CHAPTER 12

Characterization of Viral Products Expressed in the Infected Cell

CHARACTERIZATION OF VIRAL PROTEINS IN THE INFECTED CELL

All viral proteins are synthesized in the infected cell; however, the amount and nature of these proteins, and the mRNAs encoding them, change with time following infection. The synthesis of nonstructural proteins generally occurs prior to the synthesis of viral structural proteins for the following reason. The nonstructural proteins include viral enzymes that function to modify the cell for virus replication, viral genome replication enzymes, and viral regulatory proteins. These nonstructural proteins have many important functions and are important to study. For example, the enzymes involved in the replication of HSV DNA during infection are good targets for chemotherapeutic drugs because they can be specifically inhibited with little effect on cellular DNA replication enzymes (see Chapter 8).

Pulse labeling of viral proteins at different times following infection

Study of the time of synthesis and nature of viral proteins in the infected cell requires the ability to distinguish virus-encoded proteins in a background of cellular ones, and to fractionate such viral proteins away from cellular components of the infected cell. Given the large amount of mass of the biological macromolecules contained in the cell, the process of viral protein or nucleic acid purification can be difficult and requires technical ingenuity.

Although the detection of viral proteins against the background of cellular material is difficult, the task is made somewhat more tractable in many virus infections because the infection leads to a partial or total shutoff of host cell mRNA or protein synthesis while viral proteins and mRNA are synthesized at high rates. This means that if radioactive amino acids are added to infected cells to serve as precursors to protein synthesis, they will be preferentially incorporated into viral products.

The addition of radioactive precursors for a short period at a specific time after infection (a **pulse** of radioactive precursors), followed by isolation of total cellular material, will yield a mix of both viral and cellular material, but only the viral material will have incorporated significant amounts of radioactivity. Thus, size fractionation of the proteins in the infected cell provides a biochemical "snapshot" of whichever proteins are being synthesized at the time of labeling.

It is very important to remember that virus infection often leads to increased expression of some host cell proteins as part of its defenses (see Chapter 10). Therefore, the profile of proteins synthesized in a cell infected even with a virus that is extremely efficient in inhibiting host functions will not necessarily be all viral.

Examples of pulse labeling experiments are shown in Fig. 12.1. For the left panel, radiolabeled amino acids were added to poliovirus-infected cells at the time after infection shown, and then proteins were fractionated. Many of the bands of radioactivity seen by exposing the gel to x-ray film are the result of the expression of viral proteins. Some of the more notable ones are indicated, as are some cellular proteins.

Several features of this pattern of pulse labeling are readily apparent. First, the amount of the capsid protein VP2 does not appear equimolar with that of VP1 and VP3, as was seen in the frac-

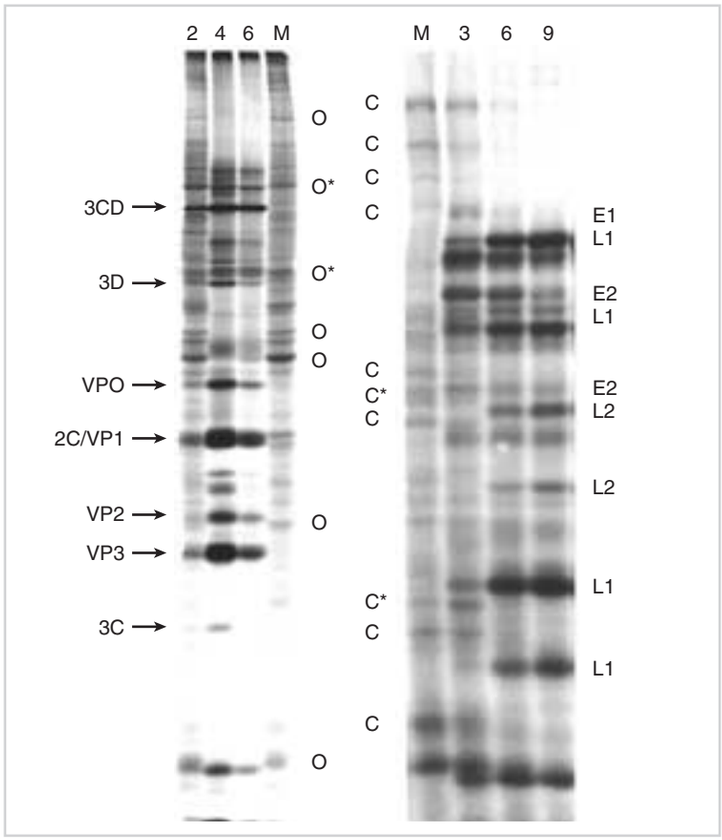

Fig. 12.1 Changes in the proteins synthesized in virus-infected cells with time after infection. The left panel shows an experiment in which HeLa cells were infected with the Sabin (vaccine) strain of poliovirus, and labeled with ^{35}S-labeled methionine for 2-hour pulses at the times (hours postinfection) shown at the top of the gel. Protein was isolated and then fractionated on a denaturing gel, and radioactive proteins were localized by autoradiography (exposure to x-ray film). The capsid proteins are indicated as are other nonstructural poliovirus-encoded proteins. Some cellular proteins whose synthesis is shutoff following infection are shown with the letter "O," while a couple whose synthesis continues are indicated by "O*." (Photograph courtesy of S. Stewart and B. Semler.) The right panel shows a similar experiment carried out by labeling HSV-1-infected Vero cells for 30-minute periods at the times shown after infection. Some cellular proteins that are rapidly shutoff are indicated with "C." "C*" marks proteins that do not appear to be shutoff or whose synthesis increases for a period following infection. Viral proteins synthesized early after infection are indicated by "E." Note that there are at least two subsets, E1 and E2, which differ in the length of time that their synthesis continues. Similarly, there are at least two subsets of late proteins ("L"); some are clearly synthesized at the earliest times while others are only synthesized later. In both panels mock-infected cells (M) show the patterns of proteins synthesized in uninfected cells. (Photograph courtesy of S. Silverstein.)

tionation of proteins found in the mature capsid shown in Fig. 11.4. The reason for this is that VP2 is derived from the processing of VP0, and therefore, some of the radioactivity that would be in the peak of VP2 is actually in the VP0 band.

Another feature is that all the viral proteins indicated are in the same relative proportions at all times measured. As described in Chapter 15, poliovirus infection is characterized by the expression of only one mRNA molecule and all proteins are derived from a large precursor that cannot be seen in this gel. However, portions of precursor proteins such as 3CD are clearly seen.

A third feature of the gel can be seen in examination of the cellular proteins labeled after infection. Although the synthesis of some is clearly shutoff, the synthesis of others persists. This is an example of the fact that some cellular genes continue to be expressed (or can be induced) following infection.

The effect of an HSV-1 infection on total protein synthesis in infected cells is shown in the right panel of Fig. 12.1. It is evident that the pattern of labeled viral proteins changes markedly with time. Some viral proteins synthesized at 3 hours following infection are no longer synthesized at later times. Conversely, some proteins are only labeled at later times after infection.

As described in Chapter 18, there are several reasons why the synthesis of some viral proteins readily detectable at one time after infection is not seen at other times. The basic reason for the temporal change in the patterns of expressed HSV proteins is that certain viral mRNAs are only expressed during a given window of time during infection; if the mRNAs are expressed at the earliest times, their synthesis declines at later times. The high constant rate of mRNA degradation in the cell (*mRNA turnover*) ensures that once the mRNA encoding a given protein is no longer synthesized, synthesis of that protein shuts off rapidly. This provides a ready means for the virus to control the timing and amount of protein synthesized at any given time.

Use of immune reagents for study of viral proteins

The immune response to viral infection in a vertebrate host is a complex process that was briefly outlined in Chapter 7. One of the major parts of this immune response is generation of antibody molecules, which are secreted glycoproteins with the capacity to recognize and combine with specific portions of viral or other proteins foreign to the host. The high degree of antibody molecule specificity, as well as the relative ease in obtaining them from immune animal serum, makes them important reagents in molecular biology. Antibody molecules isolated from the blood serum of animals following antigenic stimulation are made up of different molecules with different levels of affinity for different epitopes in the antigen. Such unfractionated preparations of antibody mixtures are often termed **antiserum** against the antigenic protein, organism, or virus in question. Although such antisera can react with many proteins, if care is used in the injected material, the immune serum will be specific for the antigen presented. Such an immune serum is **polyclonal**, as it is derived from many individual clones of antibody-secreting cells.

If an immune serum or antiserum is specific against the specific antigen in question, it can be termed **monospecific**, but this term is relative. Thus, an antiserum to HSV generated in a mouse infected with the virus is monospecific for HSV, but will contain antibodies reactive against many epitopes in any number of different HSV proteins.

Working with antibodies

The structure of antibody molecules Antibody molecules have a very specific structure that is often described as a "wine glass" shape. They are made up of two light and two heavy chains, and the two *antigen-combining sites* (made up of both heavy and light chains) are at the top of the wine glass (the **Fab region**). Antibody molecules directed against different antigens have different amino acid sequences in this *variable region*.

The stem of the wine glass (the **Fc region**) is made up of an amino acid sequence for all constant antibody molecules of a given class, no matter what the antigen with which they react is. This region serves as a signal to the cell that an antibody molecule is there. It is important to the immune reaction and can be used both diagnostically and in the laboratory. An antibody molecule is shown diagrammatically in Fig. 12.2.

Monoclonal antibodies The immune response is a result of proliferation of many different B- and T-cell types responsive to various antigenic determinants presented by the pathogen or by the antigen. Thus, each immature B cell stimulated by a specific epitope was stimulated into dividing into many daughter cells, all with identical genomes and all secreting identical antibody molecules. Such a clone of cells is short-lived in the body, but specific manipulations can be made to immortalize a single B cell so that a culture of clonally derived B cells, all secreting antibody molecules with identical sequence, can be isolated. The antibodies expressed by such a cell line are **monoclonal antibodies** and have a number of important uses in diagnostics, therapies, and research.

The generation of monoclonal antibodies involves a number of steps that are outlined in Fig. 12.3. These steps include immunizing the animal that is to be the source of the B cells (often a

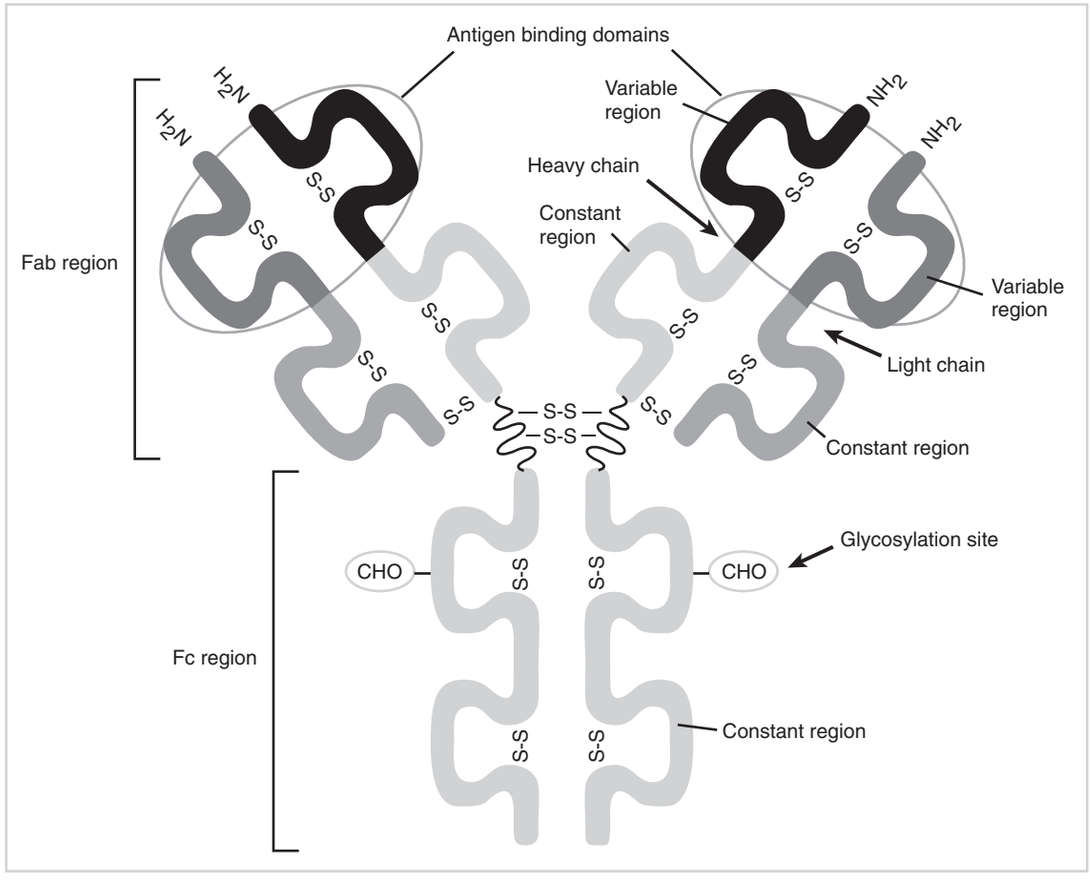

Fig. 12.2 The structure of an antibody molecule, IgG. This molecule is made up of four chains: two heavy and two light. The antigen-combining domains are at the N-terminal of the four chains and are made up of variable amino acid sequences, a specific sequence for each specific antibody molecule. The C-terminal region has a constant amino acid sequence no matter what the antibody's specificity. This is the Fc region.

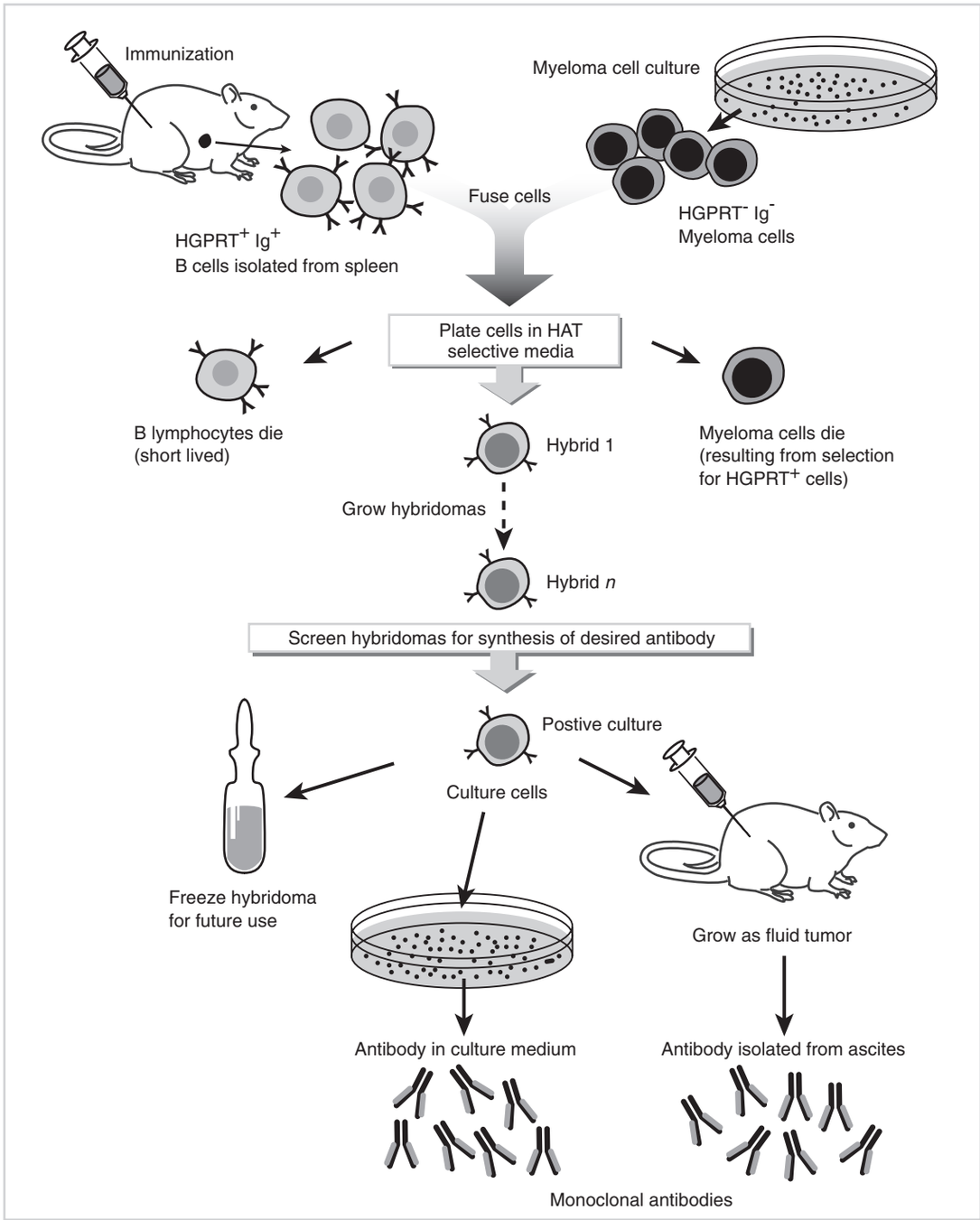

Fig. 12.3 Generation of monoclonal antibodies by making hybridoma cells between mouse immune B lymphocytes and myeloma cells that are not able to grow in selective (HAT) medium. Antibody-secreting clones are screened by testing with an antigen. Once the hybridoma cell line is made, it can be stored frozen, and then either grown in culture or injected into the peritoneal cavity of a mouse where a tumor grows as a disorganized group of individual cells and fluid (an ascites). The ascites' cells secrete the monoclonal antibody into the body cavity's fluid where it can be harvested. (HGPRT, hypoxanthine-guanine phosphoribosyltransferase; HAT, hypoxanthine, aminopterin, and thymidine.)

mouse), isolation of lymphocytes from the animal's spleen, transformation of cells to immortalize them, screening of specific populations, and selection of immortal cells that produce antibodies. Individual B cells that secrete only one antibody molecule reactive with only one determinant can be cloned by fusion of a mature B-lymphocyte population (each secreting a specific—and different—antibody) with immortal **myeloma cells** (tumor cells derived from lymphocytes that do not produce any antibody molecules).

If myeloma and B cells are induced to fuse with a very mild detergent, the cell culture contains short-lived parental B cells that will die, immortal myeloma cells, and fused cells. The key to the value of the method is that these fused cells (**hybridoma cells**) are also immortal. The job now is to get rid of the unfused cells, then *screen* the hybridoma cells for their ability to produce the desired antibody. Getting rid of unfused B cells is no trick because they have a very short lifetime in culture and will die in a few days.

Myeloma cells, however, offer a different problem because they are immortal and will continue to replicate. They are killed by using a mutant myeloma cell line that can be selected against. A convenient method uses a myeloma line that has been mutated so that it does not express hypoxanthine-guanine phosphoribosyltransferase (HGPRT negative), an essential enzyme in the biosynthesis of nucleotides. The advantage of this mutant is that since the parental myeloma cells cannot synthesize nucleotides, they need to get the nucleotides from the medium using a salvage pathway. This salvage pathway can be blocked with the drug aminopterin, which blocks the myeloma cell's ability to pick up nucleosides from the outside medium.

To understand this, remember that the hybridoma cells are not just derived from myeloma; they also have the genetic background of B cells, and the B cells are HGPRT positive. This means that adding aminopterin to the mixture of hybridoma and myeloma cells will result in the death of only the myeloma cells. The fused hybridoma cells will grow. The mixed hybridoma then can be screened by taking individual cells, growing clones from them, and testing the produced antibody for its ability to react with the antigen of interest.

Monoclonal antibodies are very useful for precise diagnosis of specific viral infections, as even closely related viruses will encode some proteins with different antigenic determinants. Each different determinant will react with only a specific monoclonal antibody generated against it. The monoclonal antibodies are also valuable tools for localizing viral proteins within the infected cell or animal, and as reagents to isolate and analyze specific viral proteins for study.

Detection of viral proteins using immunofluorescence

A number of methods to measure antibody reactions involve use of the antibody molecule's Fc region as a "handle." Figure 12.4 shows some examples using a fluorescent dye either attached directly to the antibody (direct) or attached to a second antibody that is reacted against the Fc region of the first (indirect). Methods using **immunofluorescence** are very important to localize viral antigens inside infected cells, and to generate easily measurable immune reactions.

There are a number of micrographs of immunofluorescent-tagged infected and uninfected cells in this text. A notable series is shown in Fig. 3.5 where the passage of rabies virus through an infected animal was traced. Another excellent example showing the effect of HSV infection on the cytoskeleton of an HSV-infected cell is provided in Fig. 10.3.

Immunofluorescence can also be used with two (and even three) antibodies if each is tagged with a different chromophore. Two-color immunofluorescence can provide a tremendous amount of information about the colocalization of proteins and other antigens of interest. Recently, the availability of lasers and prisms (or mirrors) that can differentially allow the passage of one wavelength of light while excluding others has allowed a technique termed **confocal microscopy** to become available for the study of cellular distribution of antigens.

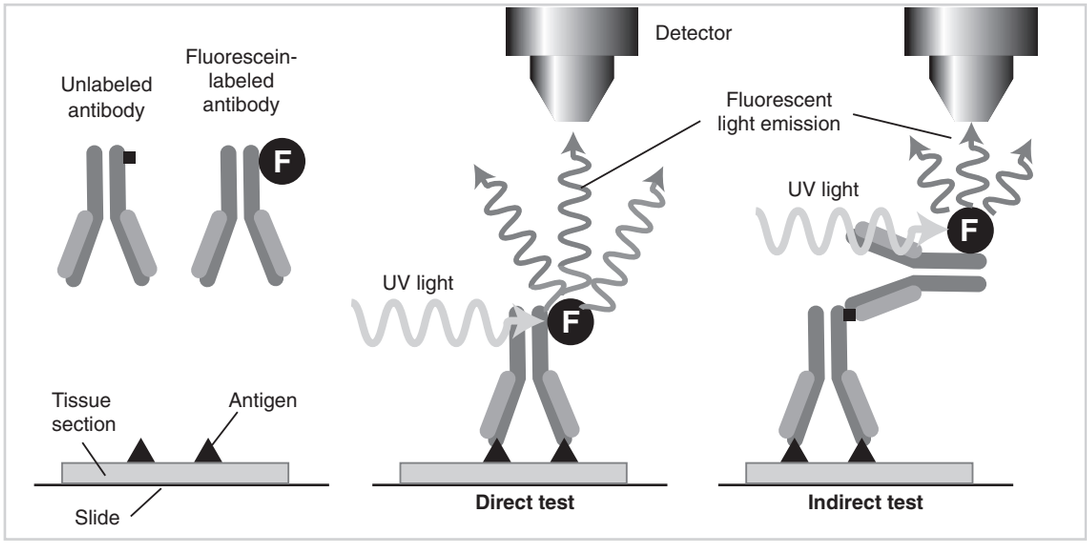

Fig. 12.4 Outline of immunofluorescence as a means of detecting and localizing an antibody–antigen complex. The antibody specific against the antigen is allowed to react. If it has a fluorescent tag on its Fc region, it can be seen directly when illuminated with ultraviolet light since the tag emits visible light. For indirect immunofluorescence microscopy, a second antibody reactive with the Fc region of the first is used, and this antibody has the florescent tag. This method is somewhat more specific and allows the same tagged antibody preparation to be used with a number of different antibodies of differing specificities.

Although there are many variations on the method, basically it depends on the ability of a laser light source to be so coherent that it can be focused to a single focal plane within a cell. This, along with the use of appropriate prisms or filters and fluorescent dyes, can allow one to visualize only the fluorescence emanating from a single plane within the cell. Since fluorescent radiation, of physical necessity, must be emitted at a wavelength longer than the incident radiation, the light path in a microscope can be used for both illumination and viewing.

The technique is shown schematically in Fig. 12.5a, and an example of the type of data that can be obtained is shown in Fig. 12.5b. For the studies shown in Fig. 12.5b, cells were infected with human cytomegalovirus (CMV), a herpesvirus with a very long replication period, and then the expression of two proteins that localize to different parts of the cell was examined.

The first protein, IE72, was detected with an antibody that was tagged with Texas red, which fluoresces red under illumination with the appropriate laser beam. This protein is synthesized in the cytoplasm, but quickly migrates to the nucleus, where it remains and serves as a regulatory protein controlling expression of other CMV genes.

The second protein, which fluoresces green due to a fluorescein isothiocyanate (FITC) tag, is pp65. This protein functions in the cytoplasm and is expressed later than IE72. The separation of the two proteins is clearly seen in the close view.

The lower photographs in Fig. 12.5b demonstrate that another herpesvirus glycoprotein, varicella-zoster virus (VZV) gE, localizes to the same region of the cell as does the transferrin receptor. This latter cellular protein is internalized into endocytotic vesicles of cells that are induced to take up iron borne by the carrier cellular protein transferrin. The fact that the VZV gE protein, which is expressed in transfected cells, colocalizes with the cellular receptor suggests that VZV may be internalized by endocytosis also. The specific glycoprotein for the virus (gE) was tagged with green fluorescent FITC-tagged antibody, while the transferrin receptor was tagged with Texas red-tagged fluorescent antibody. It is clearly evident that when both antibodies are observed, they are in the same precise location at the surface of the cell, as indicated by the color of the fluorescent light being yellow, which is a mix of the two colors.

Related methods for detecting antibodies bound to antigens

Other tags, such as enzymes, also can be bound to the Fc region of an antibody molecule. Enzyme-linked immunosorbent assays (ELISAs) were discussed in Chapter 7. A somewhat involved method is use of the enzyme peroxidase as a "tag" or indicator enzyme. Peroxidase will oxidize a soluble reagent containing a heavy metal, which then leads to precipitation of that metal near the antibody-antigen complex site. The precipitated metal can be observed in the microscope (or

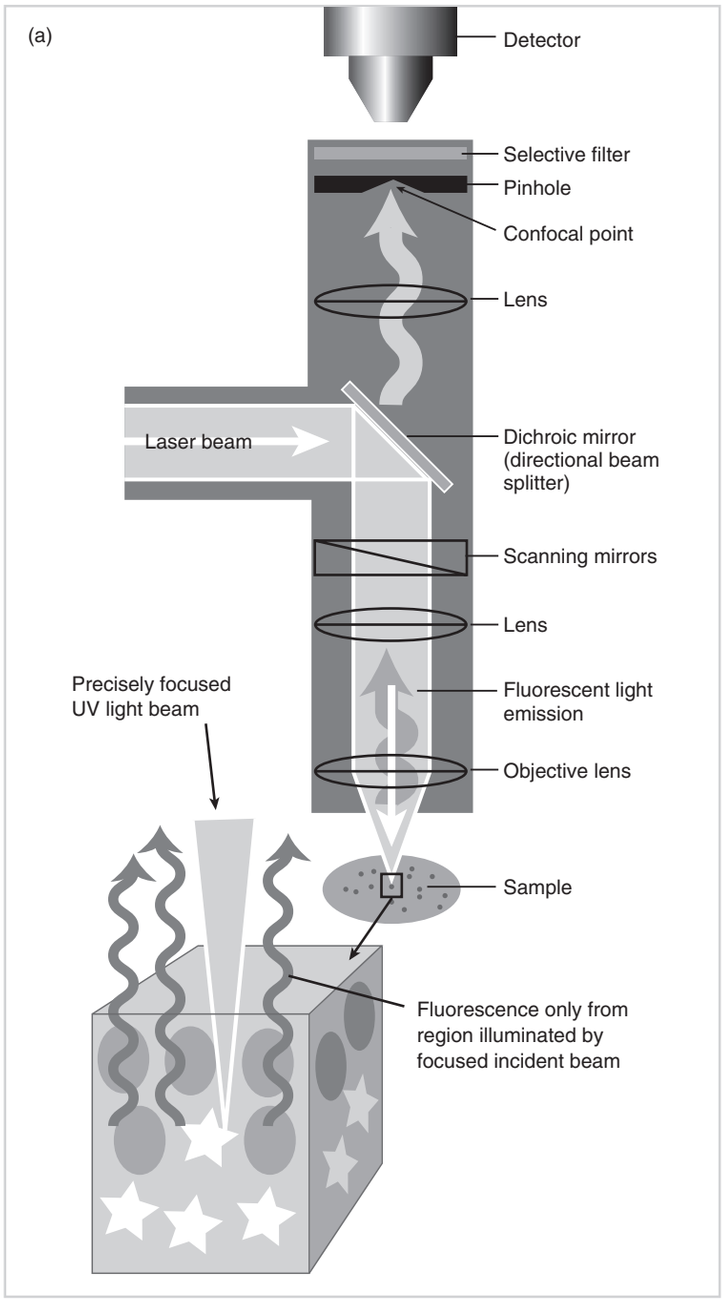

Fig. 12.5 Confocal microscopy to detect colocalization of antigens. *a.* The use of a laser beam and a specific filter to separate the incident laser light from the fluorescence that travels on the same light path. The ability to precisely focus the laser beam onto a single plane in the microscopic field allows one to observe fluorescence from proteins only in that plane. *b.* Top: Confocal microscopic visualization of two human cytomegalovirus (HCMV) proteins, IE72 (red) and pp65 (green). Primary aortic endothelial cells were infected with a strain of HCMV isolated from a human patient. This high-magnification view of a cell shows nuclear and cytoplasmic staining of the two HCMV proteins at 8 days following infection. (Photograph courtesy of K. Fish and J. Nelson.) Bottom: A series of three photographs of the identical field viewed with three different filters to localize two specific proteins to the same region. The first panel shows the association of varicella-zoster virus (VZV) glycoprotein E (gE), tagged with a green fluorescent antibody, with the surface of an infected cell. This glycoprotein was expressed in transfected cells. The second panel shows the localization of the red fluorescence due to the transferrin receptor in the same cell, and the third panel shows that both fluorescent signals are located in the same sites on the cell, indicated by the yellow color, seen when a filter that allows both colors to pass is used for viewing. See Plate 7 for color image. (Photographs courtesy of C. Grose.)

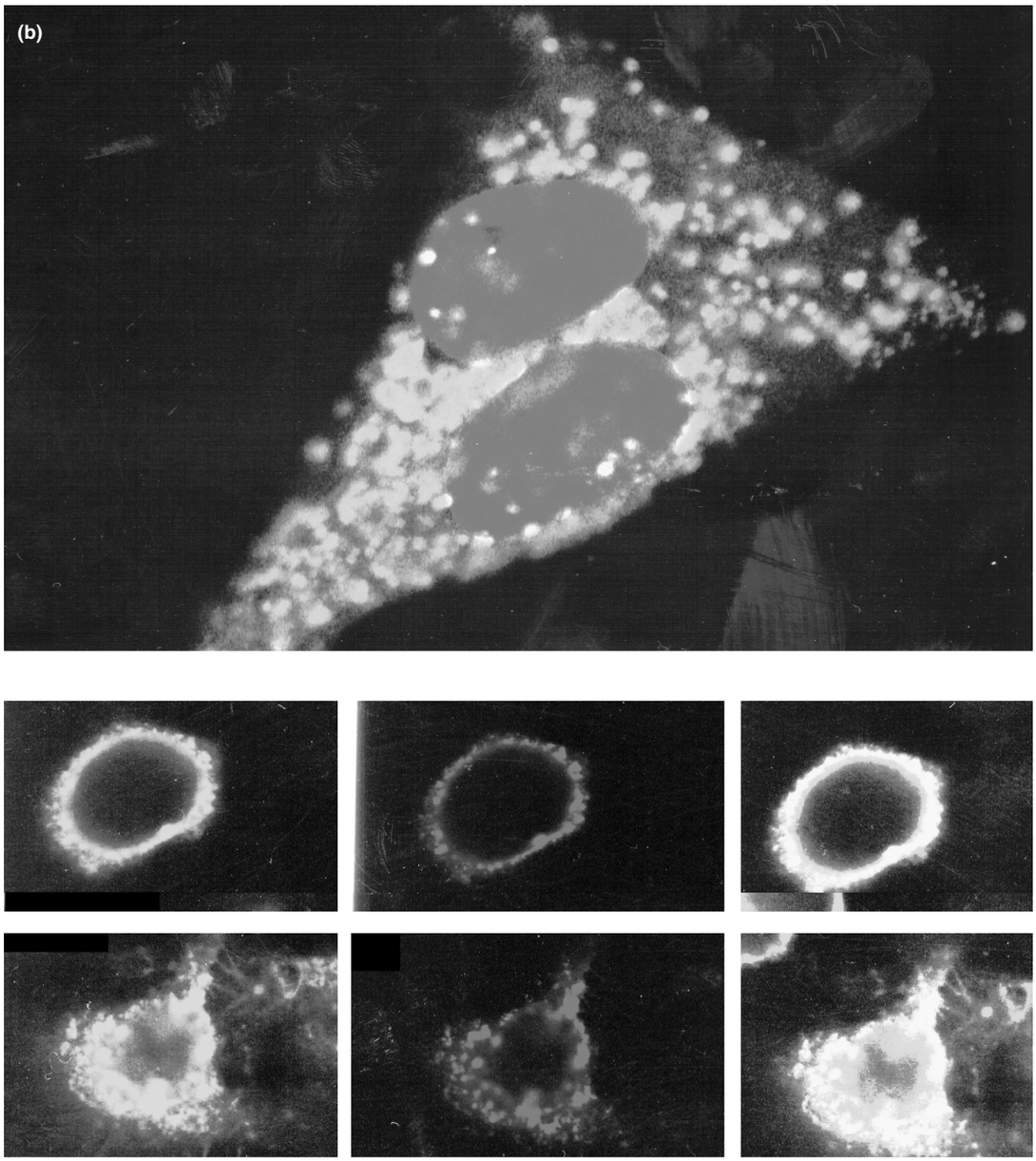

Fig. 12.5 *Continued*

electron microscope) to localize the immune reaction site. Individual antibody molecules bound to antigen can also be localized in the electron microscope using colloidal gold particles bound to the Fc region. These particles are so small as to have little effect on solubility of the antibody. An example of this technique is shown in Fig. 6.3.

Use of bacterial staphylococcus A and streptococcus G proteins to detect and isolate antibody-antigen complexes Pathogenic staphylococci and streptococci express Fc-binding proteins on their surface to bind and inactivate antibody molecules by forcing them to face away from the bacterial cell.

This reaction is quite useful in the laboratory, and the A protein of *Staphylococcus aureus* (staph A protein) and the G protein of group C streptococci (strep G protein) are commercially available for use as specific reagents to detect the presence of the Fc regions on human, rabbit, and mouse IgG molecules. An example is shown in Fig. 12.6A. Here, all the proteins from a virus-infected cell were fractionated and blotted (immobilized) onto a membrane to which they tightly stick.

This type of protein **transfer blot** is called a **Western blot** for a rather amusing reason. In the late 1960s and early 1970s, a scientist named Edward Southern developed a quantitative method for transferring gels of DNA fragments produced by restriction endonuclease digestion onto nitrocellulose filter paper. Such DNA transfer blots have ever since been called **Southern blots**. Subsequently, RNA transfer technology was developed and these type of blots were named **Northern blots** both to distinguish them from DNA blots and to establish similarity of the process. Protein blots were then named *Western blots* for comparable reasons.

In the example shown, the membrane and transferred proteins were incubated with antibodies to viral proteins. These antibodies stick only to those proteins that they "recognize." The blot was rinsed and incubated with ^{35}S-methionine-labeled staph A or strep G protein. This protein reacts with the antibody's Fc region and the area of immune complex is revealed.

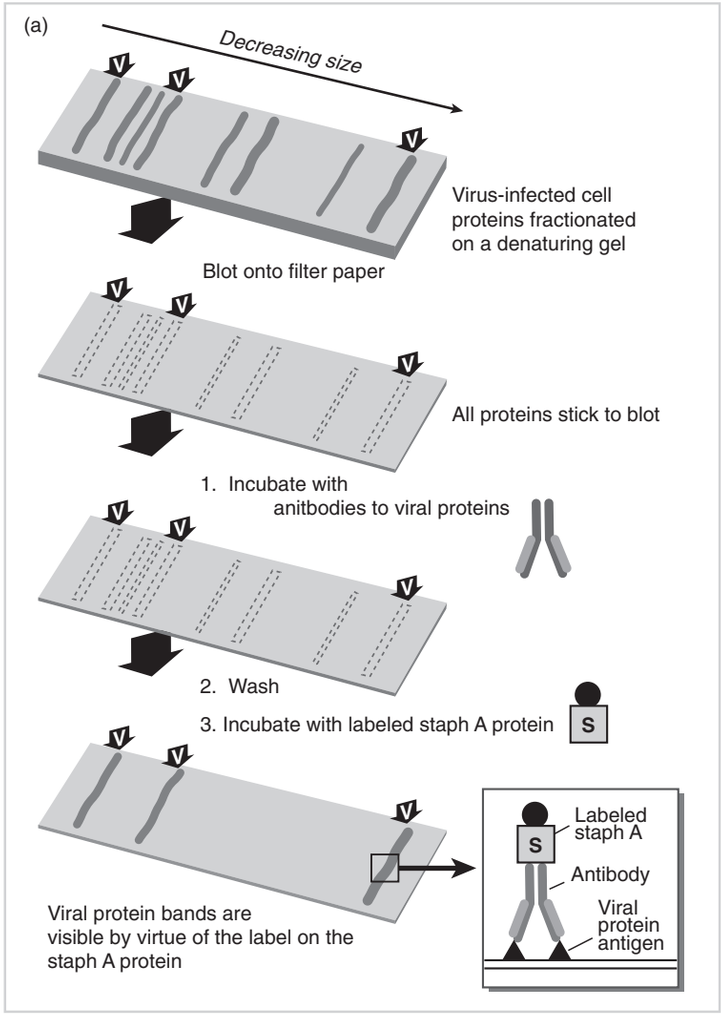

Fig. 12.6 Detection and isolation of proteins reactive with a specific antibody by use of immunoaffinity chromatography. *a.* Western blot. A mixture of viral and cellular proteins from an infected cell extract was fractionated on a sodium dodecyl sulfate gel, and the proteins blotted onto a membrane filter. The filter was then reacted with a specific antibody and washed, and then the antibody located by using radiolabeled staph A protein. *b.* The antibody and antigen mixture is incubated so that specific interaction occurs. This is followed by passing the whole mix through a column with staph A protein bound to the column matrix (sepharose). All antibody molecules bind through their Fc regions, and any antigen bound to them can be eluted with a gentle denaturation rinse that does not cause the staph A protein-Fc binding to be disrupted. *c.* A similar approach in which the antibody first is bound to the column matrix, and the proteins are washed over the column for binding. Both methods provide essentially equivalent results.

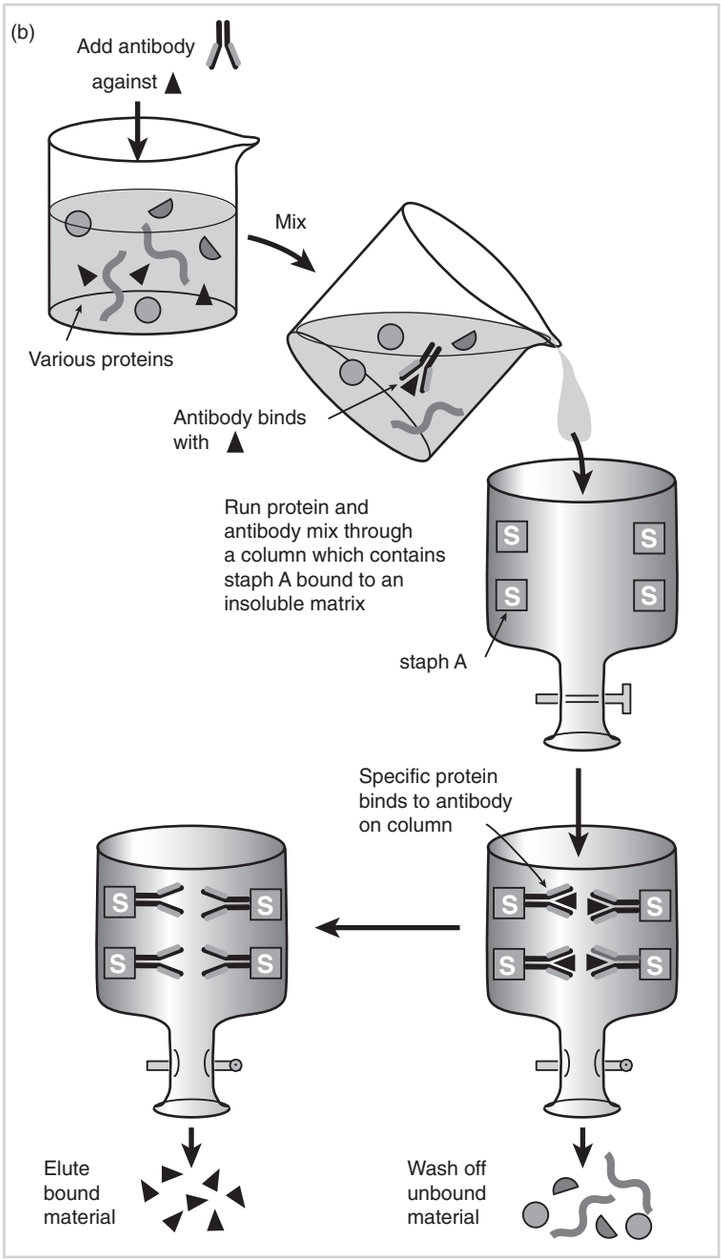

Fig. 12.6 *Continued*

Immunoaffinity chromatography Two variations on methods utilizing the binding of Fc regions to antibody molecules are frequently used to isolate specific proteins. Some methods using the affinity of staph A protein are shown in Figs. 12.6b and 12.6c. In Fig. 12.6b, an antibody against a protein in a complex mix is incubated with the protein mixture, and then passed through a sepharose column (a high-molecular-weight polysaccharide) to which the Fc-binding protein was chemically bound. All antibody molecules bind to the column, and any proteins that are bound to the antibody molecules also stick. All other proteins are washed off the column and discarded. Finally, the protein is eluted from the antibody, which is itself bound to the column via the Fc-

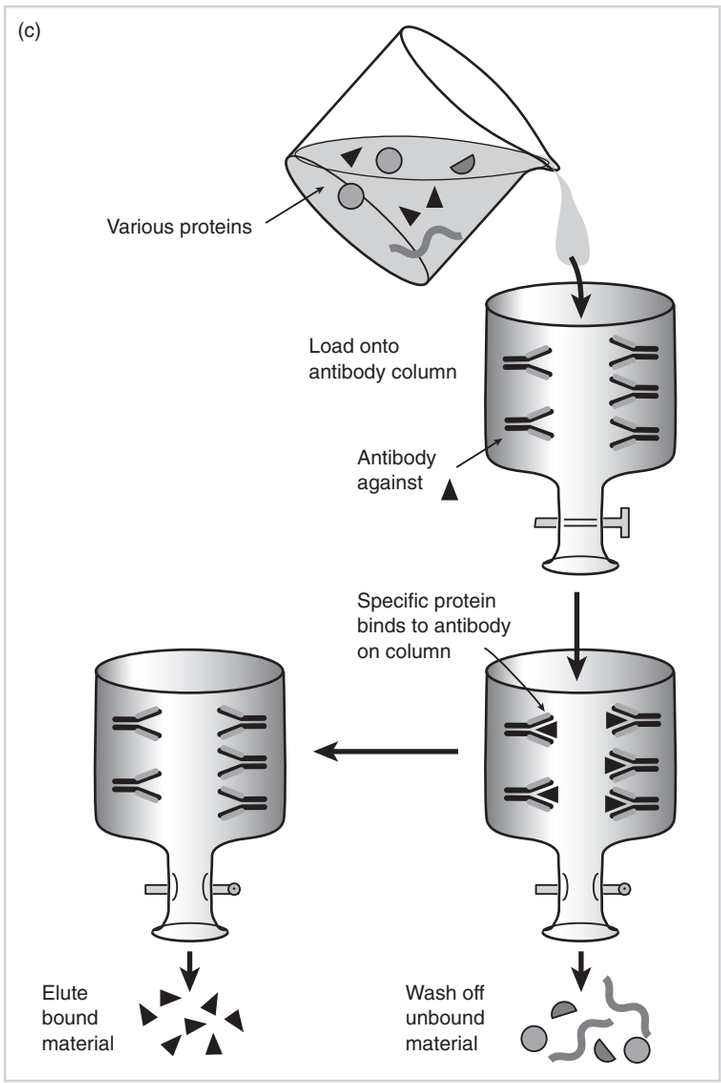

Fig. 12.6 *Continued*

binding region, using conditions that will not disturb the antibody's binding to the column, and the protein can be recovered in pure form.

In Fig. 12.6c, the antibody is first bound to the column. It then is allowed to react with antigen as the protein mix is washed through the column. It can be eluted later, after unwanted proteins are thoroughly rinsed away.

An example of one use of this method, to characterize a HSV mutant that does not express a specific glycoprotein (glycoprotein C), is shown in Fig. 12.7. Here, a polyclonal antibody against viral envelope proteins was prepared by immunizing rabbits. This antibody was allowed to bind to ^{35}S-labeled membrane proteins synthesized after infection with a wild-type and a gC$^-$ mutant of HSV. The total protein mix and the envelope proteins that bound to the antibody preparation then were fractionated on a gel and exposed to x-ray film. Absence of the protein in the mutant virus is quite evident.

These same methods can be used with antibodies against the Fc region of antibodies from a different animal. Use of such antibody-binding methods provides another degree of specificity (just as

did its use in immunofluorescence) and allows purification of even very small quantities of protein in a mix.

DETECTING AND CHARACTERIZING VIRAL NUCLEIC ACIDS IN INFECTED CELLS

Detecting the synthesis of viral genomes

Detection of viral DNA synthesized in an infected cell requires some method to separate viral material from the large background of cellular DNA. Since virus infection often shuts down cellular DNA replication, this might be accomplished by pulse labeling as described for detecting viral proteins. However, pulse labeling can lead to artifact because some viruses, such as the papovaviruses, actually stimulate cellular DNA replication upon infection. This problem can be overcome in a number of ways.

First, many viruses have genomes that can be separated readily from the bulk of cellular DNA using rate zonal centrifugation. For example, the circular genomes of papovaviruses are easily separated from larger cellular material on sucrose density gradients.

Another method that works well for many herpesviruses and other viruses with large genomes involves taking advantage of differences in the base composition of viral DNA as compared to cellular material. For example, HSV DNA has a base composition of 67% G + C while cellular DNA has a composition of 48% G + C. These differences result in the two DNAs having significant buoyant density differences in CsCl equilibrium gradient centrifugation. Here, a solution of DNA and CsCl is subjected to a high centrifugal force in an ultracentrifuge. Under these conditions, the high density of the CsCl in solution results in its forming a gradient of density with the most dense solution (as high as 1.75 gm/ml) at the centrifuge tube's bottom. Just as was shown for equilibrium banding of viral capsids, the DNA in such a solution will "float" to a region of the gradient that is equivalent to its buoyant density, and this band will be stable since the forces of buoyancy and sedimentation are balanced. The use of CsCl allows the formation of a gradient at the right density for DNA.

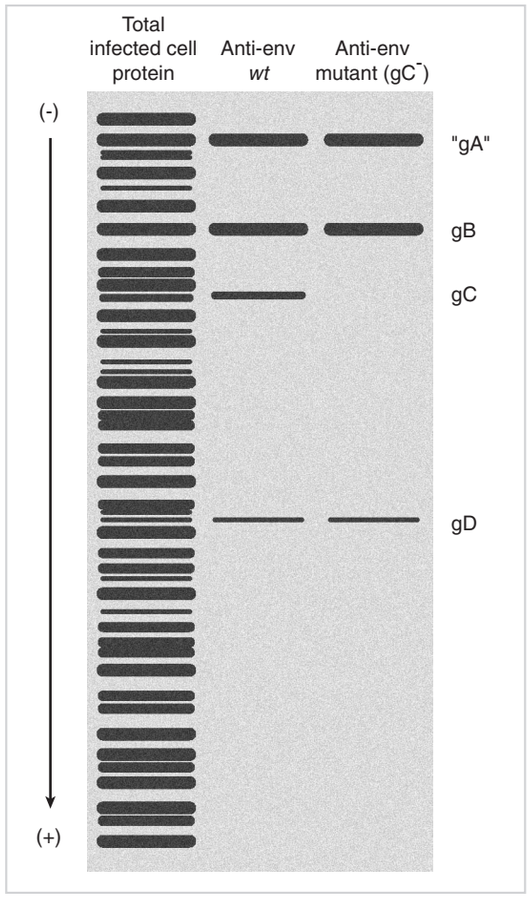

Fig. 12.7 Use of immunoaffinity chromatography to isolate HSV envelope proteins from infected cells. Total infected cell protein was labeled by incubation with radioactive amino acids. The protein then was mixed with a polyclonal antibody monospecific for viral envelope proteins. The reactive proteins were isolated as described in Fig. 12.6 and fractionated on a denaturing gel. The third column shows the results of a similar experiment where a virus unable to express glycoprotein C was used. (*wt*, wild type.)

A density gradient fractionation of HSV and cellular DNA along with a density marker is shown in Fig. 12.8. Since viral DNA can be separated from cellular DNA, its rate of synthesis can be determined readily by measuring incorporation of radioactive nucleoside precursor.

Perhaps the most convenient method for detecting viral DNA in a mixture of cellular material is through the use of restriction endonuclease digestion. If total DNA is isolated from infected cells and digested with one or a battery of restriction enzymes that produce specifically sized fragments from the viral genome, these can be readily gel fractionated and detected. Detection can be either by staining for the presence of DNA or by hybridization of a Southern blot of DNA with a radioactive probe of viral DNA. This latter will only hybridize to the viral fragments.

Detection of RNA virus genomes can be accomplished by virtue of the fact that the viral genome will have a discrete size that is different from any cellular RNA of high abundance, such as ribosomal RNA. Purification of infected cell RNA and size fractionation on a gel or sucrose gradient can then be used to detect the viral genome. If necessary, its identity can be confirmed by specific hybridization.

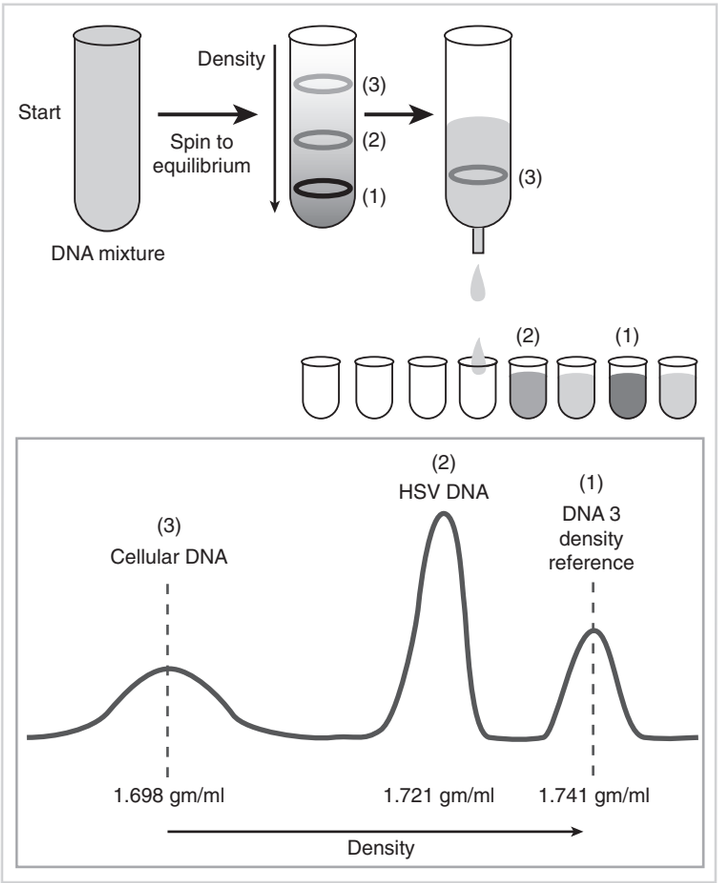

Fig. 12.8 Separation of HSV DNA from cellular DNA based on differences in base composition. The percentage of G + C residues in a given fragment of dsDNA will determine its buoyant density in CsCl. In the experiment shown, three DNA samples were mixed with a CsCl solution. One sample has a very high G + C content and serves as a density marker. HSV DNA has a lower density, but is significantly higher in G C content than cellular DNA (approximately 67% versus 48%). For this reason it has a greater buoyant density in an equilibrium gradient of CsCl. Unlike the equilibrium sucrose gradient shown in Fig. 11.1, CsCl solutions are so dense that the gradient will form under the centrifugal force available in an ultracentrifuge. Therefore, the mixture of DNA and CsCl is made and placed in a centrifuge rotor, and the mixture is allowed to form a density gradient by high-speed centrifugation. Following equilibrium, the various DNA fragments can be isolated by careful drop-wise collection of the gradient. The graph shows the position of the three DNA species at equilibrium.

Characterization of viral mRNA expressed during infection

Viral mRNA expressed during infection also can be analyzed and characterized using gel electrophoresis for size fractionation followed by nucleic acid hybridization. Without hybridization, detection of viral mRNA against the background of cellular RNA is difficult because individual mRNA molecules are not present in high abundance.

Hybridization requires a DNA or RNA probe that is complementary to the mRNA sequences to be detected. Such probes can be prepared readily by use of molecular cloning of viral DNA fragments in bacteria and one of a number of methods for making a radioactive probe. This use of recombinant DNA technology provides a convenient and inexpensive source of pure material in large quantities. Some basic methods for cloning viral DNA fragments are briefly outlined in Chapter 14. The basic hybridization method is essentially the same as the annealing of denatured double-stranded nucleic acid described in Chapter 11.

An experiment showing the different mRNAs expressed from two regions of the HSV genome is described in Fig. 12.9. In this experiment, mRNA was isolated from HSV-infected cells at 6 hours after infection. Aliquots then were fractionated by gel electrophoresis and blotted onto a membrane filter. Replicate blots were hybridized with radioactive total viral DNA probe, or with a probe made from cloned DNA fragments from specific regions of the viral genome (as shown).

In another experiment also shown in Fig. 12.9, HSV mRNA was isolated at two different times (3 and 8 hours) following infection. At 3 hours, viral DNA replication has not yet begun. At 8 hours

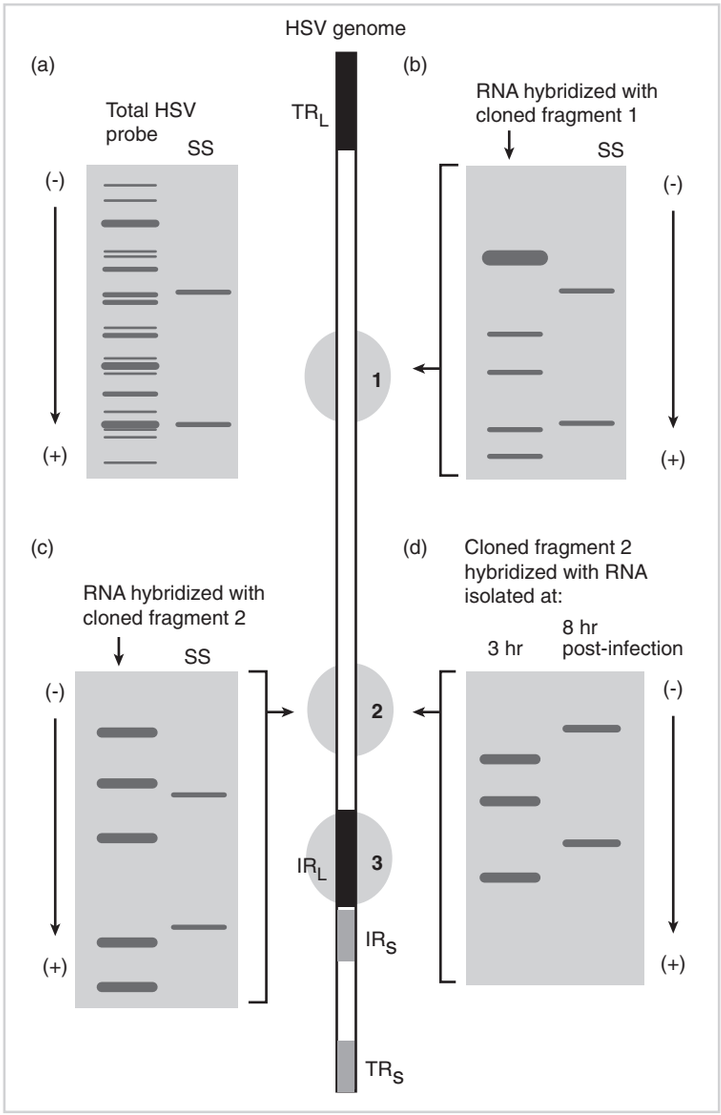

Fig. 12.9 Different viral mRNA molecules are encoded by different regions of a viral genome. The diagram shows the 150,000 bp HSV genome and the location of three cloned DNA fragments that can be used to hybridize to total infected cell RNA. More detailed information concerning the HSV genome and specific genes can be found in Chapter 18. A number of fractionation gels are shown. *a.* The total viral mRNA species expressed at 6 hours following infection. The RNA was isolated and fractionated, and a Northern blot made of the RNA. This was hybridized with radioactive viral DNA to locate the viral mRNA species. *b.* The RNA species expressed in region 1 by hybridization with radioactive DNA from this region only. *c.* The different RNAs seen with a probe for region 2. *d.* The RNA expressed from region 2 changes in character between 3 and 8 hours following infection (at the intermediate time shown in *C*, all species are being expressed). The lanes marked "SS" contain radioactive ribosomal RNA included as a size standard.

after infection, it is taking place at a high rate. The two RNA samples were fractionated by gel electrophoresis, subjected to Northern blotting, then hybridized with a fragment of radioactive DNA from a specific region of the HSV genome. One can see that the amount of RNA present at the 3-hour time point (early mRNA) is much reduced by 8 hours, and new—late mRNA—is present at this later time.

In situ hybridization

Hybridization of a cloned fragment of viral DNA to viral RNA (or DNA) in the infected cell can be achieved. The process is similar in broad outline to that for carrying out immunofluorescent analysis of antigens in a cell. In this type of hybridization, called **in situ hybridization**, the cells of interest are gently fixed and dehydrated on a microscope slide. Denatured probe DNA labeled with ^{3}H- or ^{35}S-labeled nucleosides is incubated with the cells on the slide, then the slide is coated with

liquid photographic emulsion that will detect radioactivity bound to the RNA or DNA of interest. The use of ^{3}H- and ^{35}S-labeled probes is favored because their decays are relatively low energy and the particle emitted is easily captured by x-ray emulsion near the site of its decay.

Alternatively, a nonradioactive reagent that produces a color under suitable conditions can be incorporated into the probe DNA. When the micrograph is developed and observed, areas of RNA or DNA hybridizing to the specific probe are visible.

An example of in situ hybridization is shown in Fig. 12.10. For this study, human neurons from an individual latently infected with HSV were taken at autopsy and sectioned. One set of sections was incubated with radioactive viral DNA probe from a region of the genome not expressed during latent infection, and another was incubated with a probe covering a region of the viral genome that is transcribed into **latency-associated transcripts** during latent infection. The nature of these latent-phase transcripts is described in more detail in Chapter 18. But here, it is necessary to point out that the positive hybridization signal is only seen with probes complementary to it.

Like immunohistochemical methods, in situ hybridization analysis can also be applied to larger scales. A histological section of a tissue or organ can be made, fixed, and then hybridized with an appropriate probe in order to locate areas where a specific viral transcript or viral genomes are being replicated. Indeed, the method can be applied to whole animals if they are small enough to allow sectioning.

The method was used by L. P. Villarreal and colleagues to determine the effect of site of infection on the involvement of organs in which mouse polyomavirus will replicate in a suckling mouse. An example of the approach is shown in Fig. 12.11. For this study, suckling mice were infected with polyomavirus by nasal or by intraperitoneal injection. After 6 days of replication, the mice were killed and carefully sectioned after freezing using a *microtome*, which is essentially a very sharp knife designed to cut thin sections of frozen or paraffin-embedded tissue. The slices were then placed on a membrane filter, stained, and hybridized with a radioactive polyoma-specific probe. The radioactivity was measured using a technique called *fluorography*, which is just a way of visualizing low-energy radiation.

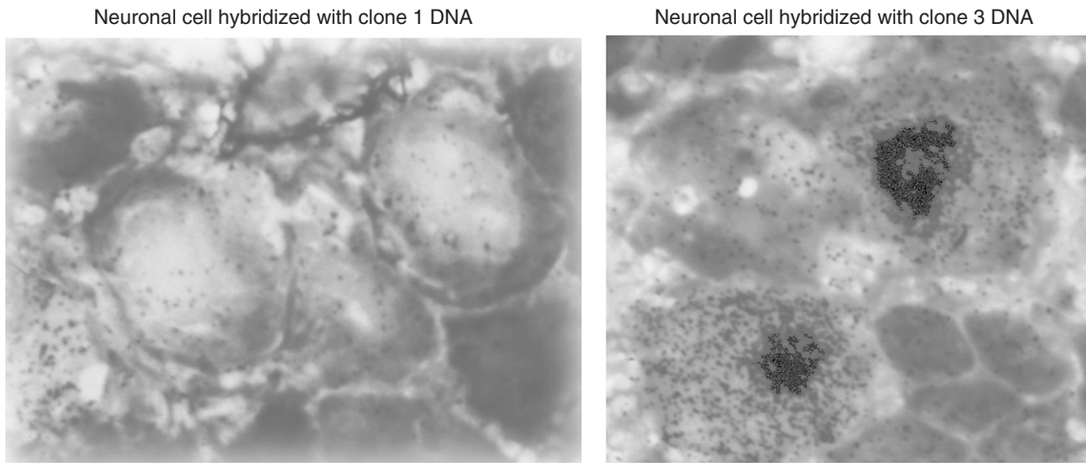

Fig. 12.10 In situ hybridization of human neurons latently infected with HSV. The trigeminal nerve ganglion was taken at autopsy from a middle-aged man killed in an automobile accident. The tissue was sectioned and individual slices incubated with labeled probe DNA from either region 1 or region 3 of the HSV genome shown in Fig. 12.9 under hybridization conditions. The left panel shows no hybridization with clone 1 DNA. The right panel shows positive hybridization with clone 3 DNA, due to the expression and nuclear localization of the HSV latency-associated transcript.

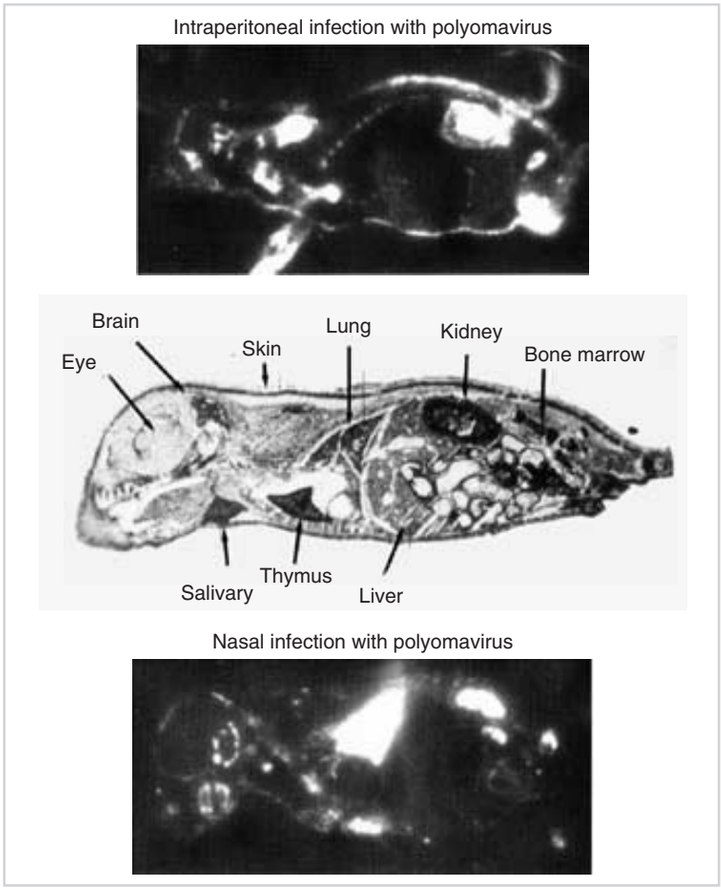

Fig. 12.11 In situ hybridization of sections of suckling mice infected with polyomavirus. A stained section showing the location of major organs of the mouse is shown in the center. Fluororadiographs of sections showing tissues in which virus is replicating are shown above and below this section. (Photographs courtesy of L.P. Villarreal.)

It is very clear from the figure that virus inoculated in the nose replicates mainly in the lung, kidney, and thymus. In contrast, virus infected into the animal's peritoneum replicates efficiently in the kidney, brain, and bone marrow.

Further characterization of specific viral mRNA molecules

Different viral mRNA molecules encode different proteins. This is shown by the technique of in vitro translation. For such an experiment, either total infected cell mRNA or a purified fraction of such RNA is combined with radioactive amino acids and mixed with an extract isolated from *rabbit reticulocytes*, which contain ribosomes and all other requirements for protein synthesis. Any synthesized proteins can be fractionated by size on denaturing gels, or the protein products reactive with a specific antibody or antibodies can be isolated using one of the techniques outlined in Fig. 12.7, and then fractionated.

An example shown in Fig. 12.12 demonstrates that the 6 kb mRNA detected with cloned DNA probe of HSV (fragment 1, shown in Fig. 12.9) encodes the 155 kd HSV capsid protein. In this experiment, the two mRNA species hybridizing to the specific region (6 kb and 1.5 kb) were subjected to in vitro translation in the same sample. The translation products were then tested for reactivity with a polyclonal antibody monospecific for this capsid protein to yield the results shown. It can be concluded that the large capsid protein must be encoded by the large mRNA because the smaller mRNA encoded in this region is not large enough.

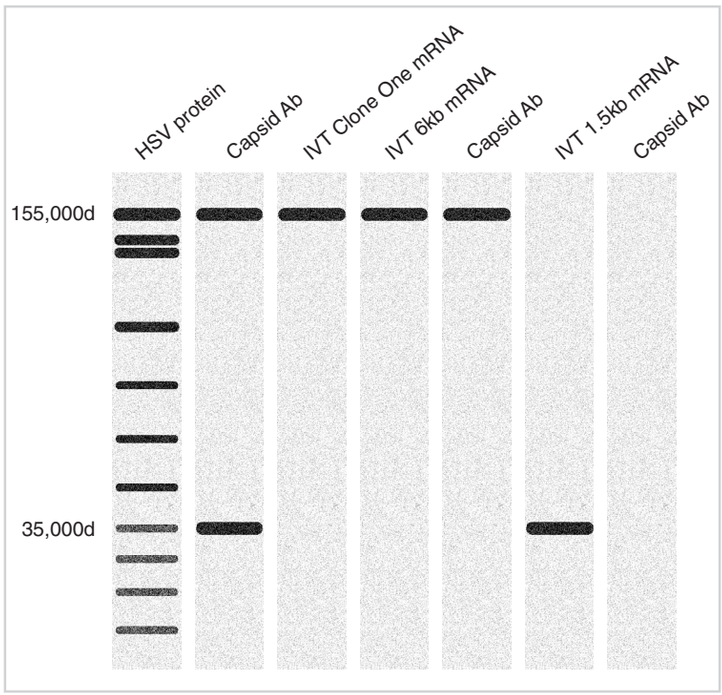

Fig. 12.12 Characterization of isolated viral mRNA by in vitro translation (IVT). Total protein labeled in a 1-hour pulse was isolated at 6 hours after infection from HSV-infected cells and fractionated on a denaturing gel. The capsid antibody (Ab) used in this experiment reacted specifically with only the 155 kd major capsid protein. The third lane shows the fractionation of protein synthesized in vitro using a rabbit reticulocyte system and mRNA hybridizing to DNA from region 1 of the HSV genome shown in Fig. 12.9. Two proteins are seen: one migrating at 155 kd and the other at 35 kd. Demonstration that the large protein is, indeed, the major capsid protein is made by use of the antibody, as shown in the other lanes.

Use of microarray technology for getting a complete picture of the events occurring in the infected cell

Early virologists called the time period between when infectious virus entered the host cell and when progeny virus was produced the **eclipse period** of infection because they could not readily determine what was going on using the techniques they had at hand. The experimental techniques outlined in this chapter have allowed modern virologists to visualize the eclipse period with the illumination of increasingly detailed knowledge. While the experimental analysis of virus infection takes time, money, and dedicated governmental interest, state of the art application of micro-robotic techniques, laser-guided detection of target macromolecules interacting with substrates, and computer-enhanced quantitative measurement of such interactions, collectively termed micro-array analysis, now provides the means of obtaining real-time measures of the intracellular environment as infection proceeds.

The basic idea behind micro-array analysis is quite simple, and one example is illustrated in Fig. 12.13. (See also Plate 6.) In the most common versions, a large number of very small samples of individual target molecules, either nucleotide sequences complementary to cellular and viral genes or peptides known or thought to interact with host and virus-modified proteins, are bound to an inert substrate such as glass or a nylon membrane. The smaller the dimensions of the spots, the more samples that can be spotted on the matrix. Currently, sizes as small as 80 μ can be spotted, which means that a microscope slide can accommodate 10,000 or more different samples that are in use. This matrix containing the test material, with each variant spotted in a known location, is known as a **microchip**.

The microchip is then incubated with a small sample of a solution containing mixtures of macromolecules known or suspected to interact with the chip substrates. This could be mRNA or cDNA synthesized from mRNA if the chip contained fragments of DNA, or it could be a mixture of proteins from infected cells if the chip contained antibodies or peptides known to

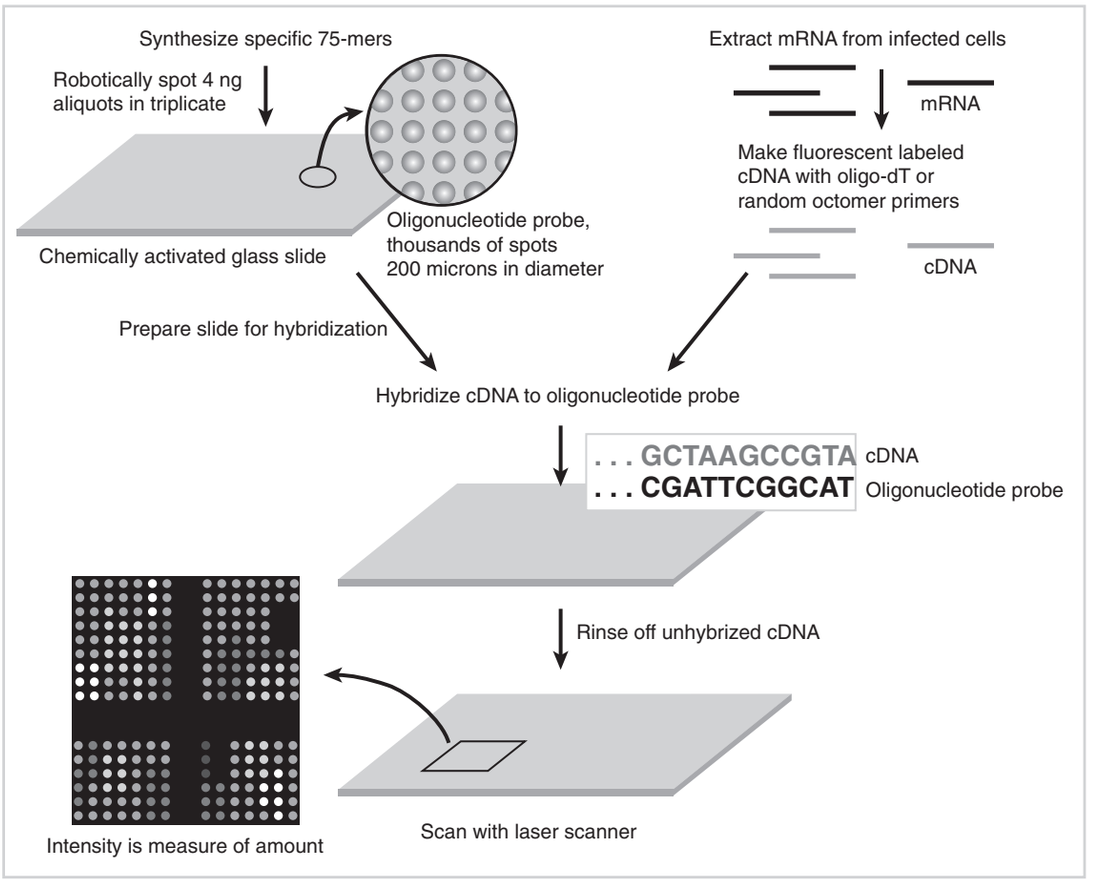

Fig. 12.13 The application of microarrays for the study of viral products produced in an HSV-infected cell. Oligonucleotides complementary to each viral transcript are bound to a glass slide along with oligonucleotides complementary to a number of diagnostic cellular transcripts. Samples of mRNA are isolated from cells under different conditions of infection, and cDNA copies are made using a dye-substituted deoxy-base; a different fluorescent dye is used for each condition. Then the cDNA is hybridized to the chip, unhybridized material washed away, and bound material is localized by scanning with a laser of a wavelength that only excites one or the other of the two dyes. The color and intensity of the signal in each spot can then be directly related to the amount of mRNA present in the original sample.

bind to a sub-set of the experimental mix. The volume of the experimental solution is kept very small by doing the incubation in a very small chamber. For example, if the probes were bound to a glass slide, layering a glass cover slip over the entire array could form the incubation chamber. Obviously, the tighter the patterning of spots in the array, the smaller the total volume needed. Ideally, a solution of materials from a few or even a single individual infected cell could be the source.

Following incubation and rinsing, interactions between chip probes and the experimental mixture added can be assayed by laser scanning. If a DNA chip was being used, fluorescent-tagged cDNA molecules made from the mRNA present in the infected cell mixture would only bind to complementary sequences on the chip after hybridization, and these could be detected by fluorescence upon laser illumination at the proper wavelength. If protein : protein interactions were under investigation, laser power could be adjusted to partially atomize some of the protein bound to each spot and its nature could be determined by mass spectroscopy.

QUESTIONS FOR CHAPTER 12

1 Antibodies against HSV-1 glycoproteins are tagged with a heavy metal in the Fc region. Virus is allowed to infect a cell, and immediately following this, the antibody is added. Then the cell is sectioned and an electron micrograph of this cell is taken. Where would you expect to see the heavy metal?

2 Which of the following methods can be directly applied to investigate the properties and characteristics of a viral protein?

a Electrophoresis in a sodium dodecyl sulfate polyacrylamide gel;
b Western blot analysis with specific antibodies;
c In situ hybridization with a specific antibody;
d Immunohistochemistry with a cloned DNA fragment;
e Determination of the sequence of the viral gene encoding it; and
f Nucleic acid hybridization

3 How is radiolabeling with amino acids used to examine the patterns of viral protein synthesis within infected cells? Give one specific example.

4 What are the ways in which a monoclonal antibody might be used in the analysis of a specific viral protein?