cDNA has been shown to be representative of the whole RNA template from which it was transcribed and was also not grossly enriched for one or more regions of the RNA template (Gould and Symons, 1977). Although we have preferred to maintain a radiolabeled dNTP concentration of 20–100 μ*M,* to ensure that this triphosphate concentration would not limit the rate of the reactions, Bisaro and Siegel (1980) showed that 2.5 μ*M* [α-^{32}P]dCTP could be used without appreciable change in the weight yield of cDNA and with the benefit of a greater counts-per-minute yield, i.e., higher specific activity cDNA.

2. *RNA Probes*

RNA probes are used to identify complementary RNA species of subgenomic RNAs that may be present in virions, or (more usually) in nucleic acid extracts of virus-infected plant tissue.

a. ^{32}P-Labeled RNA Probes. ^{32}P-labeled RNA probes can be prepared in a number of ways.

i. In vivo labeling. [^{32}P]Inorganic phosphate is taken up through the roots of virus-infected plants (Guilley *et al.,* 1975; Lot *et al.,* 1977), and

the plants are incubated for several days. ^{32}P-labeled virus is prepared from the plants, and the [^{32}P]RNA is extracted from virions. Although the RNA is uniformly labeled, some of the drawbacks to this approach are that (1) because of high concentrations of endogenous phosphate in the plant, the specific activity of the extracted [^{32}P]RNA is 2×10^6 cpm/μg or less, even when very high levels of ^{32}P (100 mCi) are used; (2) such high levels of ^{32}P require proper handling and radiation protection facilities; (3) everything coming into contact with this level of ^{32}P will become contaminated; (4) the [^{32}P]RNA will have to be prepared a number of times because of the short half-life of ^{32}P; and finally (5) the [^{32}P]RNA may not be useful as a probe because of its low specific activity.

ii. In vitro labeling with [γ-^{32}P]ATP and polynucleotide kinase. The 5′ end of an RNA is phosphorylated with [γ-^{32}P]ATP and T4-polynucleotide kinase (see Section II,F). Unfortunately, since most plant viral RNAs contain either a "capped" 5′ end or a protein covalently linked to the 5′ end (VpG), several additional steps are necessary to make the 5′ end accessible. The 5′-end cap structure can be removed with the enzyme tobacco acid pyrophosphatase (Efstratiadis *et al.*, 1977), and the 5′-terminal phosphates can be removed with a phosphatase (Chaconas and van de Sande, 1980; Efstratiadis *et al.*, 1977).* However, since many RNAs contain 5′ ends that do not readily accept ^{32}P from [γ-^{32}P]ATP (presumably owing to secondary structure) and since VpG-5′-blocked RNAs cannot be 5′ end-labeled with ^{32}P (but they can be end-labeled with ^{125}I), this procedure as such is not recommended when the RNA is to be used as a hybridization probe. Alternatively, RNA can be partially hydrolyzed by treatment with Mg^{2+} at alkaline pH (Sänger *et al.*, 1979), by boiling in formamide (Simoncsits *et al.*, 1977; Stanley and Vassilenko, 1978; Negruk *et al.*, 1980), or by heating

*Although tobacco acid pyrophasphatase (TAP) is commercially available, it has been my experience, and that of a number of other laboratories (personal communication), that the commercial TAP preparations are not very effective at decapping RNAs for the purpose of 5′ end-labeling. TAP can be prepared from a cell-suspension culture of tobacco cells (Shinshi *et al.*, 1976; Efstratiadis *et al.*, 1977); however, not everyone has such cultures available. Furthermore, the cell walls of such callus-derived cells may become extremely difficult to disrupt, possibly as a function of the number of cell passages. As an alternative, tobacco leaves can be used to prepare TAP by the following modification (my unpublished data) of a procedure used with cells (Efstratiadis *et al.*, 1977): (1) The volume of the DEAE-cellulose column is increased by a factor of 5–8 to allow for the binding of chlorophyll and other pigments (TAP does not bind to DEAE); (2) if the enzyme binding to and eluting from the cellulose phosphate column still contains appreciable RNase activity, then it can be purified further by concentration (e.g., dialysis against Ficoll or Sephadex), gel filtration on a Sephadex G-75 column (Shinshi *et al.*, 1976), and rechromatography on a (smaller) cellulose phosphate column. At this stage the TAP should be RNase free. The TAP can be assayed at each step by the hydrolysis of [^{32}P]ATP and chromatography on PEI-cellulose (Efstratiadis *et al.*, 1977) or by the hydrolysis of *p*-nitrophenyl phosphate and selection of the most active (yellow-color producing) fractions.

in alkaline buffers alone (Donis-Keller *et al.*, 1977). If the degradation is controlled such that only one or two random cleavages occur per RNA molecule, then large RNA fragments will be produced. Such RNA fragments can be labeled at their 5′ (-OH) ends with [γ-^{32}P]ATP and polynucleotide kinase (Chaconas and van de Sande, 1980; see Section II,F,2).

iii. In vitro labeling with cytidine [^{32}P]phosphate or [α-^{32}P]*ATP.* The 3′ ends of RNA molecules, or RNA fragments containing 2′-, 3′-hydroxyls (prepared by dephosphorylating chemically fragmented RNAs or by generating RNA fragments enzymatically by partial digestion with nucleases S1 or P1, which produce 5′-phosphorylated fragments) can be 3′ end-labeled with cytidine 3′,5′-[5′-^{32}P]biphosphate and RNA ligase (England and Uhlenbeck, 1978; England *et al.*, 1980). Alternatively, RNA molecules can be labeled by polyadenylation of their 3′ ends with [α-^{32}P]ATP and poly(A) polymerase (Sippel, 1973; see Section III,D,1a).

The end-labeled RNA is separated from the unincorporated radioisotope by column chromatography: either by gel filtration on Sephadex G-50 in 0.1 m*M* EDTA or 0.1% SDS, as described above for poly(A) RNA and oligo(dT)-primed cDNA, or by chromatography on CF-11 cellulose (Franklin, 1966) as follows: Add 0.1 volume of 10× STE [1× STE = 100 m*M* NaCl, 50 m*M* Tris-HCl (pH 7.0), 1 m*M* EDTA] and 1 volume of absolute ethanol. Apply this solution to a 0.1–0.2 ml CF-11 column (in a 1-ml syringe barrel that was prewashed with 0.1 *N* NaOH and then equilibrated with 1× STE:50% ethanol). Allow the sample to run in, and wash with 3–10 column volumes of 1× STE:50% ethanol. The RNA is then eluted with 3–5 column volumes of either 1× STE or water (E. Dickson, personal communication). The RNA can be recovered either by ethanol precipitation with carrier RNA or by lyophilization, if eluted with water.

b. ^{125}I-Labeled RNA Probes. These probes can be prepared by a modification of the procedure of Commerford (1971, 1980), as used by Robertson *et al.* (1973). RNA (0.3–3 μg) in 1 μl is combined with 3 μl of 42 m*M* sodium acetate–75 m*M* HNO_3 (pH 4.7), 2 μl of 1–10 m*M* thallic chloride, and 2 μl of ^{125}I (see below), and incubated at 60–70°C for 1–3 min (H. R. Robertson, personal communication) or at 70°C for 30 min (Orasz and Wetmur, 1974).

An unstable addition product formed between the nucleic acid and iodine is eliminated by heating in a neutral buffer [e.g., 0.5 ml of 0.1 *M* Tris-HCl (pH 7.0), 0.1 *M* NaCl, 1 m*M* EDTA] at 60–70°C for 20 min.

The [^{125}I]RNA is separated from the ^{125}I by either gel filtration on a Sephadex G-50 column with 50 m*M* potassium phosphate, 0.2 m*M* EDTA (pH 6.7) (Commerford, 1980), or CF-11 cellulose chromatography and ethanol precipitation, as described above for ^{32}P-labeled RNA probes.

There should be at least 6 times as much $TlCl_3$ as iodide (molar ratio);

Robertson *et al.* (personal communication) recommend 6.7 times as much Tl^{3+} as $^{125}I^-$. The molar ratio of $^{125}I^-$ to cytidine in the RNA (the site of radioiodination) should be 0.25–0.75 for maximum specific activity; the specific activity of the carrier-free $Na^{125}I$ is adjusted with KI. Using carrier-free ^{125}I, radioiodinated RNA with specific activities of 0.1 to 1 $\times$ 10^9 dpm/μg can be obtained.

Because ^{125}I emits β particles, X rays, and γ rays, special care should be taken in handling the isotope. Furthermore, radioactive iodine formed during the reaction is volatile. For a discussion of precautions to be taken when handling ^{125}I, see Commerford (1980) and Prensky (1976).

If an mRNA is labeled to low specific activity (0.5 to 1 $\times$ 10^6 dpm/μg), then the RNA contains only 1–3 iodine atoms per molecule of RNA and can still be translated *in vitro* (Kaempfer, 1979).

Since it is possible to radioiodinate protein (see Section II,C,3) attached to the 5′ end of an RNA (VpG), such ^{125}I-labeled protein-RNA molecules could also conceivably be used as probes for (−) viral RNAs.

3. *Cloned Probes*

Advantages to using cloned probes rather than the other probes mentioned above are that (1) by virtue of their method of preparation, cloned probes should not be contaminated with other viral or host plant RNA sequences; such contamination occurs to varying degrees with probes made to virion-encapsidated RNAs; (2) the problem of the preparation of radiolabeled probes to viral RNAs from viruses obtained in only very low yield is simplified; once cloned, the viral RNA sequence can be amplified to high levels in bacteria prior to radiolabeling; (3) by using restriction enzymes and "subcloning" fragments, it is possible to prepare probes to specific regions of the viral genome; and (4) single-stranded bacteriophage M13 clones can be prepared specific not only to defined regions of the genome, but also to either the (+) viral RNA molecule or the (−) viral RNA molecule.

It is not within the scope of this chapter to review the recombinant DNA techniques; however, I will briefly describe several methods for preparing radiolabeled probes, once cloned DNA from either a plasmid (e.g., pBR322) or the bacteriophage M13 (mp9) has been obtained.

a. Nick Translation (Maniatis *et al.*, 1975; Rigby *et al.*, 1977). One strand of a double-stranded plasmid DNA molecule is cleaved ("nicked") by treatment with a low concentration of DNase I. The 3′ ends of the nicked DNA can act as primers for DNA synthesis by *E. coli* DNA polymerase I. This enzyme digests the DNA 3′ to the nick in a 5′→3′ direction, as it synthesizes new (radiolabeled) DNA in its place. The net result is that the positions of the nicks migrate toward the 3′ end of that DNA strand. This

phenomenon can occur on both DNA strands and is dependent on the DNA polymerasing function being much more active than the DNase nicking activity.

Nick translation is carried out as follows: DNA (1 μg) is combined with 1 ng of DNase I per milliliter at 0°C in 50 m*M* Tris-HCl (pH 7.2), 10 m*M* $MgSO_4$, 0.1 m*M* DTT, 50 μg of nuclease-free BSA per milliliter, 20 μM of each of three unlabeled dNTPs and 2 μM of the fourth [α-^{32}P]dNTP. Five units of *E. coli* DNA polymerase I (units of Richardson *et al.*, 1964) is added, and the mixture (50 μl) is incubated at 10–16°C for 1 hr. The reaction is stopped by the addition of EDTA to a concentration of 20 m*M*. The proteins are removed by phenol:chloroform (1:1) extraction, and the [^{32}P]DNA is separated from the unincorporated label by gel filtration on a Sephadex G-50 column in 10 m*M* Tris-HCl (pH 8.0), 1 m*M* EDTA. For a more detailed explanation of the mechanism of synthesis, problems encountered, and adjustments of [^{32}P]dNTP concentrations and the ratio of DNase I to DNA polymerase I for optimization of length, yield, replacement synthesis or specific activity, see Maniatis *et al.* (1982).

b. Replacement Synthesis. In the case of cloned inserts of 600 base pairs or less, nick translation may not yield appreciable labeling of the insert, but only of the plasmid vector. In this situation, the technique of replacement synthesis might be considered. This technique makes use of the ability of the T4 DNA polymerase 3′-exonuclease function to digest linearized plasmid from both 3′ ends in a 3′→5′ orientation in the absence of deoxynucleotide triphosphates. When only a short region of overlapping double-stranded DNA remains, the digested strands are resynthesized in the presence of [α-^{32}P]dNTPs. The product DNA is thus labeled in both strands in over 90% of its length (O'Farrell *et al.*, 1980). Detailed protocols for this method are given by O'Farrell (1981) and Maniatis *et al.* (1982).

c. Excision and End Labeling. The cloned insert can be excised from the plasmid with a restriction endonuclease, purified, and end-labeled by 5′ end-labeling with [γ-^{32}P]ATP and polynucleotide kinase. If the double-stranded DNA restriction fragment contains protruding 5′ ends, then 3′ end-labeling by filling in the recessed 3′ end with the Klenow fragment of *E. coli* DNA polymerase I and [α-^{32}P]dNTPs is also possible (Drouin, 1980; Maniatis *et al.*, 1982).

d. Labeling M13 (mp9) clones. Since M13 is a single-stranded DNA bacteriophage, it has the virtue that the M13 derived clones are single-stranded and can thus be used to detect the presence of a subgenomic RNA of either (+) or (−) polarity.

There are two simple methods of labeling M13 clones. In the first method, a primer (CACAATTCCACACAAC, New England Biolabs; GTCA-

TAGCTGTTTCCTG, P-L Biochemicals) that binds to a site 5′ to the cloned insert is used to synthesize [^{32}-P]cDNA to M13. Because of the limiting deoxynucleotide triphosphate concentration, this cDNA is not near full-length, and thus the cloned insert is not copied and remains single-stranded (Hu and Messing, 1982). The double-stranded regions can be cross-linked with a psoralen to prevent denaturation, generating a ^{32}P-labeled, partially double-stranded molecule with a single-stranded insert available for hybridization (Brown *et al.*, 1982).

In the second method, the insert is cloned into the *Eco*RI site of M13 mp9. A primer, either GTAAAACGACGGCCAGT (P-L Biochemicals), AGTCACGACGTTGTA (BRL), or TCCCAGTCACGACGT (New England Biolabs), binds 3′ to the insert and is used to transcribe [^{32}P]cDNA to the insert. The product is cleaved with the restriction enzyme *Hin*dIII at its single site 5′ to the insert, boiled in formamide, and the fragments are fractionated by electrophoresis on a 5% polyacrylamide gel containing 7 *M* urea. The single-stranded ^{32}P-labeled DNA, complementary to the insert, is eluted and ethanol precipitated (Bruening *et al.*, 1982). The cDNA made to the insert contains only a few sequences of the vector (M13).

E. HYBRIDIZATION AND AUTORADIOGRAPHY: DETECTION OF SUBGENOMIC RNAS

The presence of subgenomic RNAs in virions or extracts of infected tissues is determined by the hybridization of virus-specific probes (Section III,D) to RNAs, transferred from agarose gels (Section III,B) to a binding medium (Section III,C), and autoradiography to detect the number and size distribution of the subgenomic RNAs.

1. *Hybridization Procedures*

There are as many variations of the basic hybridization and washing procedure as there are laboratories. Whether this testifies to the flexibility of the procedure or to the inability of others to duplicate results with a given protocol remains to be determined. Only two basic procedures are described here; some of the others can be found in the following publications: Alwine *et al.* (1977), Kafatos *et al.* (1979), Rave *et al.* (1979), Wahl *et al.* (1979), Smith and Summers (1980), Stellwag and Dahlberg (1980), Thomas (1980), Rezaian and Jackson (1981), Branch *et al.* (1981), Brown *et al.* (1982), Hu and Messing (1982), and Maule *et al.* (1983).

Procedure a. The nitrocellulose or DPT-paper blot in a sealed plastic bag containing prehybridization buffer (see Section III,C) is cut open across the top of the bag, and the prehybridization buffer is removed. The buffer can

be squeezed out of the bag by rolling a pipette over the bag from the bottom to the top. Hybridization buffer [30–100 μl/cm^2; either 4 parts 50% (v/v) deionized formamide, 0.75 *M* NaCl, 0.075 *M* Na_3 citrate, 50 m*M* sodium phosphate (pH 7.0), 0.1% (w/v) SDS, 0.25 mg of yeast RNA per milliliter and 0.02% (w/v) each of BSA, Ficoll, and polyvinylpyrrolidone; 1 part 50% (w/v) sodium dextran sulfate (M_r 5 × 10^5), or a solution containing each of the above at the concentrations given and sodium dextran sulfate at 10% (w/v)] containing 0.5 to 5 × 10^6 cpm of ^{32}P-labeled probe is added to the plastic bag containing the blot, and the plastic bag is sealed at the top. Air bubbles are then pushed to the top of the plastic bag and sealed away from the blot and the bulk of the hybridization buffer and probe. The plastic bag is placed inside a second bag to reduce the risk of leakage in or out, sealed, and incubated in a water bath at 50°C for 24–48 hr. Alternatively, the first bag may be sealed only once, placed in a shaking water bath or on a rolling device in an oven to ensure that air bubbles do not remain stationary over any section of the blot, and incubated as above. After incubation the bag is cut at the top, the hybridization buffer is removed, the bag is slit along the sides, and the blot is removed and washed (six times: twice for 5 min each at room temperature in 2× SSC/0.5% (w/v) SDS; twice for 5 min each, in the same buffer at 50°C; and twice for 15 min each in 0.1× SSC/0.5% (w/v) SDS at 50°C, using 100–200 ml per wash for a 12 × 12 cm bolt. The blot is enveloped in Saran Wrap and autoradiographed (see Section III,E,2).

Procedure b. This procedure is a slight modification of one described by Maule *et al.* (1983). The overall procedure is similar to that described above; however, the prehybridization and hybridization buffers are different. The prehybridization buffer contains 3× SSC, 0.08% (w/v) each of BSA, Ficoll, and polyvinylpyrrolidone and 250 μg/ml of yeast RNA or sonicated and denatured salmon sperm DNA. Prehybridization is carried out for 4–16 hr at 65°C. Hybridization is carried out in the prehybridization buffer containing 0.5 to 5 × 10^6 cpm per blot, at 65°C for 24 hr. The blots are washed as described above.

With this procedure, we have observed a 5-fold increase in hybridization to TMV RNA compared with hybridization in the formamide buffer in procedure a; Maule *et al.* (1983) claim up to a 10-fold increase in sensitivity.

The number of times a blot is washed, the volumes used, the temperature of the "high stringency" (low-salt) washes, and the duration of the washes are governed by what is "necessary" to reduce the background, i.e., improve the "signal-to-noise" ratio. In my hands, the background does not usually provide appreciable "noise" until about 10–14 days of exposure. Should there still be significant, nonspecific absorption of ^{32}P to the nitrocellulose after a brief exposure, continue washing under high or higher

stringency conditions; e.g., increase the temperature of the last washes to 55, 60, or 65°C. In order to effect the removal of ^{32}P, it is important that nitrocellose blots remain moist and do not dry out; hence, the envelopment of the blot in Saran Wrap. ^{32}P cannot be readily removed from dry nitrocellulose.

It is possible to reuse blots; i.e., either the ^{32}P-labeled probe is allowed to decay, or the blot can be washed as follows: Wash nitrocellulose in 0.1 to 0.05× wash buffer [1× wash buffer = 50 m*M* Tris-HCl (pH 8.0), 2 m*M* EDTA, 0.5% sodium pyrophosphate, and 0.02% (w/v) each of BSA, Ficoll, and polyvinylpyrrolidone] for 1–2 hr at 65°C. The blot is then prehybridized again prior to rehybridization (Thomas, 1980). Wash DPT paper or DBM paper blots in 95% (v/v) formamide, 50 m*M* Tris-HCl (pH 7.4), 10 m*M* EDTA, 0.1% (w/v) SDS, at 50°C for 1 hr followed by a 10-min wash at room temperature in 20 m*M* sodium phosphate (pH 7.7), 0.36 *M* NaCl, 2 m*M* EDTA, 0.1% (w/v) SDS. The blots are then prehybridized again. Alternatively, DBM paper can be washed by heating at 100°C for 3 min in 95% formamide, 50 m*M* Tris-HCl (pH 7.4), 10 m*M* EDTA, 0.1% SDS, followed by one wash in 20 m*M* sodium phosphate (pH 7.7), 0.36 *M* NaCl, 2 m*M* EDTA, 0.1% SDS at room temperature for 10 min, prior to reprehybridization.

2. *Indirect Autoradiography*

The detection of the radiolabeled probe is accomplished by autoradiography. In this process, β electrons or γ rays emitted by the probe convert silver halide crystals to silver atoms in a sheet of X-ray film overlaying the blot. In the case of high-energy β-emitters (e.g., ^{32}P) or γ-ray emitters (e.g., ^{125}I), the emissions pass through the X-ray film. If this excess energy can be captured and returned to the film in the form of light at wavelengths to which the film is sensitive, then the image produced by a single decay can be intensified. The excess emissions can be captured by placing a high-density fluorescent "intensifying screen" beyond the film, distal to the radioactive source. The degree of intensification obtainable varies with the isotope used (^{32}P vs ^{125}I), the temperature of autoradiography, and whether or not the film was hypersensitized; i.e., briefly preexposed to light. These variables, as well as the effects of brands of film and intensifying screens on the degree of enhancement, have been described by Laskey and Mills (1977). This process of autoradiography combined with "photography" is referred to as indirect autoradiography (see also Laskey, 1980); it can increase the sensitivity of detecting ^{32}P and ^{125}I by up to 10.5 and 16 times, respectively.

An example of an autoradiogram of a blot containing TMV genomic and subgenomic RNAs, hybridized with a TMV-specific cDNA probe is shown

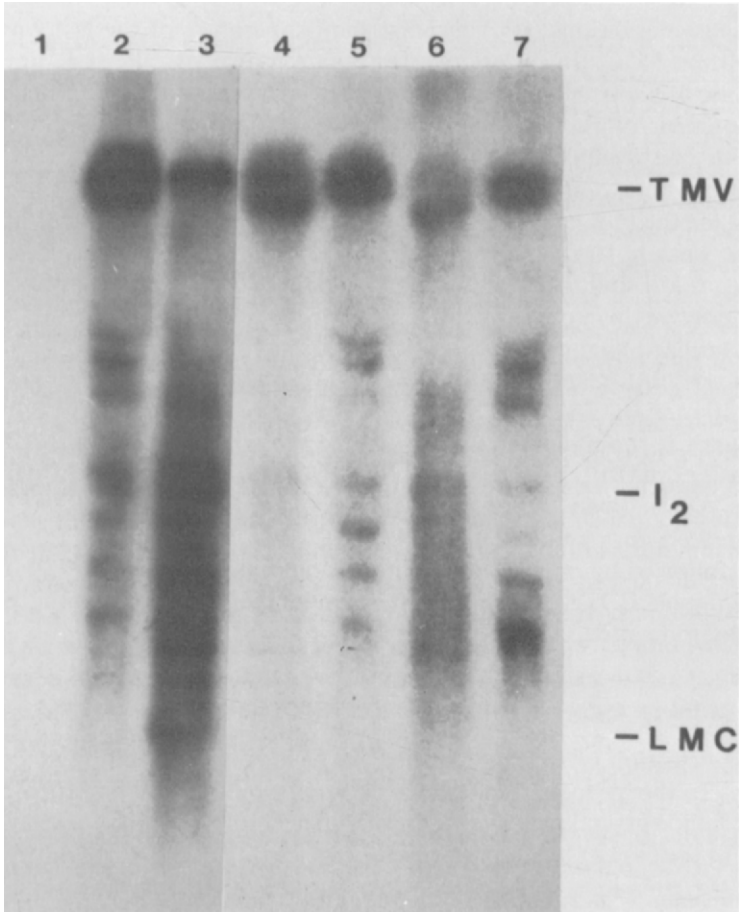


FIG. 6. Autoradiogram of RNA blots. Formylated RNAs were electrophoresed under denaturing condition in a horizontal 1.5% agarose gel containing 1× MAE and 6% formaldehyde (see Section III,B,2), blotted to nitrocellulose and hybridized with a cDNA probe specific to the 3′ end of TMV RNA. RNA samples: nucleic acid extracted from uninoculated tobacco (lane 1) or TMV-infected tobacco (lane 3); TMV RNA (lanes 4 and 6); TMV mixed with uninoculated tobacco leaves just prior to extraction (lane 2); TMV RNA mixed with nucleic acid extracted from uninoculated tobacco just prior to electrophoresis (lanes 5 and 7). Note the absence of the LMC from lanes 2, 4, 5, 6, and 7. The LMC is the subgenomic RNA coding for TMV coat protein and is not encapsidated in the U1 strain of TMV.

in Fig. 6. Although a number of apparently subgenomic RNAs are seen in RNA extracts from TMV-infected tissues (lane 3), many of these bands are artifacts resulting from the coelectrophoresis of viral RNA fragments (running as a smear in lanes 4 and 6) with plant RNAs (lanes 5 and 7). Thus, while many of the bands correspond in position to the major plant rRNAs

and their specific breakdown products, some bands are bona fide subgenomic RNAs that can be identified by preparing and analyzing polyribosomal RNAs from infected plants (Palukaitis *et al.*, 1983).

3. *Preparative Hybridization*

Hybridization can also be used to isolate and purify subgenomic RNAs. However, since this method requires relatively large amounts of cDNA to be effective, it is better suited for use with cloned probes.

The procedure is carried out as follows: Activated cellulose (epoxycellulose), either formed by the coupling of 1,4-butanediol diglycidyl ether (Eastman Kodak) and cellulose (Moss *et al.*, 1981) or obtained commercially (e.g., epoxy-activated cellulose or triazine-activated cellulose, BRL) is coupled to a cloned, single-stranded, cDNA probe (e.g., an M13 clone, or a denatured, restriction fragment clone from pBR322) of the viral genomic RNA, or a part thereof (Moss *et al.*, 1981). The cellulose (200 mg), containing 200–800 μg of cloned DNA, is combined with a nucleic acid extract from a virus-infected plant, in hybridization buffer [either the ones described in Section III,E,1 or the one described by Moss *et al.*, 1981, i.e., 10 m*M* 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, (pH 7.2), 600 m*M* NaCl, 2 m*M* EDTA, 0.2% (w/v) SDS] and incubated by shaking for 1–24 hr at either 50°C (in formamide-containing hybridization buffers) or 65°C. The cellulose is then packed into a small column placed in a water jacket maintained at the hybridization temperature, washed extensively with hybridization buffer (3–10 column volumes) to remove the unbound RNA, and then washed in 1–3 volumes of water at 65°C to remove the bound RNA, which can then be recovered by ethanol precipitation or lyophilization. The eluted RNAs can be further fractionated by sucrose density gradient centrifugation or gel electrophoresis. Alternatively, the fractionation process can be carried out prior to the affinity chromatography step. Thus, new subgenomic RNAs can be detected, isolated, purified, and readied for further characterization.

A variation of the above elution procedure used by R. H. Symons and colleagues (personal communication) is to wash the cellulose after hybridization three times with 10 m*M* Tris-HCl (pH 7), 2 m*M* EDTA, 0.1% (w/v) SDS, 50% (v/v) formamide at room temperature, and to elute the RNA in the same buffer at 80°C. The RNA is recovered by ethanol precipitation (the presence of low-molecular-weight carrier RNA may be required).

Clones can also be bound to DBM paper or DBM cellulose (Goldberg *et al.*, 1979) and used to selectively isolate or detect subgenomic RNA; however, larger volumes of liquid are required, and the recovery of the subgenomic RNAs may not be as efficient. Submicrogram levels of RNA can, however, be recovered by ethanol precipitation and ultracentrifugation (Shapiro, 1981).

IV. Genetic Mapping by Blot Hybridization

The application of recombinant DNA technology to plant virology has made it possible to map the physical locations of genes on plant virus genomes and/or to determine whether other subgenomic RNAs of a particular virus exist that had not been previously identified. For example, by using cloned DNA of the multipartite barley stripe mosaic virus (BSMV) genome, Gustafson *et al.* (1982) have shown that the smaller RNA of the type strain of BSMV actually consists of two different RNA species, and that a fourth RNA species found in three strains of BSMV is actually a subgenomic RNA derived from RNA_3 of BSMV.

When dealing with monopartite viruses containing multiple genes, the physical mapping of the genes can most unequivocally be accomplished by four steps: (1) cloning the entire virus genome; (2) determining the number of subgenomic RNAs; (3) translating the subgenomic RNAs and characterizing their translation products; and (4) determining the spatial order of the genes on the viral genome.

Step 1. The first step is the most difficult and time consuming: preparing a full-length dsDNA clone to the entire viral genome. With many viruses, however, it is difficult to obtain full-length complementary DNA. Therefore, most clones derived from double-stranded DNA prepared to plant viral RNAs will represent only the 3′ end or 3′ half or third of the viral RNA genome (e.g., Gould and Symons, 1982; Meshi *et al.*, 1982, 1983). An alternative method involves preparing random-primed cDNA to the viral genome and then either self-priming the second strand or random-priming the second strand. The random-primed dsDNA fragments are then cloned and physically mapped with respect to each other and to the viral genome. A third procedure involves preparing clones to the 3′ end of the viral genome and then using these clones as primers on the viral RNA to extend the length of the clone; i.e., "walking your way up the RNA" (e.g., Gould and Symons, 1982).

Step 2. The number of subgenomic RNAs is determined by isolating polyribosomal RNA (Section III,A,2), electrophoresing the RNAs on gels (Section III,B), transferring the RNAs from the gels to a binding support (Section III,C), and hybridizing the RNA blots with viral cDNA or cloned viral DNA (Section III,E).

Step 3. If all the viral subgenomic RNAs are also found encapsidated in virions, then it would be necessary to fractionate virion RNA on sucrose gradients and/or agarose or polyacrylamide gels (Section II,B), translate the subgenomic RNA in a cell-free lysate (Section II,C), and characterize the viral translation products (Section II,D). On the other hand, if any of the subgenomic RNAs are not encapsidated, then preparative hybridi-

zation involving either large amounts of viral cDNA, or cDNA clones of a viral genome, bound to a solid support, would be necessary to purify the viral RNAs away from the host RNAs in the polyribosomal RNA preparations (Section III,E,3). The bound RNA can be eluted and further fractionated by either sucrose gradient centrifugation, electrophoresis and elution, or further cycles of preparative hybridization to cloned DNA representing different segments of the viral genome. The fractionated viral RNAs can then be translated *in vitro* and the translation products characterized (Sections II,C,1 and II,D).

Step 4. Although cDNA can be used to determine the number of viral subgenomic RNAs, their selective purification by preparative hybridization and the physical mapping of the genes requires cloned DNA (restriction) fragments of the viral genome. The subgenomic RNAs are electrophoresed on denaturing agarose gels (Section III,B,2), blotted to a cellulose support (Section III,C) and probed with cloned DNAs (Section III,D,3) representing different regions of the plant viral genome. The cloned DNAs are either the cloned fragments of the viral genome described above, or restriction fragments of a full-length DNA clone of the viral genome. The same approach can also be used to identify subgenomic RNAs of bi- and multipartite plant viruses.

An approach that combines blot hybridization analysis with *in situ* translation of blotted RNA was described by Saris *et al.* (1982). Although we were unsuccessful in applying the procedure as described, but with TMV RNAs (unpublished experiments), it should be possible to adapt this approach to the genetic analysis of plant viral (subgenomic) RNAs. Alternatively, gel fractionation and "electroblotting" of RNA to DBM or DPT paper, incapable of covalent bond formation, followed by elution of the RNA off the paper with a "high-salt" solution (Stellwag and Dahlberg, 1980), can be used to obtain viral subgenomic RNAs that can be translated *in vitro* and further characterized.

The information obtained by ordering the genes of a virus can be of immense value in studying the temporal expression of the viral genes during the time course of a synchronous infection (e.g., in protoplasts), as well as in locating viral genes when the total nucleic acid sequence of the viral genome becomes available.

V. Concluding Remarks

The aim of this chapter is to familiarize the reader with a range of techniques that are currently available and have been used to detect and characterize subgenomic RNAs. The amount of space devoted to the description

of some methods compared with others may seem unequal; this was quite intentional. A thorough description of all the procedures mentioned would fill a volume in itself. Some of the techniques mentioned have been either described recently or adequately covered in the reference cited. (For example, analysis of viral RNAs on gels and *in vitro* translation, two of the oldest analytical techniques, have been reviewed numerous times and described in great detail elsewhere.) On the other hand, many techniques that are used are modifications of modifications, etc., and the present procedures resemble the original methods in name only. Furthermore, some techniques previously described were initially used for the analysis of double-stranded DNA molecules and have required modification for the analysis of single-stranded RNA molecules. In the latter two instances, I have seen fit to go into considerable detail. There are also instances in which conflicting data exist in the literature on the efficacy of some modification to a protocol. In these cases, I have given either my own experiences, those experiences communicated to me by others, or data and/or other observations available in the literature.

Two areas that were totally excluded from this chapter are molecular cloning of plant viruses and sequencing of RNA and/or (recombinant) DNA. These again are methodologies that are quite expansive in themselves, and many of the techniques used in these areas have been covered by Maniatis *et al.* (1982) and in Volumes 65 and 68 of "Methods in Enzymology" (Academic Press); numerous citations from these three sources appear in this chapter.

Since most plant viruses are positive-strand RNA viruses, I have described techniques suited to analyzing such systems and avoided any specific reference to detecting or characterizing subgenomic RNAs of either negative-strand RNA viruses, double-stranded RNA viruses, or DNA viruses; however, many of the same techniques described here can be used for such characterizations.

While the determination of the (5′- and 3′-) end groups of RNA plant viruses and their subgenomic RNAs was not specifically covered, the ends of RNA molecules can be determined by the use of techniques described here. Moreover, the review on RNA plant viruses by Davies and Hull (1982) contains data and references to the determination of the end groups of specific plant viruses.

Finally, it is hoped that the techniques described here will lead to a better understanding of the relationship of the components involved in the replication and spread of RNA plant viruses. With the aid of the techniques of molecular cloning and the expression of cloned genes, sufficient amounts of virus-encoded proteins should become available for study of their function and involvement in virus replication, spread, and pathology.

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