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Replication of Plant Viruses

SUMMARY

Viruses replicate using both their own genetic information and host cell components and machinery. The different genome types have different replication pathways which contain controls on linking the process with translation and movement around the cell as well as not compromising the infected cell. This chapter discusses the replication mechanisms, faults in replication and replication of viruses coinfecting cells.

One of the major features of viruses is their ability to replicate their genomic nucleic acid, often to high levels, in cells in which there are normally strict limits on the production of new nucleic acid molecules. Some viruses do this by adapting the existing cellular machinery, and others replicate their nucleic acid by mechanisms not widely used in host cells.

Our understanding of the ways in which plant viruses replicate has increased remarkably over the past few years. This is, in part, because the complete nucleotide sequences of many plant viral genomes have been established allowing the number, size, and amino acid sequence of putative gene products to be determined as well as non-coding sequences which play important roles in viral replication. We now have this information for representatives of most plant virus genera. Furthermore, a wide range of techniques (Section II) has been and is being developed which is enabling viral replication to be studied in great detail. This is revealing some of the sophistication of the integration of viral replication with other viral functions and the interactions that the virus has with the host cells in which they are replicating. These interactions will be discussed in this chapter and in Chapter 16.

I. CELLULAR COMPARTMENTS INVOLVED IN REPLICATION

Plant virus replication involves interactions with cell compartments and components, and thus it is important to have an understanding of the basic structures involved. The basic structure of a plant cell is shown in Box 7.1. All these main regions of the cell are involved in viral

Acronyms of virus names are shown in Appendix D

replication, but the actual details vary between different virus groups as will be described in this chapter.

II. METHODS FOR STUDYING VIRAL REPLICATION

Plant viruses cannot replicate without the involvement of a host plant. Two basic questions have to be addressed in studying how plant viruses replicate:

- Which parts of the viral genome are involved in replication?
- How is the plant host involved in the virus replication?

A wide range of methods is now being applied to gain deeper understanding of how viruses replicate. These can be grouped as plant *in vivo* systems, non-plant model systems, and *in vitro* systems. Because of the involvement of host proteins and pathways and the close integration with other stages of the infection cycle, it is generally accepted that a full picture of viral replication can only be obtained from plant *in vivo* systems. The use of "deep-sequencing" (Chapter 2, Section II, C, 1) and the application of various "'omics" (e.g., genomics, proteomics, metabolomics) to understanding how plants function, using plant *in vivo* systems is becoming more productive. However, this information is not yet available for many of the major plant virus hosts, and so non-plant model systems have also been productive; also many of the questions of detailed interactions and functions can be addressed by *in vitro* systems.

In this section, I am going to describe some of the systems that have yielded information on viral replication.

A. Higher Plant *In Vivo* Systems

1. The Intact Plant

In Chapter 13, Section V, C, some of the variables involved in sampling intact plants are discussed. It should be borne in mind that, in spite of these difficulties, there are certain aspects of virus replication that can be resolved only by study of the intact developing plant, for example, the relationship between mosaic symptoms and virus replication. The tissue that has been most commonly used in the study of virus replication is the green leaf blade. This tissue

BOX 7.1 Structure of the Plant Cell

The basic features of the plant cell that are involved in virus infection are shown schematically in Figure 7.1.
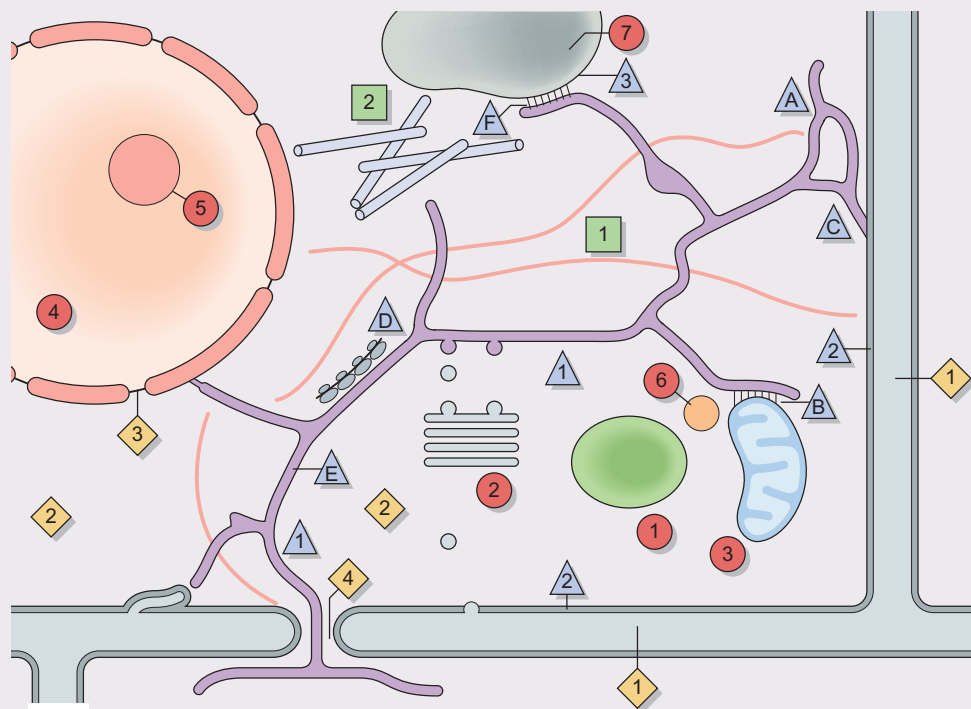
Cytoplasm

The cytoplasm is the entire content of the cell excluding the nucleus. It comprises the cytosol which is a complex mixture of ions (e.g., sodium, potassium, calcium) and macromolecules (e.g., proteins) in a water environment, together with organelles such as chloroplasts and the Golgi apparatus, and is where much of the cellular metabolism occurs. There are multiple levels of organization in the cytosol, including

concentration gradients of ions like calcium and macromolecules like enzymes. Crowding of macromolecules in the cytosol can alter the properties, proteins and nucleic acids.

Nucleus

The nucleus is a highly dynamic organelle surrounded by a double membrane which has numerous nuclear pores enabling communication with the surrounding cytosol. These pores allow free movement of small molecules and ions, but the movement of larger molecules like proteins is carefully controlled, requiring active transport regulated by carrier

Symbols

Structures
1 Cell wall
2 Cytosol
3 Nuclear pore
4 Plasmodesma

Organelles
1 Chloroplast
2 Golgi apparatus
3 Mitochondrion
4 Nucleus
5 Nucleolus
6 Peroxisome
7 Vacuole

Membranes
1 Endoplasmic reticulum (ER)
2 Plasmamembrane
3 Tonoplast

ER domains
A Action-binding domain
B Mitochondrion-attachment domain
C Plasmamembrane-anchoring domain
D Rough ER
E Smooth ER
F Vacuole-attachment domain

Cytoskeleton
1 Microfilaments
2 Microtubules

FIGURE 7.1 Schematic diagram of a plant cell depicting various structures, organelles, membranes, and cytoskeleton elements involved in virus infection.

proteins. The nucleus contains most of the cell's genetic material, organized as multiple long linear DNA molecules in complex with a large variety of proteins, such as histones, to form chromosomes. It is the site of major cellular events, including DNA replication, messenger RNA synthesis and processing, and ribosome subunit biogenesis. The structure of the nucleus is maintained by the nuclear lamina, a meshwork analogous to the cytoskeleton.

Within the nucleus is the nucleolus, which is a non-membrane-bound compartment composed of proteins and nucleic acids. It is the site of rDNA transcription, rRNA processing, and ribosome assembly and is implicated in many other aspects of cell function, such as cell cycle regulation, gene silencing, telomerase activity, senescence, stress responses, and biogenesis of multiple ribonucleoprotein (RNP) particles. Details of nucleolar structure and function are reviewed by Taliansky et al. (2010).

Chloroplast

A typical parenchyma cell contains about 10–100 chloroplasts which are organelles about 2–10 μm in diameter and 2.3 μm thick. They comprise a outer double membrane enveloping the stroma (analogous to the cytosol) and contain one or more molecules of circular DNA and ribosomes; however, most of its proteins are encoded by genes contained in the host cell nucleus, with the protein products being transported to the chloroplast. Within the stroma are thylacoids which are stacks of membrane structures termed grana and which are the the site of photosynthesis.

Mitochondria

Mitochondria are organelles about 0.5–10 μm in diameter enclosed in a double membrane and provide the energy for cellular functions like cell division. They are also involved in other functions, such as signaling, cellular differentiation, and cell death, as well as the control of the cell cycle and cell growth.

Golgi Apparatus and Other Organelles

The Golgi apparatus is a membrane-bound structure bounded by a single membrane. It is actually a stack of membrane-bound vesicles (cysternae) that are important in packaging macromolecules for transport elsewhere in the cell. The stack of larger vesicles is surrounded by numerous smaller vesicles containing those packaged macromolecules. The Golgi apparatus is integral in modifying, sorting, and packaging these macromolecules for cell secretion (exocytosis) or use within the cell. It primarily modifies proteins delivered from the rough ER but is also involved in the transport of lipids around the cell.

Peroxisomes are organelles on the periphery of the Golgi apparatus and are responsible for protecting the cell from its own production of hydrogen peroxide by oxidative enzymes to break down the hydrogen peroxide into water and oxygen.

The vacuole is a membrane-bound organelle which is essentially an enclosed compartment which is filled with water containing inorganic and organic molecules including enzymes in solution, though in certain cases it may contain solids which have been engulfed. Its functions include:

isolating materials that might be harmful or a threat to the cell; containing waste products; maintaining an acidic internal pH; and exporting unwanted substances from the cell.

Membranes and Cytoskeletal System

Plant (and other) cells have an extensive membrane and cytoskeletal systems that play major roles in virus replication and other functions. There are many interactions between the various elements.

Endoplasmic Reticulum (reviewed by Hu et al., 2011; Sparkes et al., 2009; Staehelin, 1997)

The ER forms a large interconnected network of tubules, vesicles, and cisternae within cells and is a continuation of the ONM. There are three forms of ER, rough ER which synthesizes proteins (the rough appearance being due to the presence of ribosomes) and smooth ER which synthesizes lipids and steroids, metabolizes carbohydrates and steroids, and regulates calcium concentration and attachment of receptors on cell membrane proteins. Calcium levels are also regulated in the sarcoplasmic reticulum.

Cytoskeleton

The cytoskeleton is an organized network of three types of protein filaments: microtubules, actin filaments, and intermediate fibers, and provides the cell with structure, shape, compartmentalization, and movement of macromolecules. Cytoskeletal elements interact extensively and intimately with cellular membranes.

Microtubules (reviewed by Hamada, 2007; Westeneys, 2002) are hollow cylinders about 23 nm in diameter (lumen is approximately 15 nm in diameter), most commonly comprising 13 protofilaments which, in turn, are polymers of α- and β-tubulin.

Actin filaments (reviewed by Higaki et al., 2007; Šlajcherova et al., 2012; Sparkes, 2011; Staiger and Blanchoin, 2006) are composed of linear polymers of actin subunits, and generate force by elongation at one end of the filament coupled with shrinkage at the other, causing net movement of the intervening strand. They also act as tracks for the movement of some organelles and macromolecules that attach to the microfilament and "walk" along them. Myosins are the motor proteins for organelle and macromolecule movement along actin fibers and comprise an N-terminal motor head domain responsible for actin binding and a C-terminal domain implicated in cargo binding. Plant myosins are classified into two groups: class XI and class VIII (reviewed by Sparkes, 2011); different myosin "species" carry different cargos.

There are considerable interactions between actin and microtubules (reviewed by Petrásek and Schwartzerová, 2009).

Although little work has been done on intermediate filaments in plants, there is some evidence that cytosolic intermediate filaments might be present, and plant nuclear filaments have been detected. Like actin filaments, they function in the maintenance of cell shape by bearing tension which, in contrast to microtubules, resists compression. Intermediate filaments organize the internal tridimensional structure of the cell, anchoring organelles.

constitutes approximately 50−70% of the fresh weight of most experimental plants, and final virus concentration in the leaf blade is often 10−20 times higher than in other parts of the plant. We can distinguish four types of plant system *in vivo*: the intact plant, surviving tissue samples, cells or organs in tissue culture, and protoplasts. Some plant viruses also replicate in their insect vectors.

a. Model Plant Systems

Arabidopsis thaliana (hereafter called *Arabidopsis*) was the first plant genome to be fully sequenced and as there are numerous well-characterized mutants, it is proving a useful model plant for those viruses that infect it (Whitham et al., 2003). As well as its use as a model plant for virus replication, *Arabidopsis* has been used as a model for studying virus–host co-evolution (Pagán et al., 2010).

Although the genome of *Nicotiana benthamiana* has not yet been sequenced it is the most widely used experimental host for plant viruses, due mainly to the large number of viruses that infect it. It is easily genetically transformed (reviewed by Goodin et al., 2008).

Brachypodium distachyon is a member of the *Pooideae* subfamily of the grass family and is a new model system for bridging studies between temperate cereal crops, such as wheat and barley, and also biomass grasses like *Miscanthus giganteus* (Mur et al., 2011; Vain, 2011). *Brachypodium* has a small genome (~300 Mbp), diploid, tetraploid, and hexaploid accession, a small physical stature, self fertility, a short life cycle, and simple growth requirements. Its genome has recently been sequenced (The International Brachypodium Initiative, 2010). With the rapid accumulation of knowledge, this species could be useful for studies on viruses infecting graminaceous species though it has not yet been included in host range studies of many such viruses. However, BSMV has been used as a vector for virus-induced gene silencing of *Brachypodium* (Demircan and Akkaya, 2010).

b. Inoculated Leaves

Inoculated leaves have several advantages. Events can be timed more precisely from the time of inoculation than can those from systemic infections. A fairly uniform set of leaves from different plants can be selected, and half-leaves may be used as control material. There are three major disadvantages:

i. A typical leaf such as a tobacco leaf with a surface area of $200\,cm^2$, for example, contains about 3×10^7 cells. The upper limit for the proportion of epidermal cells that can be infected by mechanical inoculation under the best conditions is not known precisely, but is probably not more than about 10^4 cells per leaf. Thus, at the beginning of an experiment, only about 1 in 10^3 of the cells in the system has been infected. Even for those that are directly infected, the synchrony of infection may not be very sharp, especially if whole virus is used as inoculum. Thus, early replication events in the small proportion of infected cells will probably be diluted out beyond detection by the relatively enormous number of as-yet-uninfected cells. Then, as infection progresses, a mixed population of cells at different stages of infection will be produced.

ii. The second major disadvantage of inoculated leaf tissue, at least for studying events over the first few hours, is that mechanical inoculation itself is a severe shock to the leaf, causing changes in respiration, water content, and probably many other things as well, including nucleic acid synthesis. Thus, the use of appropriately treated control leaves is essential.

iii. A third difficulty applies to experiments in which radioactively labeled virus is used as inoculum. Most of the virus applied to the leaf does not infect cells, and a substantial but variable proportion cannot be washed off after inoculation. The fate of the infecting particles may well be masked by the mass of potentially infective inoculum remaining on or in the leaf.

For particular kinds of experiments, two modifications in the use of the inoculated leaf have proven useful. With some leaves grown under appropriate conditions, it is relatively easy to strip areas of epidermis from the leaf surface. Very limited amounts of tissue can be harvested in this way, but the method increases by a factor of about 8 the proportion of cells infected at time soon after inoculation (Fry and Matthews, 1963). Dijkstra (1966) explored the possibility of studying TMV replication in strips of epidermis removed from leaves immediately after inoculation with TMV and floated on nutrient solutions or distilled water, but no significant progress has been made with this system.

Several workers have used micromanipulation methods to infect single cells on a leaf—usually leaf hair cells—and then to follow events in the living cells as they can be observed by phase or ultraviolet microscopy or in preparations stained with fluorescent antibody (Chapter 10, Section I, B, 5). This procedure, while it has given useful information, is limited to microscopical examination and cannot at present be used for biochemical investigations. It is mainly used for studying cell-to-cell movement of viruses and also gives information on cellular distribution of viruses and their gene products.

c. Systemically Infected Leaves

Moving from the inoculated leaf, a virus may invade the youngest leaves first, then successively infect the older and older leaves (Figure 10.2). Thus, systemically infected leaves may be in very different states with respect to virus infection. Furthermore, the time at which infectious material moves from inoculated leaves to young growth may vary significantly between individual plants in a

FIGURE 7.2 Plant protoplasts.

batch. Nevertheless, it is probable that in young systemically infected leaves (perhaps about 4 cm long at the time virus enter plants, such as tobacco and Chinese cabbage) most of the cells in a leaf become infected over a period of 1−2 days. Such a leaf has been used to study the replication of TMV (Nilsson-Tiligren et al., 1969) and TYMV (Bedbrook et al., 1974; Hatta and Matthews, 1974).

The synchrony of infection in the young systemically infected leaf can be greatly improved by manipulating the temperature. The lower inoculated leaves of an intact plant are maintained at normal temperatures (*c*. 25−30°C) while the upper leaves are kept at 5−12°C. Under these conditions, systemic infection of the young leaves occurs, but replication does not. When the upper leaves are shifted to a higher temperature, replication begins in a fairly synchronous fashion (Dawson et al., 1975). This procedure provides a very useful system that complements, in several respects, the study of virus replication in protoplasts, discussed below. The technique uses intact plants, is simple, and can provide substantial amounts of material. The main requirement is for a systemic host with a habit of growth that makes it possible for upper and lower leaves to be kept at different temperatures. Its use has been extended to some other viruses, for example, CMV in tobacco (Roberts and Wood, 1981).

d. Transgenic Plants

The expression of viral genes in plants, often coupled with mutagenesis of the viral genome (reverse genetics), is proving to be an increasingly useful approach.

e. Viral Reporter Systems

Manipulation of cloned viral genomes enables reporter molecules, usually fluorescent proteins (Chapter 10, Section I, B, 5) to be attached to specific viral gene products or expressed separately from the viral genome. This enables the virus to be studied in intact plants in real time and for details to be obtained on the exact location of the gene function being studied.

2. Surviving Tissue Samples

a. Excised Leaves

These are useful when fairly large quantities of leaf tissue are required. Petioles may be placed in water or a nutrient solution. Under these conditions, leaves vary widely in the amount of fluid they take up, and may wilt unpredictably. Tissue near the cut end of the petiole acts as a "sink" for radioactively labeled metabolites (Pratt and Matthews, 1971). On the other hand, the method minimizes the problem of the growth of microorganisms in the tissue during incubation. More commonly, leaves are placed in dishes covered with glass under moist conditions. Growth of bacteria, fungi, and protozoa is then likely to be a problem.

b. Leaf Disks

Disks of tissue 5−20 mm in diameter cut from leaves with a cork borer and floated on distilled water or some nutrient salt solution have the advantage that pieces from many leaves can be combined in one sample to smooth out leaf-to-leaf variations. The physiological state of the leaves from which disks are taken affects uptake and metabolism of radioactively labeled materials (Kummert and Semal, 1969). There may be two serious disadvantages: (i) microorganisms grow on the surface of the disks and in the intercellular spaces, so addition of antibiotics may not block all microorganisms and may well alter the biochemical situation in the cells of interest, and (ii) excised disks are not uniform in several ways (Pratt and Matthews, 1971). First, there is a "geographical" gradient from the cut edge to the center of the piece of tissue. Differences involve the uptake of labeled precursors and their utilization for nucleic acid synthesis. Second, excised tissues change with time in a complex fashion in their ability to accumulate substances from the medium. There may be a differential accumulation of labeled precursors in the cut ends of veins. Third, further variables are introduced when the excised tissue is treated with a drug like actinomycin D, which may be distributed very unevenly in the tissue.

3. Tissue Culture (reviewed by Műhlbach, 1982)

Plant cells can be grown in tissue culture in several ways, either as whole organs (e.g., roots or stem tips) or as solid masses of callus tissue growing in solid or liquid culture, or as cell suspensions. Amounts of virus produced in cultured tissue or cells are usually very much less than in intact green leaves, although tobacco callus cells disrupted in the presence of TMV inoculum produced high yields

of virus (Murakishi et al., 1971; Pelcher et al., 1972). Various methods have been tested in the study of virus replication but, except for some microscopical studies, results have been disappointing. White et al. (1977) and Wu and Murakishi (1979) have adapted the low-temperature preincubation procedure of Dawson et al. (1975) to callus cultures infected with plant viruses. The virus growth curves obtained for TMV in tobacco callus cells were comparable to that obtained with protoplasts.

4. Cell Suspensions and Tissue Minces

In principle, suspensions of surviving but non-dividing cells offer considerable advantages in the study of virus replication. Dissociated cells from callus tissue grown in culture and leaf cells separated enzymatically have been used. For example, Jackson et al. (1972) successfully used separated leaf cells to study the replication of TMV RNA.

5. Protoplasts (reviewed by Murakishi et al., 1984; Sander and Mertes, 1984; Sztuba-Solińska and Bujarski, 2008)

Protoplasts are isolated plant cells that lack the rigid cellulose walls found in intact tissue. Takebe et al. (1968), Takebe and Otsuki (1969), and Aoki and Takebe (1969) showed that metabolically active protoplasts could be isolated from tobacco leaf cells, that such protoplasts could be synchronously infected with TMV or TMV RNA and that virus replication could be studied in them. Since then, protoplasts have been prepared from many species and infected with a range of viruses.

Methods for isolating and inoculating protoplasts are given in Dijkstra and de Jager (1998). On complete removal of the cellulose wall, the cells, now bounded only by the plasma membrane, assume a spherical shape (Figure 7.2).

About 10^7 palisade cells can be obtained from 1 g of tobacco leaf in 2 h. The ability to infect protoplasts with improved synchrony enables plant virologists to carry out one-step virus growth experiments (Figure 7.3), an important kind of experiment that has long been available to those studying viruses of bacteria and mammals.

Besides improved synchrony of infection, protoplasts have several other advantages: (i) close control of experimental conditions; (ii) uniform sampling can be carried out by pipetting; (iii) the high proportion of infected cells (often 60−90%); (iv) the relatively high efficiency of infection; and (v) organelles, such as chloroplasts and nuclei, can be isolated in much better condition from protoplasts than from intact leaves.

However, a number of actual or potential limitations and difficulties must be borne in mind: (i) protoplasts are very fragile, both mechanically and biochemically, and their fragility may vary markedly, depending on the
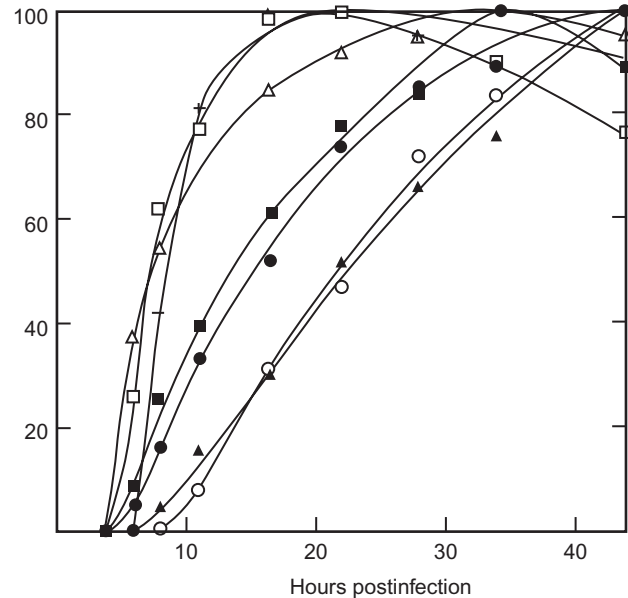
FIGURE 7.3 Time course of production of TMV-related RNAs, proteins, and progeny virus particles in synchronously infected protoplasts. One-half of a batch of protoplasts was incubated with [^{14}C] uridine in the presence of actinomycin D from the time of inoculation. The other half was incubated with [^{14}C] leucine under the same conditions. Samples were taken for analysis at the times indicated. Data are expressed as the percentages of the maximum values attained for each component during the time course studies. (□) RI; (Δ) RF; (●) TMV RNA; (■) CP mRNA; (+) 126-kDa (140-kDa) protein; (▲) CP; (○) progeny virus particles. *From Ogawa and Sakai (1984) with permission of the publishers.*

growing conditions of the plants, season of the year, time of day, and the particular age of leaf chosen. Defined plant growth conditions may improve the quality and reproducibility of the isolated preparations (Kubo et al., 1975); (ii) under culture conditions that favor virus replication, protoplasts survive only for 2−3 days and then decline and die; (iii) to prevent growth of microorganisms during incubation, antibiotics may be added to the medium. These may have unexpected effects on virus replication (e.g., gentamycin, Kassanis et al., 1975); (iv) compared to intact tissue, relatively small quantities of cells are made available; (v) cytological effects observed in thin sections of infected leaf tissue may not be reproduced in protoplasts—probably because of the effects of changed osmotic conditions on cell membranes, for example, with TMV in tobacco (Otsuki et al., 1972a), TYMV in Chinese cabbage (Sugimura and Matthews, 1981), FLSV in cowpea (van Beek et al., 1985), and CaMV in turnip (Yamaoka et al., 1982); (vi) the isolation procedure and the medium in which they are maintained must drastically affect the physiological state of the cells. Physical and chemical disturbances include partial dehydration, severing of plasmodesmata, loss of the cell wall compartment which is not metabolically inert, reversal of the cell's electrical

potential, inhibition of leucine uptake, a large increase in RNase activity, and cellulose synthesis and wall regeneration, which begin very soon after the protoplasts are isolated. In addition, tobacco mesophyll protoplasts have been shown to synthesize six basic proteins that are undetectable in tobacco leaf. Three of these are like PR proteins, a 1,3-β-glucanase and two chitinases, found in TMV-infected tobacco leaves (Grosset et al., 1990).

As a consequence of these changes, protoplasts vary with time in many properties during the period that they survive after isolation. Although little systematic study has yet been made of the changes, it is known that some features of virus replication differ in intact leaves and in protoplasts. Thus, Föglein et al. (1975) showed that, when protoplasts are prepared from leaves fully infected with TMV, vigorous viral RNA synthesis is reinitiated. Tobacco protoplasts containing the *N* gene escape necrotic cell death when infected with TMV (Otsuki et al., 1972b).

In many studies using protoplasts, it has been reported that yields of virus (virus particles per cell) are very similar to that found in intact plants. For example, Renaudin et al. (1975) found that Chinese cabbage protoplasts infected *in vitro* produced about 10^6 TYMV particles per cell which is similar to the published yields of TYMV obtained with extracts of intact leaf. These estimates were based on the assumption that all the cells in the leaf were infected, and that they were of the same size. If, however, the estimates are made on the same class of cell as used for the *in vitro* studies (i.e., palisade mesophyll), and if only infected cells are considered, then yields per cell in the intact leaf are about 10 times higher (Sugimura and Matthews, 1981). Despite these limitations, protoplast systems have contributed considerably to our knowledge.

6. Metabolic Compartmentalization

If we count a membrane as a compartment, eukaryotic cells have at least 20 compartments. In their replication, plant viruses have adapted in a variety of ways to the opportunities provided by this intracellular metabolic diversity. In thinking about experiments on virus replication (particularly those involving the use of radioisotopes and/or metabolic inhibitors) we must take account of the fact that processes take place in cells that have a high degree of metabolic compartmentalization. This exists in several forms: (i) in different cell types, which are metabolically adapted for diverse functions; (ii) in membrane-bound compartments within individual cells, e.g., nuclei, mitochondria, chloroplasts, lysosomes, peroxisomes, and vacuoles; (iii) in isolatable stable complexes of enzymes; and (iv) in microenvironments created without membranes, by means of weakly interacting proteins, or unstirred water layers near a surface.

a. Sites of Virus Synthesis and Assembly

Two general kinds of procedure have been used in attempts to define the intracellular sites of virus synthesis and assembly: (i) fractionation of cell components from tissue extracts followed by assay for virus or virus components in the various fractions and (ii) light and electron microscopy.

There are many difficulties involved in using cell fractionation procedures to locate sites of virus assembly.

  i. Chloroplasts are fragile organelles, and a proportion of these are always broken. Chloroplast fragments cover a wide range of sizes and will contaminate other fractions.
  ii. Viruses like TMV, occurring in high concentration, will almost certainly be distributed among all fractions, at least in small amounts.
  iii. Virus-specific structures may be very fragile and unable to withstand the usual cell breakage and fractionation methods.
  iv. If virus-specific structures are stable, they may fractionate with one or more of the normal cell organelles.
  v. Virus infection may alter the way in which certain cell organelles behave on fractionation.

Considerable progress has been made with some viruses using cell fractionation procedures. However, in recent years we have learned more from ultrastructural studies and most where both kinds of technique have been applied.

Viruses belonging to many different groups induce the development in infected cells of regions of cytoplasm that differ from the surrounding normal cytoplasm in staining and ultrastructural properties. These are not bounded by a clearly defined membrane but usually include some endoplasmic reticulum (ER) and ribosomes. They vary widely in size and may be visible by light microscopy. In varying degrees for different viruses, there is evidence that these bodies are sites of synthesis of viral components, replication of the viral genome, and the assembly of virus particles. They have been termed viroplasms, amorphous inclusions, "X bodies," or replication complexes. The detailed structure of the viroplasms may be highly characteristic for different virus groups, and sometimes even for strains within a group. The structure of replication complexes has been studied at super-resolution by Linnik et al. (2013).

B. Non-Higher Plant *In Vivo* Systems

1. Yeast (reviewed by Alves-Rodrigues et al., 2006; Nagy, 2008)

Yeast, *Saccharomyces cerevisiae*, is a single cell organism for which there is a considerable resource of classical and molecular genetics. Although yeast is the host for several viruses and virus-like agents, no ssRNA virus is known to naturally infect it. As will be described later, various plant

viruses, such as ss (+)-strand RNA viruses (e.g., bromo-viruses, tombusviruses) and ss DNA geminiviruses, can complete most of their intracellular replication steps in yeast cells; some animal viruses (Flockhouse virus and influenza virus) do so as well (Price et al., 1996; Naito et al., 2007). The yeast model system has enabled host factors that are involved directly in the replication of some RNA viruses, and indirectly in modifying the cell to provide replication sites, to be recognized and understood.

Advantages of the yeast system include:

- Yeast has a small eukaryotic genome, coding only for ~6000 genes, of which over 60% have been characterized.
- The yeast genome is compact with <7% of the genes carrying introns, which greatly simplifies the prediction of the expressed proteins.
- Toolboxes are available for the controlled expression of selected genes in yeast (Janke et al., 2004).
- There are high-throughput systems for studying virus replication in yeast (Pogany et al., 2010).
- Various libraries are available including: deletion libraries (YKO) covering most non-essential genes greatly facilitating genome-wide studies of gene functions (Tong et al., 2001; Ammar et al., 2009); the down-regulatable essential-gene library (yTHC) (Mnaimneh et al., 2004; Servienne et al., 2006); libraries of fluorescent tags or affinity tags to aid subcellular localization and protein purification, respectively (Ghaemmaghami et al., 2003; Huh et al., 2003; Sheff and Thorn, 2004; Gelperin et al., 2005); microarray chips with DNA oligos for most yeast genes and protoarrays with 4100 purified yeast proteins have been produced (Zhu et al., 2001).
- The databanks for yeast genes are also the most complete among eukaryotes (see The Saccharomyces Genome Database: www.yeastgenome.org).
- Because it is a simple eukaryotic cell, yeast can be expected to have extensive functional similarities with various plant cells. But, it must be remembered that plants are multicelled organisms and any possible interactions between cells in virus replication might not be recognized.

The yeast *in vivo* system has proved invaluable in gaining a detailed understanding of the replication of TBSV (Chapter 16).

2. Insect Cells

As described in Chapter 12, Section III, E, 3, some plant viruses multiply in both plants and their insect vectors. Such viruses will multiply in insect cell cultures. For instance, Kimura (1986) was able to obtain synchronous multiplication of RDV in leafhopper vector cell monolayers. However, there is no supporting material and information, such as that described above for yeast, to enable such cell systems to be used for detailed dissection of virus replication.

C. Modification and Detection Techniques

A wide range of methods are used to explore *in vivo* systems by modifying either the viral or the host genome. These include:

1. Reverse Genetics

The use of reverse genetics is described in Box 6.1.

2. Agroinfection

Viral RNA can be converted into cDNA which can be transcribed *in vitro* to give RNA that can be inoculated onto host plants. However, inoculating RNA can be very inefficient due to RNases and so, if a suitable promoter is included, the cDNA construct can be inoculated to express the viral RNA and initiate infection. Inoculations can either be direct or using *Agrobacterium tumefaciens* to introduce the viral nucleic acid into the host (Grimsley et al., 1986). This latter process, termed "agroinfection" or "agro-inoculation," (Chapter 4, Section IV, B, 2) is useful when the virus, e.g., a geminivirus or luteovirus, requires an insect (or other) vector for inoculation (Boulton et al., 1989; Leisner et al., 1992). Another situation requiring agroinfection is for members of the *Caulimoviridae*, the genomes of which are transcribed from more-than-genome length constructs (termed "one-and-a-bitmers") (Grimsley et al, 1986; Dasgupta et al., 1991; Section VII). Although *A. tumefaciens* naturally infects only dicotyledonous plants, agroinfection is equally successful with mono-cotyledonous species. However, there is some species and strain specificity of *Agrobacterium* for successful inoculations (Marks et al., 1989).

3. Radioisotopes

The use of radioactively labeled virus precursors has been essential for many studies on virus replication. There are substantial difficulties and limitations in the effective use of tracer compounds for studying the replication of plant viruses. Various ways have been used to introduce the labeled material into the tissue being studied. Whole plants can be removed from their pots, the roots carefully washed free of soil, and the isotope applied to the roots. This procedure is useful for ^{32}P-labeled orthophosphate and ^{35}S-labeled sulfate. Provided there is no delay in applying the isotope after washing the roots, uptake is rapid and efficient. With plants like actively growing Chinese cabbage, ^{32}P may be detected in leaves within minutes

of application, and uptake into the plant may be more or less complete within a few hours. With these two isotopes, uptake into leaves through the roots is much more effective than floating intact disks of leaf tissue on solutions of the isotope, even if the disks are sliced to expose more vein ends. Placing leaves with their cut petioles in the solution can lead to a highly variable and irregular uptake of isotope. However, by careful timing and attention to growth conditions, quite high specific activities can be obtained [e.g., 1 mCi ^{32}P (3.7 × 10^7 Bq)/mg viral RNA; Bastin and Kaesberg, 1975]. Kopp et al. (1981) describe a procedure in which pieces of leaf from which the lower epidermis has been stripped are floated on a solution containing the radioactive precursor. No systematic study of the best ways to introduce such precursors as amino acids and nucleotides appears to have been made. Devices are available for injecting solutions into leaves (Hagborg, 1970; Konaté and Fritig, 1983).

Most plant leaves have rather large reserves of low-molecular-weight phosphorus compounds. By various manipulations, it is possible to reduce or raise the overall concentration of phosphorus compounds not more than two- to threefold. Thus, in leaf tissue it has not been possible to carry out effective pulse-chase type experiments with phosphorus. With most organic compounds that can be used as labeled virus precursors, active leaves are continually providing an endogenous source of supply. Furthermore, plant tissues have the capacity to metabolize carbon compounds in many different ways, so that the labeled atom may soon appear in a wide range of low-molecular-weight compounds. For certain kinds of experiments, it is useful to be able to label purified virus chemically *in vitro* to high specific activity. A variety of procedures are available (Montelaro and Rueckert, 1975; Frost, 1977).

4. Fluorescent Tags

See Chapter 10, Section I, B, 5.

5. Metabolic Inhibitors

Inhibitors of certain specific processes in normal cellular metabolism have been widely applied to the study of virus replication. Three have been of particular importance: (i) actinomycin D, which inhibits DNA-dependent RNA synthesis but not RNA-dependent RNA synthesis; (ii) cycloheximide, which is used as a specific inhibitor of protein synthesis on 80S cytoplasmic ribosomes; and (iii) chloramphenicol, which inhibits protein synthesis on 70S ribosomes (e.g., in chloroplasts, mitochondria, and bacteria). Acridine derivatives, such as chlorpromazine and quinacrine, which are active against prion-based diseases, are considered to be useful tools for studying similarities in replication strategies of various RNA viruses (Sasvari et al., 2009).

Results with these inhibitors must always be treated with caution, as they may have other diverse subsidiary effects in eukaryotic cells, which may make it difficult to interpret results. For example, actinomycin D may affect the size of nucleotide pools (Semal and Kummert, 1969), can cause substantially increased uptake of metabolites by excised leaves (Pratt and Matthews, 1971), may reduce uptake by infiltrated disks (Babos and Shearer, 1969), and may not suppress synthesis of certain species of host RNA (Antignus et al., 1971).

Synthesis of the large polypeptide of ribulose bisphosphate carboxylase takes place in the chloroplasts on 70S ribosomes, while the small polypeptide is synthesized on 80S ribosomes in the cytoplasm. Owens and Bruening (1975) used these two polypeptides as an elegant internal control in their examination of the effects of chloramphenicol and cycloheximide on the synthesis of CPMV proteins.

D. *In Vitro* Systems

In vitro systems can either be to mimic *in vivo* replication or to analyze properties of molecules identified from *in vivo* or *in vitro* replication systems.

1. In Vitro *Replication Systems*

There have been various attempts to isolate competent replication complexes from virus-infected plant material. The main problems are: (i) the difficulty of separating complexes of proteins and nucleic acids from normal cell constituents. This is normally done by differential centrifugation and by gradient centrifugation. Solubilized TMV replication complexes were separated from cellular components in linear gradients of 10–40% glycerol (Watanabe et al., 1999) and membrane-bound complexes of ToMV in gradients of 20−60% sucrose (Osman and Buck, 1996); (ii) membranes are an integral part of the replication complexes of (+)-strand RNA viruses and the technology for isolating such components is not yet well developed; (iii) uninfected plant cells contain an endogenous RNA-dependent RNA polymerase (RdRp) which is often enhanced on virus infection. Care has to be taken to separate this activity from the virus-coded activity. A cell-free extract of yeast system gives authentic replication and recombination of TBSV (Pogany and Nagy, 2008).

In vitro translation–replication systems giving complete replication of some viruses have been prepared from BY-2 tobacco protoplasts and from yeast (Komoda et al., 2004; Pogany and Nagy, 2008). In the tobacco system, vacuoles (which contain proteases and nucleases) were removed from BY-2 protoplasts by Percoll gradient centrifugation and the evacuolated protoplasts are disrupted to give a cell-free extract. Amino acids, ATP, GTP, creatin phosphate, creatin phosphokinase, spermidine, RNase inhibitor, and viral RNA

are added to give a translation mix and, after translation, ribonucleotide triphosphates are added for replication of the viral RNA (Komoda et al., 2004). This system has been used for the replication of ToMV, BMV, and TCV RNAs (Komoda et al., 2004), and TBSV RNA (Gursinsky et al., 2009).

The yeast cell-free translation–replication system has been used to study the translation and replication phases of TBSV RNA (Pogany and Nagy, 2008). Extracts were prepared from cells transgenically expressing tombusviral p33 and p92 replication proteins to which amino acids, nucleotide triphosphates, and other chemicals had been added in a manner similar to the tobacco BY-2 system.

2. Primer Extension

Properties of replication complexes can be studied by adding nucleotide triphosphates under the appropriate conditions and assessing the resulting products from extension of primed strands on the existing template. The products can be analyzed by incorporating a labeled nucleotide triphosphate (radioactive or fluorescent label) or by probing the product with a labeled probe. This approach can be used to study the optimum condition for the replicase enzymes.

3. Enzyme Activities

The enzymes involved in replication have been purified by standard protein and enzyme purification techniques including size exclusion chromatography and ion exchange chromatography. The properties of these enzymes have been studied by standard enzymological techniques and other techniques like activity gels. Description of these techniques is beyond the scope of this book but can be found in standard manuals on proteins and enzymes.

4. Protein–Protein Interactions

Many processes in living cells are regulated by protein–protein interactions. Much of the effort so far has been to study interactions between individual proteins, but recently the focus has been moving to exploring global and specific protein interaction networks, termed "interactomes." Approaches to connecting viral with cellular interactomes are reviewed by Bailer and Haas (2009). There are an increasing number of techniques for studying protein–protein interactions (Brymora et al., 2004; Guan and Kiss-Toth, 2008; Williamson and Sutcliffe, 2010), some of the major ones being listed below:

a. Yeast Two-Hybrid System (reviewed by Brachmann and Boeke, 1997; Suter et al., 2008; Fields, 2009)

In the basic two-hybrid system one protein, termed the bait, is cloned so that it is fused to a DNA-binding domain (DBD, commonly derived from Gal4 or LexA). The other protein, termed the prey, is fused to an activation domain (AD, usually from Gal4 or the transactivator VP16). The two clones are introduced into a suitable yeast line and interaction between the bait and prey proteins tether the DBD to the AD allowing the activation of a downstream reporter (e.g., lacZ, HIS3, LEU2, or URA3); the basic system and some variations are illustrated in Figure 7.4.

b. Cross Linking

Proteins can be cross-linked to one another or to nucleic acids by treatment with chemicals or by UV radiation (see Ausubel et al., 1998 for details). The size of the chemical cross-linker can give information of the distances separating the proteins.

c. Sandwich Blots

In an adaptation of western blotting where the protein, immobilized on a membrane, is detected by an antiserum, a second protein can be allowed to bind to the immobilized protein and then detected by a specific antiserum. This allows protein–protein interactions to be studied. However, it must be recognized that the immobilization of the first protein may alter its conformation or hide binding sites.

5. Protein–Nucleic Acid Interactions

a. Yeast Three-Hybrid System (reviewed by Brachmann and Boeke, 1997; Wurster and Maher, 2010)

In this system, interactions between three partners are studied. These can be RNA–protein interactions and also functional activation of one partner through phosphorylation by a tyrosine kinase and the extracellular domains of transmembrane receptors. RNA–protein interactions require two interacting proteins and one interacting RNA. One part of the hybrid RNA acts in a known interaction and the other part is used to screen for RNA-binding proteins. These interactions bring together the components, which allow the activation of a downstream reported as in the two-hybrid system described above.

b. DNA and RNA Footprints

The basis of this assay is that bound protein protects the DNA or RNA from DNase- or RNase-catalyzed hydrolysis. The protected DNA or RNA fragments are separated by denaturing gel electrophoresis and analyzed by techniques, such as sequencing or autoradiography. Binding curves for each individual protein-binding site can give quantitative information and site that interact cooperatively can be identified (Ausubel et al., 1998).

c. Gel Mobility Shift

This assay using non-denaturing polyacrylamide gel electrophoresis provides a simple, rapid, and sensitive method for detecting proteins that bind to nucleic acids.
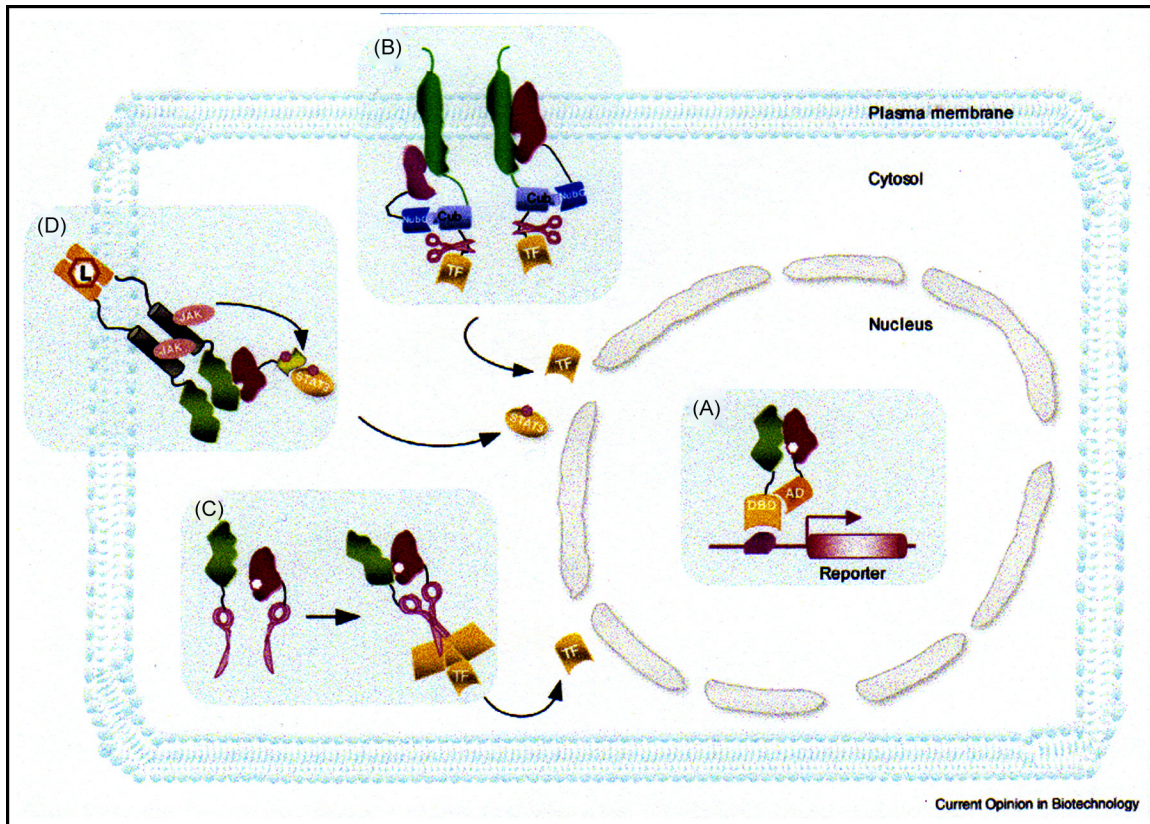
FIGURE 7.4 Yeast two-hybrid (YTH) and important variants in yeast and mammalian cells. (A) Classical (nuclear) YTH. The bait protein of interest (green) is fused to the DBD of a transcript activator, and the prey protein (red) or a library of prey proteins is fused to the AD. The interaction between bait and prey reconstitutes the functional transcription factor and results in the expression of the reporter gene for positive selection. Transcription factors in YTH are typically Gal4 or LexA activator proteins. (B) Membrane yeast two-hybrid system (MYTH). In MYTH, integral or peripheral membrane proteins (baits) are fused to the C-terminal half of ubiquitin (Cub), followed by a transcription factor (TF). Preys (membrane or cytosolic proteins) are expressed as fusions with the N-terminal half of ubiquitin (Nub). Bait–prey interaction reconstitutes native Ub, which is then cleaved by endogenous Ub-specific proteases (UBPs). The transcription factor enters the nucleus and activates reporter gene expression. (C) Split TEV system. TEV protease (scissors) is functionally reconstituted by a bait–prey interaction. TEV cleavage of the recognition sequence releases a transcription factor (TF). The activator enters the nucleus and drives reporter gene expression (transcription-coupled split-TEV). Alternatively, cleavage by TEV releases a luciferase enzyme, which is only active when liberated (proteolysis-only split-TEV, not shown). (D) Mammalian protein–protein interaction trap (MAPPIT). Recruitment and activation of STAT3 transcription factor (purple) by Janus kinases (JAKs) normally occurs when the receptor is activated and clustered by ligand (L) binding to the extracellular domain of the receptor. For MAPPIT, the bait protein is fused to a cytokine receptor variant (gray) that cannot recruit the STAT3 transcription factor. Prey proteins are fused to a functional receptor that contains docking sites for STAT3 (light green). Bait–prey interaction results in the phosphorylation (pink) of the STAT transcription factor. Phosphorylated STAT3 transcription factor translocates to the nucleus and activates a reporter gene. *From Suter et al. (2008) with permission of the publishers.*

The binding of the proteins retards the mobility of the nucleic acid fragment on gel electrophoresis that can be detected by comparing the mobilities of treated and untreated nucleic acid fragments. The retarded fragments correspond to individual protein–nucleic acid complexes and can identify specific purified proteins or uncharacterized proteins in crude extracts. Properties of the protein–nucleic acid interaction, such as affinity and binding specificity, can be studied by this technique (Ausubel et al., 1998).

d. North-Western Blot

In north-western blots, either the protein or nucleic acid under study is separated by gel electrophoresis and blotted to a membrane. The membrane is then probed with the counterpart nucleic acid or protein under study and binding is detected by methods, such as label on the nucleic acid or antiserum to the protein. As with sandwich blots, it should be remembered that immobilization might alter the conformation of the macromolecule.

III. HOST FUNCTIONS USED BY PLANT VIRUSES

Like all other viruses, plant viruses are intimately dependent on the activities of the host cell for many aspects of replication.

A. Components for Virus Synthesis

Viruses use amino acids and nucleotides synthesized by host cell metabolism to build viral proteins and nucleic acids. Certain other more specialized components found in some viruses, e.g., polyamines (Chapter 6, Section V, H, 4), are also synthesized by the host.

B. Energy

The energy required for the polymerization involved in viral protein and RNA synthesis is provided by the host cell, mainly in the form of nucleoside triphosphates (NTPs).

C. Protein Synthesis

Viruses use the ribosomes, tRNAs, and associated enzymes and factors of the host cell's protein-synthesizing system for the synthesis of viral proteins using viral mRNAs. All plant viruses appear to use the 80S cytoplasmic ribosome system. There is no authenticated example of the chloroplast or mitochondrial ribosomes being used. Most viruses also depend on host enzymes for any posttranslational modification of their proteins, e.g., glycosylation.

D. Nucleic Acid Synthesis

Almost all viruses code for an enzyme or enzymes involved in the synthesis of their nucleic acids, but they may not contribute all the polypeptides involved. For example, in the first phase of the replication of caulimoviruses, the viral DNA enters the host cell nucleus and is transcribed into RNA form by the host's DNA-dependent RNA polymerase II. In most, if not all, RNA viruses, the replication complex comprises the viral RdRp, several other virus-coded activities, and various host factors. ssDNA viruses alter the cell cycle constraints on the host DNA replication system. These aspects will be developed in greater detail in subsequent sections and in Chapter 16.

E. Structural Components of the Cell

Structural components of the cell, particularly membranes (described in Box 7.1), are involved in virus replication. For example, viral nucleic acid synthesis usually involves a membrane-bound complex. This will be described in more detail in Section IV, F.

F. Host Defense

The RNA silencing host defense system plays an important role in the replication of many viruses. This is described further in this chapter and also in Chapters 5, 9, and 13.

IV. REPLICATION OF POSITIVE-SENSE SINGLE-STRANDED RNA VIRUSES

More than 60 genera of plant viruses with (+)-sense ssRNA genomes have been established (Appendix C), but we have a significant body of information about the replication of only a few of these. However, it is likely that all viruses with this form of genome have the same basic replication mechanism though there will be variations in detail.

The basic mechanism of replication of (+)-sense RNA genomes is that the virus-encoded replicase, translated from the viral genome, synthesizes a complementary (−) strand using the (+) strand as a template and then new (+) strands are synthesized from the (−)-strand template. Synthesis of new RNA is from the 3′–5′ ends of the templates. Replication occurs in a replication complex that comprises the templates, newly synthesized RNA, and the replicase and host factors and has three phases, initiation, elongation, and termination. It must be remembered that replication itself is just one stage in the dynamic and integrated process of producing new genomes from the input virus that can spread throughout a susceptible host. These will be discussed in general terms here, and details are given of some viral systems that have attracted most study in Chapter 16.

A. Replicase

Three or more virus-coded enzymatic activities can be involved in the replication of (+)-strand RNA viruses, the RdRp, a helicase (HEL), and a methyltransferase (MT) activity. These are collectively known as the viral replicase, but sometimes this term is used (incorrectly) for the RdRp. Although the ascribed function has not been formally demonstrated for most of these activities, and technically, they should be termed RdRp-like, HEL-like, and MT-like, for the sake of simplicity I will use the functional terms in this book.

1. *RNA-Dependent RNA Polymerase* (reviewed by Ng et al., 2008; Cameron et al., 2009)

RdRps catalyze the formation of phosphodiester bonds between ribonucleotides in an RNA template-dependent fashion. For full details of the process, see Ng et al. (2008). Two features have made this enzymic activity difficult to study. First, it is usually associated with membrane structures in the cell and, on isolation, the enzyme(s) often become(s) unstable. It is, therefore, difficult to purify sufficiently for positive identification of any virus-coded polypeptides. Second, soluble fractions from tissues of healthy plants may contain low amounts of a host enzyme with similar activities. The amounts of such enzyme activity may be stimulated by virus infection.

Details of RdRps are given in Box 7.2.

BOX 7.2 Some Properties of Viral RdRps

Eight motifs have been recognized in the amino acid sequences of RdRps (Kamer and Argos, 1984; Koonin, 1991; Koonin and Dolja, 1993; Poch et al., 1989). The most conserved of these is a Gly–Asp–Asp (GDD) motif which is flanked by segments of mainly hydrophobic amino acids and is involved with the binding of Mg^{2+} which is essential for enzyme activity. Details of these motifs have led to RdRps being classified into three superfamily:

Supergroup I is sometimes called the "picornavirus (-like)" supergroup;

Supergroup II the "carmovirus (-like)" supergroup;

Supergroup III the "alphavirus (-like)" supergroup.

Supergroup I members are characterized by usually having one genome segment that has a 5′ VPg and expresses its genetic information as a polyprotein. Members of supergroups II and III have one to several genome segments, the RNA is often capped and individual genes are translated.

The supergroups extend across viruses that infect vertebrates, plants, and bacteria and there are representatives of plant viruses in each supergroup (Table 7.1). The members of each group have several properties in common.

The three-dimensional structure of the RdRp of several viruses has been determined by X-ray crystallography (Hansen et al., 1997; van Dijk et al., 2004) (Figure 7.5A).

TABLE 7.1 Organization of Domains Within the (+)-Strand RNA Virus Replication Protein ORFs

Virus Group	MT[a]	HEL Superfamily[b]	RdRp Superfamily[b]	MT and HEL Separated from RdRp			Comments
				Different ORF	RT or FS	Protease	
Secoviridae	–[c]	III	I	–	–	+	5′ VPg; replication proteins generally in C-terminal region of polyprotein
Alphaflexiviridae	+	I	III	–	–	–	No apparent separation of MT and HEL from RdRp in Potex- and Allexi-virus[b]
Betaflexiviridae	+	I	III	–	–	+	No apparent separation of MT and HEL from RdRp in Fovea-; Tricho-, and Vitii-virus[b]
Tymoviridae	+	I	III	–	–	+	–
Bromoviridae	+	I	III	+	–	–	RdRp on different RNA species HEL superfamily (Garriga et al., 2004)
Closteroviridae	+	I	III	–	+	–	–
Luteoviridae	–	+[e]	II	–	+	–	5′ VPg
Potyviridae	–	II	I			+	5′ VPg; replication proteins generally in C-terminal region of polyprotein
Tombusviridae	–	–	II	–	+	–	Polymerase parts separated by RT or FS yet no MT or HEL motifs
Virgaviridae	+	I	III	+	+	–	All genera except Hordeivirus separation by RT; Hordeivirus RdRp on different RNA species
Benyvirus	+	I	+	–	–	+	–
Cilevirus	+	+	+	–	–	+	–
Idaeovirus	+	I	III	?	?	?	Separation not determined[d]
Ourmiavirus	?	?	+	?	?	?	MT and HEL motifs not reported
Polemovirus	–	+	II	–	+	–	5′ VPg
Sobemovirus	–	?	I	?	+	?	5′ VPg; HEL motif not reported yet polymerase expressed as FS
Umbravirus	?	?	II	?	+	?	MT and HEL motifs not reported yet polymerase expressed as FS

(Data from King et al., 2012)
[a]HEL, helicase; MT, methyl transferase; RdRp, RNA-dependent RNA polymerase; FS, frameshift; RT, readthrough.
[b]HEL superfamilies described in text; RdRp superfamilies described in this Box.
[c]−, not detected; +, occurs; ?, no information.
[d]Reviewed by van der Heijden and Bol (2002).
[e]HEL present but not assigned to any superfamily.

The overall structure of the enzyme is similar to those of other polymerases [DNA-dependent DNA polymerase, DNA-dependent RNA polymerase, and reverse transcriptase (RT) (Figure 7.5B)] that have been likened to a right hand. The palm domain contains the catalytic core and is similar to that of the other three polymerases. The thumb and finger domains differ from those of the other polymerases. Using the neural net PHD (for Predict at Heidelberg) computer method (Rost et al., 1994), O'Reilly and Kao (1998) predicted the secondary structure of the RdRps of BMV, TBSV, and TMV and compared the predictions with the poliovirus RdRp structure. This analysis indicated that the RdRps of these supergroup 2 and 3 viruses have a similar structure to the supergroup 1 poliovirus enzyme, each containing a region unique to RdRps.

The palm domain contains five of the amino acid sequence motifs (A–E), which have the following functions:

A. coordination of one of the two Mg^{2+} ions required for function; possibly selection of ribose over deoxyribose;

B. possibly selection of ribose over deoxyribose;

C. coordination of the other Mg^{2+} ion;

D. completion of the palm core structure;

E. hydrophobic interaction with the thumb.

The RdRp unique region is thought to be involved in the interaction with the thumb of a neighboring polymerase.

Figure 7.5C shows models for initiation and elongation of RNA synthesis by RdRp.
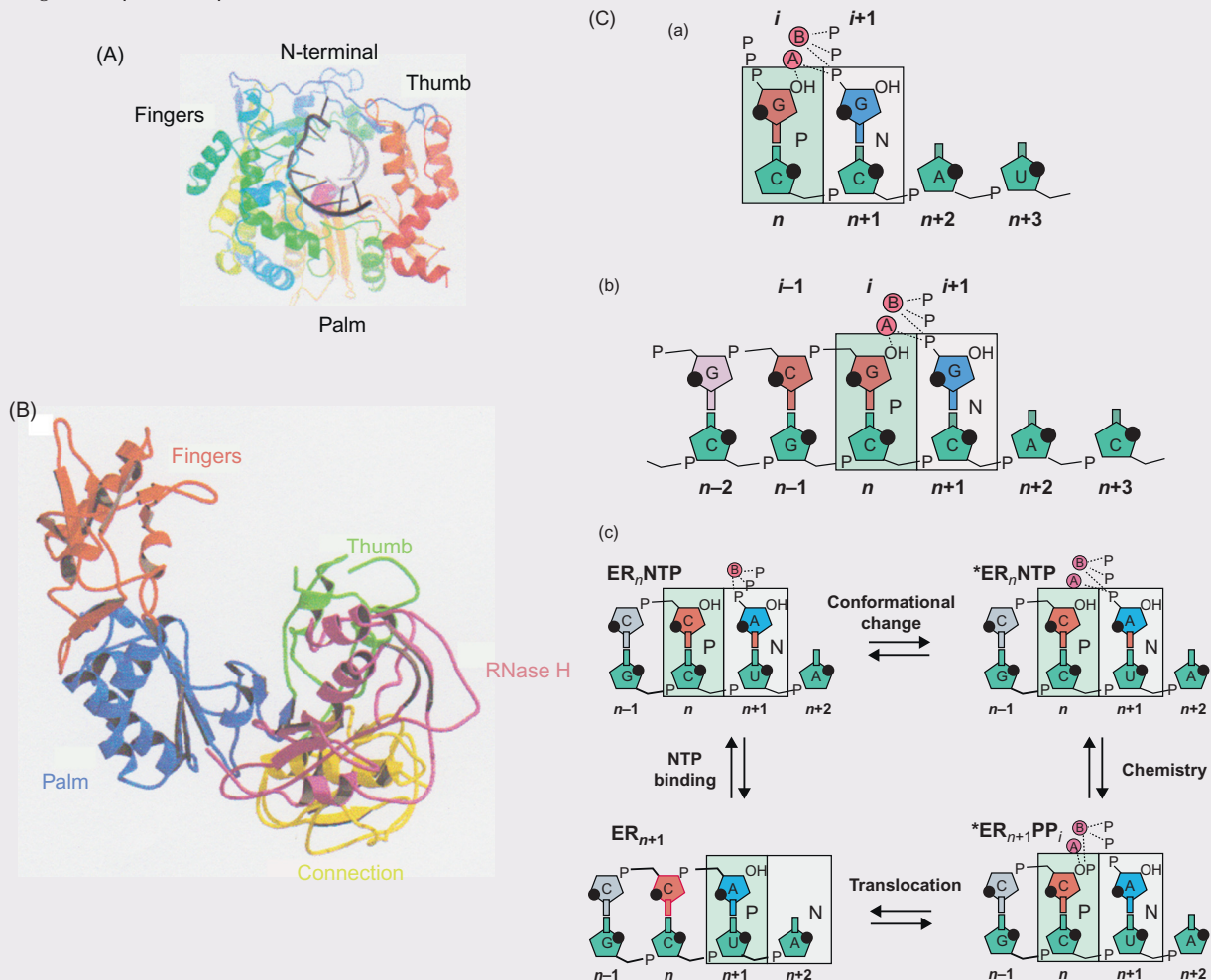
FIGURE 7.5 Structure and function of polymerases. Panel (A): Ribbon representation of FMDV (1WNE) RdRp structure (rainbow coloring with blue at the N-terminus and red at the C-terminus) bound to RNA template (black) and primer (gray) showing the finger, palm, and thumb domains (Ferrer-Orta et al., 2004). Panel (B): Crystal structure of MMTV RT shown as a ribbon diagram with the fingers domain in red, palm in blue, thumb in green, connection in yellow, and the RNaseH in magenta. Panel (C): *De novo* initiation and elongation of RNA synthesis by RdRp. Subpanel (a): *De novo* initiation of RNA synthesis involves binding of the initiating nucleotide (GTP*j*; red) at the priming or initiation site (P-site; green box), and binding of the first NTP substrate (GTP*i*+1; blue) to the nucleotide binding site (N-site; white box). Specific binding sites for divalent cations (pink circles A and B) are shown in close proximity to the α-, β-, and γ-phosphates of the first nucleotide substrate. Subpanel (b): Elongation complex. Nucleotide addition during elongation involves binding of the nascent RNA primer strand, positioning of the 3′-terminal nucleotide in the P-site, and binding of the first NTP substrate (*i*+1; blue) to the nucleotide binding site (N-site; white box). Subpanel (c): Elongation cycle. The stages of RNA synthesis can be divided into four steps: nucleotide binding (step 1), a conformational-change step, thought to be orientation of the triphosphate for catalysis (step 2), chemistry (step 3), and translocation (step 4). *Panels (A) and (C) from Ng et al. (2008) and Panel (B) from Das and Georgiadis (2004) with permission of the relevant publishers.*

The RdRp activity present in uninfected plants was thought at one time to be involved in RNA virus replication. This activity has been isolated as a cDNA clone encoding a 128-kDa protein (Schiebel et al., 1993, 1998).

2.   *Helicases* (reviewed by Kadarei and Haenni, 1997; Bird et al., 1998; Tuteja, 2000)

HELs are polynucleotide-dependent NTP phosphatases that possess ssDNA and/or RNA-displacing activity. They play a pivotal role in genome replication and recombination by displacing complementary strands in duplex nucleic acids and possibly removing secondary structure from nucleic acid templates. Some HELs require a 3′ flanking single strand of nucleic acid and others a 5′ flanking single strand; these are known as 3′–5′ and 5′–3′ HELs, respectively.

Based on conserved amino acid sequence motifs, HELs have been grouped into a number of superfamilies. Gorbalenya and Koonin (1993) recognized five superfamilies, three of which have representatives in (+)-strand RNA viruses. Superfamilies I and II have seven conserved motifs whereas superfamily III has three motifs. Two motifs common to all three superfamilies are variants of ATP-binding motifs and have the conserved sequences: GXXXXGKT/S and ΦΦΦΦD where X is an unspecified amino acid and Φ is a hydrophobic residue. Most members of superfamilies I–III are 3′–5′ HELs. The superfamily designation for various plant virus genera is given in Table 7.1.

The crystal structure for the hepatitis C virus RNA HEL (superfamily II) has been determined and mechanism for unwinding duplex RNA suggested (Cho et al., 1998). The structure comprises three domains forming a Y-shaped molecule. The RNA-binding domain is separated from the NTPase and other domain by a cleft into which ssRNA could be modeled. It is suggested that a dimer form of this protein unwinds dsRNA by passing one strand through the channel formed by the clefts of the two molecules and by passing the other strand outside the dimer. Because of the conserved motifs between the various superfamilies, it is likely that many of the features determined for the hepatitis C virus HEL are applicable to this enzyme from plant viruses. The structure of ToMV helicase (superfamily I) has recently been determined (Nishikiori et al., 2012).

Several plant virus genera appear to lack the characteristic NTP-binding motifs of HELs (Table 7.1). Several possible reasons for this have been suggested (Buck, 1996): (i) it is possible that the NTP-binding motifs have diverged so much that they are not recognizable from the primary amino acid sequence; (ii) the viral polymerase may have unwinding activity; (iii) unwinding may be effected by a helix-destabilizing protein that uses the energy of stoichometric binding to ssRNA to melt the duplex in the absence of NTP hydrolysis; and (iv) the virus may co-opt a host HEL.

Some viruses have additional HEL activities located elsewhere in their genomes. It is thought that the additional HELs in benyviruses, hordeiviruses, and potexviruses are involved in cell-to-cell movement (Chapter 10).

3.   *MT Activity* (reviewed by Schuman and Schwer, 1995)

The MT activity leading to 5′ capping of RNAs is described in Chapter 6, Section I, C, 1, a.

4.   *Organization of Replicase Functional Domains in Viral ORFs*

The presence or recognition of the three above functional domains of viral replicases in members of the (+)-stranded plant virus genera is listed in Table 7.1. From this it can be seen that not all the genera have all three domains. There are several reasons for this: (i) only those viruses that have a m^7G 5′ cap would require the MT activity and (ii) no HEL domain has been recognized in the *Tombusviridae*, the sobemoviruses and umbraviruses.

For all the viral genomes that express as polyproteins or fused protein (frameshift or readthrough), the domains appear to be in the order (N-terminal to C-terminal) MT, HEL, and the RdRp; in divided genomes it is not possible to allocate the order. However, a feature of many of the virus genera is that the MT and HEL domains are separated from the RdRp domain. This can be by the MT and HEL domains being in one ORF and the RdRp being in a separately expressed ORF, by them being in two adjacent ORFs separated by either a frameshift or readthrough translational event or them being on a polyprotein and separated by protease activity (Table 7.1). However, in the *Betaflexiviridae* (except vitiviruses) the protease domain lies between the MT and the other two domains. In the Marafi-, Viti-, and Idaeo viruses, the three domains appear not to be separated.

For many viruses, it appears that the MT and HEL domains are on a single protein. However, although these two activities are expressed on the same ORF of BYV, probing extracts from infected plants with monoclonal antibodies indicates that *in vivo* the 295-kDa protein is processed to a 63-kDa protein containing the MT domain and a 100-kDa protein containing the HEL domain (Erokhina et al., 2000).

B.   Viral Templates

Two kinds of RNA structures have been isolated from viral RNA synthesizing systems. One, known as *replicative form* (RF), is a fully base-paired ds structure, whose role is not certain. For example, it may represent RNA molecules that have ceased replicating. The other, called replicative
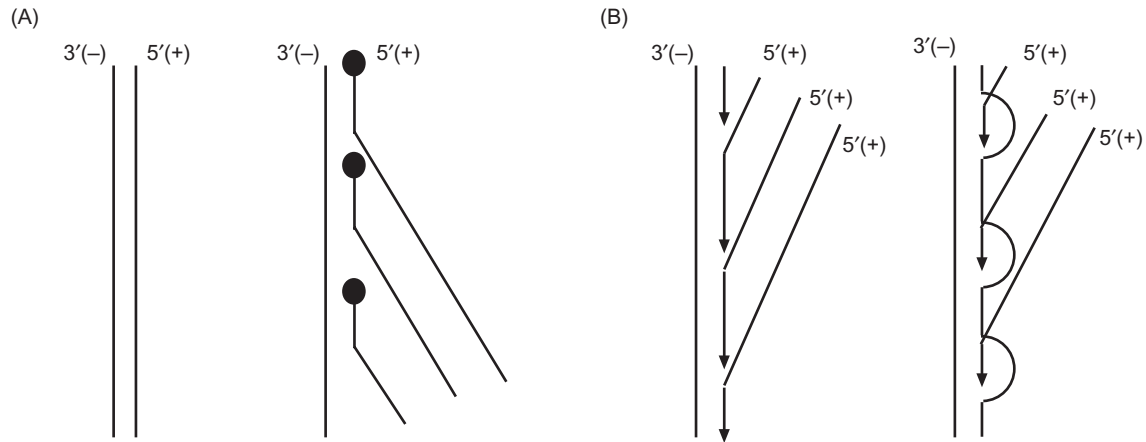
FIGURE 7.6 Replication of (+)-strand ssRNA. (A) Formation of association between positive- and negative-sense viral RNA; left, RF; right, RI. Redrawn from Hull (2002) with permission of the publishers. (B) Possible structures of RI: left, semi-conservative; right, conservative. *Redrawn from Buck (1999) with permission of the publishers.*

intermediate (RI), is only partly ds and contains several ss tails (nascent product strands) (Figure 7.6).

The structure of the RI is closely related to that actually replicating the viral RNA. It is thought that the RI as isolated may be derived from a structure like that in Figure 7.6A by annealing of parts of the progeny strands to the template.

The ds nature of RFs and RIs is apparent when these molecules have been isolated from infected cells. The nature of the structures in the infected cell is unknown but is of great significance in view of the importance of dsRNA in the plant defense RNA silencing response (Chapter 9, Section I, C, 1). It is likely that these molecules are essentially single-stranded *in vivo*, the strands being kept apart either by compartmentalization or by proteins bound to them. Cytological evidence suggests that the RI of TYMV is essentially ss (Bedbrook and Matthews, 1976).

It is suggested that the RF could arise from the initial synthesis of a (−) strand on a (+)-strand template. RI RNAs usually contain more (+) strands than (−) strands (Aoki and Takebe, 1975) which is taken to indicate that each is a single (−) strand to which is attached several (+) strands (Figure 7.6A).

There are two hypotheses as to the mechanism of (+)-strand synthesis. The semiconservative mechanism involves total displacement of the newly synthesized strand by the oncoming strand (Figure 7.6B) and, in the conservative mechanism, it is suggested that the duplex RNA is only transiently unwound at the growing end of the nascent strands (Figure 7.6B). The majority of evidence supports the semiconservative mechanism (reviewed by Buck, 1999).

The (+) and (−) forms of the viral genome contain signals that control both the specificity and timing of their replication. Information on these has been obtained from studies on genomic RNAs and defective (D) and defective interfering (DI) RNAs (Section IV, C).

1. Promoter and Control Signals

To initiate RNA synthesis at the right position, on the right template, and at the right time, the viral RdRp recognizes specific RNA elements (promoter sequences) in the (+) and (−) strands.

In general, nucleic acid replication is primed (started) at the promoter by one of three mechanisms: primed by small separate (host) RNA or DNA sequence complementary to the (+)- or (−) strand; primed by the 3′ end of the (+)- or (−) strand folding back upon itself to form a short ds segment; or being primed independently by binding of the polymerase complex (possibly including one or more host protein) onto a specific nucleic acid structure or sequence at the 3′ end of the (+)- or (−) strand. The replication of most, if not all, plant (+)-sense ssRNA viruses is primed by the latter mechanism. As well as terminal RNA elements internal elements (internal replication elements, IREs) are involved in the replication of some viruses (e.g., tombusviruses) (Nicholson et al., 2012).

One further point that needs to be taken into account is that both the (−)- and (+) strands are synthesized by the same polymerase, the virus-encoded RdRp, and thus one would expect similarity between the promoters. Surprisingly, there exists little similarity in sequence or structure between these promoters within one viral genome (Miller and Koev, 2000). The question arises as to how a single type of RdRp recognizes promoters that are different in sequence and polarity. Furthermore, as noted in Chapter 6, there needs to be a switch between translation and replication on the same initial (+)-strand input template.
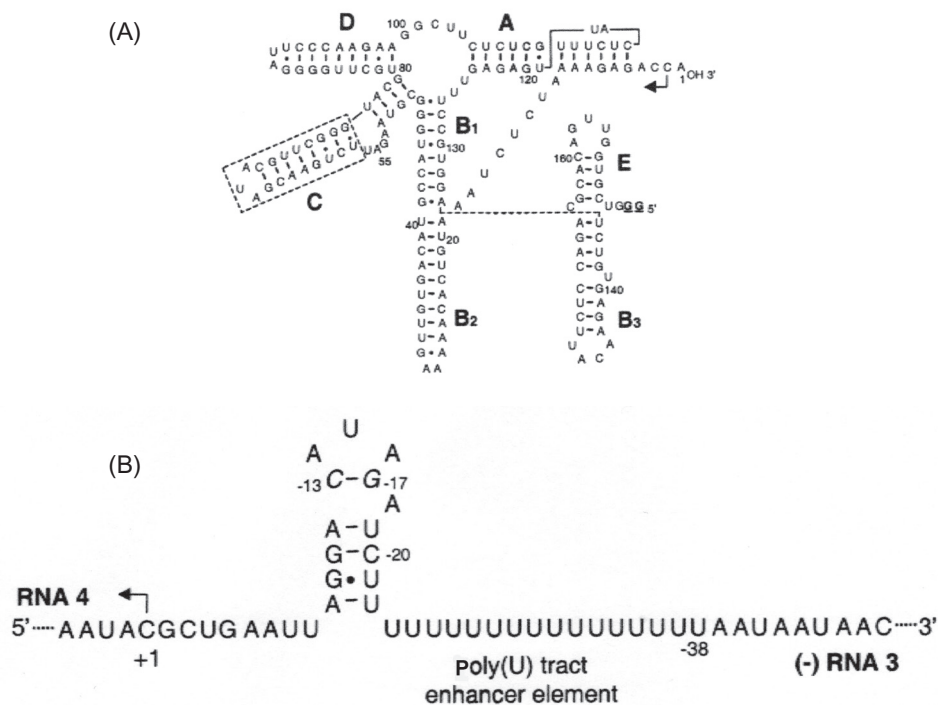
FIGURE 7.7 Structure of BMV promoters. (A) Predicted secondary structure of an RNA transcript corresponding to the 3′ terminal 173 nt of (+)-strand BMV RNA3. Stem-loop structures A–E are indicated: note that stem-loop A is involved in a pseudoknot interaction. (B) Nucleotide sequence of BMV (−)-strand RNA4 (nts 1196–1247) encompassing the sg core promoter (−20/+1) with the proposed hairpin structure and poly(U) tract enhancer element. Indicated are the RNA4 transcription initiation site +1 and the 13 nt C-13 and G-17 which have been shown to form an essential base pair. *From Haasnoot et al. (2002) with permission of the publishers.*

a. Promoters for (−)-Strand RNA Synthesis

For (−)-strand synthesis, the 3′ terminus of the (+) strand has been found to be important (reviewed by Dreher, 1999). As shown in Appendix C, there are three basic structures in plant viral RNA 3′ termini, tRNA-like structures (TLS), poly (A) tails, and non-tRNA heteropolymeric sequences. Dreher (1999) points out that there is no real correlation between the 3′ terminal structure and the super-grouping of RdRps (Box 7.2), which might indicate similar roles for dissimilar 3′ termini.

There is strong evidence that, for many (+)-strand RNA viruses (−)-strand synthesis is initiated in secondary (or perhaps tertiary) structures in the 3′ UTR of the (+) strand. The 3′ UTRs of some viruses form TLS (Chapter 6, Section I, D, 2) in which (−)-strand synthesis is initiated. For example, the 3′ 134 bases of BMV RNAs containing the TLS were identified as the minimum sequence requirement for *in vitro* (−)-strand production (Miller et al., 1986). The replicase core recognition site is the triloop AUA of stem-loop C of the TLS (Figure 7.7).

Initiation *in vitro* is primer independent and is at the penultimate C residue of the 3′-CCA. This indicates that the terminal A residue is not templated but is added to the

(+) strands by tRNA nucleotidyl transferase after replication (Rao et al., 1989). The TLS at the 3′ end of the (+) strand contains the replicase-binding site (Chapman and Kao, 1999), the specificity for the interaction with the replicase being determined by a stem-loop structure in the tRNA-like domain (Rao and Hall, 1993; Chapman and Kao, 1999; Sivakumaran et al., 2000).

As was initially shown by Bol et al. (1971), inoculation with the three AMV RNA species does not give infection unless either viral CP or the sg mRNA (RNA4) for CP is present (Box 7.3). The ilarviruses also have a requirement for CP to initiate infection and functional equivalence has been shown between their CP and that of AMV by the activation of the AMV genome by TSV coat protein (CP) and *vice versa* (van Vloten-Doting, 1975).

TYMV RNA has an 82-nt-long 3-terminal′ TLS which can be valylated and which terminates in 3′ACCA. (−)-strand synthesis initiates specifically opposite the penultimate residue of the TLS (Singh and Dreher, 1997). Binding of eEF1A GTP to the valylated TLS strongly represses (−)-strand synthesis (Matsuda et al., 2004). It is suggested that this binding occurs early in infection and helps coordinate between competing translation and

BOX 7.3 Alfalfa Mosaic Virus CP Involvement in Translation and Replication (reviewed by Bol, 2005)

The genome organization of AMV (and closely related Ilarviruses) is similar to that of BMV (Appendix A, Profile 51), but there is an involvement of CP, either from the virus particle or expressed from sgRNA4 for replication in a process called genome activation. One to three CP dimers bind through their N-terminal amino acids (Jaspars and Houwing, 2002) to a specific site near the 3′ terminus of RNAs 1–3. There is a homologous sequence at the 3′ termini of the three genomic RNAs which can assume two mutually exclusive conformers (Olsthoorn et al., 1999) (Figure 7.8).

In the CP-binding conformation (CBP) the 3′ 112 nt of the UTRs of RNAs 1–3 and of the sgRNA4 form a linear array of five stem loops (A–E) separated by the sequence AUGC (Figure 7.8A). The CP dimers bind to stem loops A and B

(Ansel-McKinney and Gehrke, 1998; Figure 7.8C). Efficient translation of AMV RNAs requires this CP binding (Neeleman et al., 2001, 2004). The CP interacts with wheat germ eIF4G and eIFiso4G components of the initiation factor complexes eIF4F and eIFiso4F (Krab et al., 2005). Extension of the AMV RNAs with a poly(A) tail circumvents the requirement for CP to initiate infection suggesting that in a wild-type infection, CP mimics the function of PABP (Neeleman et al., 2001).

In the alternative conformation (TL), the 3′UTRs of RNAs 1–3 assume a pseudoknotted structure through base pairing between the D and A loops; this resembles the bromovirus TLS (Olsthoorn and Bol, 2002; Figure 7.8B); unlike bromoviruses, this TLS does not charge with amino acids. The crystal structure of an AMV RNA–peptide complex shows that the
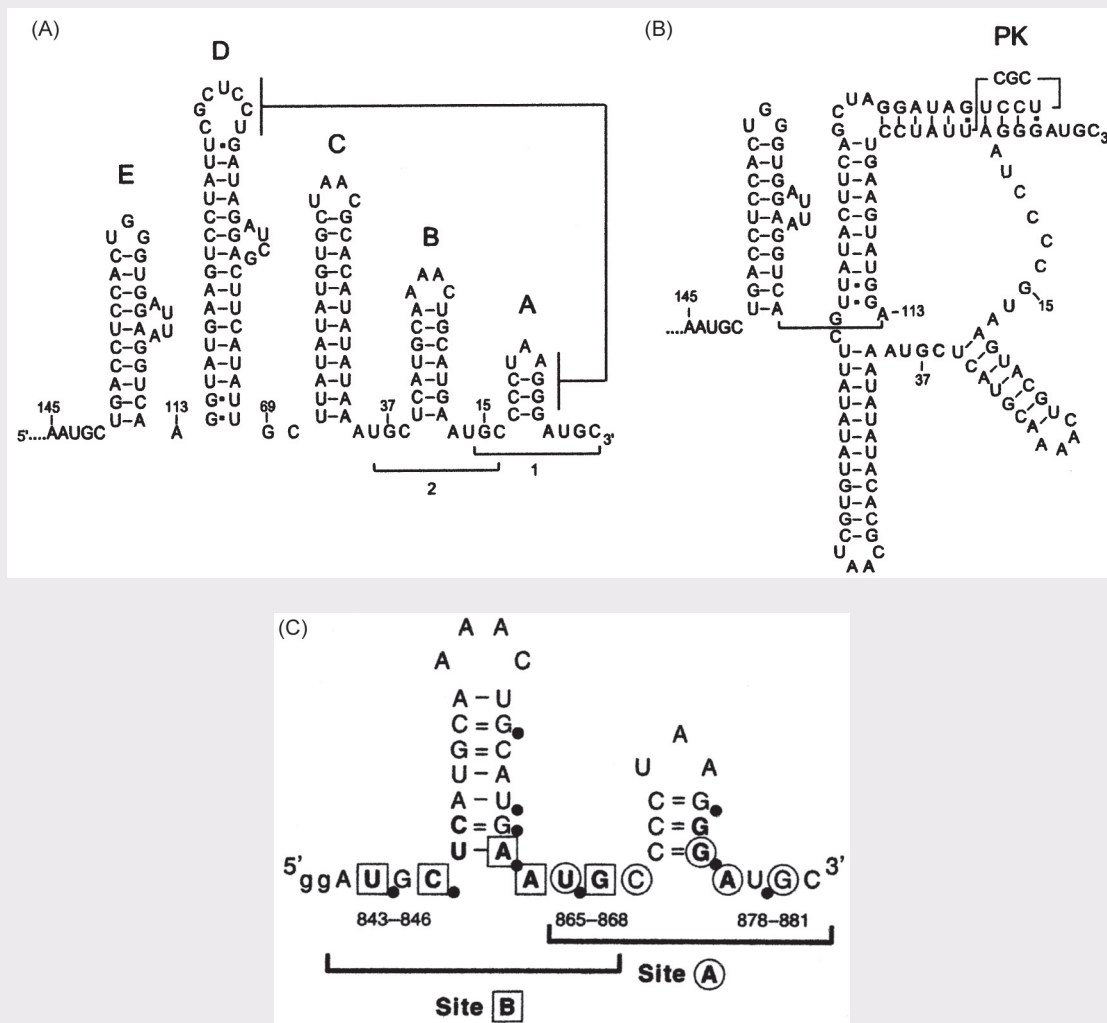
FIGURE 7.8 Structure of AMV RNA3 3′ terminus. (A) and (B) The two conformers of AMV RNA3 3′ 145 nt. (A) CBP conformer. The two major CP binding sites are indicated by brackets. Base pairing between loop D and stem A promotes TL conformation. (B) Secondary structure of the TL conformer. From Chen and Olsthoorn (2010) with permission of the publishers. (C) Secondary structure of the 3′ terminal 39 nt of AMV RNA3 with nucleotides interacting with N-terminal peptides of AMV CP indicated. Circled and boxed letters represent putative base-specific contacts for proposed peptide-binding sites A and B, respectively. Filled circles represent potential phosphate contacts. *From Ansel-McKinney and Gehrke (1998) with permission of the publishers.*

conserved AUGC repeats co-fold with Pro–Thr–x–Ser–x–x–Tyr CP amino acids giving the TLS (Guogas et al., 2004). The TLS conformation is required for (−)-strand synthesis and *in vitro* studies suggest that hairpin E binds the viral polymerase (Olsthoorn et al., 2004).

Thus, switching between translation and replication is controlled by changes in conformation involving the CP (Olsthoorn et al., 1999). The CP binding appears to have two roles, first in very early stages of virus replication most probably in translation and later in infection in shutting off (−)-strand RNA synthesis. The detailed molecular mechanism of this switch is not yet understood but at some stage could involve expression of the viral replicase. CP enhances the *in vitro* binding of RNA by the replicase proteins suggesting that the CP formed a bridge that gave accurate *de novo* initiation (Reichert et al., 2007). However, it is uncertain as to whether CP is involved *in vivo*.

replication functions of the genomic RNA (see Figure 6.9) (Matsuda et al., 2004; Hammond et al., 2010).

The 3′ terminus of the tymovirus, DuMV, lacks a TLS and terminates with –UUC which differs from that of other tymoviruses. A chimeric TYMV in which the TLS had been replaced with the DuMV 3′ UTR was capable of replication, albeit somewhat reduced, and of systemic spread through the plant (Tzanetakis et al., 2009).

The 3′ UTR of TMV and ToMV RNAs (approximately 200 nt) can be folded into three pseudoknots within its 5′ region and a 3′-terminal TLS (van Belkum et al., 1985; Felden et al., 1996). Other tobamoviruses may have longer 3′ UTRs and more pseudoknots (Gultyaev et al., 1994; Shivprasad et al., 1999; Bodaghi et al., 2000). Regions important for (−)-strand synthesis of TMV are the acceptor and anticodon arms of the TLS, the region with the three pseudoknots, and the central core sequence connecting these elements (Osman et al., 2000). Mutational analysis of the 3′-terminal four nucleotides of the TLS indicated the importance of the 3′-terminal CA sequence for (−)-strand synthesis, with the sequence CCCA or GGCA giving the highest transcriptional efficiency. Several double-helical regions, but not their sequences, which are essential for forming pseudoknots and/or stem-loop structures in the TLS and pseudoknot regions are required for high template efficiency (Osman et al., 2000).

Using UV irradiation to cross-link protein to TMV RNA, Osman and Buck (2003) showed that a 110-amino acid region just downstream of the MT domain in p126 interacts with a region of the 3′ UTR comprising the TLS anticodon arm and the central core sequence. Mutations in the p126 sequence showed that tyrosine residues 409 and 416 are essential for the cross-linking and for the ability of the virus to replicate in protoplasts. It is suggested that the synthesis of TMV (−) strand is initiated by the binding of the TLS and core sequence to the internal sequence in p126 followed by binding of p183 to this complex, thus positioning the catalytic active site of the polymerase domain close to the 3′-terminal CCCA initiation site (Osman and Buck, 2003).

Sequences at the 5′ end of TMV RNA are also important for replication (Takamatsu et al., 1991). Large deletions in the 5′ region and deletion of nucleotides 2−8 from the 5′ end abolished replication, but other small deletions in the 5′ UTR did not. This suggests that the 5′ replicase binding site may be complex.

Internal sequences in the TMV genome have been recognized to inhibit replication in *trans* (Lewandowski and Dawson, 1998). Deletion of the region between nucleotides 3420 and 4902 (sequences encoding the RdRp domain of p183) created a replication-defective RNA (dRNA) that could be replicated in *trans* by wild-type TMV.

The TLS in the 3′ UTR of some viruses (e.g., tymoviruses and hordeiviruses) are the sites of initiation of (−)-strand synthesis, but, as discussed in Chapter 6, for other viruses the TLS may be involved in switching from translation to replication rather than directly in replication initiation.

Other structures in the 3′ UTR, often forming pseudoknots, can be important in the initiation of (−)-strand synthesis as well as controlling transcription. For instance, the 3′-terminal part of the TBSV genome contains four RNA elements important for replication (Fabian et al., 2003; Na et al., 2006) which, together with the 3′ UTR sequences of carmoviruses, are described in detail in Box 6.6. The complex interactions in the replication of the two RNAs of RCNMV are described in Box 7.4.

The 5′ VPg and the 3′ poly(A) sequence are involved in potyviral replication (Figure 6.16). An internal RNA element in the P3 cistron region is possibly involved in the replication (and cell-to-cell movement) of WSMV (Choi et al., 2005). The 3′ terminal sequence of the (−) strand of BaMV contains the sequence CUUU which is involved in initiating (+)-strand synthesis (Chen et al., 2010). The 3′ UTRs of BSMV also contain a poly(A) sequence upstream of the TLS; it is considered that this poly(A) sequence is not required for replication.

b. Promoters for (+)-Strand RNA Synthesis

There is less known about priming of synthesis of (+)-strand genomic RNA from the 3′ end of the (−) strand though priming of sg mRNAs and DI RNAs on the (−) strand can give some indication of how this may be done

As noted above, BMV replication complexes can initiate the synthesis of (−)-strand RNA from a (+)-strand

BOX 7.4 Replication of Red Clover Necrotic Mosaic Virus

The genome of RCNMV is divided into two RNAs, RNA1 and RNA2. RNA1 encodes a 27-kDa protein which frameshifts an overlapping to give p88; these two proteins are required for the replication of RNA1 and RNA2 and form a 480-kDa complex (Mine et al., 2010). RNA1 also encodes the CP which is expressed from a sgRNA (CP sg mRNA). RNA2 encodes the MP.

RNAs 1 and 2 share little homology except for the 5′ first 6 nts and two stem-loop structures located at the 3′ ends of both RNAs. The RNA replication mechanisms differ between RNA1 and RNA2 (Iwakawa et al., 2011).

The 3′ UTR of RCNMV RNA1 contains sequences for both initiation of (−)-strand synthesis and cap-independent translational enhancement (the 3′ TE-DRI, see Chapter 6, Section V, E). These two cis-acting RNA elements are separated and act independently (Iwakawa et al., 2007) (Figure 7.9A).

The 3′ terminal stem-loop structure of RNA1 comprising SLF, SLDE, and SeqB is considered to be the core promoter for (−)-strand synthesis (Weng and Xiong, 2009) and SLDb and SLDc probably act as enhancers (Iawakawa et al., 2007). RNA1 replicates preferentially with p88 translated from its
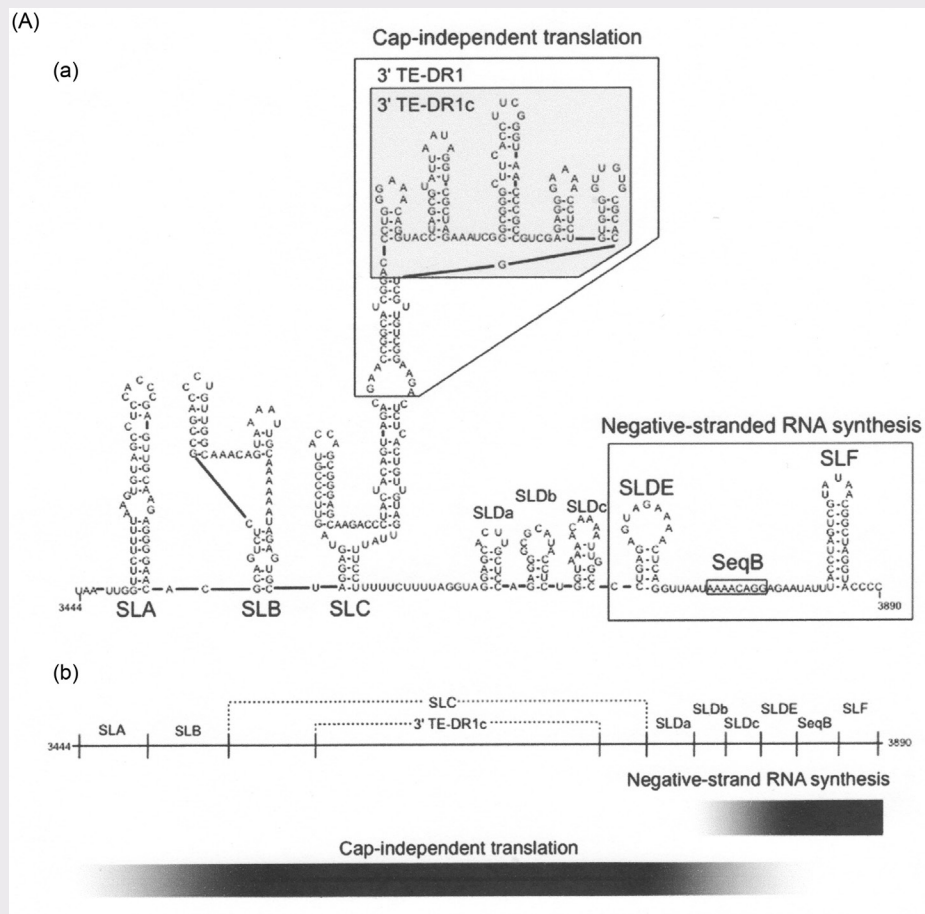
FIGURE 7.9 Panel (A): *cis*-acting RNA elements required for cap-independent translation and (−)-strand RNA synthesis of RCNMV RNA1. Subpanel (a): Essential RNA elements required for cap-independent translation and (−)-strand RNA synthesis are shown in boxes. Subpanel (b): The upper gradient shows importance with respect to (−)-strand RNA synthesis, and the lower gradient shows importance with respect to cap-independent translation of RCNMV-RNA1. From Iwakawa et al. (2007) with permission of the publishers. Panel (B): Secondary RNA structures (predicted using the computer algorithm Dynalign) in regions, in which deletions caused deleterious effects on the accumulation of RCMNV RNA2. Y-shaped RNA structures, SL10s and SL11s predicted in SCNMV and CRSV are presented above those of RCNMV. From An et al. (2010) with permission of the publishers. Panel (C): A model for the early replication process of RCNMV. The RCNMV replicase proteins p27 and p88 are translated from RNA1. For the replication of RNA1, p27 interactions with RNA1 via coupling with translation through polysome binding and recruits RNA1 to the ER membrane. p88 interacts with the 3′ UTR of RNA1 via coupling with translation. The 480-kDa replicase complex containing p88, p27, and host factors is formed at the 3′ UTR of RNA1. At the ER membrane, the RNA1, from which the polysomes have dissociated, serves as a template for (−)-strand synthesis. For the replication of RNA2, p27 and/or the 480-kDa replication complex recognizes the YRE and recruits RNA2 to the ER membrane for (−)-strand RNA synthesis. A subset of RNA1 and RNA2 is recruited to the ER membrane by using an RNA–RNA interaction or unknown mechanisms for (−)-strand RNA synthesis of the CP sg mRNA. *From Iwakawa et al. (2011) with permission of the publishers.*
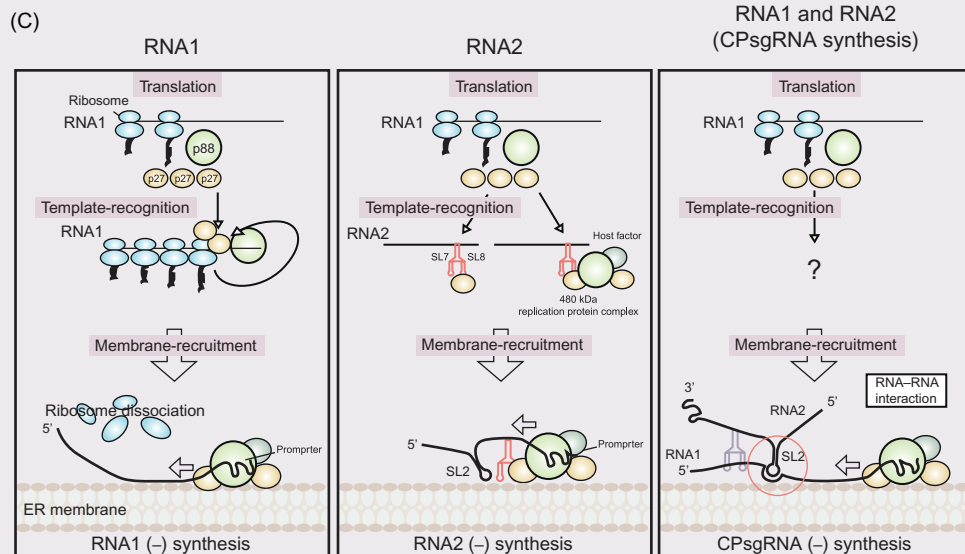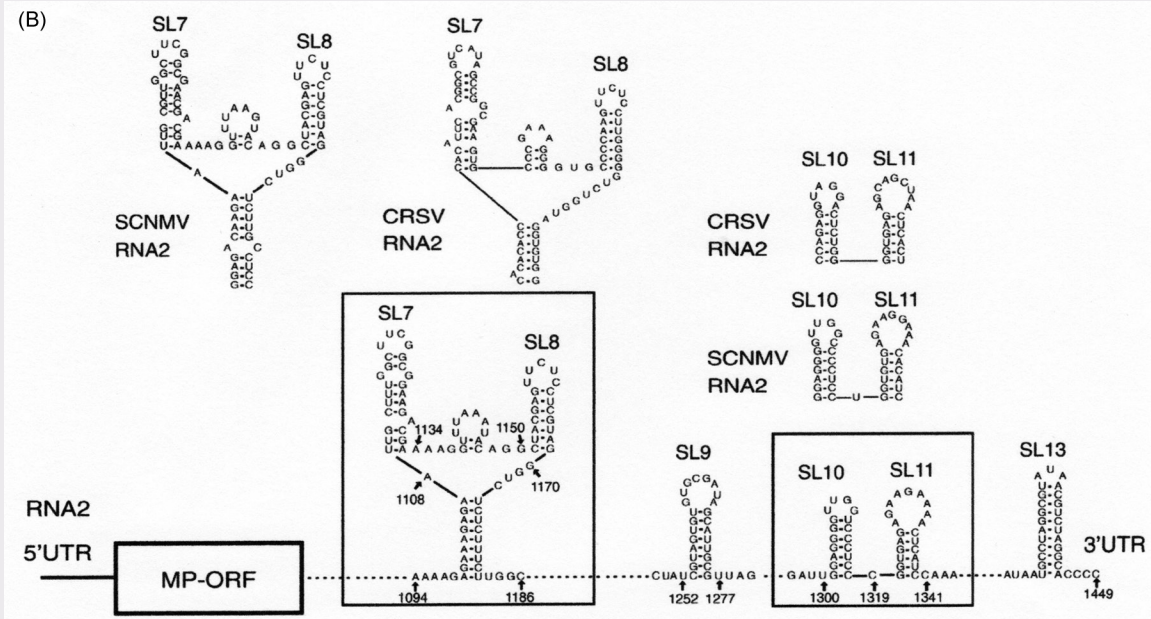
FIGURE 7.9 (Continued)

own molecule in the presence of p27, whereas RNA2 replicates effectively with p88 supplied in *trans* together with p27, presumably as the 480-kDa complex (Okamoto et al., 2008). Thus, essentially it is co-translational replication.

RNA2 requires RNA1 for its replication but, as noted above, replicates by a different mechanism with p88 and p27 supplied in *trans*. The cap-independent translational activity of RNA2 is strongly linked to RNA replication (Mizumoto et al., 2006). Therefore, RNA elements essential for RNA2 replication are also essential to enhance the cap-independent translation of RNA2. RNA2 has *cis*-acting elements that interact with the replication proteins which have been mapped to the

5′ and 3′ UTRs (Takeda et al., 2005; Turner and Buck, 1999) and to the MP ORF (Tatsuta et al., 2005). A terminal stem-loop (SL) structure and another 3′ proximal SL are well conserved between RNA1 and RNA2 and among dianthoviruses and are essential for synthesis of (−) strand of both RNAs (Figure 7.9A and B; Iwakawa et al., 2007). Nucleotide sequences in the other region of 3′ UTR differ between RNA1 and RNA2 (An et al., 2010). Such regions are also important for synthesis of (−)-strand RNA1 (Iwakawa et al., 2007) and especially a Y-shaped structure of SL7 and SL8 which interacts with p27 (Figure 7.9B) for the synthesis of (−)-strand RNA2 (Turner and Buck, 1999; An et al., 2010); it is suggested that the interaction

with p27 functions as a binding site for replication proteins possibly recruiting RNA2 to the membrane-bound replication complex (An et al., 2010).

RNA2 also contains 5′ sequences and an internal sequence in the MP ORF that are essential for replication (Turner and Buck, 1999; Sit et al., 1998). The internal sequence in the MP is a transactivator (TA) consisting of a 34 nt stem-loop structure (SL2) with an eight-nucleotide loop. The loop of the TA is complementary to and binds to an 8-nt sequence, the transactivator binding sequence (TABS), immediately upstream of the CPsgRNA promoter in RNA1 (Sit et al., 1998). This *trans* bimolecular RNA–RNA interaction is essential for transcription of the sg mRNA (Sit et al., 1998) and involves structural changes to the SL2 hairpin (Guenther et al., 2004). The same stem-loop structure (SL2) is also essential for the replication of RNA2 (Tatsuta et al., 2005) but the replication is not associated with

the RNA–RNA interaction with the TABS in RNA1. As well as the stem-loop structure itself, the nucleotide sequence of both the stem and loop are important for this function. The TA also is the origin of assembly of RCNMV virions (Basnayake et al., 2008). It is suggested that the base pairing between the RNA2 TA and the RNA1 TABS initiates co-packaging of the two RNAs. Thus, the TA sequence has at least four functions: (i) it is a *cis*-acting element for RNA2 replication; (ii) it is a *trans*-acting element for the transcription of sg mRNA on RNA1 and hence the expression of the CP; (iii) it is the origin of assembly of RCNMV virions, and (iv) it is part of the coding sequence for the MP.

Thus there are close and integrated interactions between the two RCNMV RNA involved in both their replication and expression. Figure 7.9C shows a model for the early replication events (Iwakawa et al., 2011).

template, but details are lacking on sequence and structural elements involved in the initiation of (+)-strand synthesis. Sivakumaran and Kao (1999) showed that initiation of (+)-strand synthesis requires the addition of a non-templated nucleotide at the 3′ end of the (−)-strand template. Mutational analysis indicated that this non-templated nucleotide, together with the +1 and +2 nucleotides (cytidylate and adenylate), are important for the interaction with the replicase complex. Furthermore, genomic (+)-strand RNA synthesis is affected by sequences 5′ of the initiation site but possibly not by structure in this region. This template recognition is controlled by the sequence rather than by the secondary structure at the 3′ end of the (−)-strand RNA (Sivakumaran et al., 1999).

The 5′ UTRs of bromovirus and cucumovirus RNAs share sequence similarity, the most notable region matching the box B recognition sequence of RNA polymerase III promoters and thus also the conserved TΨC loop of tRNAs (Marsh and Hall, 1987); there is also a box B consensus in the intercistronic region of RNA3. This region in RNAs 1 and 2 contain *cis*-acting factor(s) for RNA replication (Traynor and Ahlquist, 1990).

Although post transcriptional modification of the (−)-strand template of AMV by addition of a 3′-terminal noncoded guanosine and the presence of viral CP are involved in (+)-strand synthesis, they are not totally sufficient (Houwing et al., 2001).

Two domains have been found in the 5′ UTR of TBSV which is region I of the DI RNA (Figure 7.10A). A T-shaped domain (TSD) formed by the 5′ terminal 78 nucleotides (Figure 7.10B) is common to several tombusviruses (TBSV, AMCV, CIRV, CNV, CyRSV), satellite RNAs of TBSV and CyRSV, and TBSV DI RNAs. Deletion and mutation analyses showed that this structure was important for DI RNA accumulation (Wu et al., 2001). In the 3′ half of the 5′ UTR a folded structure, termed the downstream domain (DSD) (Figure 7.10C) which interacts with the TSD as a pseudoknot was also necessary for DI RNA accumulation (Ray et al., 2003).

Region II of the DI RNA (Figure 7.10A, subpanel c) contains one RNA element important for replication. This IRE comprises a functional core structure composed of two noncontiguous segments of sequence that interact with each other to form an extended helical conformation termed the RII stem loop. It is active in the (+) strand, is dispensable late in the viral replication process, and is functionally inhibited by active translation over its sequence (Monkewich et al., 2005). A C–C mismatch in this IRE binds specifically to the p33 moiety of the p33:p33 and p33:p92 replicase, and the interaction is suggested to provide a mechanism to selectively recruit viral RNAs into the cognate replicase complex (Pogany et al., 2005).

FIGURE 7.10 Panel (A): Genome and structural organization of TBSV genomic RNA and prototypical DI-73 and DI-72 RNAs. Subpanel (a): Cartoon showing structural details of the ~4800 nt TBSV genome (not to scale). The p33, p92^{pol}, p41, and the overlapping p19/p22 ORFs are depicted as black ovals and labeled accordingly. Note that p92^{pol} overlaps with p33, sharing the same initiation codon. Sequences playing role(s) in translation, genomic replication, and sgRNA transcription are shown in turquoise blue, red, and purple, respectively. Sequences involved in RNA–RNA interactions are shown in matching colors. Note that translation requires SL3–SLB interaction; UL–DL and RSE–gPR interactions are required for replicase assembly and AS1–RS1, AS2, RS2, and DE–CE interactions are crucial for sgRNA synthesis. (Abbreviations: DSD, downstream domain; TSD, T-shape domain; RSE, replication silencer element; AS, activator sequence; RS, receptor sequence; CE, core element; DE, distal element; UL, upstream linker; DL, downstream linker; CITE, cap-independent
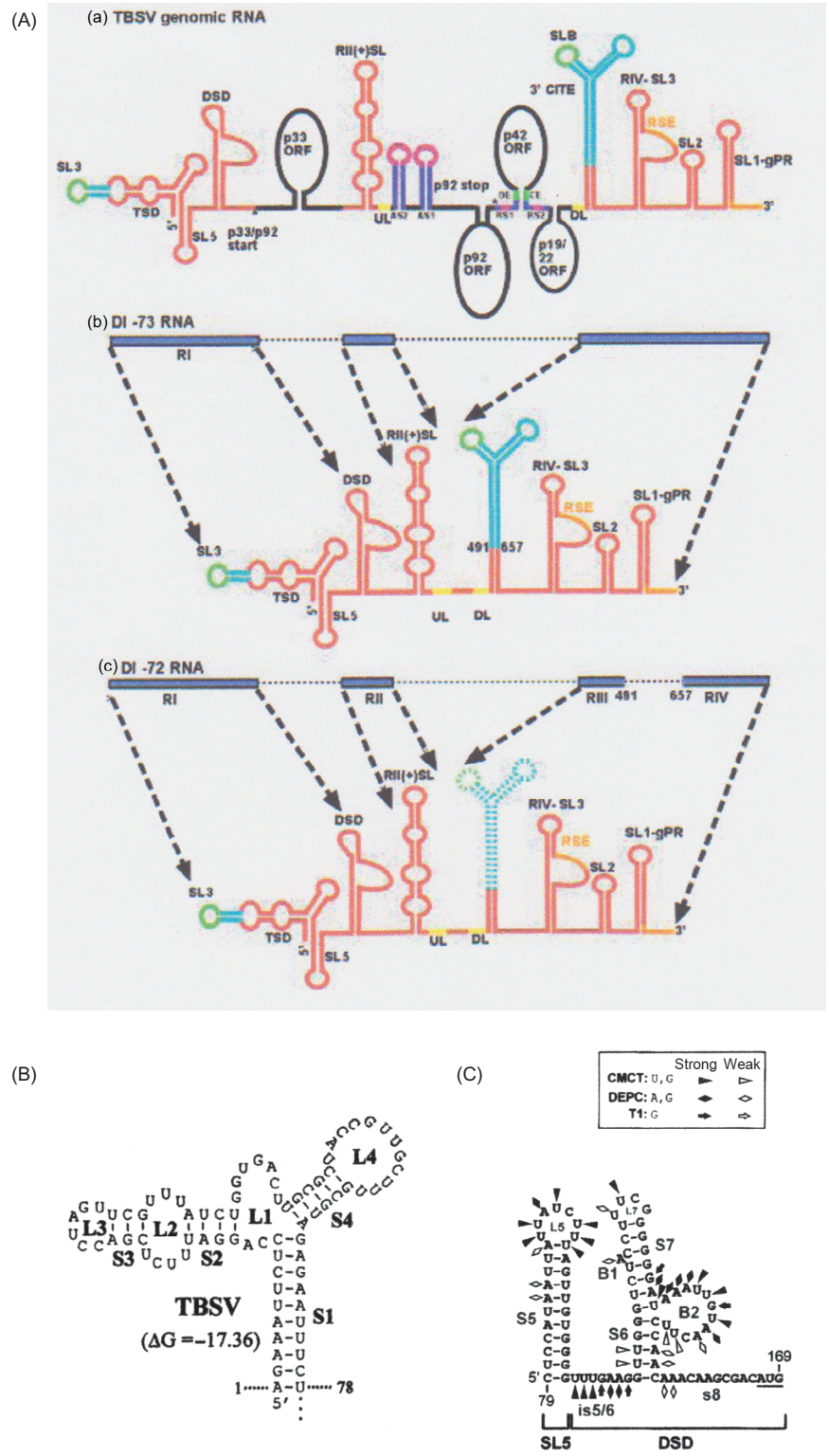
FIGURE 7.10 (Continued) translation enhancer; SL, stem loop). Subpanel (b): Structure of the ~800 nt DI-73 carrying three non-contiguous segments of the genomic RNA. Generation of DI-73 preserves critical replication elements (red) and the 3′ CITE. The blue bars and dotted arrow depict the segments corresponding to genomic RNA. Subpanel (c): Note that DI-72 RNA has an additional deletion of the 3′ CITE. From Pathak and Nagy (2009) with permission of the publishers. Panel (B): Structure of the 5′-terminal sequence of TBSV. The free energy values (ΔG) of the structure are in parentheses. Stems (S) and loops (L) are labeled. From Wu et al. (2001) with permission of the publishers. Panel (C): RNA secondary structure model for the 3′ half of TBSV 5′ UTR. Chemical and enzymatic modifications are mapped onto the MFOLD-predicted structure and labeled according to their effect. Stem-loop (SL) and bulged (B) structures are labeled. From Ray et al. (2003) with permission of the publishers.
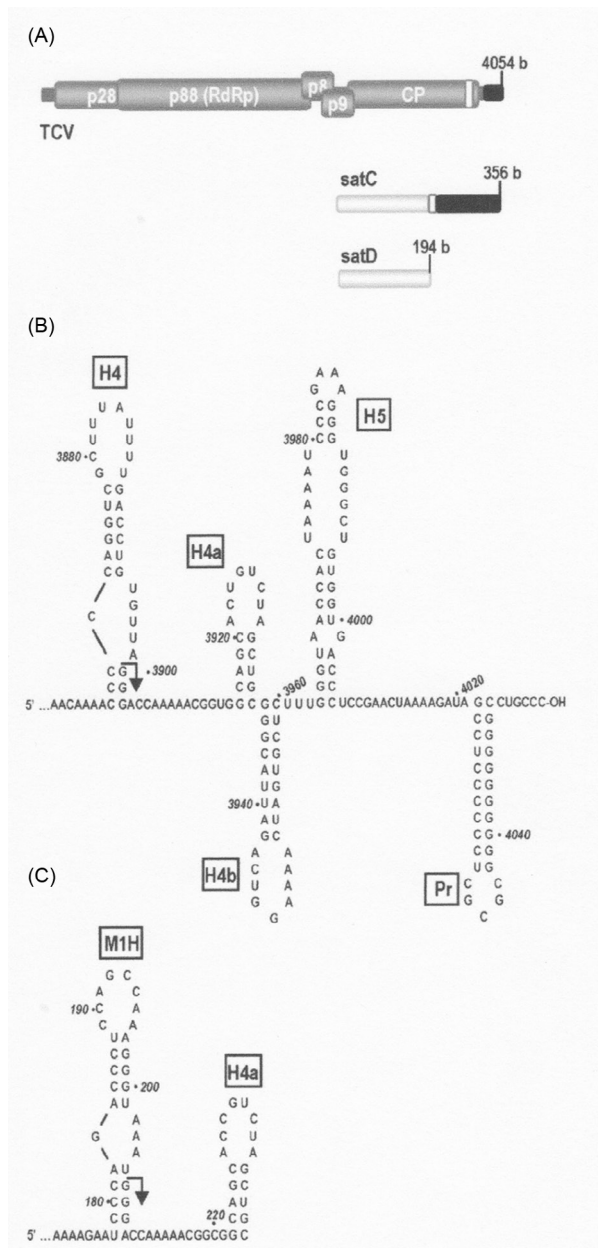
FIGURE 7.11 TCV associated RNAs. (A) Schematic representation of the TCV genomic RNA and two sat RNAs. satC is derived from satD and two regions of TCV; similar sequences are shaded alike. (B) Structure of the 3′ region of TCV showing hairpins described in Box 6.6. Arrow denoted that downstream sequence is shared with satC. (C) Structure of a portion of the 3′ region of satC. M1H is a hairpin located at the same position as H4 relative to the 3′ end of the RNA and is composed of sequence from satD and the two discontinuous regions of TCV. *From Sun and Simon (2006) with permission of the publishers.*

Several *cis*-acting elements that are important for replication *in vivo* or transcription *in vitro* have been identified on (−) strand of TCV satC (Figure 7.11).

At the 3′ terminus of (−)-strand satC is the carmovirus consensus sequence (CCS) [3′-OH-CC_{1-2}(A/U)(AU)(AU], which is conserved at the initiation sites for transcription of the genomic RNA and sgRNAs in all carmovirus (−)-strand RNAs examined (Guan et al., 1997, 2000). satC also has a second CCS adjacent to the (−)-strand 3′ terminal CCS and another promoter sequence consisting of 14 bases located 41 bases from the 3′ end of the (−) strand (a region derived from satD) which are not found in the genomic sequence (Guan et al., 1997). Thus, it is likely that the CCS is the promoter for (+)-strand synthesis of the genomic RNA and the addition promoter sequences in satC and satD are associated with the satellite activity.

TYMV RNA has two hairpin structures in the 5′ UTR each of which are required for maximum virus replication (Shin et al., 2008). The second hairpin plays a role in efficient long-distance virus movement but neither is involved in encapsidation (Shin et al., 2009).

The 5′ cap structures of the BSMV α, β, and γ gRNAs are followed by UTRs of 89–90 nt that have no obvious sequence relatedness. The 5′ UTRs of the gRNAs presumably act as *cis*-elements that regulate differences in translation and the relative rates of synthesis of each of the (+)-strand gRNAs. The 5′ UTRs of the PSLV and LRSV α, β, and γ gRNAs also have little direct sequence similarity, but similar regions of folding are evident when the individual gRNAs of each virus are compared (Solovyev et al., 1996; Savenkov et al., 1998). Sequence comparisons show that the BSMV and PSLV 5′ UTRs are more closely related than those of LRSV (Savenkov et al., 1998). Presumably *cis* elements in the 5′ UTRs play a role in initiating (+)-strand synthesis.

### c. Promoters for Subgenomic RNA Synthesis

Many (+)-strand RNA viruses also produce one or more subgenomic (sg) mRNAs to express downstream ORFs. At least four models have been proposed for the synthesis of sg mRNA from the genomic RNA (Figure 7.12).

These include:

– *De novo* internal initiation on the full-length (−) strand of the genome during (+)-strand synthesis.
– Premature termination during (−)-strand synthesis of the genome followed by the use of the truncated nascent RNA as a template for sg mRNA synthesis or on (+)-strand synthesis which gives sg mRNA.
– Initiation on the full-length (−) strand primed by a short leader from the 5′ end of the genomic DNA during (+)-strand synthesis (leader-primed transcription). This has been found for coronaviruses (Liao and Lai, 1994).
– Intramolecular recombination during (−)-strand synthesis in which the replicase jumps from the sgRNA start site on the full-length (+) strand and reinitiates near the 5′ end of the genome (discontinuous template synthesis). This also has been found for coronaviruses (Sawiki and Sawiki, 1998).

The first and second mechanisms have been proposed for plant viruses.
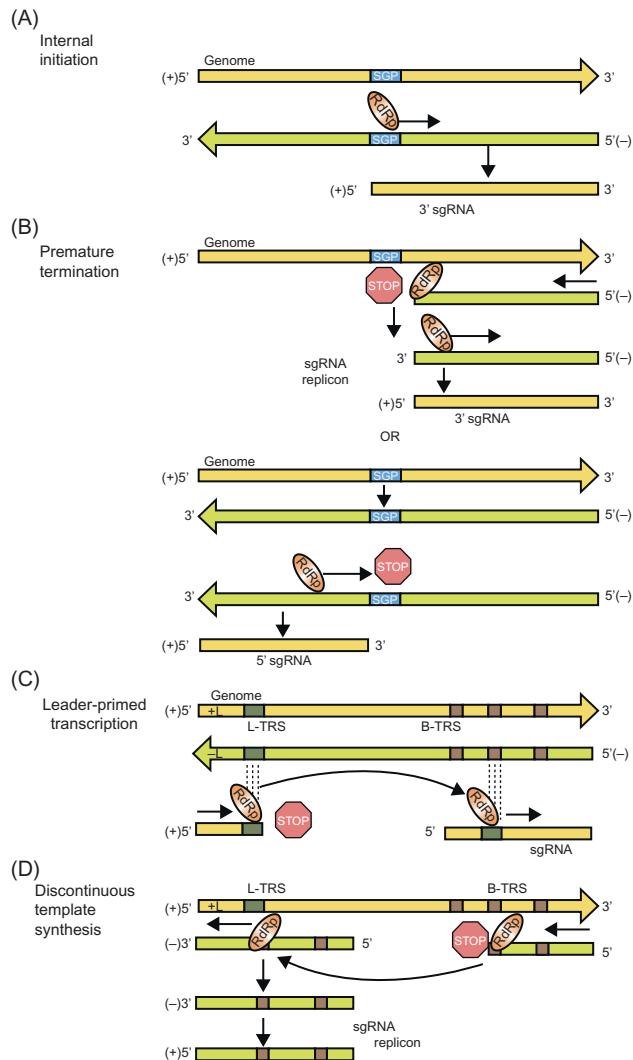
FIGURE 7.12 Schematic representation of different mechanisms for sgRNA synthesis. (A) Internal initiation model. (B) Premature termination model showing termination either during (−) and (+)-strand synthesis. (C) Leader-primed transcription model. (D) Discontinuous template synthesis. Genomic and sgRNA (+)-strands are depicted as horizontal yellow boxes, the (−)-strands are depicted as green boxes, and the ovals represent RdRp enzymes capable of starting/stopping at the internal initiation SGP promoters that are depicted as blue SGP boxes. The leader TRS (L-TRS) and the body TRS (B-TRS) are represented by dark green and brown boxes, respectively. *From Sztuba-Solińska et al. (2011) with permission of the publishers.*

Bromovirus RNA3s encode two proteins, the downstream one being expressed from an sg mRNA (Appendix A, Profile 53). A replication enhancer has been recognized in the intercistronic region (Janda and Ahlquist, 1998; Sullivan and Ahlquist, 1999). The RNA3 replication enhancer contains a box B motif that is conserved with the TCC loop of tRNAs and is essential for RNA3 replication (Sullivan and Ahlquist, 1999). The importance of this region was further demonstrated by showing that

depurination inhibited viral replication and that the region controlled (−)-strand RNA synthesis (Karran and Hudak, 2008). As well as the box B consensus sequence, bromovirus RNA3s have an oligo (A) tract (in the (+) strand) in this intercistronic regions. In the (−) strand of RNA3, adjacent to the oligo (U) tract, is a short stem-loop structure with a triloop AUA (Figure 7.7B) (Haasnoot et al., 2002) which is similar to the polymerase recognition site in the (−) strand (see above). It is noted that this triloop structure strongly resembles iron-responsive element in cellular mRNAs and may be a general protein-binding motif.

The downstream ORFs of TBSV are expressed from two sg mRNAs (Appendix A, Profile 71). The PT mechanism has been proposed to function in the transcription of two sg mRNAs in the (+)-strand RNA (White, 2002). Several observations support TBSV sg mRNAs being transcribed via a PT mechanism (Zhang et al, 1999; Choi et al, 2001; Choi and White, 2002): (i) in TBSV infections, both sg mRNA (+)- and (−) strands are detectable in total RNA extracts from infected cells; (ii) the formation of two different sets of long-distance RNA–RNA interactions, AS1/RS1 and DE/CE, involving sequences just 5′ to the two sg mRNA start sites, is required for efficient transcription of sg mRNA1 and 2, respectively (Figure 7.13); (iii) these interactions function in the (+) strand of the genome, consistent with their proposed functions as RNA-based terminators of the viral RdRp during (−)-strand synthesis; (iv) substitution of the initiating nucleotides for sg mRNA1 or 2 transcription results in inhibition of sg mRNA accumulation but not of corresponding sg mRNA(−) templates, in agreement with a PT model where the sg mRNA(−) templates are generated prior to, and independently of, their sg mRNA counterparts; and (v) the autonomous synthesis of sg mRNA1(−) [as described in (iv)] is dependent on the AS1/RS1 interaction, indicating that this interaction is involved specifically in the generation of sg mRNA1(−) templates.

The AS1 RNA element maps to the terminal loop of a predicted stem-loop structure (Choi and White, 2002) (Figure 7.13), and its base-pairing partner RS1 is positioned 3 nt 5′ to the sg mRNA1 start site.

The initiation of synthesis of sg mRNA2 is controlled by three long-range RNA interactions. AS2/RS2, DE-A/CE-A, and DE-B/CE-B and also two further elements, DE-C and CE-C, that do not base pair (Figure 7.13) (Lin and White, 2004). AS2, like AS1 is present in the terminal loop of a predicted stem-loop structure which likely facilitates its pairing with RS2. It is suggested that the DE-A/CE-A and DE-B/CE-B interactions position RS2 close to AS2 and that the DE-A/CE-a interaction might stabilize the helix formed by the AS2/RS2 interaction. The CE-C element lies immediately between RS2 and the start of sg mRNA2, and it is hypothesized that DE-C maintains non-complementarity to CE-C to allow AS2 unfettered access to RS2 (Lin and White, 2004).
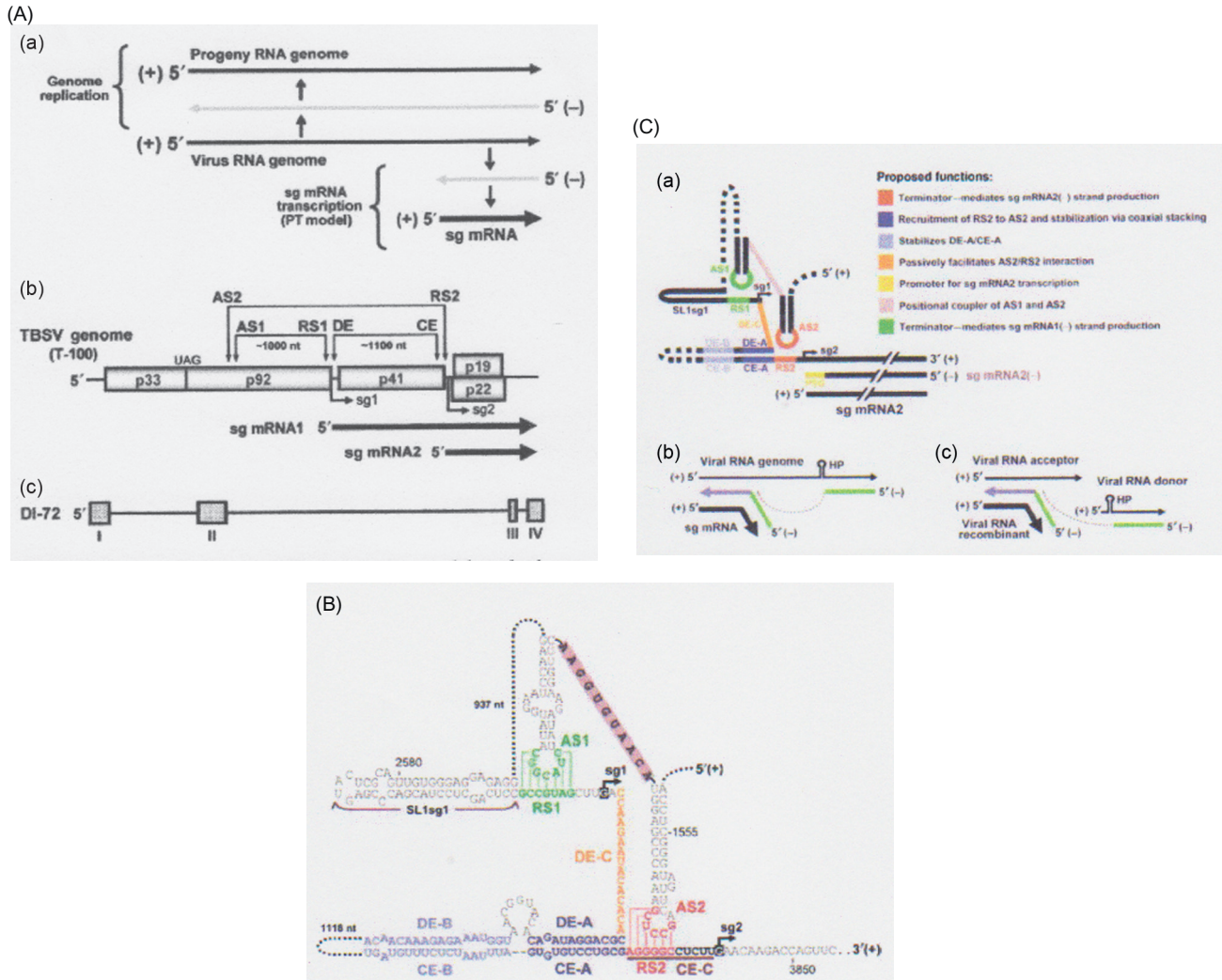
FIGURE 7.13 Panel (A): Genomic replication, premature termination (PT) model, and relevant TBSV RNAs. Subpanel (a): Simplified scheme for viral RNA genome replication and sg mRNA transcription via a PT mechanism. Subpanel (b): Linear representation of the TBSV RNA genome and coding organization. The relative positions (arrowheads) of interacting RNA elements involved in sg mRNA transcription are shown above the genome. Initiation sites for sg mRNA transcription are labeled sg1 and sg2 and corresponding structures of the two sg mRNAs are represented by bold arrows below the genome. (c) Schematic representation of DI-72. Boxes correspond to regions (I–IV) of the TBSV genome (directly above) that are present in the DI RNA, while the lines represent genomic regions that are absent. Panel (B): RNA elements that regulate sg mRNA transcription in TBSV. Relevant sequences of the TBSV genome are shown with corresponding coordinates provided. RNA elements involved in sg mRNA transcription are labeled and color-coded. The DE-B/CE-B interaction (light blue) is thought to stabilize the DE-A/CE-A interaction (deep blue) that is essential for sg mRNA2 transcription. The AS2/RS2 interaction (red) is critical for efficient transcription of sg mRNA2. Stem-loop structures containing AS1 and AS2 are connected by an 11-nt-long sequence (gray bar). DE-C (gold) and CE-C (maroon and underlined) are non-complementary elements. The AS1/RS1 interaction (green) is critical for efficient transcription of sg mRNA1. Initiation sites for the two sg mRNAs are highlighted in black and indicated by small arrows. Panel (C): Models for sg mRNA transcription and viral RNA recombination. Subpanel (a): A PT model for TBSV sg mRNA transcription. A list of the proposed functions for the color-coded RNA elements is provided (and applies only to subpanel a). Subpanel (b): A discontinuous template model for BEV sg mRNA2 transcription (van Vliet et al., 2002). Subpanel (c): Hairpin-mediated RNA recombination model for TBSV (White and Morris, 1995). *From Lin and White (2004) with permission of the publishers. A more detailed version of this figure can be found on http://booksite. elsevier.com/9780123848710.*

There is a close mechanistic link between a PT model for sg mRNA transcription and the (+)-strand synthesis step of genome replication. The AS2/RS2 helix serves as the major secondary structure-dependent RNA termination signal for the vRdRp, and the sg mRNA2 (−) template generated is used subsequently for sg mRNA2 transcription via initiation at the 3′-terminal promoter (PSG). Sg mRNA1 transcription is also thought to occur via a similar mechanism that involves the essential AS1/RS1 interaction (Figure 7.13C).

The transcription of the two sg mRNAs of TCV is initiated internally on the (−) strand (Wang and Simon, 1997).
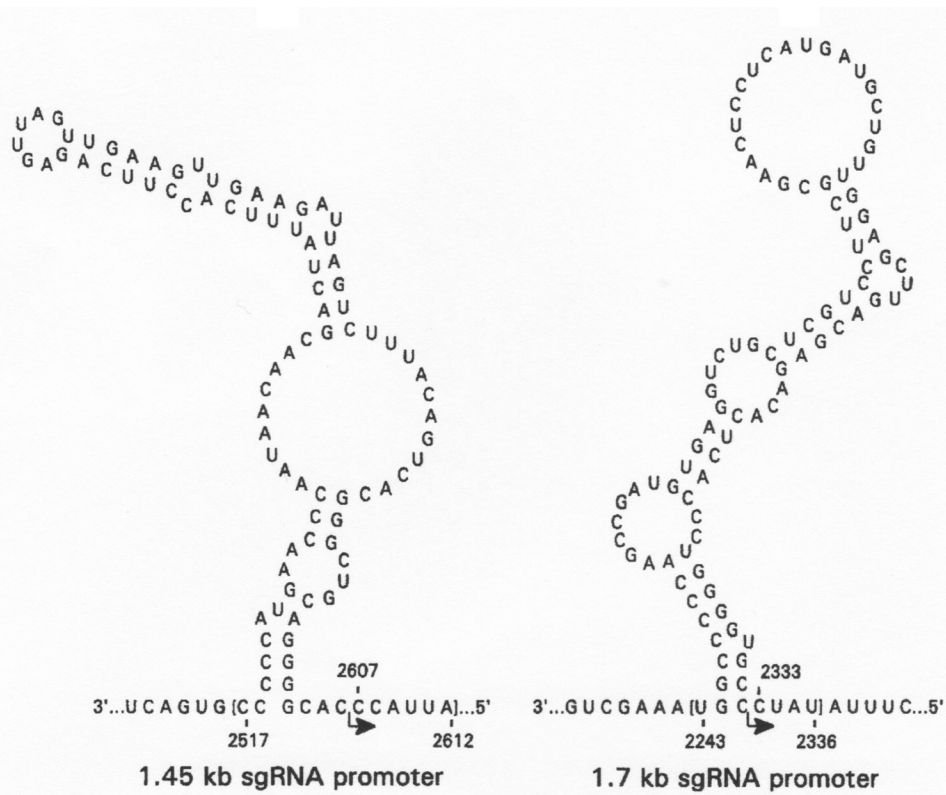
FIGURE 7.14 Computer predicted secondary structure for the 1.45- and 1.7-kb TCV sg mRNA promoters (left and right, respectively). (−)-strand sequence is shown. Brackets enclose the promoter boundaries derived from deletion analysis. Arrows denote the transcription start sites. *From Wang and Simon (1997) with permission of the publishers.*

The sg mRNA1 and sg mRNA2 promoters are located approximately 90 bases upstream to 4 bases downstream and 90 bases upstream to 6 bases downstream, respectively, of the transcription start site, but the minimal promoter for basal level transcription differs between the two promoters. The promoters have similar stem-loop structures (Figure 7.14) each consisting of a hairpin comprising most of the promoter sequence just 3′ of the transcription start site.

The 5′ termini of the BSMV sg mRNAs have been defined by primer extension mapping and their relative abundance determined by RNA blot analyses of nucleic acids (Zhou and Jackson, 1996a). A combination of differential transcription of the two RNAβ sgRNAs and translational regulation of the sgRNAβ2 ORFs results in an estimated 100:10:1 ratio of TGB1:TGB2:TGB3 protein expression.

The two BSMV sgRNAβ promoters reside upstream of their transcription start sites (Figure 7.15). The sgRNAβ1 promoter maps between positions −29 and −2 relative to the transcription start site, and this region is proximal to an internal *cis*-acting element required for RNAβ replication (Zhou and Jackson, 1996b). The core sgRNAβ2 promoter encompasses residues −32 to −17 upstream of the start codon. Maximal activity of the sgRNAβ2 promoter is also enhanced

by a hexanucleotide that spans residues −64 to −59. The BSMV sgRNAγ promoter differs from the RNAβ promoters by extending into the γb AUG. This promoter occupies positions −21 to +2 relative to the transcription start site and appears not to contain an enhancing sequence or to be adjacent to regions affecting replication (Figure 7.15).

The TYMV CP ORF is expressed from an sg mRNA (Appendix A, Profile 48). The 3′ end of the p206 ORF contains a 16-nt sequence (the "tymobox") which functions as the promoter for internal initiation of the sg mRNA (Ding et al., 1990) (Figure 6.34).

Similarities and differences between sg mRNA and (−)-strand promoters are discussed by Olsthoorn et al. (2004).

d. Transition from Initiation to Elongation

A transition between initiation to elongation of BMV (−)-strand synthesis was observed by Sun and Kao (1997a) who showed that nascent RNAs of 10 nucleotides or longer remained associated with the replication complex and could be extended into full-length RNAs whereas shorter RNAs were released from the complex. In a further analysis of this using the sensitivity of the non-templated complex to heparin, Sun and Kao (1997b) determined three
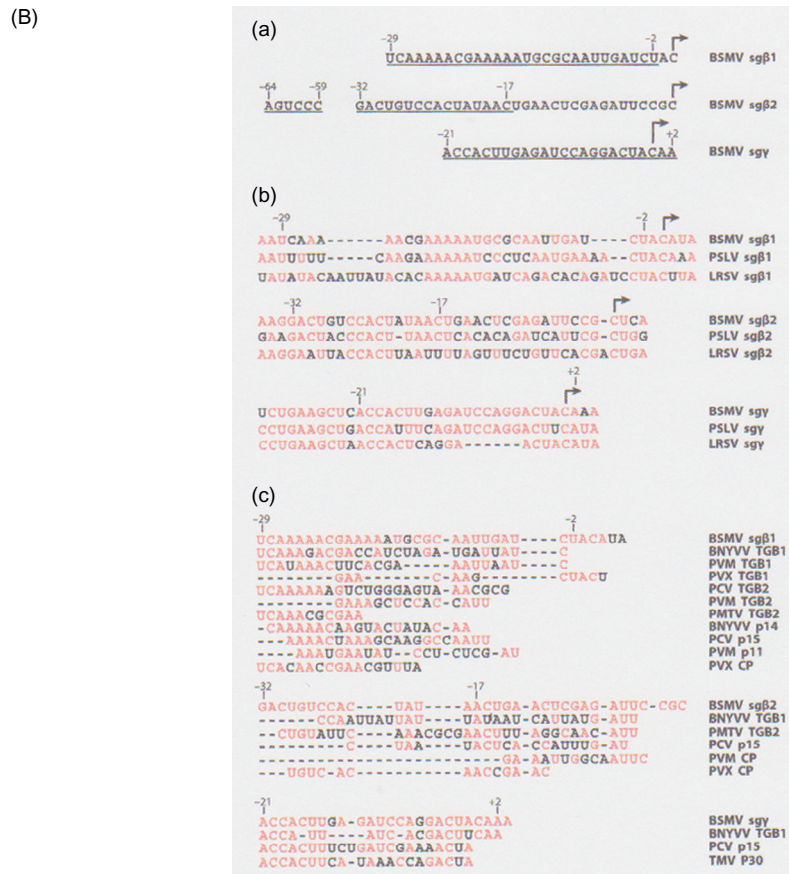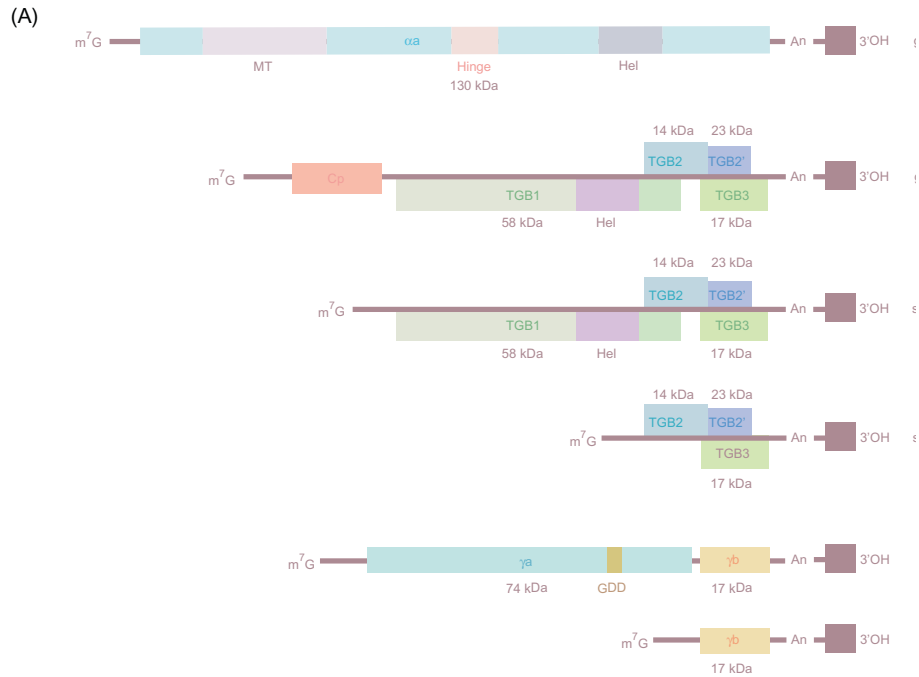
FIGURE 7.15 Panel (A): Genome organization of BSMV. The genomic RNA is capped and the open and solid rectangles represent the ORFs and 3′ terminal TLS, respectively. The genome organization is described in Appendix A, Profile 80. RNAβ produces two sg mRNAs sgRNAβ₁ which expresses TGB1 and sgRNAβ₂ which TGB2, TGB3, and TGB2′. RNAγ produces one sg mRNA, sgRNAγ, which expresses the γb gene product. Panel (B): Comparison of hordeivirus sg mRNA promoter sequences. Subpanel (a): Sequences of three BSMV sg mRNA promoters in the (−)-sense orientation. The numbers above the sequences correspond to nucleotide positions relative to the respective transcription initiation sites, which are indicated by arrows. The underlined sequences are required for sg mRNA synthesis. Subpanel (b): Alignment of sgRNAβ₁, sgRNAβ₂, and sgRNAγ promoter regions of BSMV, PSLV, and LRSV. Regions of highest sequence similarity are shown in red. The transcription start sites (arrows) for PSLV and LSRC sg mRNA promoters have not been defined experimentally, although their initiation sites were predicted. Alignments performed using MegAlign program associated within the Lasergene software package. Subpanel (c): Alignment of the sgRNAβ₁, sgRNAβ₂, and sgRNAγ promoter regions with mapped (TMV and PVX) or putative (BNYVV, PCV, PMTV, and PVM) sg mRNA promoter regions of other viruses. *From Jackson et al. (2009) with permission of the publishers; panel B modified from Johnson et al. (2003). A more detailed version of this figure can be found on http://booksite.elsevier.com/9780123848710.*
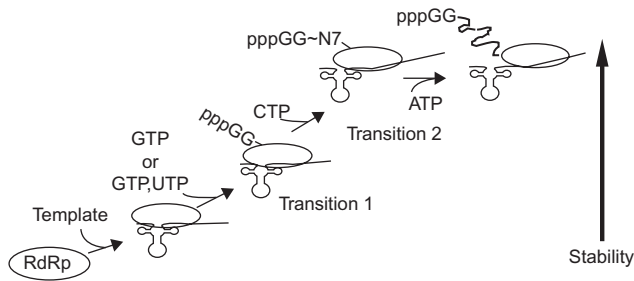
FIGURE 7.16 A model for the transitions of BMV RNA synthesis from initiation to elongation by RdRp. The stability of the RNA synthesis complex increased in three distinct steps with two transitions. First, RdRp (ellipse) binds to the tRNA structure (represented by a three-leaf clover) of the viral RNA to form a binary complex of RdRp RNA. After the synthesis of nascent RNA, the ternary complex then undergoes a transition to a structure that is more tightly associated with the template RNA, having a second stability level. A last transition occurs when further incorporation of CTP generates the short nascent RNA chains of 8–14 nucleotides in length. The latter ternary complex is now committed to template and will resist challenge with heparin and other template RNAs. *From Sun and Kao (1997b) with permission of the publishers.*

steps with two transitions in the stability of the RNA synthesis complex (Figure 7.16). They suggest that the replication proteins first bind to the 3′ TLS, and, after synthesis of nascent RNA, the complex undergoes a transition to a more stable structure. The second transition occurs when the short nascent RNA chains are 8–14 nucleotides in length.

C. DI and D RNAs (reviewed by Simon et al., 2004; Pathak and Nagy, 2009)

DI and D RNAs are subviral RNAs produced by errors in the replication of their parent (helper) virus. They do not code for all the necessary viral proteins for independent replication and thus are defective in the absence of the parent virus. The parent virus provides the missing replication protein(s) in *trans*. DI RNAs attenuate or enhance the symptoms caused by the parent virus whereas D RNAs do not interfere with the multiplication of their parent virus. DI and D RNAs comprise unmodified terminal sequences of the parent virus and, in some cases, some internal sequences. DI and D RNAs are distinct from satellite viruses and satellite RNAs (Chapter 5, Section II) which, although they depend on helper viruses for multiplication, do not have extensive sequence similarities with those helper viruses.

The D and DI RNAs are formed by recombinations during the replication of the parent virus (Section IX, B) often leading to large deletions of the parental genome. The deletion patterns in the DI and D RNAs and viruses fall into two groups (Table 7.2). In the first, the modified RNA is derived from a single internal deletion and in the second, it consists of a mosaic of the parental viral genome.

Single-deletion DI and D RNAs have been found in *Bromoviridae* (AMV, BBMV, CMV) family and in the potexvirus (ClYMV), tobravirus (TRV), furovirus (SBWMV), pecluvirus (PCV), benyvirus (BNYVV), phytoreovirus (WTV), and tospovirus (TSWV) genera (Table 7.2). All the D RNAs isolated thus far from multipartite genome viruses fall into this group.

Multiple deletion DI and D RNAs are characteristic of several members of the *Tombusviridae* family (Table 7.2) and those of TSBV and TCV have been studied in detail.

Examples of the single-deletion and multiple deletion defective molecules are described below in relation to the parent virus.

In another approach to studying important RNA elements, artificial molecules have been made that contain deletions and which can be supported by a parent virus that does not naturally have D RNAs. An example is TYMV in which molecules with small deletions in the CP gene are poorly supported by the parent virus, but those with a large deletion in the 70-kDa ORF are replicated efficiently (Dreher and Weiland, 1994). Comparison of the replication requirement of a TMV-based D RNA and its helper virus revealed different requirements for the replication of TMV RNAs in *cis* and in *trans* (Chandrika et al., 2000). Deletions of certain 3′ terminal pseudoknots decreased the level of replication of full-length TNV RNA but did not affect the replication of the D RNA. However, the 3′-most pseudoknot was required for replication of both full-length and D RNAs. Homologous 3′ sequences were important for the replication of the D RNA, the precise requirement appearing to involve the terminal 28 nucleotides and specifically the pseudoknot in the aminoacyl acceptor arm of the TLS.

Since the replication of DI and D RNAs is fully supported by the helper virus, they, and especially those that maintain critical *cis*-acting replication elements, provide useful information of the RNA elements that are important is genomic RNA replication.

1. The Mechanism of Interference by DI RNA

Many DI RNAs are replicated efficiently by the parent virus because (i) they are small; (ii) they are efficient RdRp substrates; and (iii) those that do not contain any ORF do not compete with the parent in translation. Therefore, in many cases the accumulation of the parent virus is inhibited by the most competitive DI RNAs, resulting in the symptom attenuation in host plants.

However, some DI RNAs (e.g., those from BBMV and TCV) enhance the symptoms induced by the parent virus. Three types of mechanism have been recognized that might cause symptom enhancement (reviewed by Simon et al., 2004).

TABLE 7.2 D and DI Nucleic Acids

Family/Genus	Virus	Deleted Segment	Type of Defective Element	Comments and References
Group 1				
Reoviridae	WTV	RNAs 2 and 5	D Virus	Loss of vector transmission (Graves et al., 1996, review)
Tospovirus	TSWV	L RNA	DI RNA	Encodes polymerase protein (Nagata et al., 2000)
		M RNA	D Virus	Loss of viral envelope and probable loss of vector transmission (Nagata et al., 2000)
	PBNV	L RNA	D Virus	Gowda et al. (1998)
Rhabdoviridae	SYNV			Ismail and Milner (1988)
Bromoviridae	AMV	RNA3	D RNA	Graves et al. (1996) (review)
	BBMV	RNA2	DI RNA	Exacerbates symptoms in some hosts, encodes viral polymerase (see Section IV, C, 2)
	CMV	RNA3	D RNA	Deletion in 3a protein; encodes CP (see Section IV, C, 3)
Closteroviridae	CTV	Various	D Virus	Can affect aphid transmission (Bar-Joseph et al. (1997), review; Albiach-Martí et al., 2000)
Tobravirus	TRV	RNA2	D and DI Virus	Vector transmission eliminates DI (Visser et al., 1999)
Potexvirus	ClYMV	Various	D and DI RNAs	White et al., 1991, 1992
	BaMV	Central region	D Virus	Yeh et al. (1999)
	CsCMV	Central region	D Virus	Calvert et al. (1996)
Furovirus	SBWMV	RNA2	D Virus	Loss of vector transmission (Graves et al., 1996, review)
		RNAs 3 and 4	D Virus	Loss of vector transmission and ability to infect roots (Graves et al., 1996, review)
Pecluvirus	PCV	RNA2	D Virus	Loss of vector transmission (Graves et al., 1996, review)
Benyvirus	BNYVV	RNA2	D Virus	Loss of vector transmission (Graves et al., 1996, review)
Sobemovirus	CfMV	Central region	DI RNA	Makinen et al. (2000)
Group 2				
Tombusviridae	TBSV	Various		Section IV, C, 4
	TCV	Various		Section IV, C, 5

a. Competition for Viral and Host Resources

DI RNAs share the pool of replication proteins, host factors, nucleotides, host membranes, and all other factors with the helper virus. Eventually, the helper virus is outmultiplied by the more competitive DI RNAs in a dose-dependent manner. An example is the coinfection of *N. benthamiana* protoplasts with equimolar amounts of TBSV and DI RNAs which led to a 65% suppression of TBSV genomic RNA accumulation (Jones et al., 1990).

However, high levels of DI RNA do not necessarily lead to attenuation of symptoms (Havelda et al., 1998) which indicates that the interaction may not be just simple competition.

b. Modulation of the Functions of Viral Factors

That the modulation is not due to just simple competition was shown in an experiment in which *N. benthamiana* plants were coinfected with TBSV genomic transcripts and DI RNAs (Scholthof et al., 1995). Analysis of these plants revealed that the levels of p19 (suppressor of RNA silencing), the p22 (movement protein), and sg mRNA2 were reduced much more than the level of the genomic RNA replication proteins. This suggested that the DI RNA preferentially interfered with the production and/or translation of sg mRNA2.

c. DI RNA-Triggered RNA Silencing Response of the Host

RNA silencing is an eukaryotic cellular response to the presence of dsRNAs. Plants use this mechanism as a major antiviral strategy, but viruses have means of avoiding or delaying recognition by host surveillance due to the formation of replicase complexes hidden in host membranes and by expressing suppressors of RNA silencing (Chapter 9). Thus, any effects of DI RNAs leading to a reduction of RNA silencing suppressors are likely to lead to enhanced symptoms (Havelda et al., 1995). To complicate the situation, a DI RNA may also be the target of RNA silencing if it produces enough dsRNA target. If it does not, the parent virus RNA would be targeted but not the DI RNA (Zhong et al., 2005; Hornyik et al., 2006).

2. D RNAs in the Bromoviruses (reviewed by Graves et al., 1996)

The DI RNA of BBMV is derived from, and decreases the concentration of, RNA2. Even though it decreases the concentration of RNA2, it exacerbates the severity of BBMV symptoms in some hosts. For instance, the presence of the DI RNA causes symptoms to appear earlier and to be more severe, especially in *Pisum sativum* cv Rondo where it is lethal (Romero et al., 1993). In broad bean (*Vicia faba*), yield loss due to BBMV with DI RNA is up to 65% compared with about 40% loss in the absence of DI RNA (Sandoval et al., 2007).

The DI RNAs have various sized deletions in the 2a ORF and include the GDD motif characteristic of the function of this ORF product as an RdRp (Romero et al., 1993; Pogany et al., 1995; Sandoval et al., 2008) (Figure 7.17).

The region of the ORF downstream of the deletion is in frame with that upstream suggesting that effective translation is an important feature for the production and maintenance of the defective molecules.

An artificial BBMV DI RNA in which a 60 nucleotide sequence was duplicated in another part of the molecule produced shorter RNAs if the duplication was in the reverse orientation but not if it was in the direct orientation (Pogany and Bujarski, 1996). The further deletion was at, or close to, the base of the hairpin formed by the inverse duplication suggesting that the DI molecules are formed by recombination during RNA replication.

Study of both natural and artificial BBMV DI RNAs suggests that their production is controlled by three factors: (i) the presence of the terminal regions. The DI RNAs retained the 5′-terminal 1152 nucleotides and the 3′-terminal 468 nucleotides; (ii) the overall size. The sizes of the deleted sequences are between 15% and 30% that of the wild-type RNA2; (iii) the coding capacity. The DI RNA retains a deleted form of the 2a ORF that comprises at least 79% of the molecule. BBMV DI RNAs do not accumulate and are not encapsidated in local lesion hosts or in some systemic hosts (Romero et al., 1993).

D RNAs have been reported for BMV in which there were one or two deletions in the 3a ORF of RNA3 (Figure 7.17B) (Damayanti et al., 1999; Sandoval et al., 2008). No D RNAs were found in CCMV (Sandoval et al, 2008).

3. D RNAs in Cucumoviruses (reviewed by Graves et al., 1996)

Various D RNAs have been found associated with CMV (Graves and Roossinck, 1995a; Lopez et al., 2007; Takeshita et al., 2008). Most, if not all, are derived from RNA3 and have no apparent effect on either symptom production or on virus accumulation. An example is the D RNA derived by a single deletion that removes a segment of the 3a ORF (Figure 7.17A) while maintaining the reading frame downstream of the deletion (Graves and Roossinck, 1995a). This leaves a defective cell-to-cell movement protein and a functional CP. CMV D RNAs accumulate in various *Nicotiana* species, but in tomato, zucchini squash, and muskmelon the D RNAs only accumulate in inoculated tissue and do not move systemically (Graves and Roossinck, 1995b). Furthermore, the D RNAs accumulate and are encapsidated in both inoculated cotyledons and leaves of tomato and zucchini squash and accumulate but are not encapsidated only in the inoculated cotyledons of muskmelon. This indicates that host and tissue specificity is involved in replication, cell-to-cell movement, systemic movement, and encapsidation of CMV D RNAs.

4. DI RNAs of Tombusviruses (reviewed by White and Morris, 1999; Simon et al., 2004; Pathak and Nagy, 2009)

Some of the earliest reports of DI RNAs came from work on tombusviruses. Hillman et al. (1987) and Morris and Hillman (1989) described an abnormal RNA from a culture of TBSV that met all the criteria for a DI RNA. The RNA was about 396 nucleotides long and was derived from the genomic RNA by six internal deletions, the 5′ and 3′ sequences being conserved. Two of the deletions were large (1180 and 3000 nucleotides) while the others were much smaller. Co-inoculation of the small RNA with parent virus depressed virus synthesis in whole plants and attenuated disease symptoms. Although the DI RNA could represent 60% of virus-specific RNA in leaf extracts, only about 3−4% of the encapsidated RNA was DI RNA. Experiments in protoplasts showed that the DI RNA suppresses replication of genomic TBSV RNA (Jones et al., 1990). A similar DI RNA was described from a culture of CymRSV (Burgyán et al., 1989).
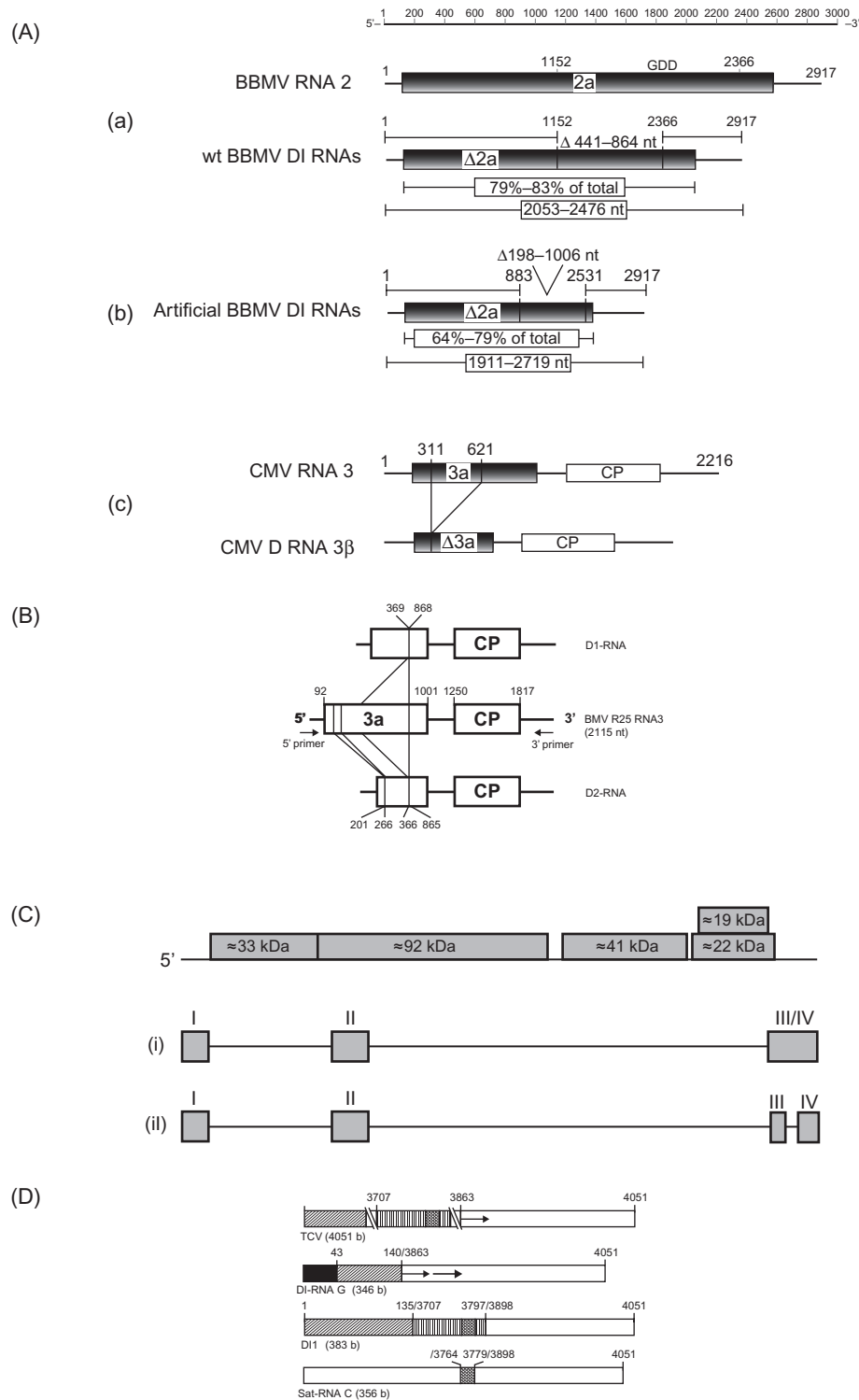
FIGURE 7.17 D RNAs of members of the *Bromoviridae* and *Tombusviridae*. Panel (A): D RNAs derived from members of the *Bromoviridae*; schematic representation of BBMV and CMV D RNAs. The 5′ and 3′ untranslated regions are shown by black lines. The 2a and 3a ORFs are represented by shaded boxes while the CP ORF is represented by a white box. Subpanel (a): BBMV genomic RNA2 (top) and wt DI RNAs (bottom). The numbered lines above the DI RNA indicate the minimum 5′ and 3′ sequences conserved in all the wt DI RNAs. The size range of the deletions is also given above the DI RNA. The top numbered line below the DI RNA indicates the length of the Δ2a ORF as compared to the entire length of the DI RNA. The bottom numbered line indicates the range in the total lengths of the wt DI RNAs. Subpanel (b): BBMV artificial DI RNAs. The artificial DI RNA is labeled as in A. The data reflect only those DI RNAs that accumulated efficiently in plants. Subpanel (c): CMV genomic RNA3 (top) and wt D RNA 3β (bottom).

DI RNAs have been found in several other tombusviruses including CIRV and CNV. These naturally occurring DI RNAs (400–800 nucleotides) are about 10–20% of the size of the parent genomes and are composed of conserved non-contiguous segments of the genome that accumulate *de novo* after serial passage of the parent virus at high multiplicity of infection. They suppress the accumulation of the parent virus and usually attenuate the severe symptoms that these viruses normally induce.

The DI RNA of TBSV has been studied in detail and has features that are common to DI RNAs of other members of the *Tombusvirus* genus. The typical molecule is composed of four non-contiguous segments (regions I–IV) derived from the parent virus (Figures 7.10A, subpanel c and 7.17C). Regions I (168 nt) and IV (about 130 nt) are derived from the 5′ and 3′ termini, respectively, Region I containing the 5′ non-coding region and start codon for the 5′ ORF and Region IV comprising non-coding 3′ sequence. Region II (200−250 nt) is from just downstream of the stop codon for the 33-kDa protein and region III (70 nt) from the C-terminal region of the 22-kDa protein. Infected plants contain two size classes of TBSV DI RNA, the larger not being deleted between regions III and IV and which is thought to be a precursor for the smaller molecule. There are also small amounts of molecules that have extra duplications such as of region II or are direct repeat dimers of the entire DI RNA.

The importance of the conserved regions for the replication of the molecules has been determined by manipulating natural DI RNAs or making artificial ones (Chang et al., 1995; Havelda and Burgyan, 1995; Havelda et al., 1995). Deletion of region I, II, or IV abolishes the ability of the DI RNA to be replicated by the parent virus. Region IV contains secondary structure that plays an important role in DI RNA accumulation (Havelda and Burgyan, 1995). Region III is critical for CymRSV DI RNA (Havelda et al., 1995) but not for those of CNV or TBSV (Chang et al., 1995).

TBSV DI RNAs show host specificity (Omarov et al., 2004). After several passages through *N. benthamiana*, the DI RNAs co-inoculated with a pepper isolate of TBSV (TBSV-P) accumulate to significant levels and attenuate symptoms. However there are no detectable levels of DI RNAs in pepper (*Capsicum annuum*) and no mitigation of the TBSV symptoms. Co-inoculation of TBSV-P with a DI RNA from CIRV gives apical necrotic symptoms in pepper. Chimeras for DI RNAs from TBSV and CIRV show that the 5′ proximal sequence element (region I) is an important symptom determinant, but the whole DI RNA is involved in symptom modulation (Hornyik et al., 2006).

5. DI Carmovirus RNAs (reviewed by Simon et al., 2004; Pathak and Nagy, 2009)

In addition to the genomic RNA, infections with TCV are often associated with satellite (Chapter 5, Section II, B, 2, c) and DI RNAs. Two TCV DI RNAs have been characterized (Li et al., 1989), DI RNA G and DI RNA 1, which were generated *de novo* (Figure 7.17D). DI RNA 1 is composed of a mosaic of the 5′ 135 nt of the genomic RNA (the 5′ UTR and 74 nt of ORF1), nts 3707–3797 from the C-terminus of the CP and the 3′ 153 nt (3′ UTR). The 5′ 41 nt of DI RNA G differ from the genomic sequence; the rest of the DI RNA is composed of nts 43–140 (5′ UTR and N-terminus of ORF1), nts 3863–4051 (3′ UTR) with a direct repeat of nts 3863−3898. Thus, both contain the 3′ terminal sequence and the N-terminus of ORF1, but they differ in other parts of their composition.

6. Other D RNAs Associated with RNA Viruses

A putative DI RNA has been described for the comovirus, BPMV (Sundararaman et al., 2000). This was found in a cDNA library from mRNA from apparently healthy soybean pods and appeared to be a deleted form of BPMV RNA2.

Viruses other than those with (+)-strand RNA genomes can have DI-like RNAs. For those with (−)-strand RNA genomes, Adam et al. (1983) described a population of DI-like particles associated with a plant rhabdovirus that arose after 30 passages. Ismail and Milner (1988) isolated DI particles from *Nicotiana edwardsonii* plants chronically infected with SYNV. Most of the DI particles were 73−86% as long as the standard virus. Alone they were non-infectious,

FIGURE 7.17 (Continued) The region deleted from the 3a ORF is indicated. From Graves et al. (1996) with permission of the publishers. Panel (B): Schematic representation of BMV D RNAs based on the nucleotide sequences of two cDNA clones and comparison with the parental RNA3. The deleted region in the 3a ORF is indicated by vertical lines. D1 RNA contains one deletion, at nts 369–868. D2 RNA contains two deletions, at nts 201–266 and 366–865. The first nucleotide of the initiation codon and the third nucleotide of the termination codon of the 3a and CP genes are shown above the RNA3 diagram. From Damayanti et al. (1999) with permission of the publishers. Panel (C): Schematic representation of the RNA genome of a typical tombusvirus and of naturally occurring DI RNAs. The organization of the coding regions in the ~4.7 kb genome is shown at the top with the approximate sizes of the encoded proteins. Regions from which the TBSV DI RNAS were derived are shown below as shaded boxes with the deleted intervening regions depicted as lines. The four regions that are conserved, to some degree, in all characterized naturally occurring DI RNAs are indicated by roman numerals. (i) A larger size-class DI RNA containing three non-contiguous regions (note: region III/IV represents a contiguous 3′ terminus which includes the segment between regions III and IV. (ii) A proteotypical DI RNA containing four distinct regions. From White (1996) with permission of the publishers. Panel (D): Sequence similarity among TCV, DI RNA G, DI1 RNA, and sat-RNA C. The arrow represents a 36-base repeated sequence in DI RNA G corresponding to nucleotides 3863–3898 of TCV. Numbering in the DI RNAs and sat-RNA C refers to TCV nucleotides involved in junction sequences. *From Li et al. (1989) with permission of the publishers.*

TABLE 7.3 Cellular Membranes Associated with Replication of Some (+)-Strand RNA Viruses

Virus Group	Replication Membrane						
	ER	Outer Nuclear Membrane	Golgi Apparatus	Chloroplast	Peroxisome	Tonoplast	Mitochondrion
Bromovirus	+	(+)[a]					
Dianthovirus	+	(+)[a]					
Tombusvirus	+				+		
Tobamovirus	+						
Tobravirus							+
Potyvirus	+		+	+			
Comovirus	+						
Nepovirus	+						
Alfamovirus							
Cucumovirus						(+)[a]	
Carmovirus							+
Tymovirus				+			
Hordeivirus				+			
Benyvirus							(+)[b]

[a]*Associates with both ER and ONM which are contiguous.*
[b]*Early in infection.*

but when co-inoculated with complete virus they were replicated to a greater extent than the infectious particles.

Shorter than normal dsRNA segments are associated with transmission-defective isolates of WTV. Nuss and Summers (1984) showed that these RNAs are formed by the deletion of up to 85% of a genomic RNA segment, giving rise to terminally conserved RNAs that are functional with respect to transcription, replication, and packaging. These isolates interfere with standard virus production in leafhopper cell monolayers (Reddy and Black, 1977).

D. Sites of Replication (reviewed by den Boon and Ahlquist, 2010; Laliberté and Sanfaçon, 2010)

(+)-strand RNA virus genome replication is invariably associated with extensive rearrangement of intracellular membranes. Studies on a range of viruses have revealed some common features of these replication sites:

- RNA replication occurs with rearrangements of intracellular membranes and frequently within numerous virus-induced vesicles invaginated into, or elaborated from, a continuous membrane network.
- Viruses often target the membranes of specific organelles for their replication (Table 7.3) but, in some

cases, may be redirected to an alternative intracellular membrane (see Heinlein et al., 1998).
- The viral factors responsible for modeling membrane and organelle alterations are integral or peripheral membrane proteins. They are also multifunctional and interact with host and other viral factors thus assembling the replication complex.
- The replication vesicles of, at least some, viruses appear to be lined by a capsid-like shell of self-interacting membrane-bound viral replication proteins which could provide a scaffold for anchoring the replication complex.
- The membrane compartments concentrate and sequester viral and host replication factors and templates, coordinate replication steps, and most probably protect the dsRNA intermediates of replication from the RNA silencing host defense system.
- Each vesicle often contains just one or a few genome replication intermediates together with many copies of viral non-structural proteins.
- The vesicles usually have necks opening to the cytoplasm which enables ribonucleotides to be imported and product RNA to be exported.
- Genome replication is often closely associated with virion assembly within these compartments.

- The cellular remodeling associated with viral replication can be associated with other later functions such as intra- and inter-cellular movement (Chapter 10).

Most of the membrane replication sites involve the ER (Table 7.3). For several viruses, e.g., tobamoviruses, comoviruses, and nepoviruses, the cytoplasmic ER appears to be the only membrane involved, but, for some, membranes associated with other organelles are also implicated; Figure 7.1 shows that many of the membrane systems are inter-connected. Cucumoviruses and alfamoviruses (Ibrahim et al., 2012) modify the tonoplast membrane, tobraviruses and carmoviruses, the mitochondrial outer membrane (Harrison and Roberts, 1968) and tymoviruses and hordeiviruses, the outer chloroplast membrane; BNYVV particles localize to the mitochondria early in infection but later are found in the cytoplasm (Erhardt et al., 2001). Other viruses appear to modify more than one membrane. Bromoviruses and dianthoviruses are associated with the perinuclear ER and also the outer nuclear membrane (ONM) which is contiguous with the ER. TBSV forms multivesicular bodies derived from peroxisomes in a variety of plant species (reviewed by Martelli et al., 1988 and for vertebrate viruses, Lazarow, 2011). However, TBSV replicates as efficiently in yeast which had a single deletion in peroxisome biogenesis (pex) genes as in wt yeast (Panavas et al., 2005). Using confocal microscopy on yeast cells missing either PEX3 or PEX19 genes which are absolutely essential for peroxisome biogenesis, Jonczyk et al. (2007) showed that the site of TBSV replication switched to the ER. This supports the strong links between peroxisomes and ER with peroxisomes being generated from domains in the ER (Hoepfner et al., 2005) and suggests that TBSV can exploit one or more common feature between the two membranes but for some reason prefers peroxisomes. Wei et al. (2010) found that PPV replication-associated proteins initially associated with the ER but then moved predominantly to the Golgi apparatus and then, via actin microfilaments, to the periphery of chloroplasts where they docked on the outer chloroplast envelope and induced chloroplast invaginations. The chloroplast-associated vesicles contained viral replicase components and dsRNA and were concentrated with viral RNA. They suggested that plant potyviruses sequentially recruit the ER and chloroplasts for their genome replication.

The localization to specific membrane sites and the modification of the membrane to contain the replication complex involve both viral and host proteins. Details of these sites of replication and restructuring of membranes are given for the relevant virus in Chapter 16.

E. Host Factors Involved in RNA Virus Replication

Viruses co-opt a wide range of host factors in the replication of their genomes, thereby reducing the information that they need to encode. Most of these host factors interact with viral gene products or with the viral nucleic acid. The host factors involved in the replication of two RNA viruses have been studied in detail using the yeast *in vivo* system. About 100 genes were identified whose absence significantly inhibited or stimulated BMV RNA replication and/or gene expression (Kushner et al., 2003) and more than 250 yeast host factors have been identified that affect the replication and recombination of TBSV or are bound to the viral replicase, replication proteins, or the viral RNA (Nagy and Pogany, 2010). For several other RNA viruses, the involvement of smaller numbers of host proteins has been recognized and for yet others (e.g., AMV and CPMV) application of inhibitors of host protein synthesis like actinomycin D affects viral replication. Some of the host factors involved in the replication of specific viruses are described in Chapter 16.

The effects of host factors on replication are either required, or stimulatory or inhibitory. Functions of factors have been characterized in detail for bromoviruses (Noueiry and Ahlquist, 2003), tombusviruses (Nagy and Pogany, 2010), and tobamoviruses (Ishibashi et al., 2010), and it is likely that many of these functions are likely to applicable to other RNA viruses. However, it is becoming evident that factors specific for the functioning of one virus may not be specific for another virus. The basic functions thus far identified are listed below together with some examples of how they may be involved in viral replication.

1. Translation Factors and Cellular Proteins Involved in Protein Biosynthesis

At least some of the incoming viral genomic (+)-strand RNA is translated to provide the replication protein(s) before replication uses the same template RNA. Therefore, translation and replication must be coordinated to regulate and switch these processes temporally and spatially and prevent collision between the ribosomes and replicase(s). Several host translation factors have been identified to bind either to viral replication proteins or the viral RNA and are good candidates to be involved in the switch. For example, translational factor eEF1A is a permanent resident of the tombusvirus replicase complex (Li et al., 2009) and, among other functions, may be involved in promoting (−)-strand synthesis by the replication complex (Nagy and Pogany, 2010); the p41 subunit of wheat germ eIF-3 binds strongly and specifically to BMV protein 2a (Quadt et al., 1993).

The interaction of the potyvirus VPg with the translational eukaryotic initiation factor eIF4E/eIF(iso)4E is an integral part of the replication of these viruses.

2. Protein Modification Enzymes

Posttranslational modification is important in switching protein molecules between active and inactive forms,

in regulating their stability, their subcellular localization, and their interactions with other proteins, nucleic acids, or membranes.

As viruses are known to induce high-level expression of stress-related proteins, such as chaperones, including heat-shock proteins (HSPs) it is not surprising that such proteins are identified in genome-wide screens of yeast. However, as well as being a plant stress response, such proteins play a significant role in virus replication.

Replication complexes of several viruses contain HSPs which are chaperones. The tombusvirus replicase complex contains Hsp70, and Hsp70 and Hsp90 are implicated in BMV replication. These Hsp chaperones seem to play multiple and essential roles during viral replication: (i) it is suggested that they are involved in the folding and localization/transportation of the viral replication proteins; (ii) the binding of replication proteins to Hsp70 results in shielding the hydrophobic transmembrane domains in the replication protein thus preventing their aggregation and promoting binding to membrane transport protein(s); (iii) HSPs function by inserting the replication proteins into intracellular membranes; and (iv) HSPs assist the assembly of replication complexes (Pogany et al., 2008).

Phosphorylation which can modulate protein properties, such as enzymatic activity, stability, subcellular localization, or interaction with binding partners, is becoming increasingly recognized as a regulator of RNA virus replication (reviewed by Jakubiec and Jupin, 2007). For example, it is suggested that phosphorylation of CNV p33 replication protein by a host kinase leads to the release of viral RNA from the replication complex (Stork et al., 2005).

As well as the virus-coded MT activity involved with capping viral RNAs, host MT activity appears to regulate some viral replication functions. For example, CMV 1a MT domain interacts with a tobacco MT, Tcoi1, which methylates both the MT and HEL domains of 1a (Kim et al., 2008). It is suggested that methylation may regulate the MT and HEL activities of the 1a protein but not its interaction with 2a protein.

Many proteins in the ubiquitin (Ub) pathway interact with TBSV p33 suggesting that ubiquitination plays a critical role in the replication and/or recombination of this virus (Barajas et al., 2009).

3. RNA-Binding Proteins, RNA Modification Enzymes, and Proteins Involved in RNA Metabolism

The large number of host proteins in this group indicates that they are likely to play important and diverse roles in RNA virus replication. Examples include Nsr1p (nucleolin) which probably inhibits TBSV RNA recruitment and the 5′–3′ exoribonuclease, Xrn1 which is likely to play a central role in TBSV replication, recombination, and viral

RNA degradation. BMV replication in yeast requires the host protein, LSm1p, which is part of the LSM1−7 complex. The LSM1−7 complex directly binds to the BMV TLS in the 3′ UTR and two internal A-rich ss regions (Galão et al., 2010). As both regions regulate the translation and replication of the BMV genome, it is suggested that the RNA-binding properties of the LSm1−7 complexes control the translation/replication switch.

4. Proteins Involved in Lipid/Membrane Biosynthesis and Metabolism

As discussed above (Section III, D) RNA viruses replicate on cellular membranes which are extensively remodelled. The genome-wide screens in yeast reveal host genes involved in lipid biosynthesis/metabolism, fatty acid biosynthesis/metabolism, and sterol biosynthesis (sterols affect membrane rigidity, fluidity, and permeability). Two roles have been identified as functions provided by sterols during tombusvirus replication: (i) facilitating the assembly of the viral replication complex and (ii) stabilizing p92pol replication protein. BMV replication in yeast is affected by *OLE1*, an essential gene required for synthesis of unsaturated fatty acids.

5. Cellular Proteins Involved in Vesicle-Mediated Transport/Intracellular Protein Targeting

The replication proteins, translated from viral RNA, together with the viral RNA must be localized to the membrane replication site. A number of host genes have been identified, which are involved in TBSV intracellular protein targeting and vesicle-mediated transport, but the functions of these genes are not yet fully understood (Nagy and Pogany, 2010). The *Arabidopsis Tom1* gene product is a membrane protein and interacts with a long α-helix in the N-terminal region of the HEL domain of tobamovirus replication proteins (Nishikiori et al., 2012); it is thought that this interaction tethers the replication proteins onto the membrane sites where replication complexes are assembled.

6. Membrane-Associated Cellular Proteins

As viral replication of several viruses takes place on cellular ER membranes (Table 7.3), it would seem likely that some membrane-bound host proteins could affect viral replication directly or indirectly. Several host proteins in this group affected TBSV replication. BMV protein 1a co-precipitates with reticulon homology proteins (RHPs) (Diaz et al., 2010) which are a family of membrane-shaping proteins that normally induce positive curvature (towards the cytoplasm) of peripheral ER membrane tubules. These interactions of RHPs with BMV proteins are thought to shape the vesicular structures in the ER in which the viral replication complexes function.

7. Proteins Involved in General Metabolism of the Cell

As noted in Section II, virus replication depends on the resources provided by the host cells. Many host proteins have multiple functions, and viruses might exploit alternative, less characterized functions of such proteins. A large number of host general metabolism proteins are being shown to affect RNA virus replication. One example is GAPDH (glyceraldehyde-3-phosphate dehydrogenase), a component of tombusvirus replication complexes (Serva and Nagy, 2006), which is proposed to play a role in asymmetric viral RNA synthesis by selectively retaining the TBSV (−)-strand RNA template in the replicase complex (Wang and Nagy, 2008).

8. Cellular Transcription Factors/Cellular Proteins Involved in DNA Remodeling/Metabolism and Cellular and Hypothetical Proteins with Unknown Functions

The screens identified various yeast proteins with these functions as affecting TBSV replication. However, it is not yet known if any of these effects are direct or indirect.

9. Host Defense Responses

As described in Chapters 9 and 11, plant hosts can mount either general or specific resistance to viruses. These can involve host factors that interact directly with viral gene products modulating or inhibiting viral replication. For example, the *Tm*-1 gene product from *Solanum habrochaite* binds to the HEL domain of ToMV inhibiting viral replication. Similarly, the tobacco *N* gene product interacts with the HEL domain of TMV replication protein giving a hypersensitive response to TMV infection (Chapter 11, Section III, B, 1).

10. Host Factors Missed During the Global Genomics and Proteomics Screens

Genome-wide screening in yeast may miss host factors which are identified by other approaches. For instance, as noted above TBSV replication complexes relocate from peroxisome to ER membranes in yeast lacking the host shuttle protein Pex19p (Panavas et al., 2005). However, this was not identified in the genome-wide screens as it did not affect the replication of the virus *per se*.

It is important to demonstrate the relevance of the host factors identified in yeast in a natural plant host as well.

F. Replication Factories (reviewed by Mackenzie, 2005; den Boon and Ahlquist, 2010; den Boon et al., 2010; Laliberté and Sanfaçon, 2010)

As described above, most, if not all, (+)-strand RNA viruses replicate in organelle-like structures (termed replication factories or replication complexes) associated with extensively rearranged intracellular membranes. These virus factories are virus induced and the detailed structure and membrane(s) involved are usually specific to a virus group. Many are in the form of vesicles with a neck opening to the cytoplasm. Within the replication factory is the replication complex comprising virus and host proteins together with the viral RNA(s). It is thought that virus factories function to (i) increase the local concentration of components required for replication; (ii) provide a scaffold for assembling and anchoring the replication complex; (iii) the vesicle neck filters what goes into and come out of the factory; and (iv) the factories confine the process of RNA replication to a specific location that prevents the activation of host defense functions (Laliberté and Sanfaçon, 2010).

The steps leading to the assembly of the replication factories of BMV and TBSV have been suggested by den Boon and Ahlquist (2010) and Nagy and Pogany (2010) and are described in detail in Chapter 16, Section II, D; it is likely that most if not all (+)-strand RNA viruses follow similar steps. Essentially, there are nine sequential steps most of which involve both viral and host factors: (i) uncoating of the input virions; (ii) translation of the genomic RNA to give the viral replication proteins; it should be noted that the ORFs for these proteins are at the 5′ end of most viral RNAs; (iii) switch from translation to replication and template selection; (iv) recruitment of the RdRp/viral RNA complex to the appropriate membranes and membrane reconformation; (v) assembly and activation of viral replicase; (vi) (−)-strand RNA synthesis; (vii) (+)-strand RNA synthesis; (viii) release of (+)-strand RNA progeny; and (ix) disassembly of the replication complex.

PVX replication complexes have been studied at "super-resolution" using 3D-structured illumination super-resolution microscopy (Figure 7.18) (Linnik et al., 2013). They identified a previously unrecognized membrane structure induced by the PVX triple gene block proteins.

G. Coordination of Replication Events

Replication is just one stage in the virus infection cycle, and the various steps both in (+)-strand RNA virus replication and the events before and after replication are strictly coordinated to facilitate the efficient production of progeny virus and infection of the host. As noted in Chapter 6, Section III, uncoating of the input virus is closely linked to, and in several cases facilitated by, initial translation of the virus genome. Translation is in a 5′–3′ direction and replication from 3′ to 5′ direction on the same RNA template; thus, there has to be a switch from translation to replication to prevent the polymerase colliding with the ribosomes. Mechanisms for controlling and coordinating this switch are noted above and in Chapter 16. The coordination of assembly and the composition of the replication
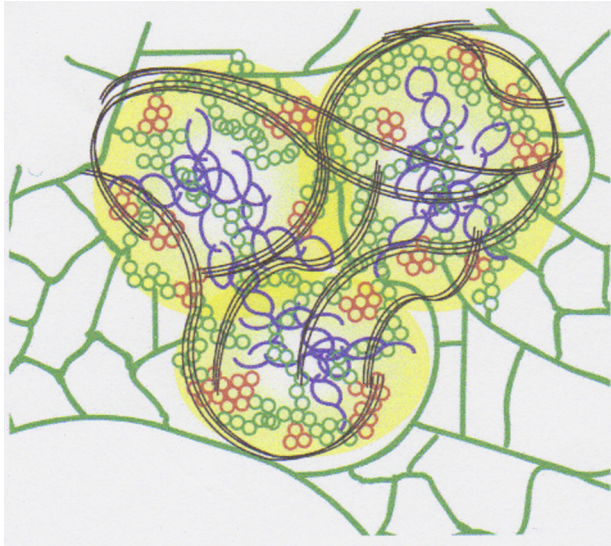
FIGURE 7.18 Schematic model of the PVX replication complex (X body) (not to scale). The triple gene block 1 (TGB1) "beaded sheets" (purple) are localized in the center of the X body. Non-encapsidated vRNA (yellow) surrounds the TGB1 inclusions (Tilsner et al., 2009, 2012). Host ER (green) is remodeled into arrays of small membrane hoops by TGB2 which are wrapped around the TGB1 aggregates within the X body. Some patches of these TGB2 loops also contain TGB3 (red) and may constitute the replication sites of the virus (Bamunusinghe et al., 2009). Bundles of encapsidated virions (black) accumulate at the periphery and form "cages" around the X body. *From Linnik et al. (2013) with permission of the publishers.*

complexes are controlled not only by the interactions between the component proteins (and nucleic acids) but also by the way that they are expressed. Thus, in some cases, some of the component proteins are expressed from frameshift or readthrough, and in other cases, they are expressed from a polyprotein processed in a defined manner. Some aspects are not yet fully understood, such as why frameshift and readthrough occur in only about 5−10% of the times that the ribosome reaches the site of this feature yet the products (at least of TMV) are assembled in a 1:1 ratio in the replication complex. It seems likely that (−)-strand and (+)-strand syntheses are highly coordinated and that once the (+)-strand input template has been "captured" by the *in vivo* replication complex, the full round of RNA replication will occur. Furthermore, much more (+)-strand RNA is synthesized than (−)-strand RNA. The lack of (+)-strand synthesis in most *in vitro* replication systems indicates either that an important factor is lost during extraction or that there are conformational constraints imposed by the location of the complex *in vivo*.

Replication is coincident with or followed by (especially in the case of sg mRNAs) translation of other viral gene products, such as cell-to-cell movement proteins and CP, which takes place in the cytoplasm. This is presumably on rough ER which is, in many cases, close to replication

factories. The next step is encapsidation and/or cell-to-cell movement of the newly synthesized (+)-strand RNA. There is increasing evidence that encapsidation occurs within or near replication factories which could explain why there is more (+)-strand RNA than (−)-strand RNA. Replication factories are linked by cytoskeletal structures enabling translocation of particles or RNA to plasmodesmata (Chapter 10).

H. Detailed Replication of (+)-Strand RNA Viruses

Further descriptions to details of (+)-strand RNA virus replication are given in Chapter 16.

I. Discussion

There have been rapid advances in the understanding of how (+)-strand viruses replicate. The evidence for the involvement of membranes is incontrovertible, but the reasons why different viruses use different membranes (Table 7.3) are not yet understood. It could be that these represent different "ecological" niches within the cell (Chapter 8, Section V, E).

The replication complexes comprise several virus-coded proteins with different functions. These are assembled onto the relevant membrane by a membrane-binding protein(s) or domain(s) that then interacts with the other components.

Most of the RNA elements involved in replication operate in *cis* showing that the template RNA is an integral part of the replication complex. The elements at the 3′ end of the template RNA that initiates (−)-strand synthesis appear to be well defined. This is in contrast to those at the 5′ end of the genomic RNA, which initiates (+)-strand synthesis.

It is likely that long-range RNA–RNA interactions are involved. A network of long-distance interactions could offer certain advantages such as (i) enabling a switch from (−)-strand to (+)-strand synthesis; (ii) controlling strand synthesis so that more (+)-strand RNA is produced rather than (−)-strand RNA; (iii) providing a more efficient system for sg mRNA transcription; (iv) allowing better regulation of transcription; and (v) facilitating and/or coordinating additional viral processes (Lin and White, 2004).

Details of the assembly of replication complexes are discussed in Chapter 16.

V. REPLICATION OF NEGATIVE-SENSE SINGLE-STRANDED RNA VIRUSES

A. Plant *Rhabdoviridae* (reviewed by Jackson et al., 2005; Redinbaugh and Hogenhout, 2005)

Rhabdoviruses have large membrane-bound particles containing a single species of (−)-sense ssRNA (Appendix A,

Profiles 22 and 23). Basically, the virion RNA is associated with the nucleocapsid protein (N), a phospho protein (P), and a large protein (L), considered to be the replicase to form a coiled nucleocapsid. The nucleocapsid is encased in the matrix (M) protein which, in turn, is enveloped in a membrane to form the bacilliform particle. Virus-encoded glycoproteins (G) extend through this membrane. It has been suggested (Cartwright et al., 1972) that there are structural interactions among the N, M, and G proteins. The overall structure and replication resembles that of animal rhabdoviruses but there are some differences. For instance, all vertebrate rhabdoviruses replicate and assemble in the cytoplasm as do some plant rhabdoviruses (the cytorhabdoviruses) but other plant rhabdoviruses (the nucleorhabdoviruses) replicate in the nucleus.

The (−)-strand genome of rhabdoviruses has two functions, as the template for transcription of mRNAs for individual genes (described in Chapter 6, Section VI, A) and as the template for replication via a full-length (+) strand. The polymerase complex undertakes both functions but the switch mechanism is not fully understood, even for the much studied animal-infecting vesicular stomatitis virus (Rodriguez and Nichol, 1999).

1.   Cytological Observations on Replication

Because of their large size and distinctive morphology, the rhabdoviruses are particularly amenable to study in thin sections of infected cells. Morphologically they appear to fall into three groups:

  i. The nucleorhabdoviruses that accumulate in the perinuclear space with some particles scattered in the cytoplasm. With some viruses of this group, structures resembling the inner nucleoprotein cores have been seen within the nucleus (see Box 7.1 for details of the nucleus). The envelopes of some particles in the perinuclear space can be seen to be continuous with the inner lamella of the nuclear membrane. Figure 7.19 illustrates this group.

Immunogold labeling with an antiserum against the five structural proteins of PYDV showed that viral proteins accumulate mainly in the nucleus (Lin et al., 1987). *In situ* hybridization demonstrated that (−)-strand genomic RNAs are found only in nuclei of infected plants whereas the (+)-strand RNA sequences are in both nuclei and cytoplasm (Martins et al., 1998). Immunofluorescence and immunogold labeling showed that N and L proteins are in viroplasms in the nucleus and the M2 protein is more generally distributed within the nucleus.

  ii. In the second group, the cytorhabdoviruses, e.g., LNYV, maturation of virus particles occurs in association with the ER, and particles accumulate in vesicles
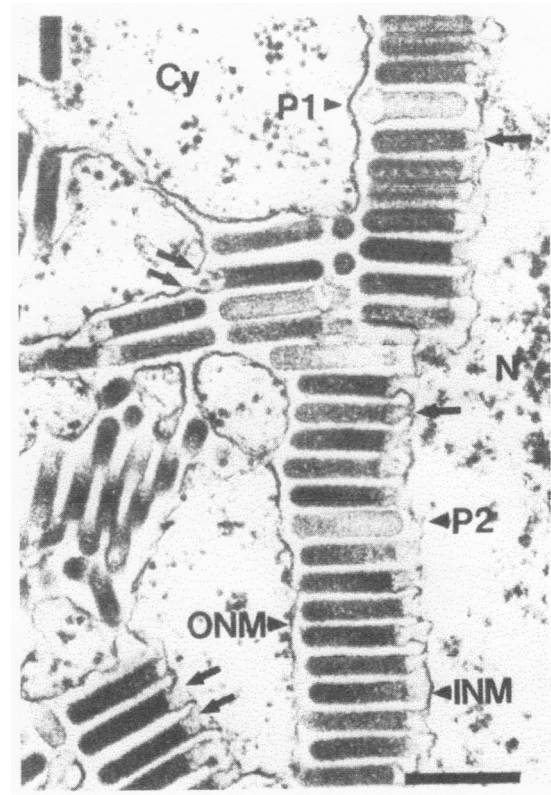
FIGURE 7.19   Electron micrograph of a thin section of a maize leaf infected with a Hawaiian isolate of maize mosaic rhabdovirus. Virus particles apparently budding through the inner nuclear membrane (INM) and through intracytoplasmic extensions (double arrows) of the outer nuclear membrane (ONM); single arrows indicate constriction of the INM. Alignment of particles at P1 and P2 suggests budding on the ONM. Cy, cytoplasm; N, nucleus. Bar = 0.3 μm. *From McDaniel et al. (1985) with permission of the publishers.*

in the ER. Biochemical evidence suggests that the nucleus might be involved in the early stages of infection by members of this group.

  iii. This group comprises structures that appear to be rhabdovirus nucleocapsid cores lacking the surrounding membrane (Francki et al., 1985).

When examining rhabdoviruses in the cell it must be remembered that the ONM is contiguous with the ER. Thus, nucleorhabdoviruses budding through the INM into the perinuclear space may further be included in vesicles derived from the outer membrane and be found in the cytoplasm. Similarly, cytorhabdoviruses that associate with the ER may affect the ONM giving an appearance of a nuclear involvement.

2.   Nucleorhabdoviruses

Most of the studies have been performed on SYNV for which a reasonably detailed picture of its replication has been developed.
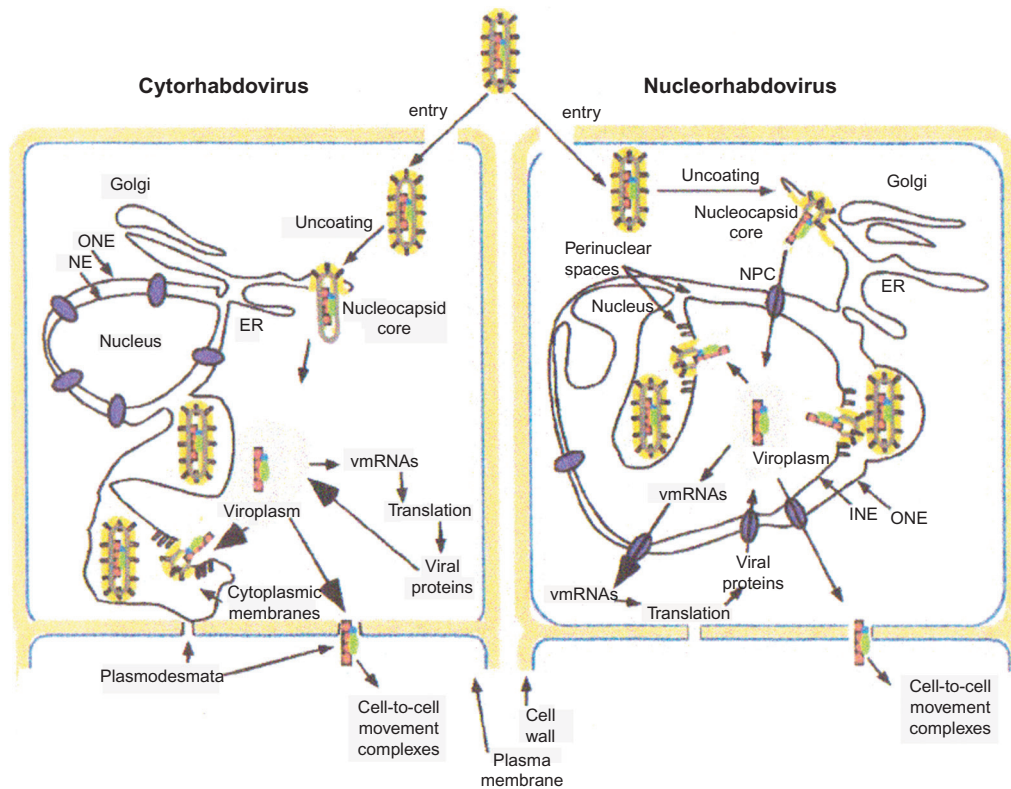
FIGURE 7.20 The replication cycles of cyto- and nucleo-rhabdoviruses. Most rhabdoviruses gain entry into host cells during insect vector feeding. Uncoating is believed to take place on ER membranes, followed by release of the nucleocapsid core into the cytoplasm. At this point, the replication cycles of the two genera diverge. For cytorhabdoviruses, the newly released core become transcriptionally active and associate with the ER to establish viroplasms that function in transcription of viral mRNAs (vmRNAs) and replication of genomic and antigenomic viral RNAs. Following translation of the vmRNAs, the viral proteins involved in replication accumulate in the viroplasm. Viral glycoproteins are targeted to cytoplasmic membranes or, possibly, the outer nuclear envelope (ONE). Maturation of cytorhabdoviruses takes place via matrix protein-mediated condensation of cores at sites of G protein accumulation in the ER. For nucleorhabdoviruses, released cores are transported into the nucleus through NPCs. Following transcription and export, vmRNAs are translated and viral proteins are imported into the nucleus, where they participate in replication and formation of large viroplasms. Morphogenesis of members of both genera occurs near the end of active transcription and replication and involves interaction of the M protein to coil the viral nucleocapsid and form associations with membrane-associated G protein. In the cytorhabdoviruses, EM observations suggest that budding occurs into proliferated ER associated with the viroplasms. At least two models are proposed for morphogenesis of nucleorhabdovirus virions. One model suggests that the inner nuclear envelope (INE) proliferates due to the redistribution of cytoplasmic membranes and invaginates to form intranuclear spherules, into which viral budding occurs. In the classical model, virus budding occurs through intact INE resulting in an expansion of the ONE. In both models, mature virions accumulate in the perinuclear spaces of infected cells, where they may be reacquired during subsequence vector feeding. *From Jackson et al. (2005) with permission of the publishers.*

a. *In vitro* Studies

A salt extraction procedure proved effective in isolating an active polymerase complex from SYNV-infected *N. edwardsonii* leaf tissue (Wagner et al., 1996). The products of the *in vitro* polymerase reactions included full-length, polyadenylated N and M2 mRNA and (+)-strand leader RNA. Animal rhabdoviruses do not polyadenylate their (+)-strand leader transcript and it is suggested that this feature of SYNV may reflect its replication in the nucleus. The polymerase complex comprises the N, M2, and L proteins (Wagner and Jackson, 1997) and addition of antibodies to L protein inhibits the *in vitro* system. The reaction condition of this system favors sgRNA transcription over (−)-sense replication possibly due to depletion of N protein during extraction. Some small virus-sense RNAs were formed and it is suggested that the formation of genomic (−) strand is inhibited by specific signal sequences in the (+)-strand RNA (Wagner and Jackson, 1997).

b. Replication

The following steps have been described for the replication of nucleorhabdoviruses (Figure 7.20) (Jackson et al., 1999, 2005):

- On entry into the cell virus particles associate with the ER and release the nucleocapsid cores into the cytoplasm.
- The nucleocapsid cores enter the nucleus through the nuclear pore complexes (NPC).

- Primary transcription takes place using the L protein incorporated in the nucleocapsid core to give mRNAs that are transported to the cytoplasm and translated.
- The core polymerase proteins, N, P, M2, and L are transported back to the nucleus where they initiate genomic RNA replication and further mRNA synthesis.
- Granular electron-dense viroplasms, containing N, P, M2, and L proteins, form near the periphery of the nucleus and are the site of viral replication.
- In the late stages of replication, M protein associates with the newly synthesized nucleocapsid cores coiling them. This complex then associates with G protein that is concentrated at sites on the INM.
- Newly synthesized virus particles bud into the perinuclear space.

Goodin et al. (2007) demonstrated that infection with SNYV results in invaginations of the INM and that the virus-induced intranuclear membranes are contiguous with the ER. It is suggested that the N protein complexed with the P protein associates with the newly synthesized RNA in the viroplasm and, after removal of the P protein, the complex moves to the intranuclear membranes. The M and G proteins are associated with these membranes and together with the N protein–RNA complex form core particles. Thus, replication (in the viroplasms) and particle assembly are spatially separated and an M protein-containing complex moves from the nucleus to ER membranes. The movement protein, sc4, accumulates at punctuate loci on the periphery of cells (Chapter 10, Section VI, B, 1, j). The localization and interactions of PYDV proteins are compatible with this morphogenesis model based on SNYV (Bandyopadhyay et al., 2010).

3. Cytorhabdoviruses

Replication of cytorhabdoviruses passes through the following steps (Figure 7.22):

- As with nucleorhabdoviruses, on entry into the cell cytorhabdovirus particles associate with the ER and release the nucleocapsid cores into the cytoplasm; the pathways then diverge.
- The newly released cores become transcriptionally active and associate with ER to establish viroplasms.
- Transcription of viral mRNAs occurs in the viroplasms.
- The viral mRNAs are translated and the viral proteins involved in replication accumulate in the viroplasms.
- Genomic RNA replication and further mRNA synthesis occur in the viroplasms.
- Viral G proteins are targeted to the cytoplasmic ER or possibly to the ONM.
- Mature virus particles form by M protein-mediated condensation of cores at sites of G-protein accumulation of the ER.

The exact details of the interaction involved in the assembly and budding of plant rhabdoviruses are unknown, but they may be similar (but happening on different membranes) to those of animal rhabdoviruses (Jayakar et al., 2004).

Replication of plant rhabdoviruses in their insect vector is discussed in Chapter 12, Section III, E, 3.

B. Tospoviruses

The tospovirus genome consists of three RNA segments: L, M, and S enclosed in a membrane-bound particle (see Appendix A, Profile 24 for genome organization). RNA L is (−)-sense and M and S have an ambisense strategy (Chapter 6, Section VI, C, 1). There are four structural polypeptides. Two are glycosylated (G_1 and G_2) and are at the surface of the virus particle (Figure 3.43). The N protein binds to the RNA, and there is a large protein, the RdRp occurring in a minor amount that is encoded by RNA L. These and other properties place tospoviruses in the *Bunyaviridae*, a large family of viruses replicating in vertebrates and invertebrates.

Purified TSWV particles have been shown to support either genome replication or transcription of mRNAs *in vitro*, depending on the conditions chosen (van Knippenberg et al., 2002). However, little is known about the details of tospovirus replication except that it occurs in the cytoplasm but it is thought to be similar to other bunyaviruses (for an account of bunyavirus membrane-associated replication, see Schmaljohn and Nichol, 2007). As noted in Chapter 3, Section VII, B, 2, tospovirus assembly involves the enwrapment of the ribonucleoproteins with viral glycoprotein-containing Golgi stacks (Ribeiro et al., 2008) with the glycoprotein Gc associating with the ER and then moving to the Golgi apparatus in association with the Gn. Thus, it is likely that tospovirus replication is membrane-associated and linked closely to particle assembly.

VI. REPLICATION OF DOUBLE-STRANDED RNA VIRUSES

Plant members of the *Reoviridae* family are placed in three genera: *Phytoreovirus* with 12 dsRNA genome segments, the type member being WTV, *Fijivirus*, with 10 dsRNA genome segments, the type member being FDV, and *Oryzavirus* with 10 dsRNA genome segments, the type member being RRSV (see Appendix A, Profiles 19, 20, and 21 for details of genome organization). Little is known about the molecular aspects of plant reovirus replication, but it is likely to be similar to that of animal reoviruses (described by Schiff et al., 2007).

A. Intracellular Site of Replication

Plant reoviruses replicate in the cytoplasm as do those infecting vertebrates (Wood, 1973). Following infection, densely staining viroplasms appear in the cytoplasm. Viroplasms are present in cells of various tissues of leafhopper vectors infected with WTV as well as infected plant cells (Shikata and Maramorosch, 1967). Immunofluorescence demonstrates the presence of viral antigen in the cytoplasm of cultured leafhopper cells (Chiu et al., 1970). It is not yet possible to relate the *in vitro* studies on the replication of WTV to the structures seen cytologically.

Enzyme digestion experiments and radioautographic assay of the incorporation of ^{3}H-labeled uridine into maize cells infected with the fijivirus, MRDV, indicate that much of the viroplasm is made up of protein—probably viral proteins. Viral RNA appears to be synthesized in the viroplasm, where the mature particles are assembled. The mature particles then migrate into the cytoplasm where they may (i) remain as scattered particles, (ii) form crystalline arrays, or (iii) become enclosed in or associated with tube-like proteinaceous structures (Bassi and Favali, 1972; Favali et al., 1974). The autoradiographic studies failed to implicate the nucleus, mitochondria, or chloroplasts in virus replication.

A detailed electron microscopic study supports the view that the viroplasms caused by FDV in sugarcane are the sites of virus component synthesis and assembly (Hatta and Francki, 1981a, 1981b). The viroplasms are composed mostly of protein and dsRNA. Some areas contain numerous isometric particles 50−60 nm in diameter. Some appear to be empty shells while others contain densely staining centers of dsRNA. These particle types appear to be incomplete virus particles or cores. Complete virus particles are seen only in the cytoplasm.

Viroplasms containing RDV nonstructural proteins, Pns6, Pns11, and predominantly Pns12, are found within 6 h of infecting an insect cell culture (Wei et al., 2006). Later, the core proteins, P1, P3, P5, and P7, were identified in the inner region of the inclusions with the outer capsid proteins (P2, P8, and P9) together with intact virus particle accumulating in the peripheral regions of inclusions. These observations suggest that core particles are constructed inside the inclusions and the mature particles assemble at the inclusion edges.

B. Replication

The (−)-sense strands of vertebrate reoviruses are synthesized by the viral replicase on a (+)-sense template that is associated with a particulate fraction (Acs et al., 1971). These and related results led to the proposal that dsRNA is formed within the nascent cores of developing virus particles, and that the dsRNA remains within these particles. If true, this mechanism almost certainly applies to the plant reoviruses. It implies that the mechanism that leads to selection of a correct set of 12 genomic RNAs could involve the ss (+) strand.

Xu et al. (1989) constructed a series of transcription vectors that allowed production of an exact transcript of WTV S8 RNA and of four analogues that differed only in the immediate 3′ terminus. Their experiments provide three lines of evidence supporting the view that the 5′- and 3′-terminal domains interact in a functional way: (i) nuclease T1 sensitivity assays show that even a slight change in the 3′-terminal sequence can affect the conformation of the 5′ terminus; (ii) translation *in vitro* is slightly decreased by alterations in the 3′ terminus, which extends the potential for 3′–5′ terminal base pairing, and is increased by changes that reduce potential base pairing; and (iii) computer modeling for minimal energy structures for six WTV transcripts predicts a conformation in which the terminal-inverted repeats were base paired.

Dall et al. (1990) developed a gel retardation assay with which they demonstrated selective binding of WTV transcripts by a component of extracts from infected leafhopper cell cultures. Using terminally modified and internally deleted transcripts, they established that the segment-specific inverted repeats present in the terminal domains were necessary but not sufficient for optimal binding. Some involvement of internal sequences was also necessary. There was no evidence for discrimination in binding between transcripts from different segments. The binding component or components present in extracts of infected cells, which are not present in those of healthy cells, have not yet been characterized.

VII.   REPLICATION OF REVERSE TRANSCRIBING VIRUSES

The *Caulimoviridae* is the only family of plant viruses with dsDNA genomes (see Appendix A, Profiles 7−12 for description of family). In 1979, very little was known about the replication of this group, but since then progress has been very rapid. There have been two main motivating factors. First, it was hoped that these viruses, because of their dsDNA genomes, might be effective gene vectors in plants (Chapter 15, Section III, A, 1, a). Second, the realization that the DNA is replicated by a process of reverse transcription made their study a matter of wide interest.

The minimal replication time of CaMV was examined in two permissive hosts, *Arabidopsis* and turnip and in *N. benthamiana* where the virus replicates slowly (Khelifa et al., 2010). The kinetics of replication are the same in all three hosts with the first progeny virus being detected about 21 h after transfection.

The family comprises six genera that form two groups, the "caulimoviruses" and the "badnaviruses." These two groups differ in genome organization but have essentially the same replication methods. Most experimental work has been carried out on the "caulimovirus" CaMV and the "badnavirus" RTBV. Reviews include Hohn et al. (1985), Hull et al. (1987), Pfeiffer et al. (1987), Mason et al. (1987), Hull (1996), and Hohn and Fütterer (1997).

Although the replication of members of the *Caulimoviridae* is by reverse transcription and, in many respects, is similar to that of retroviruses, it does differ from that of retroviruses in several important points. These differences include:

- The replication does not involve integration into the host genome for transcription of the RNA but is from an episomal minichromosome.
- The virus does not encode an integrase gene.
- The template for replication is circular dsDNA and not the linear DNA with long terminal repeats characteristic of retroviruses. (The two points above relate to the lack of integration).
- The DNA phase of the replication cycle is encapsidated rather than the RNA phase, which is encapsidated in retroviruses. Thus, the *Caulimoviridae* are known as pararetroviruses.

As with retroviruses, the replication cycle of pararetroviruses has two phases, a nuclear phase where the viral DNA is transcribed by host DNA-dependent RNA polymerase and a cytoplasmic phase where the RNA product of transcription is reverse transcribed by virus-encoded RNA-dependent DNA polymerase or reverse transcriptase (RT) to give DNA. In retroviruses, the RT activity is part of the *pol* gene which also includes the RNase H activity that removes the RNA moiety of the RNA:DNA intermediate of replication. The *pol* gene is part of the gag–pol polyprotein that is cleaved by an aspartate proteinase, the gag being analogous to CP. In pararetroviruses, the RT and RNaseH activities are closely associated. In badnaviruses, the CP and pol are expressed from the same ORF but in caulimoviruses they are expressed from separate ORFs. All plant pararetroviruses encode an aspartate proteinase.

A. Reverse Transcriptase (reviewed by Götte et al., 2010)

Most studies have been performed on retrovirus pol. RT has a characteristic motif of tyrosine–isoleucine–aspartic acid–aspartic acid (YIDD) and several amino acid motifs identify the RNase H domain. Processing of the 66-kDa pol region of human immunodeficiency virus (HIV) by the aspartate protease removes the RNase H domain giving a heterodimer of 66- and 51-kDa proteins. This enzyme complex has three activities for the conversion of ssRNA to dsDNA, RNA-dependent DNA polymerase,

DNA-dependent DNA polymerase, and RNase H. The crystal structure of the RTs from two viruses (HIV and Moloney murine leukaemia virus) has been determined (Cote and Roth, 2008) showing a structure resembling a right hand as described above for RdRp (Box 7.2).

The first indication that the product of CaMV ORF V is analogous to the retrovirus *pol* gene came from sequence comparison (Toh et al., 1983). The N-terminal domain of ORF V has an aspartate protease motif (Toruella et al., 1989) that autocatalytically cleaves an N-terminal doublet of polypeptides (20 and 22 kDa) from *in vitro* translated ORF V transcript. Mutants of the protease active site are not processed. This fits with other features of the replication cycle described below. ORF V was expressed in yeast as a 60-kDa protein that had RT activity on a synthetic template (Takatsuji et al., 1986). In contrast, expression of ORF V in *E. coli* gave a protein of 78 kDa, the size expected from that ORF, but it did not have any RT activity. An activity gel analysis revealed that RT activity associated with CaMV particles is also 60 kDa (Takatsuji et al., 1992). Deletion analysis showed that removal of between 143 and 185 N-terminal amino acids from the *E. coli*-expressed protein gave RT activity similar to that of the yeast-expressed protein. This suggests that CaMV RT is translated as an inactive precursor form that is converted to the active form by proteolytic processing. It is presumed that this is by the N-terminal aspartate protease activity.

The pol motifs are in the C-terminal part of ORF III of "badnaviruses" and that of RTBV has been studied in insect cells (Laco and Beachy, 1994). The predicted 87-kDa product was detected and was processed to give 62-kDa and 55-kDa proteins. Sequencing showed that these proteins were N-coterminal. Both proteins exhibited RT and DNA polymerase activities but only the 55-kDa protein had RNase H activity. The precise weights of the 62- and 55-kDa proteins were determined by mass spectrometry (Laco et al., 1995) enabling the C-termini to be identified. Mutagenesis of the putative active site of the aspartate protease prevents the 87-kDa protein being processed in insect cells. Using antisera to specific fragments of the RTBV ORF III product, Hay et al. (1994) detected a 13.5-kDa protein corresponding to the aspartate protease in extracts from infected plants. The protease antibody labeled the surface of the virus particle. Antibodies against the RT domain identified proteins of 68, 65, and 56 kDa, the latter two probably corresponding to the 62- and 55-kDa protein found on expression of this ORF in insect cells (Laco and Beachy, 1994).

Mutation of the Y^{1339}, D^{1341}, or D^{1342} residues of the RT core motif abolishes RT activity whereas that of the I^{1340} did not (S.-C. Lee and R. Hull, unpublished observation).

B. Replication of "Caulimoviruses"

There are a large number of publications concerned with CaMV nucleic acid replication and the phenomenon of

reverse transcription. Many of these are referred to in the review articles noted above. Here, only a few key or recent references will be given.

By 1983, various aspects of CaMV nucleic acid replication led three research groups to propose that CaMV DNA is replicated by a process of reverse transcription involving an RNA intermediate (Guilley et al., 1983; Hull and Covey, 1983; Pfeiffer and Hohn, 1983). Some of the observations that led to the model were:

- the fact that a full-length RNA transcript is produced that has terminal repeats (Covey and Hull, 1981);
- the fact that DNA in virus particles has discontinuities (Section VII, B, 3) while that found in the nucleus does not, but is supercoiled and is associated with histones as a minichromosome (Ménissier et al., 1982; Olszewski et al., 1982);
- the existence of dsDNA in knotted forms (Ménissier et al., 1983); and
- the existence of other forms of CaMV DNA in the cell that are not encapsulated, such as an ss molecule of 625 nucleotides with the same polarity as the α strand covalently linked to about 100 ribonucleotides (Covey et al., 1983).

Since 1983, a detailed picture of the replication of CaMV has been built up.

1. Replication Pathway

The replication pathway is outlined in Figure 7.21.

Essentially, the replication has two phases, transcription of an RNA template from the virion DNA and then reverse transcription of the RNA template to give dsDNA. The transcription phase occurs in the nucleus and the reverse transcription phase in the cytoplasm. In the first phase of replication, the virus particles dock to nuclear pores via a nuclear localization signal which is close to the N-terminus of the CP (Karsies et al., 2002). It is suggested that there are specific receptors on the nuclear membrane for the CP which interacts with the import receptor, importin α. The virus particles disassemble and the dsDNA of the infecting particle moves into the nucleus, where the overlapping nucleotides at the gaps are removed, and the gaps are covalently closed to form a full dsDNA. The covalently closed DNA associates with host histones to form minichromosomes that are the template used by the host enzyme, DNA-dependent RNA polymerase II, to transcribe two RNAs of 19S and 35S.

The two polyadenylated RNA species migrate to the cytoplasm for the second phase of the replication cycle that takes place in the viroplasms (inclusion bodies) (Mazzolini et al., 1985). The 19S RNA is the mRNA for gene VI product that is translated in large amounts to produce the viroplasm protein. Gene VI is the only
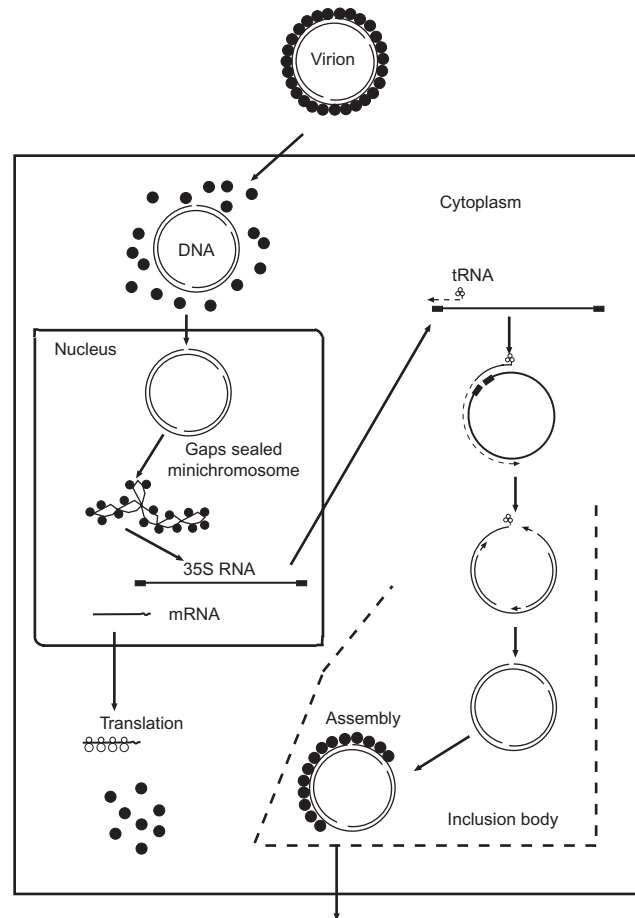

FIGURE 7.21 Diagram of the replication cycle of CaMV. *From Hull (2002) with permission of the publishers.*

Caulimovirus gene to be transcribed as a separate transcript from its own promoter, suggesting that it may have an important role at an early stage following infection (Gowda et al., 1989). Mutagenesis of the coding part of gene VI showed that it was the protein product rather than the mRNA that was responsible for transactivation which is described in more detail in Chapter 6, Section IV, C, 2, g.

To commence viral DNA synthesis on the 35S RNA template, a plant methionyl tRNA molecule forms base pairs over 14 nucleotides at its 3′ end with a site on the 35S RNA corresponding to a position immediately downstream from the D1 discontinuity in the α-strand DNA (see below). The viral RT commences synthesis of a DNA (−) strand and continues until it reaches the 5′ end of the 35S RNA with the RNase H activity removing the RNA moiety of the RNA:DNA duplex giving what is termed "strong-stop DNA." At this point, a switch of the enzyme to the 3′ end of the 35S RNA is needed to complete the copying. The switch is made possible by the 180-nt direct repeat sequence at each end of the 35S RNA which enables the

3′ end of the strong-stop DNA to hybridize with the 3′ end of the 35S RNA. When the template switch is completed, reverse transcription of the 35S RNA continues up to the site of the tRNA primer, which is displaced and degraded to give the D1 discontinuity in the newly synthesized DNA.

The rest of the used 35S template is removed by an RNase H activity. In this process, two polypurine tracts (PPTs) of the RNA are left near the position of discontinuities D2 and D3 in the second DNA strand (+ strand). Synthesis of the second (+) strand of the DNA then occurs, initiating at these two PPT RNA primers. The growing (+) strand has to pass the D1 gap in the (−) strand, which again involves a template switch.

There are several observations that support and enhance this model for CaMV replication (reviewed by Hohn and Fütterer, 1997): (i) both (−)- and (+)-strand DNA synthesis are resistant to aphidocolin, and inhibitor of DNA>DNA synthesis; (ii) RT activity is associated with viral inclusion bodies and virus particles; and (iii) various unencapsidated nucleic acid molecules are interpreted as being replication intermediates have been isolated. These include strong-stop DNA which has ribonucleotides at the 5′ end, DNA molecules that are partially double- and partially single-stranded compatible with being products of defective replication and hairpin structures (Turner and Covey, 1988); (iv) the association of replication intermediates with apparently incomplete virus particles (Thomas et al., 1985; Marsh and Guifoyle, 1987).

The findings of RT activity in inclusion bodies and virus particles and of replication intermediates in virus particles indicate that, as with retroviruses, the reverse transcription of CaMV occurs in particle-like proviral structures.

2. Inclusion Bodies

As noted in an earlier section, CaMV (and other caulimoviruses) induce characteristic inclusion bodies or viroplasms in the cytoplasm of their host cells (Figure 7.22).

There are two forms of inclusion bodies, electron-dense ones that are made up of ORF VI product and electron-lucent ones that are made up of ORF II product (Espinoza et al., 1991); virus particles are found in both types of inclusion bodies. The electron-dense inclusion bodies are the site for progeny viral DNA synthesis and for the assembly of virus particles; it is not known if virus replication takes place in the electron-lucent inclusion bodies which are involved in aphid-transmission of CaMV (Chapter 12, Section III, B, 2). Viral CP appears to be confined to them and most virus particles are retained within the inclusion bodies.

At an early stage in their development, the ORF VI product inclusion bodies appear as very small patches of electron-dense matrix material in the cytoplasm,
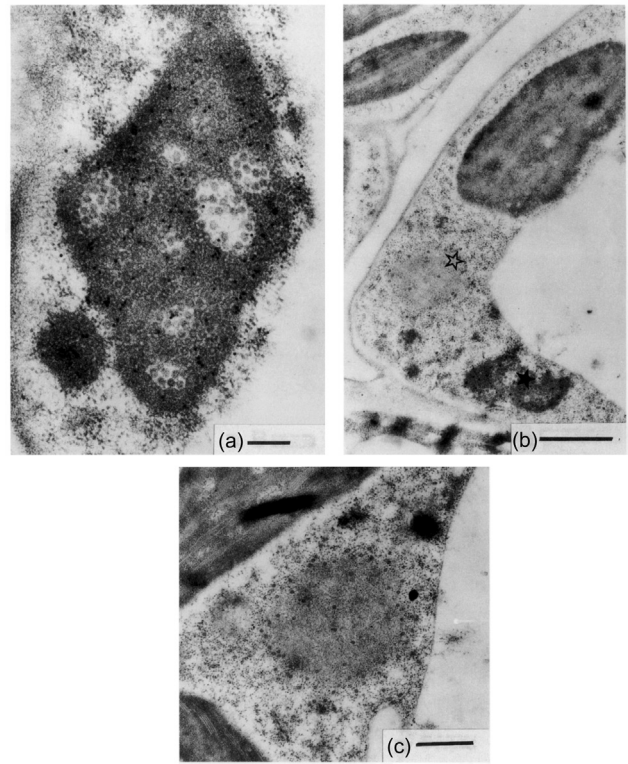
FIGURE 7.22 Electron micrographs of the inclusion bodies of CaMV Cabb B-JI in infected turnip leaves immunogold labeled with anti-P62 (ORF VI product) antiserum. (a) Electron-dense inclusion body with gold particles preferentially labeling the inclusion body matrix (bar = 200 nm). (b) Cell showing an electron-dense inclusion body (filled-in star) heavily labeled and an electron lucent inclusion body (open star) without gold particles contained within the same cell (bar = 1 μm). (c) An electron lucent inclusion body showing the lack of gold particles (bar = 500 nm). *From Espinoza et al. (1991) with permission of the publishers. A more detailed version of this figure can be found on* http://booksite.elsevier.com/9780123848710.

surrounded by numerous ribosomes. Larger inclusion bodies are probably formed by the growth and coalescence of the smaller ones leading to mature inclusion bodies that vary quite widely in size from about 0.2 to 20 μm in diameter. They are usually spherical and are not membrane bound. They often have ribosomes at the periphery and consist of a fine granular matrix with some electron-lucent areas not bounded by membranes. Virus particles are present in scattered or irregular clusters in the lucent areas and the matrix.

Little is known about the way CaMV particles are assembled. No empty virus shells are found in infected tissue. These observations suggest that encapsulation may be closely linked to DNA synthesis. The role of glycosylation and phosphorylation of the CP remains to be determined.

3. Discontinuities

The DNA of "caulimoviruses" has gaps or discontinuities at specific sites, one (D1) in one strand (the + or α strand)

FIGURE 7.23 Structure of CaMV gaps or discontinuities. G_1 (also referred to as D1) is on the transcribed α strand and G_2 (D2) and G_3 (D3) are on the complementary strand. For each of the gaps the upper and lower sequences are those of discontinuous strand and the middle sequence the unbroken strand. These sequences are the most common found but for each the 5′ terminus is at a fixed position, the 3′ terminus may vary from the shown position. The numbers above each sequence are the positions on the CaMV sequence. *From Richards et al. (1981) with permission of the publishers.*

and one or more in the other strand. Those of CaMV have been studied in detail and shown to comprise an overlapping sequence with the 5′ end being in a fixed position and that of the 3′ end being in a variable position giving an overlap varying between 8 and 40 nucleotides (Figure 7.23; Richards et al., 1981).

As explained above (Section VII, B, 1), these discontinuities arise from replication where the advancing DNA strand reaches the priming site. Thus, D1 is at the tRNA priming site for (−)-strand synthesis, and the discontinuities in the other strand are at the PPTs of RNA, generated by RNase H cleavage that give (+)-strand priming. In an analysis of the PPT-associated (+)-strand priming, Noad et al. (1998) showed that altering the length of the 13-base pair PPT by ±25% significantly reduced priming efficiency but did not affect the site of the 5′ end of the new (+)-strand DNA which is 3 nucleotides from the PPT 3′ end. There is a short pyrimidine tract 5′ to the PPT that plays an important role in PPT recognition *in vivo*. Noad et al. (1998) proposed a model for pararetroviral (+)-strand priming in which the pyrimidines enhance the PPT recognition during RNase H cleavage and suggest that the fidelity of primer maturation involves PPT length measurement and 3′ end recognition by the RNase H.

C. Replication of "Badnaviruses"

The replication of RTBV, the most studied of the "badnaviruses" is similar to that of CaMV in most respects and is supported by the detection of characteristic replication intermediates (Bao and Hull, 1994). RTBV CP has several NLSs located within both N- and C-terminal regions (Guerra-Peraza et al., 2005). As with CaMV, RTBV CP interacts with importin α, but it is the C-terminal NLS

that interacts and not the N-terminal one as with CaMV. It is thought that the N- and C-terminal NLSs may both be involved with importing the DNA into the nucleus, one effecting docking and the other the actual entry. The "strong stop" DNA has a methionine initiator tRNA at its 5′ end ((Bao and Hull, 1993). However, the discontinuity at the (+)-strand priming site does not map to the PPT site predicted from the sequence but to a site about 1400 base pairs away (Bao and Hull, 1992). Furthermore, both the 5′ and 3′ termini at this discontinuity are heterogeneous in position giving structures varying from a gap of 10 nucleotides to an overlap of 103 nucleotides.

VIII. REPLICATION OF SINGLE-STRANDED DNA VIRUSES

There are two families of plant viruses that have ssDNA genomes, the *Geminiviridae* and the *Nanoviridae*. The replication of members of these two families is ssDNA>ssDNA via a dsDNA stage. Many of the features of replication of the two families are similar but there are some differences. Most is known about replication of the *Geminiviridae*.

There are four genera in the *Geminiviridae*, the genome organizations of which are described in Appendix A, Profiles 1−4. Three of the genera, the mastreviruses, the curtoviruses, and the topocuroviruses have monopartite genomes whereas many of the begomoviruses have bipartite genomes. However, DNA A of bipartite begomoviruses contains all the information necessary for virus replication, while the genes on DNA B encode proteins involved in movement to the nucleus and between cells (as described in Chapter 10). Therefore, with the caveat that the nuclear localization properties of DNA B BV1 ORF are required for nuclear shuttling, for discussing replication, DNA A of these bipartite viruses can be considered to be comparable to the DNAs of the monopartite viruses.

A. Methods for Studying Geminivirus Replication

Two methods have been of great use in elucidating the details of geminivirus replication, agroinfection of whole plants (for review, see Annamalai and Rao, 2006) and transfection of protoplasts.

As mentioned in Chapter 4, Section VI, B, 2, Grimsley et al. (1987) showed that *Agrobacterium* containing tandem repeats of MSV DNA inoculated to whole maize plants led to symptoms of MSV infection. Since MSV DNA is not mechanically transmissible, and intact virus can infect only by means of an insect vector, this experiment provided a very sensitive demonstration that *Agrobacterium* could interact with a monocotyledon. Elmer et al. (1988a, 1988b) adapted the agroinfection procedure of Grimsley et al. (1987) to provide a simple and efficient assay for TGMV replication. They produced transgenic *N. benthamiana* plants containing

multiple tandem copies of TGMV B-DNA. They found that an inoculum containing as few as 2000 *Agrobacterium* cells containing TGMV A-DNA could produce 100% virus infection. Agroinfection is also a highly efficient way of introducing DNAs A and B together into *N. benthamiana* (Hayes et al., 1988b). The technique has also been extended to *Digitaria* streak virus (Donson et al., 1988), MSV in various species of *Poaceae* (Boulton et al., 1989), and ACMV (Morris et al., 1988; Klinkenberg et al., 1989).

The use of protoplasts to study plant viruses is described in Section II, A, 5. Both protoplasts and agroinfection allow the effects of mutations on the viral genome to be explored.

B. *In Vivo* Observations on Geminiviruses

Geminivirus particles usually accumulate in the nucleus, and with some, such as MSV, large amounts of virus accumulate there. In some infections, fibrillar rings, which must be part of a spherical structure, appear in the nucleus (Francki et al., 1985) but their composition and significance are not known. Nuclei isolated from *Nicotiana* tissue infected with TGMV synthesized variable amounts of (+) and (−) strands of both DNAs A and B (Coutts and Buck, 1985).

C. Rolling-Circle and Recombination-Dependent Replication (reviewed by Novik, 1998; Martin et al., 2011a)

Two initial observations pointed to geminiviruses using a rolling-circle mechanism of replication. One came from two-dimensional electrophoresis of extracts of ACMV-infected plants that revealed five putative replication intermediates (Saunders et al., 1991). These included sg (−)-strand DNA associated with genomic (+)-strand DNA, unit length (−)-strand DNA, and virion DNA ranging from 1 to 2 genome lengths, concatemeric virion ssDNA, dsDNA, and partially ssDNA. The other came from the replicational release of the BCTV genome that had been agroinoculated to *N. benthamiana* as a tandem construct (Stenger et al., 1991).

Rolling-circle replication is common in the replication of bacterial viruses and plasmids. It is a two-step process, in the first phase of which the ss (+) strand is the template for the synthesis of (−) strand to generate a ds RF. This RF has two functions. It is the template for transcription as described in Chapter 6, Section VIII, B and it is the template for (+)-strand synthesis generating free ssDNA. The priming of (−)-strand synthesis is often by an RNA molecule that is generated through RNA polymerase or DNA primase activity. (+)-strand synthesis is primed by a site-specific nick in the (+) strand of the RF.
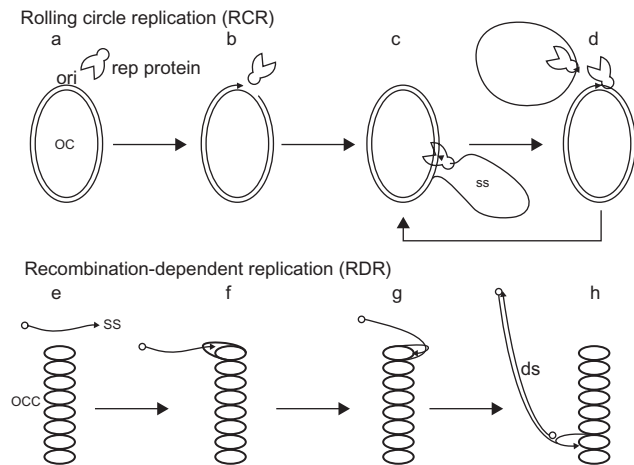
FIGURE 7.24 Models for RCR and recombination-dependent replication (RDR). Step a: Binding of a replication-associated protein (Rep: corresponding to ORF AC1, C1 or C1–2 for geminiviruses), to the origin of replication (ori). Step b: Nicking of DNA and covalent binding of Rep to the 5′-end of DNA. Step c: ssDNA displacement and replication. Step d: New nicking, ssDNA closing, and Rep release. Step e: Incomplete ssDNA interacts with cccDNA at homologous sites. Step f: Homologous recombination. Step g: Loop migration and ssDNA elongation. Step h: ssDNA elongation and complementary strand synthesis resulting in dsDNA. *From Jeske et al. (2001) with permission of the publishers.*

Rolling circle replication (RCR) of viroids and some satellites is also described in Chapter 5, Section I, D.

However, not all the DNA intermediate forms found in plants infected with some geminiviruses are compatible with the rolling-circle replication model. Jeske et al. (2001) concluded AbMV replication intermediates fitted a recombination-dependent replication (RDR) model. A comparison of the two models is shown in Figure 7.24.

RDR has been suggested for other begomoviruses and for curtoviruses and mastreviruses (Pilartz and Jeske, 2003; Preiss and Jeske, 2003; Alberter et al., 2005; Jovel et al., 2007; Erdmann et al., 2010).

D. Geminivirus Replication (reviewed by Hanley-Bowdoin et al., 1999; Gutierrez, 1999, 2000a, 2002)

Many features of the geminivirus RCR cycle have recently been elucidated though there are still several points that are poorly understood. The elucidation of the replication cycle has revealed several aspects of the normal cell cycle as geminivirus replication depends upon many host functions. Of especial interest is that geminiviruses replicate in differentiated cells that are in the G phase and have shut down most of their DNA replication activities. Thus, geminiviruses reactivate the replication activities that they require and convert the cell back to S phase.

The geminivirus RCR cycle is outlined in Figure 7.25.
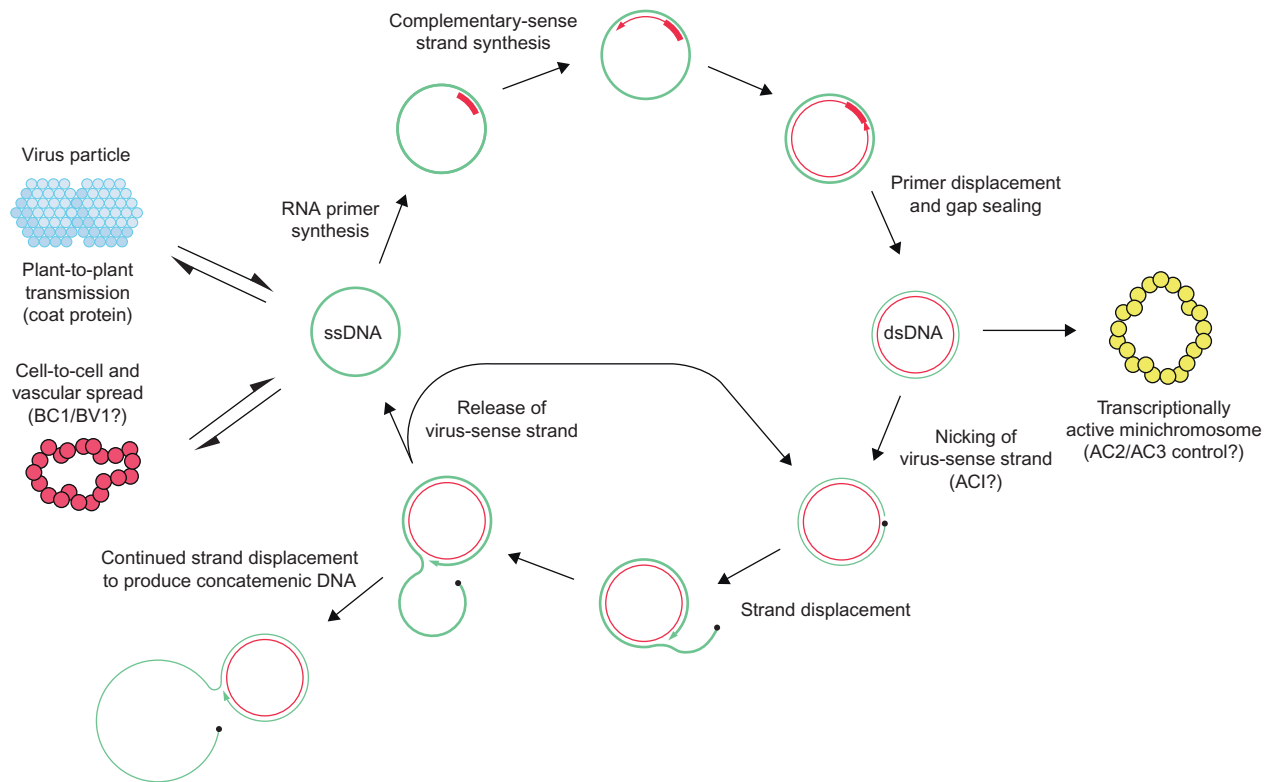
FIGURE 7.25 Diagram of the replication of a geminivirus. *Kindly provided by J. Stanley. From Hull (2002) with permission of the publishers.*

1. Minus-Strand Synthesis

A small oligonucleotide complementary to the 3′ intergenic region has been isolated from several mastreviruses (Donson et al., 1984; Hayes et al., 1988a; Morris et al., 1992). This oligonucleotide can be extended by DNA polymerase *in vitro* and may be the *in vivo* (−)-strand primer. This is supported by the finding that sequences in the 3′ intergenic region of WDV have been implicated as being involved in replication (MacDonald et al., 1988b; Kammann et al., 1991). No analogous molecules have been found in curtoviruses or begomoviruses. The two-dimensional electrophoretic analysis of ACMV replication intermediates (Saunders et al., 1992) indicated that (−)-strand synthesis is primed within the 5′ intergenic region. The primers found in the mastreviruses and begomoviruses contained ribonucleotides.

Little is known about the proteins and mechanisms of (−)-strand synthesis. It is most likely that the virus particles are targeted to the nucleus (Chapter 9, Section II, E) where this stage of the replication occurs. As no viral proteins other than the CP have been detected in virus particles and as the CP is not required for replication (Elmer et al., 1988a; Woolston et al., 1989), it is generally believed that (−)-strand synthesis is effected by host factors like the DNA-dependent DNA polymerase.

2. Plus-Strand Synthesis

The priming of geminivirus (+)-strand synthesis is through a DNA cleavage at a specific site *in vivo*. The progeny of plants infected with heterodimers of different strains of BCTV, WDV, or ACMV (Stenger et al., 1991; Heyraud et al., 1993a, 1993b; Stanley, 1995) was shown to comprise predominant genotypes dependent on the arrangement of the parental genomes. The sequences of the progeny are consistent of (+)-strand DNA synthesis by rolling circle initiating or terminating within the conserved hairpin sequence in the 5′ intergenic region (termed the common region). The initiation site was mapped to the conserved nonanucleotide sequence, TAATAATT↓AC, in the loop of the hairpin (Stanley, 1995). The geminivirus Rep protein is a site- and strand-specific endonuclease that nicks and ligates (+)-strand viral DNA at the same position *in vitro* (Laufs et al., 1995; Orozco and Hanley-Bowdoin, 1996). The nick leaves a 3′-OH end that is used as a primer for leading-strand synthesis by the host DNA polymerase.

There are two major categories of molecular organization of the (+)-strand DNA replication origin. The replication origin for mastreviruses consists of a large *cis*-acting region where the Rep protein forms multiple complexes (Castellano et al., 1999) and that for the begomoviruses contains one

binding site for Rep (Lazarowitz et al., 1992; Fontes et al., 1992, 1994b; Orozco and Hanley-Bowdoin, 1998).

3. Plus-Strand Origin

The origin of TGMV (+)-strand synthesis has been studied in detail (reviewed by Hanley-Bowdoin et al., 1999) and has been compared with those of other begomoviruses and of curtoviruses. The features of the TGMV (+)-strand origin are illustrated in Figure 7.26. The origin is in the left-hand side of the common region and overlaps the promoter for AC61 (also termed AL61) (described in Chapter 6, Section VIII, B, 1, a). Six *cis* elements have been identified in this region.

- The hairpin element is common to all geminivirus genomes (Arguello-Astorga et al., 1994). This comprises a GC-rich stem and an AT-rich loop, and mutagenesis demonstrates that it is the structure of the stem rather than the sequence that is essential for its activity. The stems of mastreviruses are much longer than those of members of the other genera. The 5′-TAATATTAC loop is conserved in all geminiviruses and is found in the (+)-strand origins of other nucleic acids that replicate by rolling circle. There is some sequence flexibility in the loop sequence but the cleavage is between the TT|AC as noted above.
- The binding site for rep has several features: (i) it is virus specific (Table 7.4) but has some conserved sequence; (ii) the GGAT repeat is an absolute requirement; (iii) the spacing between the repeats is important; and (iv) the spacing between the binding site and the cleavage site is important. Less is known about how the Reps of mastreviruses recognize their origins. No sequences analogous to the binding sites described above have been found and mastreviruses do not have the same degree of specificity, as do the other two genera. It has been suggested that mastrevirus Rep proteins bind to reiterated sequences in the stem of the hairpin (Arguello-Astorga et al., 1994), but electron microscopy of WDV Rep/DNA complexes indicated that the binding site is in a similar site to those of the other two genera (Sanz-Burgos and Gutierrez, 1998).
- Begomovirus and curtovirus (+)-strand origins share binding sites for two transcription factors, the TATA box and the G box. Neither of the host transcription factors binding sites is required for virus replication.
- The two other elements are the AG motif and the CA motif (Figure 7.26A). The AG motif is essential for virus replication (Orozco et al., 1998) but has no detectable role in transcription of AC61. Deletion of the CA motif reduced TGMV replication 20-fold, and mutagenesis suggested that it acts as an efficiency element. Although the mechanism by which these elements operate has not been determined, it has been suggested that they may bind plant factors required for replication (Hanley-Bowdoin et al., 1999).

| TABLE 7.4 Rep Binding Sites	
TGMV	GGTAGTAA-GGTAG
BGMV	GG-AG-ACTGG-AG
ABMV	GG-AGTATTGG-AG
Consensus	GG-AGTAYYGG-AG

4. Geminivirus Rep Proteins

The Rep protein is the only geminiviral protein essential for replication (Elmer et al., 1988a; Schalk et al., 1989). In the curtoviruses, begomoviruses, and the topocuvirus, Rep is encoded by ORF C1 and in mastreviruses it is expressed from ORFs C1:C2 through a spliced mRNA; unspliced RNA gives RepA from ORF C1.

Rep and RepA are multifunctional proteins in that they: (i) localize within the nucleus (Nagar et al., 1995); (ii) have specific DNA recognition sites (Fontes et al., 1994a); (iii) have site-specific endonuclease and ligation activity for (+)-strand viral DNA (see above); (iv) have ATP/GTPase activity (Desbiez et al., 1995); (v) the Reps of TYLCSV and MYMIV have DNA HEL activity (Clerot and Bernardi, 2006; Singh et al., 2008). It is not known if Reps of other geminiviruses have a similar activity; (vi) those of some mastreviruses and begomoviruses have been shown to activate the promoter for the CP gene mRNA (Sunter and Bisaro, 1991; Hayley et al., 1992; Hofer et al., 1992; Zhan et al., 1993); (vii) the begomovirus Rep can repress its own promoter (Sunter et al., 1993; Hong and Stanley, 1995).

The domain structure of TGMV Rep protein has been determined (reviewed by Hanley-Bowdoin et al., 1999; Figure 7.27A). It is likely that other begomovirus and curtovirus Rep proteins have a similar structure (Figure 7.27B). Although the mastrevirus Reps differ from those of the other genera, they do have some motifs in common and they are likely to have domain structure similarities.

The replication of geminiviruses initiates with the sequential binding of Rep to a set of iterative sequences or "iterons" located at variable distances from the stem-loop containing the conserved nonanucleotide 5′-TAATATTAC-3′, where Rep cleaves the (+) strand of viral DNA to initiate the RCR process (Londoño et al., 2010). The iterons generally differ in nucleotide sequence among viral species (e.g., see MSV; Willment et al., 2007) and are the major (but not the only) *cis*-acting determinants of virus-specific replication. A comparative study revealed a strong similarity in the relative position of putative specificity determinants in Rep proteins from a wide range of ssDNA viruses of eukaryotes (Londoño et al., 2010).

The solution NMR structure of the catalytic domain of TYLCV Rep shows that it is composed of a central five-stranded anti-parallel β-sheet flanked by a small two-stranded
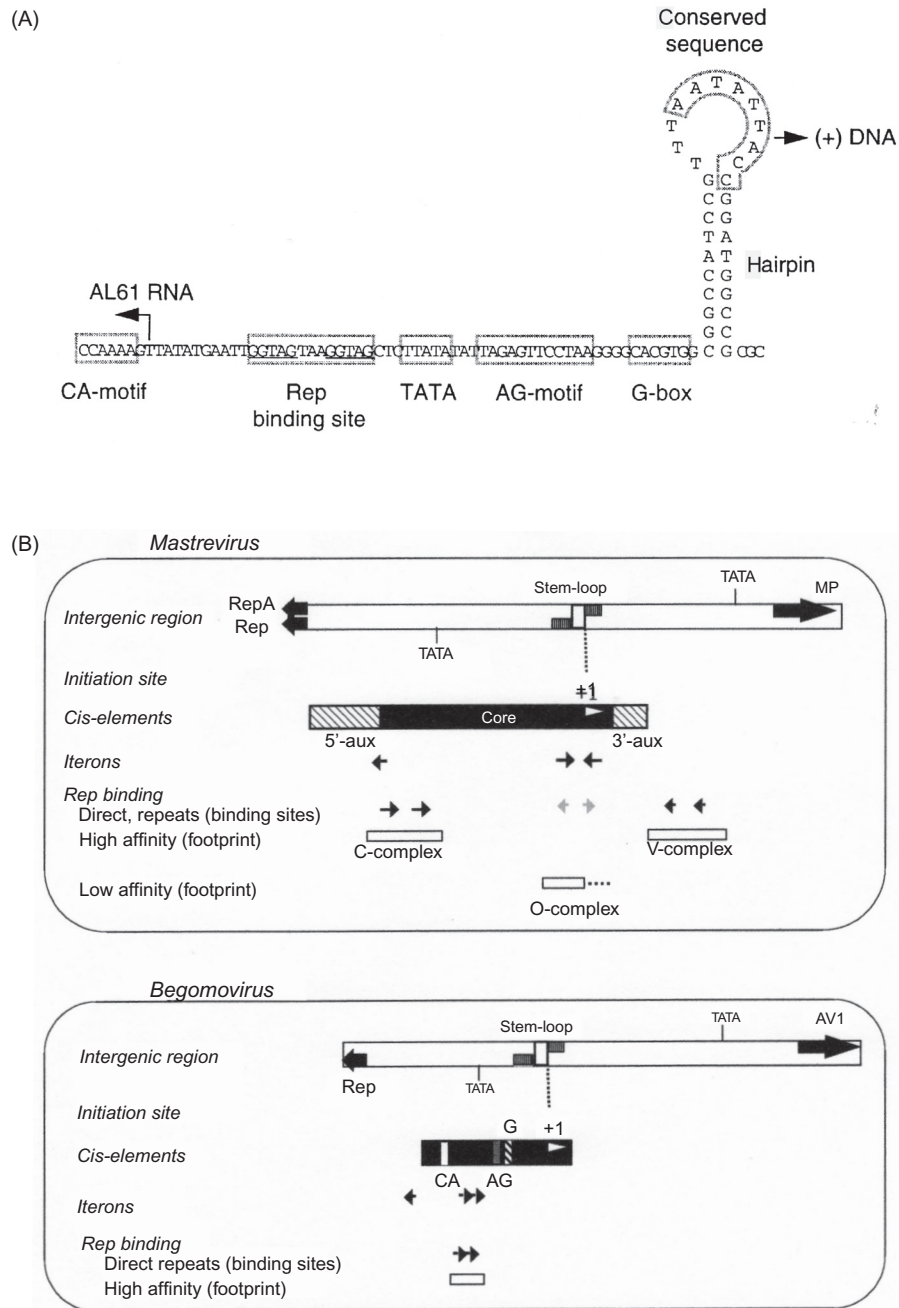
FIGURE 7.26 Replication origins of geminiviruses. Panel (A): The TGMV (+)-strand origin of replication and AC61 (AL61) promoter. The DNA sequence corresponding to the TGMV DNA A positions 54–153 is shown. Only the top strand of the duplex DNA is given. The initiation sites and the directions of synthesis for (+)-strand DNA replication and AC61 transcription are indicated. Other functional elements are boxed. The hairpin structure is drawn and the conserved nonananucleotide loop sequence is marked. From Hanley-Bowdoin et al. (1999) with permission of the publishers. Panel (B): Functional organization of geminivirus DNA replication origin; comparison of the origins of DNA replication (RCR stage) of mastreviruses and begomoviruses. In each box, the uppermost bar represents the intergenic region of WDV (upper box) or TGMV (lower box), and indicates their main features, namely: (1) in the middle, the inverted complementary sequences (small boxes with stripes) flanking the invariant 9 nt sequence (white small box) thought to represent the loop of a stem-loop structure; (2) the TATA boxes of the two promoter elements controlling complementary-sense (left) and viral-sense (right) transcription; (3) the start of the ORFs. Features that define the functional organization of the origin are shown below, namely: (1) the initiation site, +1 being the A residue after the nicking in the 9 nt invariant sequence of the loop; (2) the *cis* elements including the 5′- and 3′-auxiliary sequences (MSV) and the CA-, Ag-, and G-rich motifs (begomoviruses); (3) a small white triangle at the initiation site denoting the direction of RCR; (4) the rep binding sites defined by mutational, DNase 1, and footprinting studies. *From Gutierrez (2002) with permission of the publishers.*
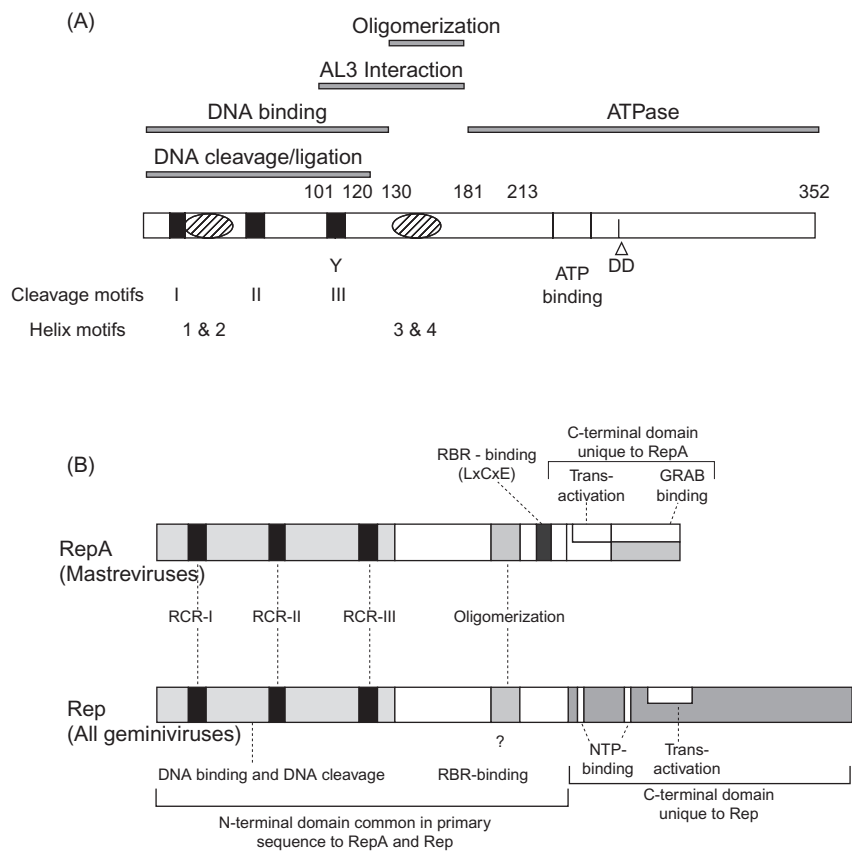
FIGURE 7.27 Domain organization of Rep and RepA proteins. (A) TGMV Rep domains and predicted motifs. Solid boxes mark the locations of the three conserved DNA cleavage motifs in the Rep protein. The active site tyrosine residue is shown in Motif III. The stippled box shows the location of the ATP-binding motif which, in combination with the indicated conserved aspartic acid residues, are related to DNA HELs. The hatched circles indicate predicted sets of α-helices. Helix 2 is strongly amphipathic in character. Solid lines above the protein mark the location of the functional domains for oligomerization, AC3 (AL3) interaction, DNA binding, and DNA cleavage/ligation. The numbers correspond to amino acid positions in Rep. From Hanley-Bowdoin et al. (1999) with permission of the publishers. (B) Diagram comparing the organizations of RepA of mastreviruses and Rep of all geminiviruses. The different domains correspond to a composite based on data available for different geminiviruses and, therefore, the location of each domain is approximate. RCR-I, -II and -III refer to the amino acid motifs conserved in proteins involved RCR. *From Gutierrez (2000b) with permission of the publishers.*

β-sheet, a β-hairpin, and two α-helices (Figure 7.28) (Campos-Olivas et al., 2002).

This structure is similar to those of a large group of proteins that bind RNA and DNA. The endonuclease domain of TYLCSV exhibits a fold similar to that of porcine circovirus type 2 but differs in some of the secondary structural elements (Vega-Rocha et al., 2007a) and is similar to that of fabavirus Rep (Section VIII, E).

Rep proteins have several protein:protein interactions. They form large oligomers of about eight subunits in a virus non-specific manner (Orozco et al., 1997). It is thought that the DNA-binding activity of Rep is dependent on this multimerization (Orozco and Hanley-Bowdoin, 1998). MSV Rep dimerizes and it is suggested that, as the monomer has only one active tyrosine for cleavage, the additional tyrosine in the dimeric form would be available for a second cleavage or a ligation reaction (Horváth et al., 1998). In a study on WDV Rep and RepA using DNase footprinting and chemical cross-linking, Missich et al. (2000) showed

that these replication proteins form large nucleoprotein complexes near the TATA boxes of the virion-sense and complementary-sense promoters. Oligomerization of both proteins is dependent on pH with octomers being formed at pH ≤ 7.0 while at pH ≥ 7.4 the predominant form is a monomer. Preformed oligomers interact very poorly with DNA. They suggest that there is a stepwise assembly of the protein–DNA complex with the monomers interacting with the DNA and then with other monomers to assemble the oligomeric structure.

Rep interacts with some other geminivirus proteins. For example, the (A)C3 protein locates to the nucleus and enhances DNA accumulation of begomo- and curtoviruses. It is thought that this enhancement is through the binding to Rep (Hanley-Bowdoin et al., 1999). The nicking activity of the begomovirus MYMIV is enhanced by the presence of AV2 protein (Rouhibakhsh et al., 2012).

Interactions between Rep and some host protein are described in Section VIII, D, 6.
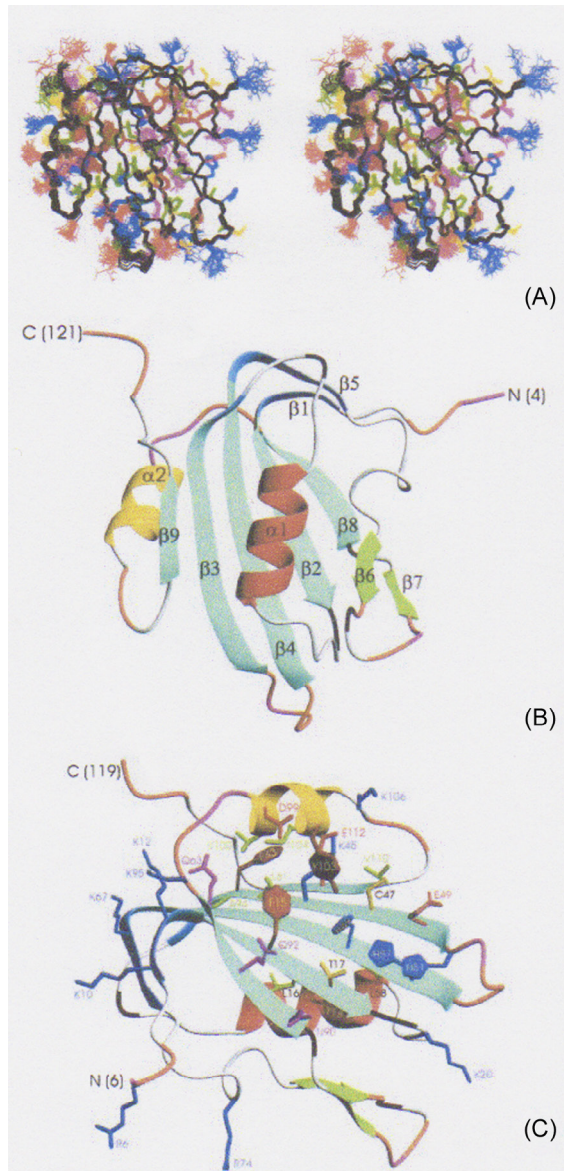
FIGURE 7.28 Three-dimensional structure of TYLCV Rep$_{4-21}$. (A) Stereoview displaying best-fit superimposition of the final ensemble (residues 6–119) of 30 conformers with the lowest $_{\text{DYANA}}$ target function (PDB ID 1L51). The protein backbone (N, Cα, CO) is shown in black, and the side chains are colored according to the residue type (Y, F, W: brown; D, E: red; K, R, H: blue; A,V,L, I, P: green; T, S, C: yellow; N. Q: magenta). The coordinate precision for the protein backbone heavy atoms is 0.48Å. (B) and (C) Ribbon representations of TYLCV Rep$_{4-21}$ regularized mean structure (PDB ID 1L2M). The central 5-stranded β-sheet is shown in blue, the small extension sheet in dark blue, the helix covering the β-sheet in red, the small two-stranded sheet in green and loops in gray. The helix carrying the catalytic tyrosine is colored yellow. The strands and helices are numbered and the N and C termini labeled. Loop residues exhibiting substantial flexibility (low ^{15}N heteronuclear NOE) or non-detected NH resonances are colored in orange and magenta, respectively. In (C), selected amino acid side chains are displayed as well. They either belong to the conserved sequence motifs or occupy equivalent positions to those implicated in ss- or dsRNA/RNA binding of structurally related protein. *From Campos-Olivas et al. (2002) with permission of the publishers.*

## 5. Geminivirus Control of the Cell Cycle
(reviewed by Gutierrez, 2000b, 2002; Hanley-Bowdoin et al., 2004)

As noted above, geminiviruses replicate in differentiated plant cells in which host DNA replication has ceased. The viral replication is dependent upon host DNA replication factors and thus the cell cycle has to be modified. Various geminivirus gene products have been shown to re-program the host cell cycle.

There are several lines of evidence suggesting that the Rep protein is involved in this modification (reviewed by Hanley-Bowdoin et al., 1999). Many Rep proteins are recalcitrant to stable constitutive expression in transgenic plants. In those plants in which expression does occur, and in plants infected with TGMV, the nucleus becomes round and migrates to the cell center, features associated with dedifferentiation. Rep proteins (or RepA of mastreviruses) bind to retinoblastoma (RB) proteins from a variety of sources including plants (Horváth et al., 1998; Liu et al., 1999).

Animal RB proteins regulate cell growth most probably through control of the transition of the G_0/G_1 into S phase of the cell cycle (reviewed by Gutierrez, 1998; Gutzat et al., 2012). It is thought that the plant analogs of RB proteins (retinoblastoma-related, RBR, proteins) have a similar function. Various animal DNA viruses control their host cell cycle through the binding of a virus-encoded protein with the host RB protein through an LXCXE motif (reviewed by Hanley-Bowdoin et al., 1999; Gutierrez, 2000a). Curtovirus, topocuvirus, and begomovirus Rep proteins have this LXCXE motif as does the RepA/Rep protein of mastreviruses. However, the Rep proteins of some geminiviruses, e.g., CSMV and TGMV, do not have an LXCXE motif (Horváth et al., 1998; Arguello-Astorga et al., 2004) and, although mutagenesis of this motif in BYDV reduces the Rep binding capacity, it does not abolish the replication of the virus (Liu et al., 1999). A binding site for Rb has been identified within predicted helix in TGMV Rep with a leucine (L_{148}) in the helix playing an important role (Arguello-Astorga et al., 2004).

As noted above, the curtovirus and begomovirus Rep proteins and the mastrevirus RepA protein bind RBR proteins from various sources, and it is likely that this binding is in a similar manner to that of animal viral proteins to their host RBR protein. Thus, the suggestion is that the binding inhibits the RBR protein control that maintains the host cell in the G phase of the cell cycle enabling it to return to S phase and produce the factors required for viral replication. However, for this to occur, Rep must be expressed from the incoming virus. Therefore there must be enough capability in the newly infected cell to initiate (−)-strand synthesis to give the dsDNA for transcription of the mRNA for Rep. Details of this initial event have not yet been determined but a model is shown in Figure 7.29.
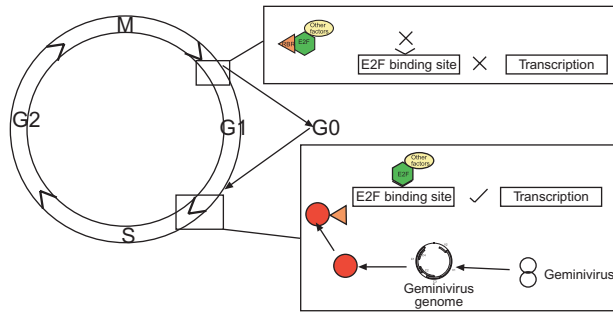
FIGURE 7.29 The left part of the diagram shows the cell cycle: M, mitosis phase; G_1, (growth) interphase; G_0, quiescent phase; S DNA synthesis phase; G_2, interphase. The top box to the right shows how the retinoblastoma (RBR) protein interacts with the E2F protein preventing it binding to its binding site necessary for transcription leading to the G_1 phase. The bottom right-hand box shows how the viral Rep (or RepA) protein prevents the RBR protein from interacting with E2F, thus enabling E2F to bind to the DNA, overcoming the G_1/S phase checkpoint and initiating transcription. *From Hull (2009) with permission of the publishers.*

BCTV C2 protein confers a positive effect on the replication of TYLCSV (Caracuel et al., 2012). Expression of C2 upregulates the cell cycle-related genes on cell cycle re-entry restoring the DNA replication competency of infected cells and creating a favorable environment for viral spread.

TYLCV C3 protein greatly enhances viral DNA accumulation (see Settlage et al., 2005 for references). C3 interacts with itself and the viral Rep and with host proliferating cell nucleus (PCNA) and RBR proteins. Although the C3-RBR protein interaction is not essential for viral replication, it is suggested that the C3 protein plays a role during infection of differentiated cells in host plants (Settlage et al., 2005).

BCTV C4 protein causes ectopic cell division when expressed in transgenic *N. benthamiana* (Latham et al., 1997) and radically alters tissue layer organization in *Arabidopsis* (Mills-Lujan and Deom, 2010). The expression of BSCTV C4 protein in *Arabidopsis* leads to abnormal host cell division and induction of a RING finger protein, RKP, which is a homolog of human cell cycle regulator KPC1 (Lai et al., 2009); it is postulated that the induction of RKP by C4 regulates the host cell cycle. Exogenous application of brassinosteroid and abscisic acid weakly rescues the C4-induced phenotype in *Arabidopsis* suggesting that the effects of C4 expression are due to disruption of multiple hormonal pathways.

As well as being essential for geminivirus replication, the reprogramming of infected cells also induces host DNA replication (Nagar et al., 2002). It is quite possible that some of this reprogramming of the host cell DNA replication is to the advantage of the virus.

6. Interactions between Rep and Host Proteins

Many of the interactions between Rep and host proteins relate to the major functions of the Rep protein, initiation of replication and control of cell cycle. Among the Rep interacting host proteins that are likely to be involved in viral replication, apart from the DNA-dependent DNA polymerase, are proliferating cell nuclear antigen (PCNA) (Nagar et al., 1995; Castillo et al., 2003; Bagewadi et al., 2004) and the replication factor C complex, the clamp loader that transfers PCNA to the replication fork (Luque et al., 2002); these interactions are likely to represent early steps in the assembly of a DNA replication complex on the geminivirus origin. The C-terminus of MYMIV Rep interacts with the 32 kDa subunit of the host replication protein A (Singh et al., 2006); MYMIV Rep also interacts with host RAD54, a recombination/repair protein, enhancing its nicking ability (Kaliappan et al., 2012); the interaction with this protein might reflect an RDR strategy.

The involvement of the interaction of Rep with RB factor from both plants and animals on control of the cell cycle is described above. RepA binds to GRAB proteins (Geminivirus RepA-binding proteins) (Xie et al., 1999). Using the WDV RepA protein as bait in the YTH system, a family of GRAB proteins was isolated from wheat suspension cultured cells. The 37 amino acids at the C terminus of RepA, a region conserved among other mastreviruses, are involved in the interaction with an N-terminal domain of the GRAB proteins. The expression of GRAB1 or GRAB2 proteins in wheat cells inhibits WDV replication. GRAB proteins have significant amino acid homology with the NAC domain of proteins involved in plant development and senescence.

A variety of other proteins that Rep interacts with are revealed by YTH studies. Kong and Hanley-Bowdoin (2002) showed that CbLCV Rep (AL1) interacted with host Ser/Thr kinase, a kinesin and hostone H3 and Castillo et al. (2004) showed that TGMV and TYLCSV interacted with the *N. benthamiana* sumoylation enzyme NbSCE1; sumoylation is a posttranslational process that modifies function, activity, or localization of the target protein by the covalent attachment of a Ub-like polypeptide (Ubl) called SUMO. The interacting region lies between Rep aas 85–114 and mutagenesis of this region does not interfere with other Rep functions such as oligomerization, DNA binding or cleavage, or interaction with AC3 or Rb protein (Sánchez-Durán et al., 2011). It is suggested that Rep alters the sumoylation of selected host factors to create an environment suitable for virus infection.

A reverse genetics approach identified 11 *N. benthamiana* genes required for full infection with TYLCSV (Lozano-Durán et al., 2011); almost half of these genes play a role in posttranslational modification.

It is not yet understood if many of these Rep protein:host protein interactions revealed by YTH and reverse genetics

studies actually impact primarily on viral replication or cell cycle control.

E.   Nanovirus Replication

As described in Appendix A, Profiles 5 and 6, the genomes of nanoviruses are distributed over at least six small circular ssDNA species, each of which (with one exception) has a single ORF. Although they have not been studied in as much detail as geminiviruses, nanoviruses have several features in common with geminiviruses that suggest that their replication mechanisms are very similar.

Each of the nanovirus DNA species has a common region that is predicted to form a stem-loop, the loop containing the sequence 5′-TANTATTAC-3′ which is found in the origin of geminivirus (+)-strand synthesis (Burns et al., 1995). Herrera-Valencia et al. (2006) showed that the BBTV DNA-R common region contained iterons that are important in viral replication.

At least one of the DNA species (DNA-R) is inferred from the amino acid sequence to encode a Rep protein (Harding et al., 1993; Wu et al., 1994; Sano et al., 1998; Timchenko et al., 1999). *In vitro* tests with *E. coli*-expressed protein show that the BBTV Rep protein has site-specific cleaving and joining activity (Hafner et al., 1997a). In FBNYV, five of the DNA segments appear to encode a Rep protein (Timchenko et al., 1999). Site-specific DNA cleavage and nucleotide transfer activities have been shown *in vitro* for those from DNAs 1 and 2 (Reps 1 and 2) and the essential tyrosine residue that catalyzes these reactions has been identified by mutagenesis. Rep 1 and 2 proteins hydrolyze ATP, and this activity is essential for multiplication of the viral DNA. Each of the five Rep proteins initiated replication of the DNA species by which it was encoded but only Rep2 was capable of replication of all the six DNAs that did not encode a Rep protein. Thus, only one of the Reps is a master Rep (M-Rep), and this is capable of triggering replication of heterologous nanovirus DNAs (Timchenko et al., 2000).

The solution NMR structure of FBNYV M-Rep shows that the endonuclease domain comprises amino acids 2−95 and that its global fold is similar to those of geminivirus and circovirus Rep endonuclease domains (Vega-Rocha et al., 2007b). It consists of a central five-stranded antiparallel β-sheet, an α-helix and irregular loops on one side and by an α-helix containing the catalytic tyrosine residue on the other side. The binding site for the catalytic metal is on the exposed face of the central β-sheet.

Nanoviruses face the same problem as geminiviruses in that they need to start replicating their DNA in cells that are not transcriptionally active. The DNA of SCSV is able to self prime (−)-strand synthesis (Chu and Helms, 1988). Using self-primed extension with a DNA-dependent DNA polymerase, Hafner et al. (1997b) showed that all six DNAs of BBTV had endogenous primers bound to the genomic DNA. These primers were heterogeneous in size and appeared to be derived from DNA-5 and that DNA self-primed more efficiently than the other DNAs. It suggests that the function of the protein encoded by DNA5 is important early in the infection process.

The product of BBTV DNA-C (DNA5) contains the LxCxE motif characteristic of the RBR protein described above (Section VIII, D, 5) (Wanitchakorn et al., 2000). The YTH system shows that this protein has RBR-binding activity and that the activity is dependent upon the LxCxE motif. None of the five Rep proteins of FBNYV contains the LxCxE motif. However, the 20-kDa protein encoded by DNA-C (DNA-10) does contain this motif and also an F-box associated with binding to a ubiquitin ligase (a plant SKP1 homolog) (Aronson et al., 2000). The protein from DNA-C, named Clink (cell cycle link), binds to RB and stimulates viral replication; the product of BBTV DNA-C (DNA-5), described above, is a homolog of Clink. From studies on the expression of Clink in *Arabidopsis*, Lageix et al. (2007) showed that it interacts with retinoblastoma-related proteins and affects cell cycle regulation. However, Clink is not an absolute requirement for infection of *N. benthamiana*. From its association with a constituent of the Ub–protein turnover pathway it is suggested that, as well as blocking the action of the RBR protein, it targets that protein for processing.

IX.   MUTATION AND RECOMBINATION

The main two ways by which faults arise in replication is by mutation and recombination. In this section, I will discuss the mechanisms that lead to these faults and in Chapter 8, I will discuss the impact that these faults in replication have on the variation and evolution of plant viruses. Recombination is also involved in various other viral phenomena, which will be described here.

A.   Mutation (reviewed by Domingo and Holland, 1997; Drake et al., 1998)

Replication mutations can be base substitutions, base additions, or base deletions. In discussing mutations, one has to distinguish between *mutation frequency* and *mutation rate* (Domingo, 1999). Mutation frequency is the proportion of mutants (averaged for an entire sequence or specific for a defined site) in a genome population. Mutation rate is the frequency of occurrence of a mutation event during genome replication. Here we will discuss mutation rate but the frequency is important in the analysis of variation and evolution.

The rate of mutational errors depends on the mode of replication, the nucleotide sequence context, and environmental factors. As shown in Figure 7.30, nucleic acids that

10⁻³–10⁻⁴

$10^{-3}–10^{-4}$

DNA ← RNA

10^{-11} $10^{-3}–10^{-4}$

DNA → RNA

$10^{-3}–10^{-4}$
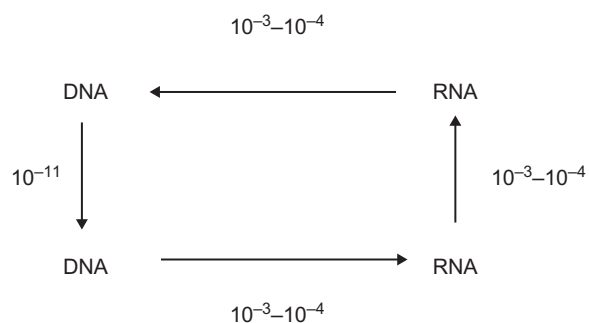
FIGURE 7.30   Error rates of transcription within and between RNA and DNA. *From Hull (2002) with permission of the publishers.*

TABLE 7.5 Upper-Limit Estimate for the Mutation Rate (μ_{max}) for Several Plant Viruses (Sanjuán et al., 2009)

Virus	Host	μ_{max} (±s.e.m.)×10⁻⁴
CMV	*Capsicum annuum*	15.34 ± 0.71
	C. annuum	1.39 ± 0.07
	N. benthamiana	6.64 ± 0.95
	N. tabacum	0.20 ± 0.09
CCMV	*N. benthamiana*	5.29 ± 4.93
TMV	*C. annuum*	11.02 ± 0.12
	Collinsia heterophylla	4.74
	Fagopyrus esculentum	4.55
	Lycopersicum esculentum	1.45 ± 0.51
	N. benthamiana	4.21 ± 0.69
	N. tabacum	4.14
	N. tabacum[a]	0.24 ± 0.00
	Phacelia campanularia	16.81
	Plantago sp.	8.50
	Solanum nigrum	4.21
	Tagetes erecta	8.15
WSMV	*Zea mays*	9.01 ± 0.90

[a]*This study reported a μ estimation instead of μ_{max} upper limit.*

replicate DNA→DNA have much lower mutation rates than those that replicate by other pathways. This is because DNA-dependent DNA polymerase has a proof-reading ability that checks that the correct nucleotide has been added whereas the other polymerases (DNA-dependent RNA polymerase, RdRp and RT) do not (Steinhauer et al., 1992). The crystal structures of RNA replicases and RT do not reveal the 5′–3′ exonucleolytic proofreading domain present in DNA-dependent DNA polymerases (Kohlstaedt et al., 1992; Joyce and Steitz, 1994; Hansen et al., 1997).

Most of the studies on error rate have been undertaken *in vitro* and these have shown that parameters, such as ionic composition of the medium and relative concentration of NTP substrates, can have significant effects (Domingo and Holland, 1997). Similarly, *in vitro* studies show that the sequence context being copied can have an effect with some regions being hypermutagenized.

There is little evidence on which to judge the significance of these effects on plant viruses *in vivo* where there are various selection pressures, such as bottle-necks associated with cell-to-cell movement, long-distance movement, and vector transmission (Sanjuán et al., 2009). Mutation rates are measured as μ=mutations/base/replication round (m/b/r) and, as well as technical difficulties (discussed by Tromas and Elena, 2010), one of the difficulties of obtaining realist values *in vivo* is determining the number of replication rounds. Table 7.5 lists the estimated μ_{max} for four viruses in various hosts; the median μ_{max} is 4.74×10^{-4} m/b/r (Sanjuán et al., 2009). Sanjuán et al. (2009) estimate a μ_{max} of 3×10^{-5} for TEV infection of *Nicotiana tabacum*. In comparing this value with previously published ones, they point out that the region of the genome that they studied is more constrained than those of the other viruses and that they were looking at short-term rather than long-term variation. Tromas and Elena (2010) obtained a similar value for the spontaneous mutation rate of TEV ($10^{-5}–10^{-6}$ m/b/r) with 2/3 of the mutations being transitions and non-synonymous (including some stop codons and small deletions) and 1/3 transversions.

B.   Recombination (reviewed by Bujarski, 1999; Hammond et al., 1999)

Recombination is the formation of chimeric nucleic acid molecules from segments previously separated on the same molecule or present in different parental molecules. It usually, but not always, takes place during replication and can be a repair mechanism for aberrations resulting from mutation. It is also a major source of variation as is discussed in Chapter 8.

In many of the experiments on recombination, the design is to restore an important function by recombination between two nucleic acids with lost or depleted function. In this approach, there is strong selection for the recombination event that may distort measurement of recombination frequency. A more realistic picture of the "natural" situation is given if one performs the experiments under reduced or non-selective conditions. Thus, although it is recognized that the rates of recombination, especially that of RNA, are high, there are few estimates as to the actual values under "natural" conditions.

There are differences and similarities in the mechanisms of recombination of RNA and DNA viruses.

1. RNA Virus Recombination (reviewed by Lai, 1992; Nagy and Simon, 1997; Sztuba-Solińska et al., 2011)

RNA recombination was considered to be a rare event, but studies over the last 10–15 years have shown that it is relatively common and associated with viral replication. However, only a few recombination events are selected and "fixed" into natural viral populations. Evidence for RNA recombination has been found in (+)-ssRNA viruses including many infecting plants and also in vertebrate and bacterial (−)-ssRNA and dsRNA viruses (Khatchikian et al., 1989; Onodera et al., 1993).

Recombinants have been detected intra- and inter-specifically between plant viruses and between viral and host RNA (Tanne and Sela, 2005).

a. Mechanisms of RNA Recombination

Initially RNA recombination was categorized in the same manner as DNA recombination into homologous recombination (HR) and non-HR (King, 1998). Because of the range of variation in HR, Lai (1992) divided this category into homologous and aberrant homologous. Homologous recombinants have no sequence alterations in comparison with the parental molecules whereas aberrant homologous recombinants contain mutations, deletions, or insertions at, or close to the insertion site. In a further analysis of RNA recombinants, Nagy and Simon (1997) proposed three classes of recombination (Figure 7.31A).

- Class 1, termed *similarity-essential recombination*, has substantial sequence similarity between parental RNAs. There can be two types of products, precise and imprecise recombinants, similar to the homologous and aberrant homologous of Lai (1992).
- Class 2 recombination, *similarity-nonessential recombination*, occurs when there are no similar regions between the parents. It is thought that features, such as transesterification, RdRp binding sites, and secondary structure, play a role in the recombination event (reviewed by Chetverin, 1999).
- Class 3 recombination, *similarity-assisted recombination*, combines features from both classes 1 and 2 recombination. In this class, there are sequence similarities between the parental RNAs, but additional RNA determinants on only one of the parental RNAs are required for efficient recombination. This form of recombination has been reported for PVX (Draghici and Varrelmann, 2010).

Three mechanisms have been proposed for RNA recombination (reviewed by Nagy and Simon, 1997).

i. The replicase-driven template switching model involves four elements, three RNAs [the primary RNA template (donor strand), the strand synthesized from the primary strand (nascent strand), and the acceptor strand], and the replicase complex (Figure 7.31B). Synthesis of the nascent strand on the donor strand is halted or slowed temporarily which enables either the RdRp or nascent strand to interact with the acceptor strand leading to template switching. Thus, there are two types of signal on the donor or nascent strand, one (pausing or arrest signal) that halts the RdRp but from which it can escape and the other (terminator signal) that releases the RdRp from the RNAs. It is thought that these signals may be similar to those involved in template switching by DNA-dependent DNA polymerases and RT and to be the sequence and/or secondary structure of the donor or nascent RNA. These regions that promote RdRp pausing or termination will constitute recombination hotspots. To enable template switching, the RdRp must be able to bind to the acceptor RNA and use the 3′ end of the nascent RNA as a primer to reinitiate RNA synthesis. Little is known about the actual mechanism of the switching of template by the RdRp though there are several models. In the processive model, it is suggested that when the RdRp approaches the heteroduplex region it pauses and either switches to the acceptor sequence or slides backwards 10–20 nucleotides enabling the nascent strand to hybridize to the acceptor strand. In the non-processive model, the RdRp is suggested to dissociate from the donor strand and then reassociates with the nascent and acceptor RNAs.

ii. The RNA breakage and ligation model is similar to the well-characterized DNA breakage and ligation system. It has not been formally demonstrated for RNA recombination.

iii. The breakage-induced template switching model is similar to the replicase-driven template switching model with the switch being induced by pausing of the replicase at a break in the template RNA. Because of the lability of RNA, it can be difficult to distinguish this model from the replicase-driven template switching model.

Recombination is thought to occur in most, if not all, RNA viruses. The evidence for recombination is chimeric molecules, defective and defective interfering molecules.

b. Factors Affecting RNA Recombination

A wide range of techniques have been used to identify factors that are involved in recombination (Sztuba-Solińska et al., 2011). In general, *in vitro* approaches determine factors that can lead to recombination and *in vivo* approaches reveal factors that pass selection criteria.

i. RNA Sequences RNA sequences that determine recombination are likely to be involved in early steps to induce pausing and dissociation of the replicase and in late stages to promote the rebinding of the replicase (White and
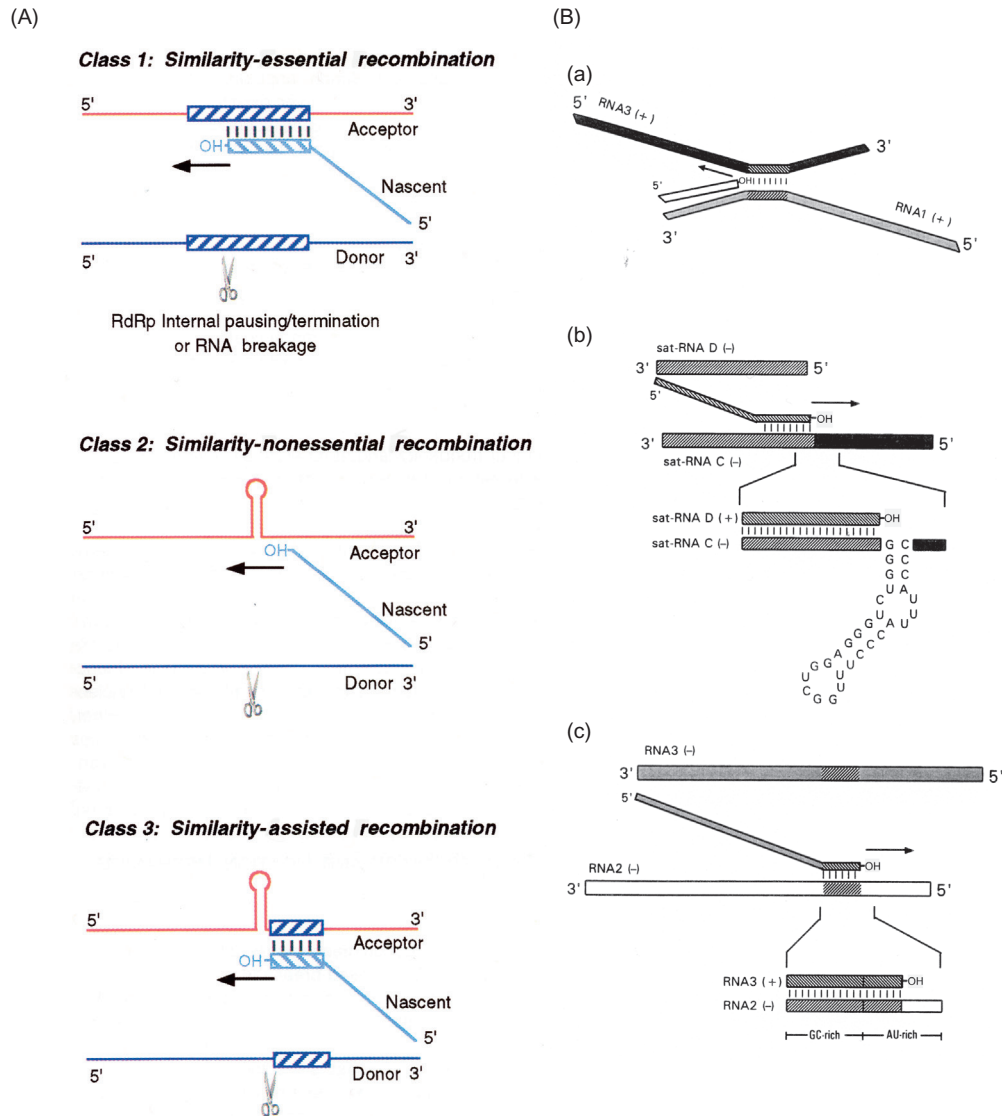
FIGURE 7.31 RNA recombination. Panel (A): Three classes of RNA recombination. Replicase-mediated RNA synthesis after the template-switch event is shown by an arrow. The hairpin structure shown on the acceptor RNA symbolically represents various RNA structures that are required for class-2 and -3 recombination. From Nagy and Simon (1997) with permission of the publishers. Panel (B): Replicase-mediated template-switching models of RNA recombination in TCV and BMV. Subpanel (a): Heteroduplex-mediated recombination between (+) strands of BMV RNA1 and RNA3. Subpanel (b): Recombination between satellite RNAs associated with TCV. The sequence of the required motif 1-hairpin is shown. Subpanel (c): Recombination events within the identical regions of BMV RNA2 and RNA3. Recombination is favored when GC-rich and AU-rich sequences are located as shown. *From Nagy and Simon (1997) with permission of the publishers.*

Nagy, 2004). Among the determinants for early stage dissociation are:

- truncated template formed say by limited RNA degradation by a cellular replicase;
- the sequence composition of the template. The major factor promoting HR appears to be complementarity between the acceptor and the nascent strand (Figlerowicz and Bujarski, 1998). On the other hand, the formation of local RNA–RNA heteroduplexes between recombining substrates appears to force the replicase switch during non-HR (Figlerowicz, 2000).

Weak A–U base pairing between the template and nascent strands has been suggested to promote replicase pausing/dissociation of BMV (Nagy and Bujarski, 1996, 1997).

- Strand of template. Homologous crossovers occur more readily during (+)-strand synthesis rather than during (−)-strand synthesis (Olsthoorn et al., 2002).
- Secondary and tertiary structures in the RNA. There is evidence that recombination sites in BMV and tombusviruses are associated with secondary structure in the template RNA that would induce pausing of the replicase

(Nagy and Bujarski, 1993; White and Morris, 1995; Kim and Kao, 2001). Replication enhancers, such as the RIII region of TBSV DI RNA (Figure 7.17) appear to be involved in recombination (Cheng and Nagy, 2003); the RIII region has a high affinity for viral replicase proteins which could help in the "landing" of a dissociated replicase/nascent RNA complex (White and Nagy, 2004).

ii. Viral Proteins

The properties of the viral replicase protein play a major role in recombination (reviewed by White and Nagy, 2004). For example, mutations at different parts of BMV replication proteins unevenly influenced both homologous and non-homologous crossovers (Nagy et al., 1995; Figlerowicz et al., 1997). Mutations within the HEL-like domain of BMV 1a protein affected the nature of recombinants, possibly due to altered frequency, location, and/or duration of pausing during RNA synthesis (Nagy et al., 1995); these mutations within the HEL region may affect the ability to unwind secondary structures or may influence viral proteins 1a–2a binding, which could affect the overall stability of the replicase, its interactions with the RNA, and its ability to switch between templates. A mutation within the polymerase domain of 2a inhibited heteroduplex-mediated non-HR while increasing homologous crossovers (Figlerowicz et al., 1997).

CNV p33 replication protein also seems to direct recombination possibly through its ability to hold and release the viral RNA (Panaviené and Nagy, 2003). The hydrophilic linker between the N-terminal MT and the C-terminal HEL-polymerase (HEL-POL) domains of the PVX replicase possesses a distinct recombination function vital for maintenance of genome integrity (Draghici and Varrelmann, 2009).

iii. Host Factors

The host protein Rpb11p, a part of the polII complex, affects TBSV replication and recombination via regulating viral p33 and p92 polymerase protein levels (Jaag et al., 2007).

As noted above (Section II, B, 1), gene-knockout yeast strains serve as a useful host for identification of cellular proteins essential for RNA recombination (Serviene et al., 2005). The deletion of a set of yeast genes involved in RNA metabolism accelerates RNA recombination, whereas the deletion of host genes involved in cellular transport reduces the level of viral RNA recombination (Pathak and Nagy, 2009). The lipid content/structure of the membranous compartment, which hosts the viral RdRp (Section III, D), could be altered in the absence of these genes, resulting in reduced crossover efficiency.

c. Recombinational Hotspots

If the RNA sequence and/or structure is involved in recombination, one would expect hotspots of recombination. Many hotspots have been reported for (+)-strand RNA viruses and hotspots have also been detected in a (−)-strand RNA virus (Kumar et al., 2010). Clusters of crossovers at the potyviral CP ORF suggest that such ORFs encoding structural proteins are more prone to recombination or selection. However, cases of recombined non-structural proteins were also found (Moreno et al., 2004; Tan et al., 2004; Ramírez-Rodríguez et al., 2009; Seo et al., 2009; Tugume et al., 2010).

d. Frequency of RNA Recombination

Measurements of frequency of recombination depend on how much selection there is of any recombinant. Obviously, there is less selection involved in *in vitro* approaches which gives an estimate of the "intrinsic" frequency of recombination; however, it may not cover all the factors controlling recombination and thus not give a maximum frequency. *In vitro* studies have mainly focused on bromoviruses and tombusviruses and it is suggested that these have a higher recombination frequency than many other virus groups. In the *in vivo* situation, there can be strong selection pressures and many approaches use strong positive selection to obtain recombinants. One way of overcoming this is to use viral RNAs containing silent markers. Using this approach with BMV, Bruyere et al. (2000) and Urbanowicz et al. (2005) showed an overall frequency per 100 nt of about 10% with higher frequency in RNA3 than in RNAs 1 and 2.

A range of factors, especially ecological and the mechanistic factors discussed above, influence the frequency of recombination in natural situations. For interspecies recombination, there will be significant homology between two viral variants. As described in Chapter 14, Section IV, A, these variants are likely to cross-protect against each other and so unless they are co-inoculated, it will be unlikely that they will replicate in the same cell compartment. However, viral populations exist as a "cloud" of quasispecies (Chapter 2, Section III, A) which will be replicating together, and thus there is a strong likelihood of recombination. To support intraspecies, intragenus, and/or intergenus RNA recombination, multiple virus strains must coinfect the same host cell and replicate in the same membrane compartment. Coinfections may be rare, especially when the virus strains are ecologically or geographically isolated. If there is successful coinfection, mechanistic restraints, such as the level of sequence dissimilarity, or the specificity of viral RdRp may prevent the formation of viable hybrids. Even if recombination occurs, the recombinants are likely to be deleterious and thus purified from the population (Pinel-Galzi et al., 2009).

Selection of recombinants may depend upon the host and on the symptoms induced in that host. For example, *Chenopodium hybridum* supports the formation of BMV and CMV recombinants within the limited area of a local lesion, which isolates the recombinant from other recombinants in other local lesions, reducing the selective

pressure on the individual recombinant (Escriu et al., 2007).

A phylogenetic survey detected evidence for recombination in 12 out of 36 (+)-sense plant RNA viruses (Chare and Holmes, 2006). The alignments were made between different sized fragments from different regions of the viral genomes and recombination frequencies (normalized to 100 nt) ranged from 3.8×10^{-2} for BCMV to 2.3×10^{-6} for CTV; however, another region within the CTV genome showed a frequency of 8.6×10^{-3}. The authors concluded that many of the exchanges were of modules (Chapter 8, Section II, B) reflecting modular evolution. Such evolutionary footprints of successful recombination are found in many other virus groups, especially the *Luteoviridae*.

2. DNA Virus Recombination

There are two basic forms of recombination in DNA viruses; HR occurs between two DNA sequences that are the same or very similar at the crossover point and non-homologous or illegitimate recombination that occurs at sites where there is either microhomology or no obvious homology; the latter usually occurs during double-strand break repair (Sargent et al., 1997). In animal and bacterial viruses, non-HR is a rare event and is usually mediated by a virus- or host-encoded protein. HR can require specific host or viral proteins but can also be due to template switching during replication.

Five families of plant viruses have DNA genomes, but basically there are two genome replicating types, the geminivirus type that replicates DNA>DNA and the caulimovirus type that replicates by reverse transcription DNA>RNA>DNA.

a. Recombination Among Geminiviruses (reviewed by Martin et al., 2011a)

Various types of recombination occur within geminiviruses including HR during which sequences within one genome are replaced with homologous sequences from another genome, non-HR during which genome regions are rearranged, duplicated, deleted, or are inserted into the genomes of host cells, and reassortment (or pseudo recombination) during which whole genome components of multicomponent ssDNA virus genomes get exchanged between strains or species.

Recombination is common among geminiviruses, especially begomoviruses, and is a major driving force in the evolution of this virus family (Chapter 8, Section VIII, H, 8). Some of the early evidence came from insertion or deletion mutagenesis of the two large ORFs of ACMV DNA-B (DNA2), which destroyed infectivity, but infectivity was restored by co-inoculation of constructs that contained single mutations in different ORFs (Etessami et al., 1988). Frequent intermolecular recombination produced dominant

parental-type virus. Infection of *N. benthamiana* with uncut cloned tandem dimers of TGMV DNA components gives rise to genome-length ssDNA species of both components (Hayes et al., 1988b). As noted above (Section VIII, C), there is evidence for RDR. Lethal mutations within the conserved stem-loop of ACMV are rapidly corrected by recombination (Roberts and Stanley, 1994).

Recombination has been found both within and between species of geminiviruses. The replicational release from tandem constructs agroinoculated into plants (Stenger et al., 1991) is presumably due to HR. It is difficult to distinguish whether interspecies recombination is homologous or non-homologous. The apparent recombination site in the complex of begomoviruses found in cotton is often close to the origin of (+)-strand synthesis (Sanz et al., 2000). As the sequence of the origin of (+)-strand synthesis is conserved between virus species, the recombination crossover could be due to homologous sequences. On the other hand, there is strong evidence for non-HR from reversion of deletion mutants to wild-type genome size, deletion of foreign sequences from geminivirus vectors, synthesis of wild-type molecules from two mutants, synthesis of sg defective molecules, and release of infectious virus DNA from recombinant plasmids containing monomer genome inserts (reviewed by Bisaro, 1994).

There are both intercomponent and intracomponent recombination in the babuvirus, BBTV (Stainton et al., 2012).

i. Mechanisms of DNA Recombination
The processes by which recombination occurs within geminiviruses are still quite poorly characterized but probably involve a number of different mechanisms. Recombination breakpoints in geminiviral genomes are generally not randomly distributed (Martin et al., 2011b) and cluster either within discrete recombination hot spots less frequently in cold spots. These uneven breakpoint distributions are in many cases caused by underlying differences in the rates of recombination in different parts of ssDNA virus genomes. Mechanistic factors that might influence site-to-site variations in basal recombination rates across these genomes include:

- Replication origins of geminiviruses, but not nanoviruses, are recombination hot spots (Lefeuvre et al., 2009). These sites comprise a 10- to 30-nt-long inverted repeat sequence capable of forming a hairpin structure that contains within its loop a highly conserved nonanucleotide sequence that defines the actual replication origin. Recombination experiments in geminiviruses (Stenger et al., 1994) have shown that this genome site is a mechanistically predisposed recombination hot spot because of the replicational release of viral genomes from genomic concatemers. When these concatemers either arise following a copy-choice mediated polymerase strand switch or break and are rescued by host

double-stranded break repair pathways (i.e., by RDR), recombinants that are replicationally released will have one breakpoint at the site of the strand switch/breakage and another at the origin.

- Sequence similarity. The efficiency with which HR can be used to replicationally repair broken ss and dsDNA molecules is strongly dependent on the degrees of similarity between broken sites and those of the unbroken molecules used as templates during recombinational repair. Recombination in geminiviruses tends to occur more frequently at genome sites where the two parental genomes share between 5 and 14 identical nucleotides than at sites where they share longer runs of identical sequence; recombination can also occur at quite a low frequency between two nucleotides that are non-identical in both parents (Garcia-Andres et al., 2007).

- ssDNA secondary structure. As noted above the geminivirus replication origin is a recombination hotspot and forms a stable hair-loop structure. Computational predictions indicate that additional uncharacterized ssDNA structures probably exist within many ssDNA virus genomes (Garcia-Andres et al., 2007) and the possibility exists that these too may facilitate recombination. Although the overall genomic secondary structures of recombinant genomes can vary quite substantially from those of their parents, there is evidence of strong selection pressures in geminiviruses for recombinants to maintain parent-like secondary structures (Martin et al., 2011b).

- Transcription–replication clashes. Geminivirus genes are expressed from both the virion and complementary sense strands. The complementary sense genes are transcribed in the opposite direction to RCR leading to the possibility for replication complex–transcription complex clashes. Geminiviruses tend to have more detectable recombination events and measurably higher estimated population-scaled recombination rates in their complementary sense genes than they do in their virion sense genes (Martin et al., 2011a). The imbalance between recombination rates in the virion and complementary sense genes is particularly apparent when considering only recombination occurring between very closely related sequences suggesting that strongly homology dependent copy-choice recombination may be a particularly important mechanism of replication re-initiation following interruption due to transcription–replication complex clashes.

- Differential degrees of ssDNA exposure within minichromosomes. Transcriptionally active geminiviral covalently ccDNA forms associate with host histones forming minichromosomes (Pilartz and Jeske, 2003). Recombination breakpoint hot spots in begomovirus genomes co-localize very closely with genome sites that are exposed within minichromosomes to host transcription and replication factors. These sites are also apparently the most common

sites of dsDNA breakage during begomovirus infections (Jeske et al., 2001) and it is therefore likely that at least part of the reason that these regions are recombination hot spots is that they are hypersensitive to either physical breakage or host nuclease attack.

b. Recombination in CaMV DNA

Recombination is also common in CaMV and probably in all the *Caulimoviridae*. As CaMV replicates by reverse transcription both DNA and RNA recombination have been found.

The fact that CaMV DNA is converted to a covalently closed ds circle to allow transcription shows that there must be an early involvement of host plant DNA repair enzymes following infection. This idea is reinforced by the fact that cloned DNA, excised from the plasmid in linear form, is infectious and that the progeny DNA is circular. Coinfection of plants with non-overlapping defective deletion mutants usually leads to the production of viable virus particles (Howell et al., 1981). Analysis of the progeny DNA demonstrated that the rescue is by recombination rather than complementation. Lebeurier et al. (1982) showed that pairs of non-infectious recombinant full-length CaMV genomes integrated with a plasmid at different sites regain infectivity on inoculation to an appropriate host. In the progeny virus, all the plasmid DNA was eliminated and the viral DNA had a normal structure. Walden and Howell (1982) provided further evidence for intergenomic recombination.

Based on experiments with pairs of heterologous genomes, Geldreich et al. (1986) proposed a model for recombination in CaMV mediated by the 35S RNA. In this model, just after inoculation two different DNAs with identical cohesive ends can be ligated together to give a dimer DNA. This dimer is then transcribed to generate a hybrid 35S RNA that is responsible for the formation of the recombinant genome by reverse transcription.

To study the mechanisms of recombination, Vaden and Melcher (1990) inoculated turnip plants with pairs of mutated CaMV DNAs and analyzed the progeny by restriction fragment patterns and sequencing. They found evidence for both DNA:DNA and replicational recombination. Several of the chimeras had junctions between the parental sequences at, or near, the site for initiation of (−)-strand DNA synthesis or near the initiation sites for 35S or 19S RNA transcription. These were taken as being indicative of strand switching during reverse transcription. Other junctions were found that did not bear any obvious relationship with (−)-strand DNA synthesis suggesting that they arose from DNA:DNA recombination. The deletion of inserts from the large intergenic region of CaMV DNA also suggested illegitimate recombination (Pennington and Melcher, 1993). Similar forms of recombination have

TABLE 7.6 Sequences of Plant Viruses Detected Integrated in Host Genome

Virus	Family	Genus	Host	Activatable[a]	Reference
TGMV-like	*Geminiviridae*	*Begomovirus*	Tobacco	No	Berjarano et al. (1996)
BSV	*Caulimoviridae*	*Badnavirus*	*Musa* spp.	Yes/No	Harper et al. (1999) and Ndowora et al. (1999)
PVCV	*Caulimoviridae*	*Petuvirus*	Petunia	Yes	Richert-Pöggeler and Shepherd (1997)
TVCV	*Caulimoviridae*	*Cavemovirus*	Tobacco	Yes/No	Lockhart et al. (2000)
TVCV-like	–	–	*Nicotiana* spp., Tomato	No	Jakowitsch et al. (1999) and Staginnus and Richert-Pöggeler (2006)
RTBV	*Caulimoviridae*	*Tungrovirus*	Rice	No	Kunii et al. (2004)
PBV	*Caulimoviridae*	*Badnavirus*	Pineapple	No	Gambley et al. (2008)
DMV	*Caulimoviridae*	*Caulimovirus*	Dahlia	No	Pahalawatta et al. (2008)
Pararetrovirus-like sequences	–	–	Potato	No	Hansen et al. (2005)

[a]*Episomal infection generated from integrated sequence.*

been found on the interaction of episomal CaMV with integrated viral sequences (Gal et al., 1992).

To estimate the recombination frequency of CaMV, Froissart et al. (2005) distributed four neutral markers along the viral genome, and co-inoculated host plants with marker-containing and wild-type viruses. On average, over 50% of viral genomes recovered after a single host infection 21 days postinoculation were recombinants and all regions of the genome were equally affected by this process. Assuming that 10 viral replication cycles occurred during the experiment, the recombination rate per base and replication cycle was of the order of 2–4×10^{-5} indicating that recombination is very frequent in the infection cycle of this virus. It is not known if this is DNA and/or RNA recombination.

c. Recombination in a Tungrovirus

There are two major strains of RTBV mainly differing in the isolates from the Indian subcontinent having a deletion in the non-coding region when compared with those from S.E. Asia (Fan et al., 1996); it is not known if this difference is due to the deletion or insertion of a sequence. Comparison of RTBV isolates from different regions in India shows a possible recombinant in the CP gene (Sharma et al., 2011).

3. Integrated Viral Sequences in Plant Genomes (reviewed by Harper et al., 2002; Staginnus and Richert-Pöggeler, 2006; Iskra-Caruana et al., 2010; Liu et al., 2011; Teycheney and Geering, 2011)

An increasing number of viral sequences have been found to be naturally integrated into plant genomes (Table. 7.6).

Thus far, they have all been "DNA viruses" (*Caulimoviridae* and *Geminiviridae*) that have at least one phase of their replication cycle as DNA in the nucleus. The first reports were of multiple direct repeats of partial geminivirus sequences in *N. tabacum* but there was no associated virus infection (Berjarano et al. (1996)). An analysis of geminivirus-related DNA in *Nicotiana* spp. showed that there is a recurrent dynamic interplay between geminivirus and plant DNA during evolution (Murad et al., 2004). In a systematic search of eukaryotic genome databases, sequences related to geminivirus Rep protein ORFs were found in *Populus trichocarpa* (not a known host of geminiviruses) and in transposable elements in fungi and protists (Liu et al., 2011). As these viruses do not encode integrases, it is considered that they are inserted into the host genome by non-homologous end-joining (illegitimate recombination).

Viruses from three of the six genera (*Badnavirus*, *Caulimovirus*, and *Petuvirus*) of the family *Caulimoviridae* have been reported to have sequences integrated into plant genomes (see Iskra-Caruana et al., 2010; Teycheney and Geering, 2011 for references); based on the *pol* gene sequence, Geering et al. (2010) propose two further genera, Orendovirus and Solendovirus in the family *Caulimoviridae* for integrating viruses OsatV (Kunii et al., 2004) and TVCV, respectively. Teycheney and Geering (2011) list more than 25 endogenous caulimovirus-type sequences integrated into the genomes of about 20 plant species in 9 plant families. Many of the viral sequences have not yet been assigned to known virus species.

a. Integrated Viral Sequences Causing Diseases

The integrated forms of only the petuvirus ePVCV (Richert-Poggeler et al., 2003), the Solendovirus eTVCV (Lockhart

et al., 2000), and the badnaviruses eBSOLV, eBSImV, and eBSGFV (Harper et al., 1999; Ndowora et al., 1999; Safar et al., 2004; Gayral et al., 2008) have been shown to initiate episomal infection. There is no evidence for an integrase in the genomes of these viruses, and they replicate episomally without an integration phase in their replication cycle (Section VII).

Integrated sequences of ePVCV are found in *Petunia axillaris* and *P. hybrida* but not in *P. parodi*, *P. inflata*, or *P. violaceae* with four loci in *P. hybrida* chromosomes (Richert-Poggeler et al., 2003). At least one of these integrants comprises a tandem arrangement of the PVCV genome which would allow direct release of the virus by transcription. The production of episomal virus from the integrant is activated by nutritional stress and wounding.

eTVCV was identified from *N. edwardsonii* (Lockhart et al., 2000) which is an autotetraploid between *N. glutinosa* (which contains eTVCV) and *N. clevelandii* (which does not contain TVCV sequences). TVCV sequences are found in many loci in *N. edwardsonii*, the sequences being highly variable and much rearranged. The activation of the integrated sequence is thought to be induced by the light regime.

Activatable integrants of three virus species causing banana streak disease have been identified and several others are suspected (Box 7.5).

The release of activatable endogenous pararetroviruses giving episomal virus from the integrant is induced by a stress event, such as tissue culture, micropropagation (Dallot et al, 2001), and nutrition.


BOX 7.5 Integration of Banana Streak Viruses

Activatable integrants of three virus species causing banana streak disease have been identified and several others are suspected. In certain banana (*Musa*) cultivars there have been apparently spontaneous outbreaks of BSV, especially during tissue culture and breeding programs. The first example was in tetraploids from variety Obino l'Ewai (AAB genome) crossed with variety Calcutta 4 (AA genome). The evidence for episomal infections arising from integrated sequences is: (i) many of the tetraploid lines had up to 100% infection of BSOLV after crossing symptomless parent plants. Tissue culture plantlets of Obino l'Ewai from symptomless mother plants had lower rates of infection and those of Calcutta 4 no infection; (ii) PCR of total DNA from Obino l'Ewai using BSV primers and Southern blotting of that DNA probed with BSV sequences gave positive results even though no virus could be detected by immuno-electron microscopy or by immune-capture PCR; (iii) sequencing of genomic clones from Obino l'Ewai revealed

a complex insert of BSV (Figure 7.32B), the sequence of which was >99% homologous to that of the episomal virus; (iv) the cloned products prepared by the sequence-specific
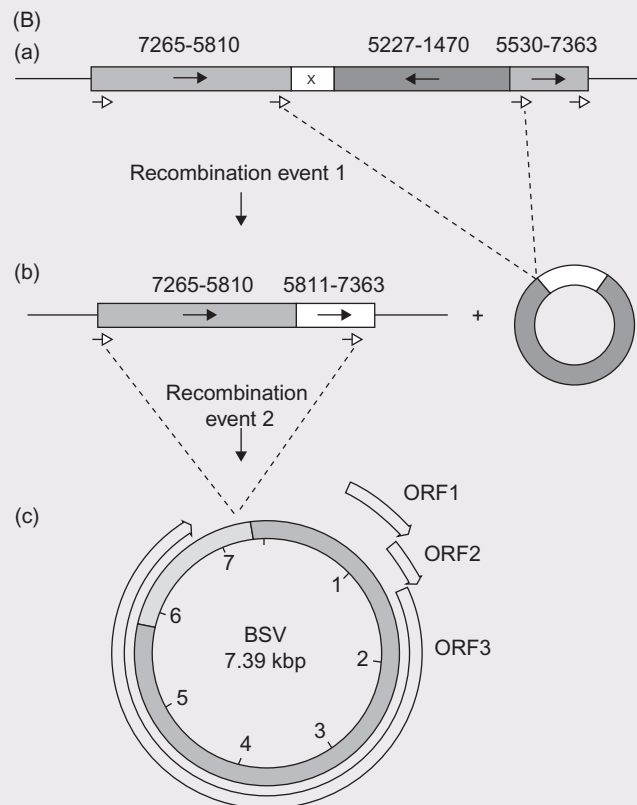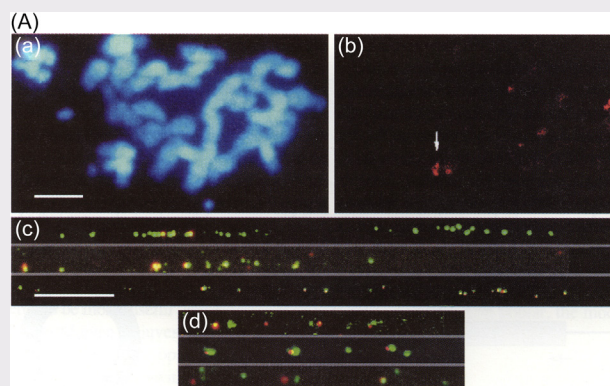
FIGURE 7.32 Activatable integrants of BSV. Panel (A): *In situ* hybridization of *Musa* Obino l'Ewai chromosomes. Subpanels (a and b): Hybridization of metaphase chromosomes. (a) Chromosomes stained blue with the DNA stain DAPI (4,6-diamidino-2-phenylindole). (b) Hybridization sites of BSV (red) showing one major site in each metaphase (arrowhead) and at least one minor site near the limits of hybridization sensitivity (arrow). Scale bar = 5 μm. Subpanels (c and d): Hybridization of extended chromosomal DNA fibers. Two different length hybridization patterns, with chains of dots probe hybridization sites, were detected with BSV (green) and *Musa* flanking sequence (red probes). (c) Three independent aligned long fibers. Both BSV and *Musa* flanking sequence are present in multiple copies in a 150 kb structure.
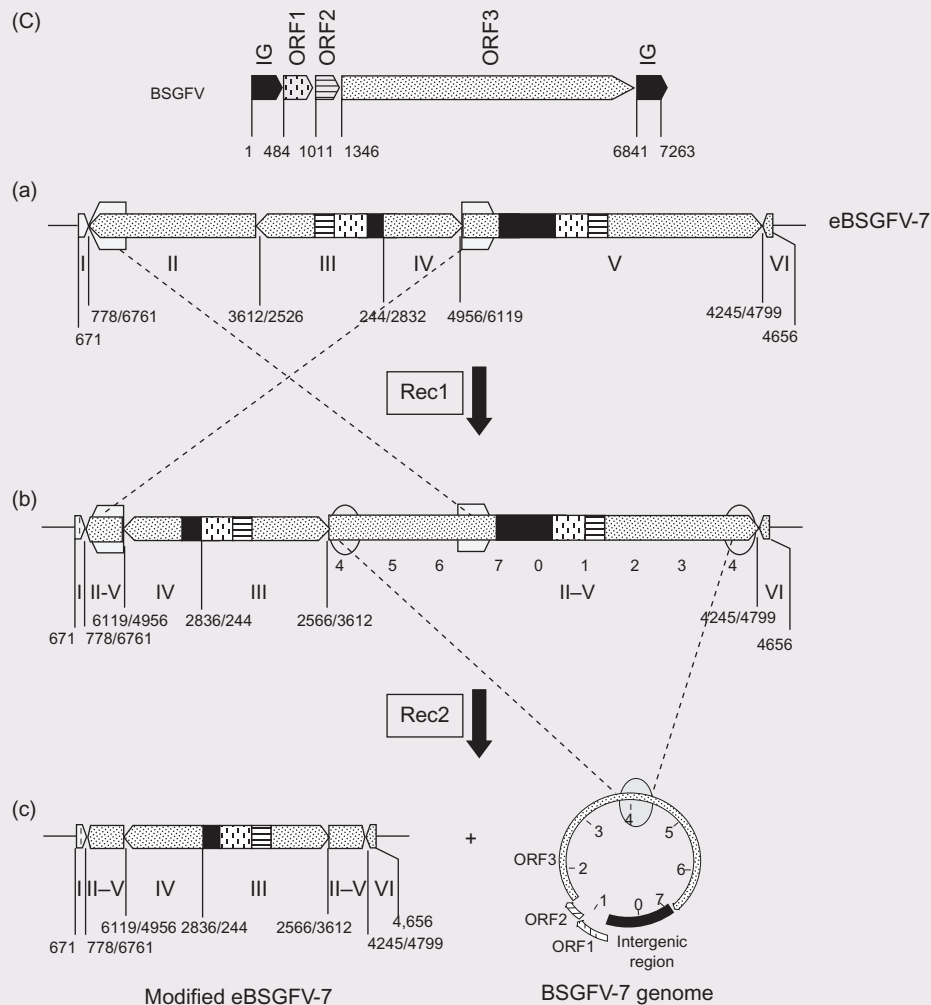
FIGURE 7.32 (Continued) (d) Three independent aligned short fibers in a 50kb structure. Scale bar = 5 µm. From Harper et al. (1999) with permission of the publishers. Panel (B): Model for recombination from integrated BSV sequence to give episomal virus. Subpanel (a): Structure of an integrated BSV sequence in *Musa* Obino l'Ewai nuclear genomic DNA. Filled arrows and numbers represent the BSV sequence and their directions relative to the episomal virus. X is a rearranged assortment of short BSV sequences; open arrows represent direct repeats. Subpanel (b): Hypothetical intermediate after a first recombination event between 280 bp direct repeats at BSV 5530–5810. Subpanel (c): Episomal BSV sequence produced after a second recombination between 98 bp direct repeats at BSV 7265–7363. Predicted ORFs are shown. From Ndowora et al. (1999) with kind permission of the publishers. Panel (C): HR-based model of the release of BSGFV genome from endogenous counterpart, eBSGFV-7. Thin vertical lines delimit internal fragments of eBSGFV (I–VI) and numbers below vertical lines (Bp) and below fragments (numbered 1–7; kbp) refer to positions in the BSGFV genome. Thin horizontal lines represent flanking regions of eBSGFV in genomic *Musa* sequences. Repeats used as templates for each HR event are indicated by gray symbols. Subpanel (a): Schematic representation of native eBSGFV-7. Subpanel (b): Theoretical structure of recombined molecule of BSGFV-7 after the first recombination event (Rec1). Subpanel (c): Theoretical structures of recombined molecules after the second recombination event (Rec2). Left subpanel: Residual eBSGFV remaining in the Musa genome; right subpanel: circular full-length viral molecule excised from BSGFV-7. *From Iskra-Caruana et al. (2010) with permission of the publishers.*

amplification polymorphism (S-SAP) approach fell into three classes, one of which comprised *Musa* sequence interfacing BSV sequence; (v) fluorescent *in situ* hybridization revealed a major and a minor locus; (vii) fiber-stretch hybridization, in which chromosomes are denatured, spread, and then hybridized with fluorescent probes showed that the integrants were complex (Harper et al., 1999; Figure 7.32A). Two allelic copies of BSGFV have also been found in the *Musa balbisiana* (B) genome and none in the *M. accumunata* (A) genome which can be activated in triploid (AAB) and tetraploid (AAAB)

varieties (Gayral et al., 2008); eBSImV is present as a single allelic form (Gayral et al., 2010). Only one of the allelic copies of BSGFV is replication competent (Gayral et al., 2008); both alleles are very similar in structure but the replication-competent form has many less mutations.

eBSOLV and eBSGFV have complex integration patterns comprising long stretches of contiguous viral sequence interspersed by fragments of non-contiguous and inverted sequences and possibly host sequences (Gayral et al., 2008; Ndowora et al., 1999; Figure 7.32B and C).

b. Discussion

The large amounts of sequence data of plant genomes being produced are showing that the integration of DNA viral sequences is a relatively common event. In some cases, these integrants can be activated to give episomal infections. Activation appears in many cases to be associated with the production of hybrids giving allopolyploids and stresses. Teycheney and Geering (2011) discuss possible mechanisms of epigenetic control of activation induced by these factors.

The rapid fluxes of "foreign" DNA into and out of the plant nuclear genome raise the question as to why these sequences are maintained. It is suggested that the integrants provide an RNA silencing defense for the host (Hull et al., 2000; Mette et al., 2002) but as Teycheney and Geering (2011) point out (i) most of the ancestral viruses that gave rise to the endogenous viral sequences appear to now be extinct and (ii) the episomal virus likely contains a silencing suppressor that would overcome any silencing. Thus there are likely to be other factors that lead to the maintenance of the integrated sequences.

X.   MIXED VIRUS ASSEMBLY

Mixed virus assembly can be shown to take place *in vitro* between the RNA of one strain of a virus and the CP of another (Okada, 1986; Okada et al., 1970), between RNA and protein from unrelated viruses (Matthews, 1966), and between one kind of RNA and two different CPs (Wagner and Bancroft, 1968; Taliansky et al., 1977). Mixed infections are discussed by Hammond et al. (1999). Of more interest is the formation of mixed virus particles *in vivo*.

When two viruses multiply together in the same tissue, some progeny particles may be formed that consist of the genome of one virus housed in a particle made partially or completely from the structural components of the other virus. Among enveloped viruses infecting animals, mixed infections may lead to the production of nucleoprotein cores of one virus enclosed in an envelope of the other. Such mixed particles, called *pseudotypes*, have not been observed with enveloped plant viruses. They will probably be found among the plant *Rhabdoviridae*.

Other kinds of mixed particle may be formed. Where the genome of one non-enveloped virus is encased in a protein shell made entirely of subunits of another virus (or strain) the phenomenon has been called *genomic masking*, *heterologous encapsidation*, *heteroencapsidation*, or *transencapsidation*. When the protein coat consists of a mixture of proteins from the two viruses, it has been called *phenotypic mixing*. The potential for encapsidation can depend upon origins of assembly and the specific protein/RNA mass ratio (Cadena-Nava et al., 2012).

Dodds and Hamilton (1976) give an account of the methods used to study phenotypic mixing. Various studies on phenotypic mixing have been carried out with defective mutants of TMV whose protein will not form rods with the RNA when plants are grown at high temperature. When such strains are grown in mixed infections with type TMV (or some other strain able to form virus rods at the higher temperature) then a proportion of the progeny contains the mutant strain RNA in a rod made with the protein of the competent strain (Schaskolskaya et al., 1968; Sarkar, 1969; Atabekov et al., 1970b).

Such mixing may take place in leaves only under conditions where two viral RNAs are present and one functional CP is made (Atabekova et al., 1975). On the other hand, Otsuki and Takebe (1978) showed that when protoplasts are inoculated with TMV together with ToMV, some of the individual progeny rods are coated with a mixture of the two CPs.

Strains of BYDV show aphid vector specificity (Chapter 12, Section III, D, 1, a). When a strain of the virus normally transmitted in a particular vector was grown in oats in a double infection with a serologically unrelated strain not normally transmitted by the aphid, this latter strain was transmitted. Rochow (1970) showed that this transmission was because some of the RNA of the second strain had been assembled into protein shells of the normally transmitted strain. In an analysis of mixed infections of four isolates of cereal-infecting luteoviruses, Wen and Lister (1991) demonstrated heterologous encapsidation between various combinations of CYDV-RPV, BYDV-MAV, BYDV-PAV, and BYDV-RMV. In most combinations the heterologous encapsidation was in both directions, but in two of the cases, CYDV-RPV + BYDV-PAV and CYDV-RPV + BYDV-MAV, it was only in one direction with CYDV-RPV providing the capsid.

A novel immuno-hybridization procedure has been developed to demonstrate directly that, in mixed infections in the field, an aphid non-transmitted strain of BYDV became encapsulated in the protein of an aphid-transmitted strain (Creamer and Falk, 1990). Similarly, phenotypic mixing has been demonstrated in potyviruses. Bourdin and Lecoq (1991) showed that an isolate of ZYMV that was not aphid transmissible because of a defect in its CP was aphid transmitted from plants coinfected with a transmissible strain of PRSV. Immunosorbent electron microscopy revealed particles that were heteroencapsidated (or transencapsidated) by the CPs of both viruses (Figure 7.33).

Phenotypic mixing can occur between two unrelated helical viruses with different dimensions (TMV and BSMV in barley) as shown by Dodds and Hamilton (1974). It has even been found between a helical virus (BSMV) and an icosahedral one (BMV) (Peterson and Brakke, 1973).

The encapsidation of umbravirus genomes by luteovirus CP is described in Chapter 12, Section III, D, 1, a, iv and heteroencapsidation by transgenically expressed CP in Chapter 15, Section I, G, 1.
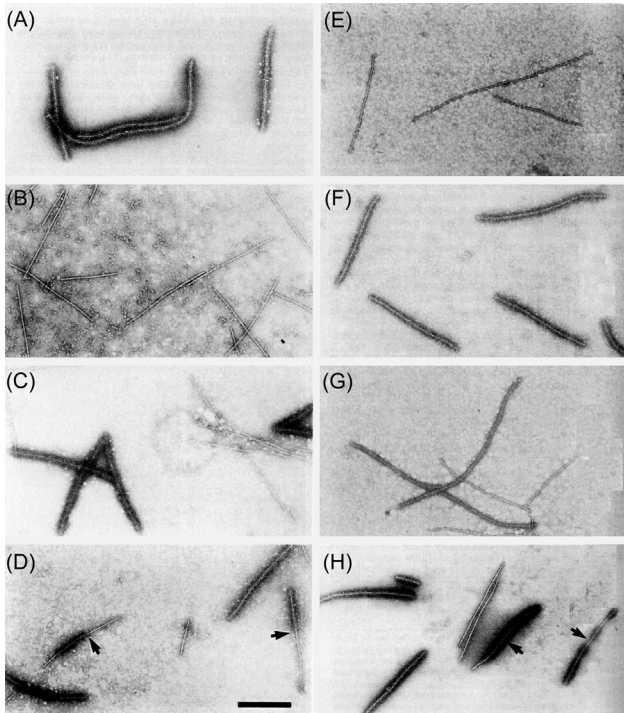
FIGURE 7.33 Electron micrographs of purified preparations of (A), (E) PRSV (-E2); (B), (F) ZYMV (-NAT) viruses; (C), (G) a 1:1 mixture of these virus preparations; or (D), (H) a purified preparation from plants coinfected with PRSV-E2 and ZYMV-NAT. Grids were coated with a mixture of PRSV and ZYMV antisera, and trapped particles were decorated with PRSV (A–D) and ZYMV (E–H) antisera. In artificial mixtures, particles were decorated or not decorated (C, G), whereas in preparations from doubly infected plants some particles appeared partially decorated (arrows) regardless of which antiserum was used for decoration (D, H). Bar = 400 nm. *From Bourdin and Lecoq (1991) with permission of the publishers.*

The existence of phenotypic mixing also suggests that two unrelated viruses or two related strains can replicate together in the same cell at least under some conditions.

The formation of distinctive inclusion bodies has been used to confirm that two unrelated viruses can replicate in the same cell, for example, TMV and TEV in tobacco (Fujisawa et al., 1967), TuMV and CaMV in *Brassica perviridis* (Kamei et al., 1969), and SMV and BPMV in soybean (Lee and Ross, 1972).

In tobacco leaves doubly infected with TMV plus PVX or PVY plus PVX, no assembly of one viral RNA in the CP of another could be detected (Goodman and Ross, 1974b). A likely reason for this is that, whereas closely related strains of a virus might replicate in the same region of the cell, different viruses may be assembled from components accumulated in separate sites or membranes in the same cell. Such separation may not always be complete.

Efficient and specific virus assembly would be favored by the localization of the RNA and protein subunits in a compartment within the cell. There are several reasons for this. First, if *in vivo* assembly is due to random meeting between protein subunits, then maintenance of a high local concentration of these would favor efficient assembly. Second, since subunits can pack around non-viral RNA of appropriate size, and since insignificant amounts of nonviral RNA are usually present in virus particles, free host RNA must be largely excluded from the assembly sites. Third, *in vitro* studies show that aggregation of subunits is markedly dependent on ionic environment and pH. These specific conditions differ *in vitro* for different viruses. Fourth, uncoated RNA must be protected from attack by nucleases.

Nevertheless, with some viruses significant amounts of host RNA may be incorporated into virus-like particles or pseudovirions. Such particles have been reported as making up to 2.5% of preparations of various strains of TMV (Siegel, 1971; Rochon et al., 1986). Most of the encapsulated host RNA is the 5′ region of 18S ribosomal RNA (Rochon et al., 1986). The site for initiation of this packaging has been located within a 43-nucleotide region beginning at position 157 from the 5′ terminus of the rRNA. This sequence has limited similarity to the TMV assembly initiation sequence (Figure 3.13), but it can fold to give a stem-loop structure (Gaddipati et al., 1988).

The mRNA for the large subunit of ribulose 1,5-bisphosphate carboxylase, which is coded by chloroplast DNA, is encapsulated in TMV CP in infected cells. This mRNA was found to contain at least three sites that are capable of reacting with CP aggregates *in vitro* to initiate rod formation (Atreya and Siegel, 1989). The most reactive site had significant sequence similarity to the initiation site in TMV.

XI. DISCUSSION

There are basically three types of viral replication mechanisms, making DNA directly from DNA, alternating between DNA and RNA, and making RNA from RNA. Each of these faces different problems as well as the overall problem of replicating to a level that would ensure propagation to a new host without causing irreparable damage to the current host.

The viruses that replicate DNA→DNA use the host machinery for this process; however, this machinery is usually only active during cell division. As described in detail in Section VIII, D, geminiviruses and nanoviruses have mechanisms that "switch on" the host DNA replication enzymes, thereby overcoming this limitation.

The replication of nucleic acid by the routes DNA→RNA→DNA and RNA→RNA is not found to any great extent in uninfected plant cells. Although plant genomes contain retrotransposons, most are inactive due to mutations, and the active ones appear to only replicate under certain stresses. Furthermore, their replication is thought to be controlled by host defense systems (Chapter 9). The *Caulimoviridae* use the DNA→RNA→DNA route to replicate their genomes with

the DNA→RNA phase being effected by a host enzymic system and the RNA→DNA phase by virus-coded enzyme activities. How these viruses overcome the host constraints directed at the unrestrained replication of retrotransposons is unknown. However, the fact that the episomal replication mechanism does not involve integration into the host genome may play a part in this, as will virus-encoded suppressors of RNA silencing. The replication of viruses, such as BSV and TVCV, is episomal, and the integration of the viral sequences into the host genome only supplies the inoculum and is not an integral part of the replication mechanism. However, in considering the constraints on the reverse transcription route of replication it must be recognized that several animal retroviruses involve an integration stage and there is little, if any, episomal replication. It may be that other host defense systems, such as immune surveillance, not found in plants, play a role.

The synthesis of RNA from RNA has not been considered to be a major mechanism in uninfected plants. As is described in Chapter 9, this route of nucleic acid replication is used in a host defense system. However, the majority of plant viruses replicate by this route using virus-coded enzyme systems together with some host-coded factors. Why this mechanism is so relatively common in plant viruses when compared with those infecting hosts in other kingdoms (Table 8.1) is unknown.

As noted above, there are controls on the unfettered replication of viruses that limit their detrimental effects on their hosts. There is selective pressure on viruses not to overly damage their natural hosts (Chapter 8). In evolutionary terms, the damage that viruses cause to crops is to the disadvantage of the virus. Thus, there are controls built into the replication of viruses that we are just beginning to understand. These include controls on gene expression so that virus-coded factors and enzymes are produced in certain amounts at certain times and also the sequestering of newly synthesized viral genomes by encapsidation in viral CP.

As well as synthesizing new genomes, viral replication also produces variants that form the basis of virus adaptation and evolution. This is discussed in Chapter 8.

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