14 CHAPTER

The Molecular Genetics of Viruses

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Virus replication requires that the information encoded in the viral genome be expressed in the infected cell using cellular machinery. Despite this, many viral genomes are not made up of double-stranded (ds) DNA, the cellular genetic material. This and other factors have resulted in viruses evolving and maintaining various unique ways of fitting into the cellular flow of genetic information. The best way to tie all the various details together is to remember that *a virus must express at least some of its genetic material as mRNA that can be readily translated by unmodified translational machinery* so that the cell can translate this into viral protein. Such proteins then are able to convert the cell into a virus replication factory, or at least alter the cell in virus-specific ways.

The type of genome used by a virus defines the way mRNA is generated. As originally formulated by David Baltimore (Nobel Prize winner and the co-discoverer of reverse transcriptase), the way viruses must generate mRNA provides an operationally useful classification scheme, such as that described in Chapter 5. Such a scheme also provides a scientifically defensible and generally reliable means of predicting the relationship between two viruses. It is not too hard to see that two viruses that use very different genetic material, or that generate mRNA during infection in very different ways, may not be particularly closely related!

A second major benefit in organizing viruses by genetic material is that such an organization will allow mastery of the various mechanisms by which the virus gets the cell to replicate its genome. For DNA viruses, the pattern is often similar to cellular DNA replication; for RNA viruses, the patterns

are quite different since the cell does not use RNA as genetic material, and does not replicate RNA from an RNA template.

Viral genomes

Since all the information required for a virus to replicate itself ultimately must be maintained as genetic information in the viral genome, it is important to represent this genetic information in a standardized way. Generally, the viral genome is represented as a schematic of general genomic organization (i.e., single- or double-stranded nucleic acid, linear or circular, etc.), with viral genes and *cis*-acting genetic elements represented. The schematic represents, then, both a physical and a genetic map of the virus (a shorthand summary of encoded genomic sequence information).

Viral genomes can also be expressed in terms of *map units* (mu). Map units vary in size depending on the genome in question; in other words, a map unit is a relative term. The term still has a great deal of use when describing a genome, especially when locating a specific genetic function within a genome. Map units can be on a scale from 0.0 to 1.0 or from 1 to 100. If one knows the size of the genome in bases or base pairs, one can determine the size of a map unit for any genome.

If the genome is linear, one end is arbitrarily defined as the start of the map (0.0 map unit) and the other is the end (1 or 100 map units). If the virus has a circular genome, a specific site within the genome must be designated by convention as the start and endpoint of the map. This may be in relation to a known genetic element, or in some cases, it is defined as the site where a highly specific restriction endonuclease cuts the genome.

A relatively straightforward example is the genetic and transcription map of SV40 virus shown in Fig. 17.1. This 5243 base pair circular genome is cleaved once by the restriction enzyme *Eco*RI, which arbitrarily defines the start and end of the map. Since the map is divided into 100 map units, a single map unit is 52 base pairs. As another example, the HSV-1 genome is 152,000 base pairs, and the map is expressed from 0.0 to 1.0 map unit. Thus, 0.01 map unit for HSV would be 1520 base pairs. The HSV genetic map is shown in Chapter 18.

Most proteins found in a eukaryotic or prokaryotic cell—while more difficult to generalize—are larger than 100 but smaller than 1000 amino acids; therefore, one could estimate that a virus with a genome made up of 7000 bases could encode 10 to 20 "average"-size proteins.

Such estimates are only that, and will not include protein information encoded in overlapping translational reading frames. However, these estimates are very useful when trying to determine whether or not all the new or novel proteins seen expressed in a cell following virus infection are encoded by the virus. Further, it is possible to use such information to make inferences about whether certain viral proteins might be derived from precursors, or expressed unmodified.

Locating sites of restriction endonuclease cleavage on the viral genome – restriction mapping

As discussed in Chapter 8, restriction enzymes have a very high specificity for DNA sequence and thus, cut DNA in only a limited number of locations. Locating sites on a viral genome where restriction endonucleases cleave relative to other genetic "landmarks" is of fundamental importance because the enzymes can be used to specifically break the genome into discrete pieces. Further, these pieces can be individually *cloned* into one or another bacterial or eukaryotic plasmid vectors so that they can be produced in large amounts. Cloned fragments of DNA can then be used for specific modifications, for probes for detecting viral genes and gene products as described in Chapters 11 and 12, or for expression of viral proteins for a variety of uses.

Mapping restriction fragments within a viral genome is like solving a jigsaw puzzle. The fragments are shuffled around until a consistent fit is obtained. A simple example for a linear viral genome is illustrated in Fig. 14.1. Consider a viral genome with a size of 8 kbp, and two restriction

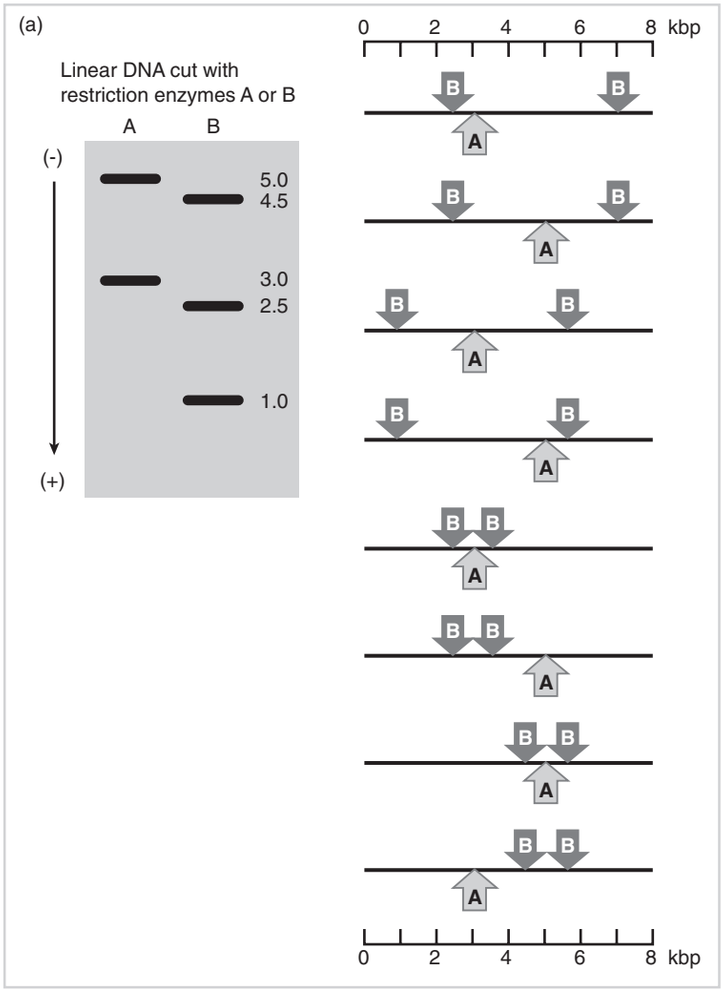


Fig. 14.1 Mapping restriction endonuclease cleavage sites on a viral genome. The basic methods for mapping restriction sites resemble those used in putting a puzzle together. Essentially, all possible combinations are tried until one is found that satisfies the results of all combinations of cuts with the restriction enzymes being mapped. *a.* The eight (8) possible arrangements of sites for enzyme A, which cuts the genome once, and enzyme B, which cuts the genome twice. Since the two enzymes can cut the DNA into three pieces, there are 2^3, or 8 possible arrangements. *b.* Cutting the DNA with both enzymes together followed by measuring the size of the resulting fragments eliminates all but two of the possible arrangements posited in *a. c.* Finally, a marker specific for one region of the DNA will allow choice of a single arrangement of sites.

enzymes (called "A" and "B" in the figure) that cleave this genome at a few sites. Cleavage of the viral DNA with enzyme A produces two fragments that are 5 and 3 kb and which can be readily separated by gel electrophoresis. This means that this enzyme cuts the DNA at only one site: either 3 or 5 kb in from one end. Cleavage of the same DNA with enzyme B generates three fragments that are 4.5, 2.5, and 1 kb. This means that this enzyme cuts the genome twice. A series of the eight possible arrangements of the sites in relationship to the ends of the viral genome and each other are shown in Fig. 14.1a.

Further analysis can resolve the problem into one correct arrangement of the sites as shown in Fig. 14.1b. First, cutting with both enzymes together results in the generation of four fragments: 4, 2.5, 1, and 0.5 kb. This means that the viral genome can have the restriction sites located in one of two possible arrangements vis-à-vis each other and its unique ends. Finally, if one end can be specifically labeled, say by locating a specific gene there, then a single, unique, arrangement can be deduced.

Since cleavage of a circular DNA molecule will result in its becoming linear, the same principles can be applied to mapping circular genomes. Also, depending on the size of the genome in question and specificity of the restriction enzymes being mapped, a few large fragments or many small

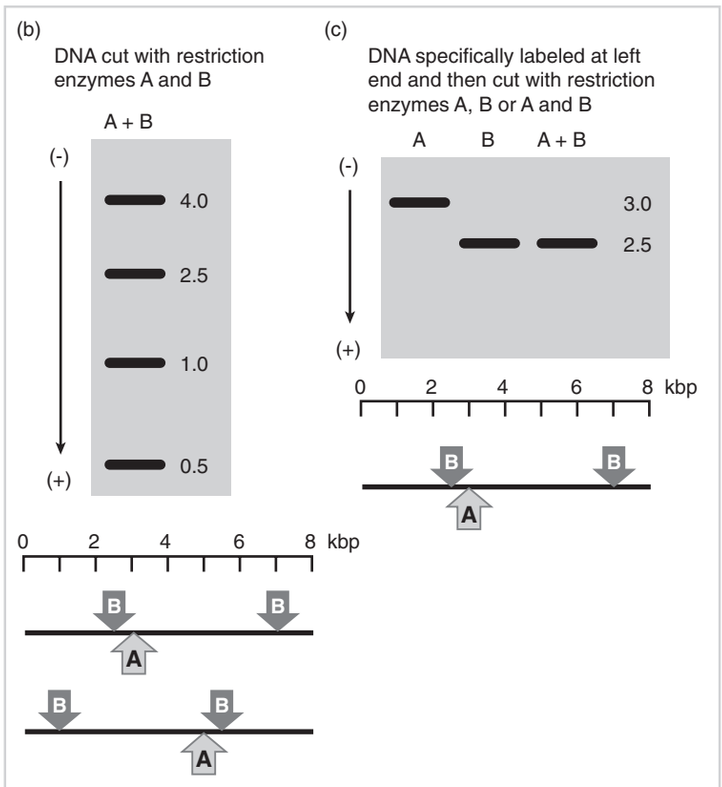


Fig. 14.1 *Continued*

fragments can be generated by digestion with restriction enzymes. Separate digestion of large fragments can be used to provide information about the arrangement of sites of enzymes that cut the genome more frequently, so that continuing the process as described can be used to build a map of any genome and any specific enzyme.

While restriction enzymes do not generally cleave single-stranded (ss) DNA or ssRNA, restriction maps can still be generated from genomes utilizing these types of nucleic acid. Single-stranded genomes can be enzymatically converted to double-stranded forms with DNA polymerase, and RNA genomes can be converted into DNA forms using reverse transcriptase isolated from retroviruses.

Cloning of fragments of viral genomes using bacterial plasmids

Generation of specific fragments of viral genomes with restriction enzymes allows them to be cloned and maintained separately from the genome itself. This process utilizes the fact that many bacteria maintain extrachromosomal plasmids bearing drug resistance markers (see Chapter 8). These plasmids contain a bacterial origin of replication and one or several genes encoding enzymes or other proteins that mediate the drug resistance. A number of plasmids can be grown to very high copy numbers inside bacteria, and many carefully engineered variants can be maintained in *E. coli* and are laboratory standards.

The genetic maps of three widely used plasmids, pBR322, pUC19, and pGEM, are shown in Fig. 14.2. Plasmid pBR322 was the first widely used high-copy-number plasmid in *E. coli*, and the other two plasmids have been specifically constructed to incorporate a number of convenient features. The pGEM plasmids, for example, contain promoters from T7 and Sp6 bacteriophages.

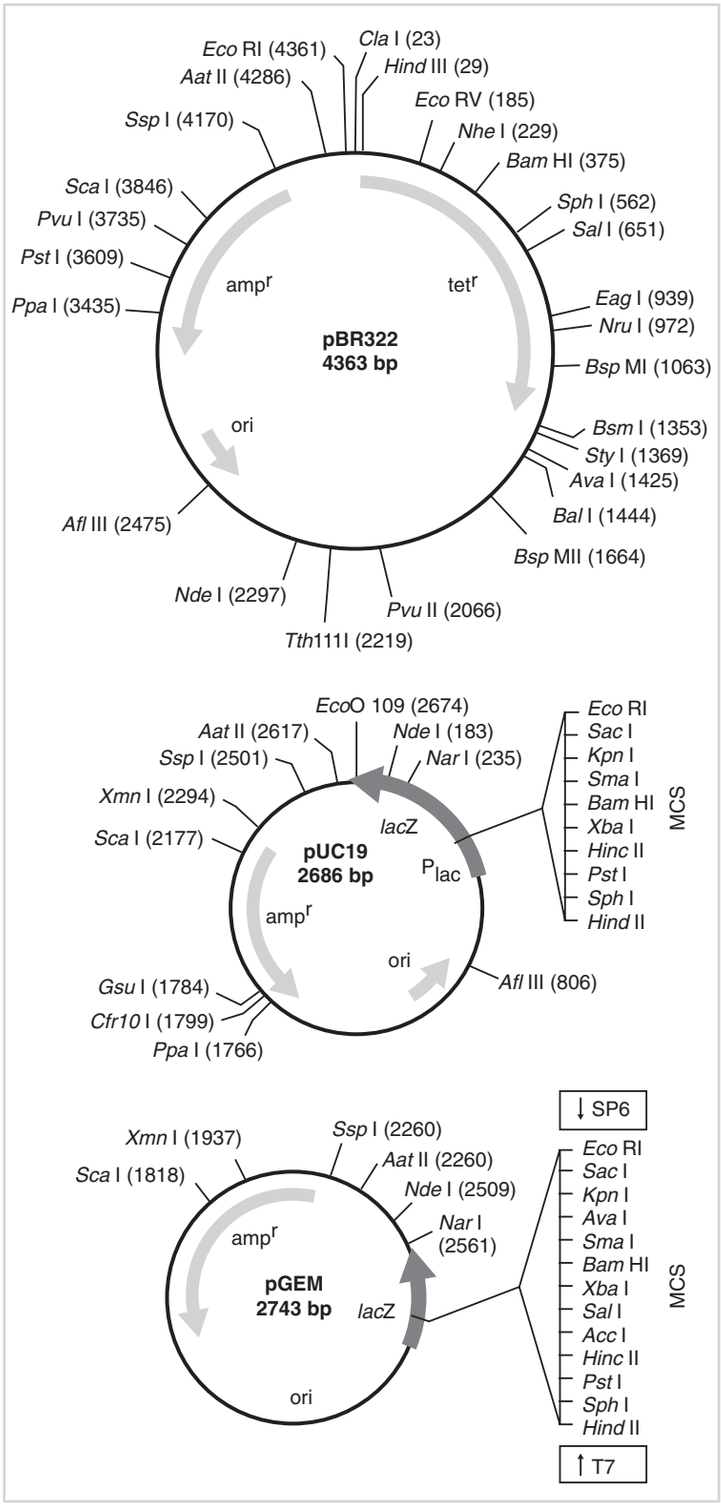


Fig. 14.2 Three widely used cloning plasmids that replicate in *E. coli*. These plasmids contain drug resistance markers that can be used for screening or selection, high-copy-number origins of replication, and genetic markers for screening. Different variants of pUC-based plasmids have different restriction sites in their multiple cloning sites. The pGEM plasmid contains two bacteriophage promoter elements that can be used in conjunction with commercially available bacteriophage-encoded RNA polymerases to make specific transcripts from the cloned sequences. (MCS, multiple cloning site.)

These promoters are absolutely specific for virus-encoded RNA polymerases; therefore, one can selectively transcribe RNA from cloned genes in either direction.

These and most other plasmids have four important general features. The first feature is the *cis*-acting origin of replication, which allows plasmid replication in the carrier cell. This origin of replication determines the actual number of plasmid genomes produced in each bacteria. Usually, one wants to maximize this number, but low-copy-number origins are useful for specialized purposes (e.g., for maintaining large segments of cloned DNA).

The second component is one or more drug resistance markers that can be used to allow the bacteria in which the plasmid is present to grow in the presence of a drug such as ampicillin or tetracycline that inhibits growth of *E. coli*, which does not contain the plasmid. This is a *selectable* genetic marker.

The third feature is the presence of one or more restriction sites that can be used for cloning the viral (or other) DNA fragment of interest. Both the pUC and pGEM plasmids have sites into which a large number of specific restriction enzyme cleavage sites have been incorporated. Such **multiple cloning sites (MCSs)** are very convenient for cloning a variety of fragments.

The fourth component of the plasmid is an enzymatic marker that can be used as a genetic "tag" to differentiate the plasmids containing the cloned fragment from those that do not.

A favorite marker seen in the pUC and pGEM plasmids is the bacterial β-galactosidase enzyme, which can turn a colorless substrate blue and thus cause bacterial colonies in which the enzyme is present to become blue under the proper growth conditions. When a fragment is cloned into the MCS, this enzyme is inactivated. This property allows one to rapidly **screen** a plate for the presence of either blue or white colonies in the background of the other.

The pBR322 plasmid does not have the β-galactosidase as a screenable marker. Despite this, there are a number of unique cloning sites within one or the other of the drug resistance markers, and the ability of a bacterial colony to grow in the presence of one drug but not in the other can be used to screen for inserts.

To clone a fragment of viral DNA, a suitable plasmid is purified and cleaved with a restriction enzyme that interrupts a screenable marker. Then this linearized fragment is mixed with a fragment of DNA that has been cleaved from the viral genome with the same (or occasionally another) restriction enzyme. The two fragments are then ligated using bacteriophage T4 DNA ligase to form a plasmid in which the desired fragment has been inserted. The resulting mixture of religated plasmid without fragment, plasmid with fragment, and other pieces of DNA that may be ligated are then mixed with bacterial cells under conditions in which cells will efficiently take up and internalize large circular fragments of DNA. This process, which is (unfortunately) often called transformation in bacteria, is just transfection—briefly described in Chapter 6.

Individual bacterial cells are then plated on selective media and those able to grow in the presence of the drug will form colonies. It should be clear that such colonies must have come from bacteria that have incorporated either modified or unmodified plasmids, since all other bacteria will be drug sensitive. Finally, the colonies are screened either for their inability to produce blue colonies in the case of the β-galactosidase screening marker or for their inability to grow on tetracycline or ampicillin in the case of the pBR322 plasmid.

Ligation of the fragments into the plasmid is often aided by the fact that restriction enzymes cleave at palindromic DNA sequences to generate a break in the DNA with a short stretch of complementary single-stranded DNA at each end. For example, in the following sequence, where N is any nucleotide and lowercase indicates the complementary base on the antiparallel strand, the restriction enzyme *Eco*RI cleaves at the sequences:

5′-NNNNNG*AATT C NNNNNN-3′
3′-nnnnnc ttaa*gnnnnn-5′

to produce ends with 5′-single-stranded overlaps:

5′–NNNNNG–3′ 5′–*AATTCNNNNN–3′
3′–nnnnncttaa*–5′ 3′–gnnnnn–5′

The overlapping sequences allow any DNA fragments digested with this enzyme to be annealed together at the complementary overlaps and then religated to form a complete DNA strand. Note that this ligation will regenerate the restriction site at both ends of the insert:

Plasmid 5′–NNNGAATTCNNN–3′
3′–nnncttaagnnn–5′

Insert: 5′–***NNGAATTCCACGGTCAGCGATTCNN***–3′
3′–***nncttaaggtgccagtcgctaagnn***–5′

Cleavage:

Plasmid 5′–NNNG AATTCNNN–3′
–nnncttaa gnnn–

Insert: 5′***AATTCCACGGTCAGCG*** –3′
ggtgccagtcgcttaa 5′

Religation and generation of two *Eco*RI sites:

5′–NNNG***AATTCCACGGTCAGCG***AATTCNNN–3′
3′–nnncttaa***ggtgccagtcgcttaa***gnnn–5′

Some enzymes produce blunt ends, but mixing relatively high concentrations of such blunt-ended fragments together at relatively low temperature can optimize ligation. Even where two different restriction enzymes are used to generate the open plasmid and the DNA fragment, any overhanging ends can be filled in with the appropriate enzymes to produce blunt ends, and these can be ligated together. When such different enzymes are used, however, the restriction site will be lost when the ligation takes place.

An example of cloning a DNA fragment is outlined in Fig. 14.3. In this experiment, HSV DNA was digested with the restriction enzyme *Sal*I, which cleaves at the sequence GTCGAC and generates more than 20 specific fragments ranging in size from larger than 20 kbp to less than 1 kbp from the 152 kbp HSV genome. A portion of the restricted DNA with a size range of approximately 4 to 9 kbp was then ligated with pBR322, which had also been cut at its single *Sal*I site, which is within the tetracycline resistance gene. The mixture was then ligated, and transfected into *E. coli*. Cells were plated on nutrient agar plates containing ampicillin, and nine colonies were chosen at random for screening. Each was stabbed with a sterile toothpick and bacteria were then **replica plated** by streaking the toothpick onto a nutrient agar plate containing ampicillin and then onto a nutrient agar plate containing both ampicillin and tetracycline.

Bacterial colonies growing on the ampicillin-containing medium, but not the medium containing both ampicillin and tetracycline, were then chosen and further characterized. In the figure, plasmid from a tetracycline-sensitive colony was extracted, digested with *Sal*I, and fractionated on an agarose gel by electrophoresis next to a sample of the HSV DNA that had been digested. Two DNA species are present in the digested plasmid: the 4363 bp pBR322 molecule, and a 6.3 kbp DNA fragment corresponding to a specific fragment of HSV DNA. This DNA then can be characterized further by sequence analysis, and so on.

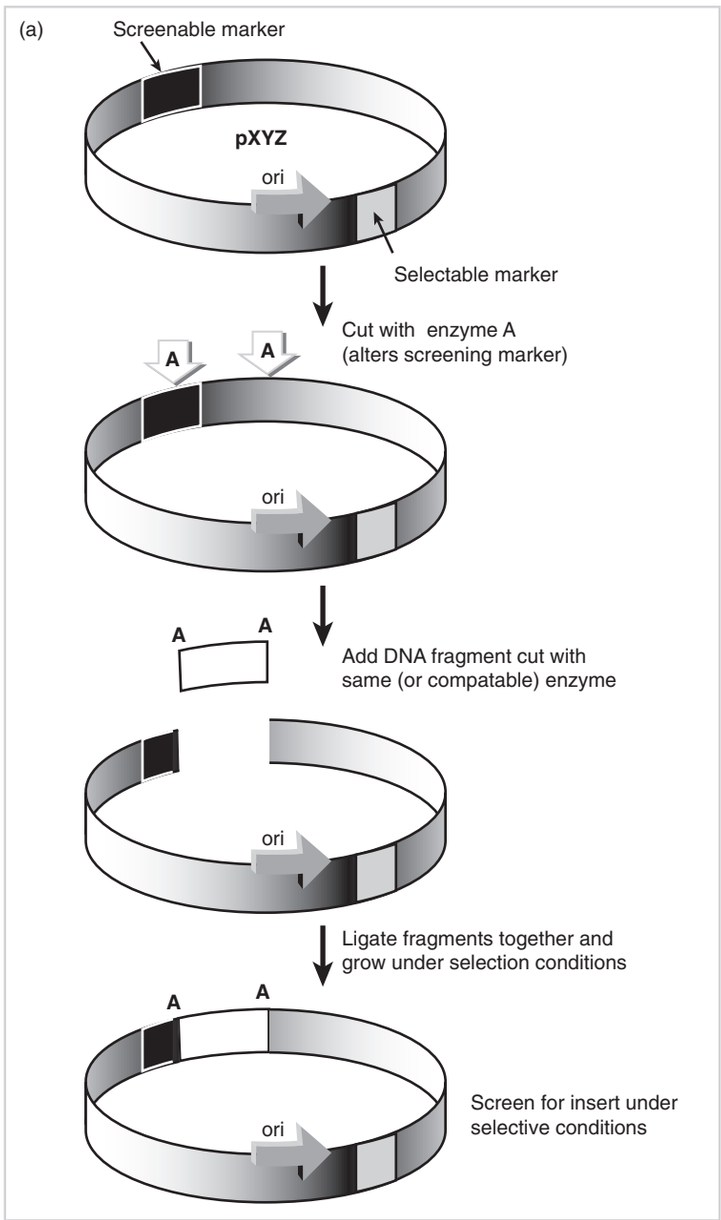


Fig. 14.3 Isolation of a specific restriction fragment of viral DNA cloned into a bacterial plasmid. *a*. Outline of the process of cloning a DNA fragment into a plasmid using selection and screening. *b*. Cloning of a 6.3 kbp HSV DNA fragment. The electrophoretic separation of 20 µg aliquots of *Sal*I-digested HSV DNA, *Hind*III-digested bacteriophage λ DNA, and a pBR322 plasmid containing the cloned fragment are shown. The digestion of λ DNA with *Hind*III generates the six (6) fragments ranging from 23 to 2 kbp in size, and these serve as a convenient size marker. The screening of individual ampicillin-resistant colonies of bacteria that were formed by transformation (transfection) of a sensitive strain with pBR322, which had been ligated with a mixture of HSV DNA fragments, is shown. The cloned DNA fragment was isolated by *Sal*I digestion of plasmid DNA isolated from a bacterial colony that is ampicillin resistant but tetracycline sensitive.

GENETIC MANIPULATION OF VIRAL GENOMES

Mutations in genes and resulting changes to proteins

Sometimes, as nucleic acids replicate, a mistake occurs. This is a very rare event in organisms, but in viruses that replicate so rapidly and whose replication enzymes are often error prone, such changes occur with appreciable frequency. With some viruses, like HIV, the polymerase can generate one mistake for every 10,000 bases transcribed so that many changes are generated. Indeed, as outlined in Chapter 20, some of these changes have a role in the virus being able to avoid the body's immune defenses.

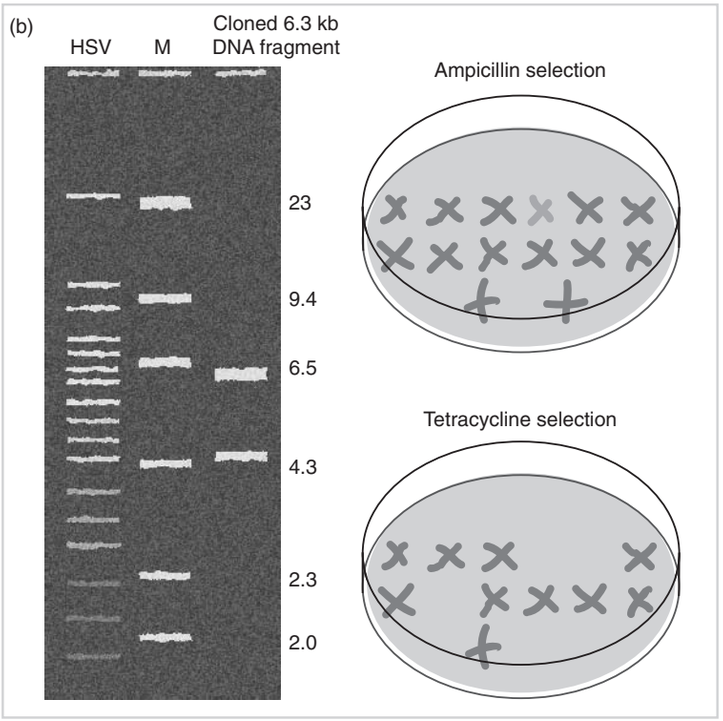


Fig. 14.3 *Continued*

Changes in the genome usually result from insertion of the wrong base during genome replication. For example, a mismatched A : C pair could be formed during replication of DNA instead of A : T, or an A : T pair could be miscopied into G : T. More rarely, a piece of nucleic acid could be lost due to some slippage of polymerase.

Such changes in genomes are **mutations** and can have effects on the function of the gene in which they occur. Despite this, mutations often lead to very minor changes in proteins. Over very many generations (usually over long time spans), these changes accumulate and lead to the formation of related but distinct organisms. In viruses, changes like these lead to the generation of small antigenic differences between strains or serotypes.

The effect of a mutation on the function of a protein can be profound if that protein is vital to the replication of a virus or has a very critical structure. Sometimes the change can be beneficial to the virus. For example, a viral DNA polymerase might be mutated so that the enzyme is no longer sensitive to a nucleoside analogue; hence, the virus is not able to be controlled by an antiviral drug using this analogue.

Some examples of mutations are shown below where a hypothetical, very short, unspliced open reading frame is mutated. Only the mRNA sense strand is shown, but remember that the DNA is double stranded:

Gene: ATG-GTT-GAT-AGT-CGT-TAT-TTA-CCT-CAA-TGG-CAG-TAA
Protein: Met-Val-Asp-Ser-Arg-Tyr-Leu-Pro-Gln-Trp-Gln

A mutation in the seventh codon (TTA) to TTC would lead to the substitution of phenylalanine for leucine. Such a change might affect the way the protein folds and change its function.

Met-Val-Asp-Ser-Arg-Tyr-*Phe*-Pro-Gln-Trp-Gln

A mutation in this same codon to GTA would lead to a protein with one aliphatic amino acid changed for another; this might have no effect at all on the protein's structure.

Met-Val-Asp-Ser-Arg-Tyr-*Val*-Pro-Gln-Trp-Gln

A mutation in this codon to TGA (a stop codon) would lead to a shorter protein; this would almost certainly destroy the protein's function, and if very close to the N-terminal amino acid, would result in the loss of all protein from the gene. Such translation termination mutations are sometimes termed *amber*, *ocher*, or *opal* mutations by geneticists for essentially historical reasons.

Met-Val-Asp-Ser-Arg-Tyr!

A mutation in the second codon (GTT) to GAT would lead to a change in protein charge, substituting asparagine for valine. This would almost certainly significantly alter the function of the protein:

Met-*Asp*-Asp-Ser-Arg-Tyr-Leu-Pro-Gln-Trp-Gln

All such mutations could be mutated further or mutated back by another base change. Such mutations are called *revertible*. If, however, a base were lost, the change is essentially permanent. Indeed, this type of mutation (addition or loss of genomic material) can be inferred if a mutation has essentially no ability to revert.

An example of a nonrevertible change can be seen if the first two bases of the second codon were lost. This **frame shift** mutation would lead to loss of the protein because the second codon is now a translation stop codon:

ATG-***TGA***-TAG-TCG-TTA-TTT-ACC-TCA-ATG-GCA-GTA-A

Other frame shifts can completely change a protein, and thus can completely change or lose the protein's function.

Analysis of mutations

Complementation Many mutations lead to significant differences in virus plaque morphology, growth rates, host range, cytopathic effects, interaction with the host immune system, and so on. Thus, one can distinguish a mutant virus from a normal or wild type (*wt*) parent. Mutant viruses are useful because the lack or alteration of the function of a specific viral gene can lead to changes in virus replication, among other things, and this generates information about the function of mutated genes.

An example of the use of **complementation** to maintain a replication-deficient virus mutant is shown in Fig. 14.4. If a virus, P^-, with a mutation in its polymerase gene, is coinfected into a cell with a virus, C^-, containing a mutation in a capsid protein, for example, each virus can supply the function that the other virus is missing. Complementation allows enumeration of important genes in a virus and manipulation of mutant viruses. *In complementing infections, you get back the same virus that you put in.*

Recombination Sometimes, two viruses that have different mutations can physically entangle each other during the infection process. This close association can lead to formation of a *recombinant* viral genome. The precise mechanism for the formation and resolution of recombinant genomes is still being investigated, but in many cases, the process happens most frequently as

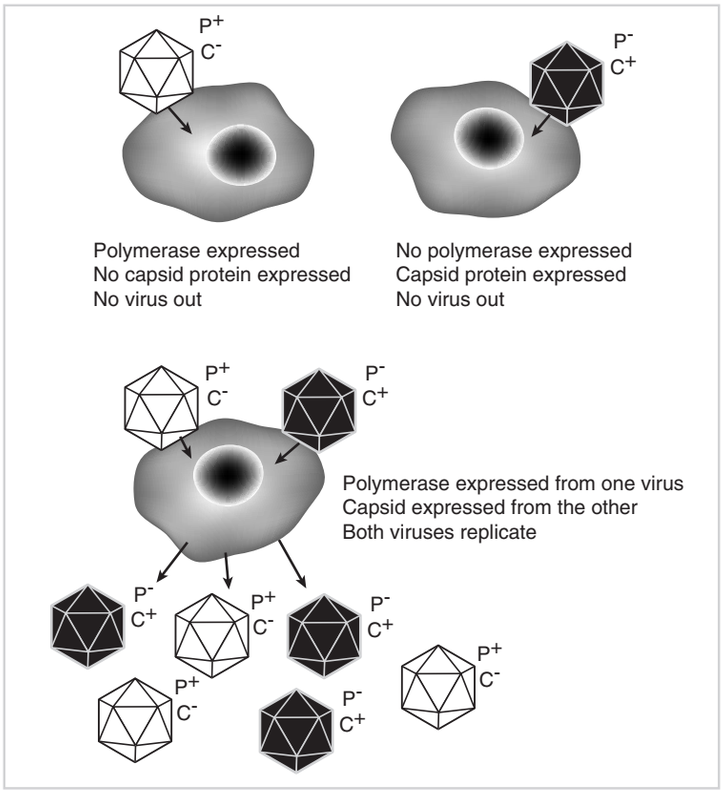


Fig. 14.4 Complementation. Neither of two mutant viruses shown can replicate because each contains a lethal mutation in a required gene encoding an enzyme or structural protein. Still, if the two mutant viruses are infected into the same cell, each can supply functions missing in the other. This means that the infected cell will have all the necessary viral gene products for the replication of both mutant viruses – they can complement each other's growth in a mixed infection.

genomes are replicating. Also, the farther apart on the parental genomes the mutations lie, the more probability there is of forming a recombinant. Even so, closely separated mutations — even two different ones separated by only a few base pairs within a single gene — can recombine, albeit rarely.

A recombination event leads to two novel viral genomes (one has both mutations, one has neither and is, thus, *wt*). Such an infection then leads to generation of four types of virus: two single mutant input viruses, *wt*, and double mutant virus from recombination. An example using HSV is shown in Fig. 14.5. A virus with a temperature-sensitive (*ts*) mutation in DNA polymerase (U_L30 Pol^{ts}) is coinfected with a virus with a mutation in the thymidine kinase gene (U_L23 TK^-). Thus, one parent virus is not able to grow at an elevated (nonpermissive) temperature, and the other parent is resistant to inhibition of DNA synthesis with a nucleoside analogue.

Recombination results in four different viral genotypes being produced from the mixed infection: *wt*, double mutant (Pol^{ts}, TK^-), and the two single mutant parents. Each genotype will produce plaques that can be distinguished from the others with proper **screening** or **selection** technique. Replica plating of virus plaques and incubation under different conditions can distinguish all genotypes.

In the experiment, progeny viruses are plated and plaques allowed to form. Then four identical replicas are made on other plates. These are incubated under various conditions that allow the genotypes of the viruses to be distinguished:

1 At high (nonpermissive) temperature, only *wt* and TK^- will make plaques.
2 At normal (permissive) temperature, *wt*, Pol^{ts}, TK^-, and $Pol^{ts}TK^-$ all make plaques.
3 At permissive temperature with an antiviral nucleoside analogue drug, only virus lacking the TK gene (TK^- and $Pol^{ts}TK^-$) will make plaques.

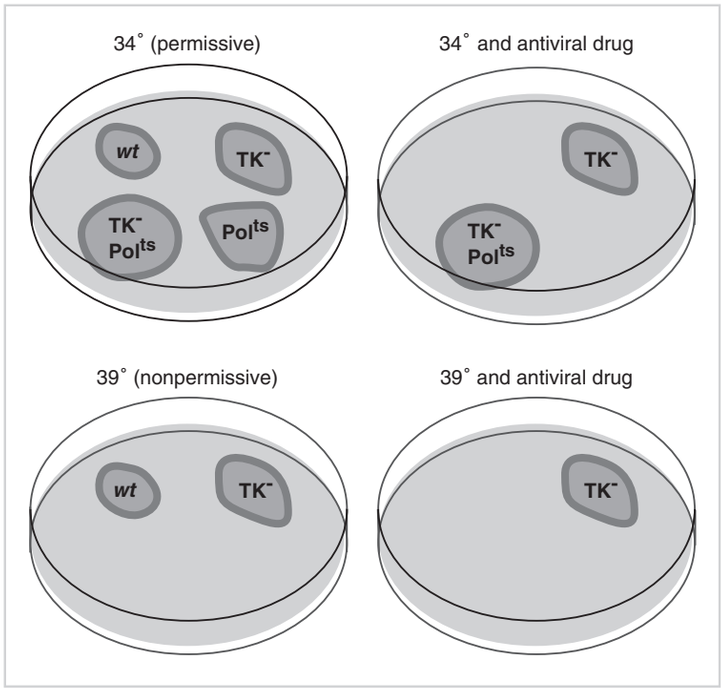


Fig. 14.5 Replica plating of virus plaques to distinguish genotypes produced by recombination following a mixed infection. Mixed infection of two genotypes will (rarely) produce novel genotypes by recombination. The example shows replicas of virus plaques that were developed under different selective and screening conditions as described in the text.

4 At nonpermissive temperature with antiviral drug, only the mutant (TK^-) virus will form plaques.

Isolation of mutants

Selection If a mutation in a virus leads to a significant alteration in growth properties, or resistance to a drug, or resistance to a neutralizing antibody, then the mutant can be selected by growth under conditions where the *wt* virus will not replicate or will replicate poorly. This is an ideal situation for isolation of viruses, but unfortunately, it is not easy to come up with appropriate selective conditions for many desirable mutations. In animal cells (as in bacteria), many mutations change aspects of the virus but do not markedly change growth properties.

HSV thymidine kinase – a portable selectable marker

The ability of the HSV TK^- mutants to replicate in the presence of an antiviral drug is an example of a selective advantage for the virus under these conditions of growth. Further, a selection scheme that was discussed in Chapter 12 allows for a selective advantage for growth of TK^+ organisms. The easy selection of cells that are TK^+ or TK^- is an important tool because the HSV TK gene can be removed from the viral genome and used essentially as a portable selectable marker.

The HSV TK gene has been exploited for the past two decades to apply the power of selection to generation of recombinant viruses and cells bearing foreign genes. This is a result of the fact that the HSV TK gene is of convenient size (it is contained within a small piece of DNA—2.3 kbp) that can be isolated and readily cloned from the HSV genome. This gene contains both the structural gene for the enzyme and the promoter controlling expression of the mRNA encoding this structural gene. Of equal significance, this promoter is expressed in uninfected cells and in viruses other than HSV.

To use the HSV TK gene for selection, a cell line that is constitutively TK^- is constructed. This is accomplished by culturing cells in the presence of a toxic nucleoside analogue, as has been described in the selection process for hybridoma cells outlined in Chapter 12 and Fig. 12.3. Since only TK^- cells can replicate under these conditions, the cell lines can be readily established.

To construct a recombinant virus or cell line, one can incorporate the HSV TK gene into a plasmid bearing the gene of interest for transfection, or a TK plasmid can be added to the plasmid of interest. The process of transfection strongly favors cells taking up relatively large aggregates of DNA, so both plasmids will be incorporated.

After recovery from the transfection process, those cells or viruses that have undergone recombination with the added genome, and thus are TK^+, can be selected. For the selection of recombinant virus, the total virus recovered from the original transfection can be used to infect TK^- cells under selective conditions; only the viruses that express TK will be able to replicate because only those cells will survive the selection.

The TK selection technique can also be used in the generation of recombinant HSV. This follows from the fact that the TK gene is dispensable for HSV replication in most cells. Thus, a cloned plasmid of recombinant DNA that contains HSV sequences from either side of the TK gene recombined with the gene of interest, which replaces the viral TK gene itself, can be cotransfected with *wt* virus DNA. When the progeny virus of this transfection is used to infect TK^- cells being grown in the presence of the toxic nucleoside analogue, all cells infected with TK^+ parental virus will die. Thus, plaques formed will be greatly enriched for TK^- virus bearing the inserted gene of interest.

Screening

If the mutation leads to an observable change in the plaque morphology or some other aspect of infection, the virus mutant can be screened or picked. Here, one looks for the change and picks out the viruses with it. For example, a mutation in a single gene might alter the efficiency of packaging of virus and thus, lower the burst size of virus from an infected cell. This mutant virus would make smaller plaques than would the *wt* parent, and one could easily pick out either large or small plaque-producing virus in a background of many of the others.

Of course, the HSV *ts* mutants described earlier, which do not replicate at 39°C, are also examples. Since they *do not* replicate under restrictive growth conditions, they cannot be selected for, but they can be picked or screened by going back to the replica plaque that does form at 34°C.

DELIBERATE AND ACCIDENTAL ALTERATIONS IN VIRAL GENOMES AS A RESULT OF LABORATORY REPLICATION

Virulence and attenuation

As noted in Chapter 7, many of the mutations accumulated by viruses lead to changes in virulence of the virus, that is, changes in the severity of symptoms and course of viral infection in the host. Such changes occur when a disease-causing virus is passaged for long times in cell cultures or in a nonnatural animal host. This attenuation of virus strains allows the generation of live-virus vaccines as well as providing convenient sources of viruses safe enough to use in the laboratory.

Generation of recombinant viruses

Many techniques of molecular biology allow us to make directed mutations in specific viral genes, and to generate recombinant viruses bearing foreign or specifically altered genes. Recombinant

viruses can be used to study the specific blockage of replication caused by interruption of a particular viral gene. If an "indicator" protein such as bacterial β-galactosidase is recombined into a virus, the presence of the virus in specific histological sections of tissue could be determined by localization of a diagnostic enzymatic reaction. A very important potential use of recombinant viruses is to introduce specific genes into tissues or tumors for therapeutic uses. Some examples of this latter use are outlined in the last chapter of this book.

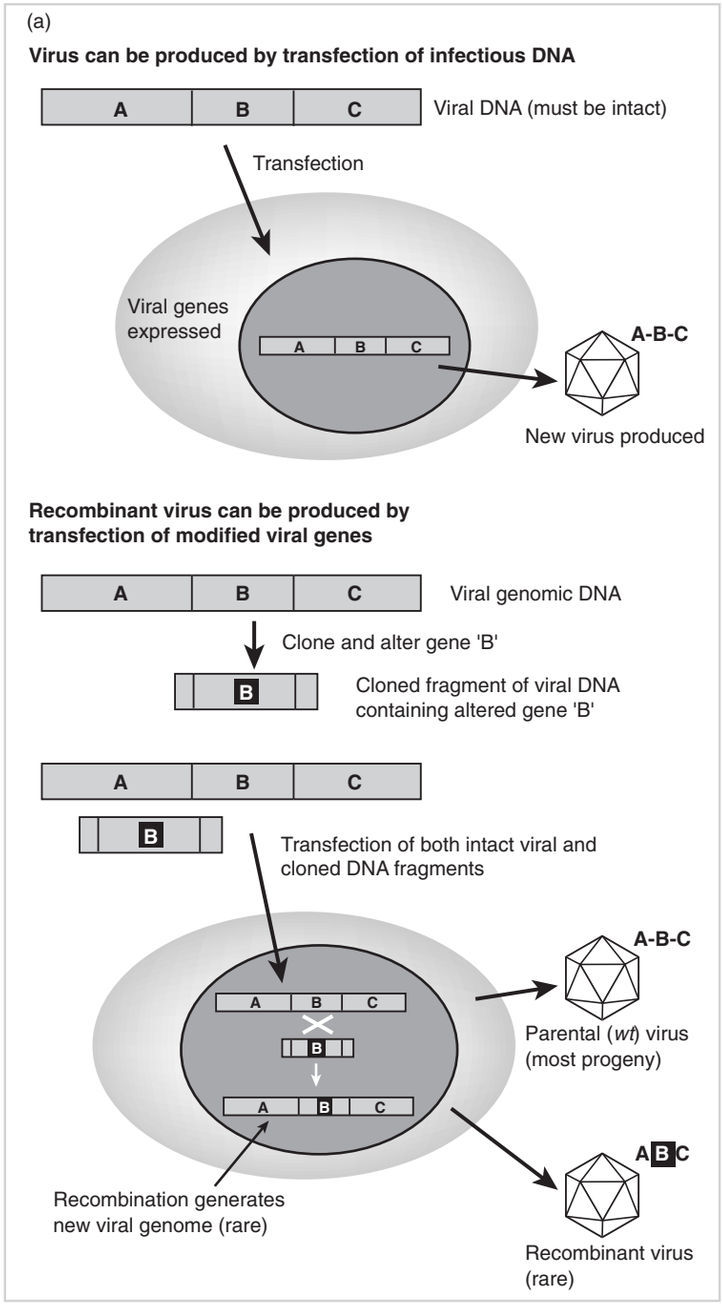


Fig. 14.6 Generating and isolating recombinant viruses. *a.* As outlined in Chapter 6, transfection of infectious viral DNA into a permissive cell leads to gene expression. If a full-length viral genome is transfected into a cell, production of infectious virus will ensue. If a fragment of homologous DNA containing a modified or foreign gene is included in the transfection, recombination can occur. While this is a rare event, the appropriate combination of selection and screening for recombinant virus can result in isolation of pure stocks. *b.* One approach toward screening for a recombinant virus. In this example, hybridization was used to detect the presence of virus containing the bacterial β-galactosidase gene. This requires physically picking plaques from an infected dish and testing the viral DNA present. The presence of the desired gene was confirmed by the fact that insertion of the 4 kbp β-galactosidase gene results in formation of an altered restriction fragment that can be identified by Southern blot hybridization. *c.* The DNA fragment was inserted into a 3.5 kb *Sal*I fragment of HSV-1. The altered fragment size can be seen by hybridization of blots of electrophoretically separated *Sal*I-digested DNA isolated from recombinant viruses. Hybridization was either with the DNA sequences specific for the region of viral DNA used for the homologous recombination, or with a probe specific for the inserted β-galactosidase gene. Hybridization of the blot with the latter probe, however, does not produce a signal with the *wt* fragment into which the gene was inserted.

The generation of recombinant viruses involves first and foremost the ability to isolate and separately manipulate specific portions of the viral DNA using the cloning and restriction analyses outlined in earlier sections of this chapter. This DNA can be modified to introduce mutations into the genes being studied. When a desired foreign or modified viral gene has been altered in a desired way, the gene can be cloned into a fragment of viral DNA so that it will be bounded by regions of DNA that are homologous to the viral genome.

The gene can then be introduced into the virus by transfection of the DNA containing it along with full-length viral DNA into cells under conditions where the cell is encouraged to use endocytosis to incorporate large amounts of DNA. If one or a few copies of the viral DNA remain intact in the recipient cell, transcription of the genome can lead to initiation of a productive replication cycle.

The recombinant virus is formed by random homologous recombination between the piece of viral DNA carrying the modified viral gene and the full-length infectious DNA. Once the replica-

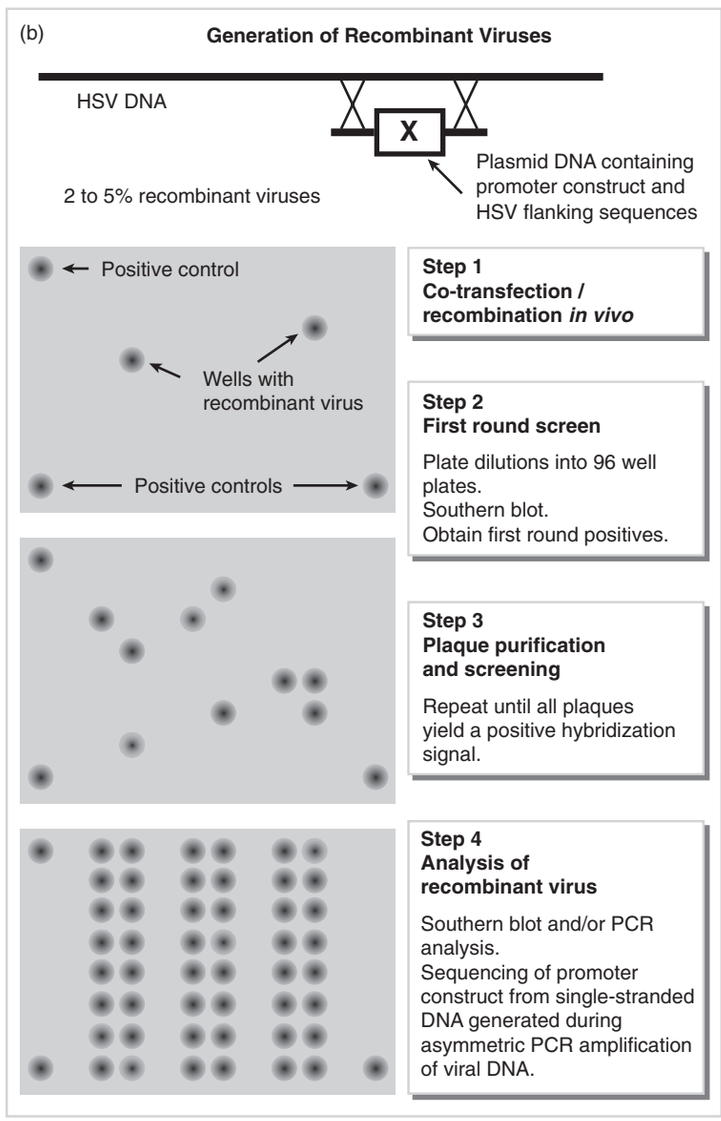


Fig. 14.6 *Continued*

tion cycle is begun, the generated virus will be normal in its ability to spread efficiently to neighboring cells. The recombinant virus can then be isolated either by selection, if possible, or by screening if the new gene does not provide a growth advantage to the virus.

The isolation of a recombinant HSV containing the bacterial β-galactosidase gene inserted into a specific region of the genome is shown in Fig. 14.6. In the method shown, a cell culture was infected with virus isolated from plaques formed by virus produced in a transfected cell culture. Some virus can be isolated from individual plaques by probing them with a sterile toothpick or disposable pipette. This virus was used to infect small cultures of cells, and following development of cytopathology and progeny virus, some viral DNA was spotted on a membrane filter and hybridized with a probe for the gene of interest. A positive signal indicates the presence of recombinant virus, and the process is repeated until the virus is pure. Identity of the recombinant can then be confirmed by Southern blotting (shown) or PCR analysis.

Defective virus particles

The process of viral genome replication and packaging is not tightly controlled during productive infection by many viruses. If a viral genome produced contains a replication origin and a packaging

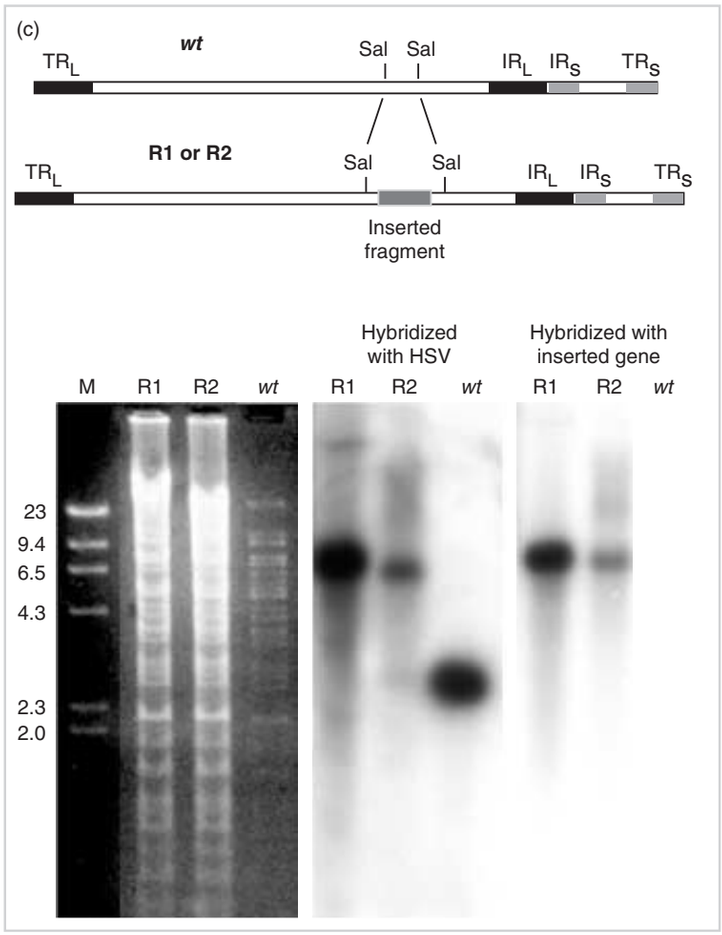


Fig. 14.6 *Continued*

signal, but lacks one or a number of essential genes, this *defective* genome can still be packaged and released from the infected cell. The virion produced will be a defective particle in that while it can initiate infection in a cell, its genome does not contain all the required genes for replication, and thus the infection will be abortive.

If, however, a cell is infected with both the defective particle and an infectious virus particle, both genomes will replicate in the infected cell, and both can be packaged and released. In such a mixed infection, the normal virus serves as a *helper* for the defective particle.

It should be apparent that propagation of defective particles will be favored by repeated high-multiplicity infections of cells with stocks of virus recovered from such mixed infections. Further, since the defective virions contain genomes that are smaller and less genetically complex than those of the infectious virus, these genomes will be replicated a bit faster than full-length ones. This gives them a replication advantage. Eventually, the proportion of defective particles will become so high as to swamp out the replication-competent virus, and infectivity will be lost.

Some defective viral genomes, notably vesicular stomatitis virus (VSV), have such a replication advantage that they compete for the virus-encoded replication machinery in the infected cell and exacerbate the loss of infectivity. Such particles are called **defective interfering particles** (DI particles).

The process of generation of defective DI particles may have a role in naturally limiting virus infection in the host, although this is not certain. What is important about the phenomenon, however, is that the defective genomes can serve as vectors for carrying other genes. Such defective viral vectors require a helper virus for replication, but in principle at least, can serve to introduce a gene into cells without the attendant cytopathology and virus-induced cell death that are caused by infection with the nondefective virus. The generation of defective virus vectors and their potential therapeutic uses are briefly described in the last chapter of this book.

QUESTIONS FOR CHAPTER 14

1 A virus such as bacteriophage T7 has a linear, dsDNA genome. Why is it correct to say that a schematic description of this DNA represents both a physical and a genetic map of the genome?

2 Describe the differences between complementation and recombination.

3 You wish to clone a specific fragment of the SV40 dsDNA genome. Using one of the cloning plasmids shown in Fig. 14.2, design an experiment to do this.

4 You wish to prepare temperature-sensitive mutants of bacteriophage T4 that are defective in their ability to attach and enter their host cells at high (nonpermissive) temperature. Describe an experimental protocol and selection method you might employ to do this.

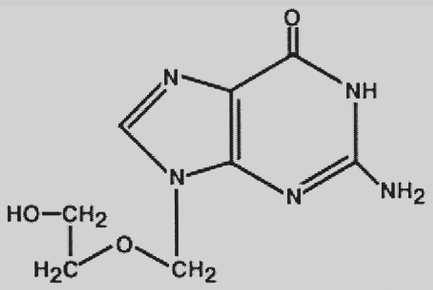
5 You have isolated a circular, dsDNA genome of 5000 base pairs (5 kbp) from a virus. You digest this DNA with one of three restriction enzymes or with combinations of the three. The fragments are separated by agarose gel electrophoresis and their sizes determined. The results are shown in the table below:

Restriction Endonuclease	Fragments and Lengths (kpb)
*Eco*RI	5.0
*Pst*I	3.5, 1.5
*Hinf*1	1.8, 1.75, 1.45
*Eco*RI + *Pst*I	3.5, 1.0, 0.5
*Eco*RI + *Hinf*1	1.8, 1.75, 1.2, 0.25
*Pst*I + *Hinf*1	1.8, 1.0, 0.75 (double-intensity band), 0.7
*Eco*RI + *Pst*I + *Hinf*1	1.8, 1.0, 0.75, 0.7, 0.5, 0.25

From these data, draw the circular restriction map of this viral genome.

PART III Problems

1 The drug acycloguanosine, sold as acyclovir, has been one of the most successful antiviral compounds produced. Acyclovir is used in the treatment of herpes simplex virus infections. These viruses replicate their double-stranded DNA genomes using a virus-specific DNA polymerase. The structure of acyclovir is show below:



acycloguanosine (Acyclovir)

a Given the structure of this drug, what is the specific effect of this nucleoside analogue on herpes virus DNA replication? Your answer should refer to a particular structural feature of the drug.

b Acyclovir is administered to patients in the form shown. What must happen to this drug inside the cell before it can inhibit viral DNA replication? Again, your answer must refer to a particular structural feature of the drug.

2 You have prepared two highly purified suspensions of poliovirus, each grown on a different host cell. The first (stock A) was grown on HeLa cells (a human cell line). The second (stock B) was grown on AGMK cells (a monkey kidney cell line). Each suspension has a total volume of 10.0 ml. The virus stocks were titered by diluting the stocks and performing plaque assays. Dilutions were made by taking 1 part of the virus stock and mixing it with 9999 parts of buffer (dilution 1). A further 1 to 10^4 dilution was made, using this same procedure. The resulting suspension (dilution 2) was plaque assayed in duplicate on a lawn of susceptible cells. In addition, you measured the optical density of the original virus stocks at a wavelength of 260 nm. You know that an optical density (OD_{260}) of 1.0 equals 10^{13} poliovirus particles/ml. The data you obtained are shown in the following table.

Virus stock	Host cell	Plaques in 1 ml of dilution 2 (replicate plates)	OD_{260} of virus stock
A	Hela	190 and 210	0.1
B	AGMK	48 and 52	0.5

a What are the plaque-forming unit (PFU) to particle ratios for the two viral stocks?

Stock A = _____
Stock B = _____

b Which host cell line produced the most *total virus particles*?
c Which host cell line produced the most *total infectious virus*?

3 The Svedberg equation that describes the motion of a molecule through a solution under the influence of a centrifugal field is:

$$S = \frac{v}{\omega^2 r} = \frac{M(1 - \bar{\nu}\rho_{sol})}{N_{AV} f}$$

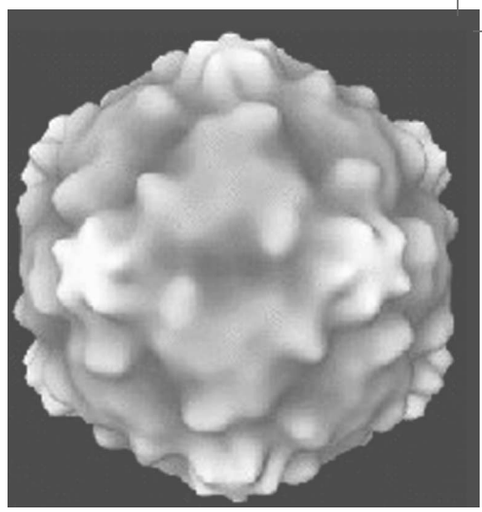
where S, the Svedberg coefficient, is a function of the molecular weight (M) and the frictional coefficient (f). The constants in the equation are Avagadro's number (N_{av}), the partial specific volume of the molecule (ν), and the density of the solution (ρ_{sol}). The table below gives some relevant data for several DNA molecules:

DNA	Molecular weight	Configuration*
PBR322 DNA	2.84×10^6	ds, circular†
PBR322 DNA digested with *Eco*RI‡	2.84×10^6	ds, linear
Phage T4 DNA	1.12×10^8	ds, linear
Phage T7 DNA	2.5×10^7	ds, linear
Phage ΦX174 RF DNA	3.76×10^6	ds, circular

* Assume that $f_{ds,linear} \gg f_{ds,circular}$.
† Assume the same degree of supercoiling for all of the circular molecules.
‡ pBR322 has only one recognition site of *Eco*RI.

Predict the sedimentation behavior (sedimentation rate) of the following pairs of molecules. In each case, state whether the indicated molecule of the pair will move "faster" or "slower" *relative to the other member of the pair*. (Note: you do not need to calculate a sedimentation rate. You need to determine the relative behavior of the pair of molecules in each case.)

Molecules		Relative sedimentation rate	
1	2	1	2
Phage T4 DNA	Phage T7 DNA		
pBR322 DNA	Phage ΦX174 RF DNA		
pBR322 DNA	pBR322 DNA digested with *Eco*RI		
Phage T7 DNA	pBR322 DNA digested with *Eco*RI		



Additional Reading for Part III

Note: see Resource Center for relevant websites.

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Volume 1.

Section 1. *Escherichia coli*, plasmids, and bacteriophages: Part II. Vectors derived from plasmids.

Section 2. Preparation and analysis of DNA: Part IV. Analysis of DNA sequences by blotting and hybridization.

Section 3. Enzymatic manipulation of DNA and RNA: Part I. Restriction endonucleases.

Part II. Enzymatic manipulation of DNA and RNA: restriction mapping.

Section 4. Preparation and analysis of RNA: Part IV. Analysis of RNA structure and synthesis.

Section 7. DNA sequencing: Part I. DNA sequencing strategies.

Section 9. Introduction of DNA into mammalian cells: Part I. Transfection of DNA into eukaryotic cells.

Volume 2.

Section 10. Analysis of proteins: Part III. Detection of proteins: sub-section 10.7. Detection of proteins on blot transfer membranes.

sub-section 10.8. Immunoblotting and immunodetection.

Section 11. Immunology: Part I. Immunoassays: subsection 11.2. Enzyme-linked immunosorbent assay (ELISA).

Section 14. In situ hybridization and immunohistochemistry: subsection 14.3. In situ hybridization to cellular RNA.

Section 15. The polymerase chain reaction: subsection 15.1. Enzymatic amplification of DNA by the polymerase chain reaction: standard procedures and optimization.

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Chapter 6:300–72. Basic genetic mechanisms: RNA and protein synthesis.
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Chapter 8:179–212. Protein synthesis.

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