30

Geminivirus DNA Replication

David M. Bisaro
Plant Biotechnology Center and
Department of Molecular Genetics
The Ohio State University
Columbus, Ohio 43210

Diseases caused by geminiviruses have long been recognized as a limitation to the cultivation of several important crops, including maize, cassava, bean, squash, cucurbits, and tomato, in tropical and subtropical regions of the world. More recently, geminivirus diseases, particularly those transmitted by whiteflies, have become an even greater threat to agriculture due to the appearance of a new and more aggressive whitefly biotype (Brown et al. 1995). This has renewed interest in the study of geminivirus pathogenesis and epidemiology and has stimulated work on the development of virus-resistant crop plants. For quite different reasons, geminiviruses have also attracted the attention of researchers concerned with fundamental aspects of plant molecular biology. In particular, their small DNA genomes and extensive reliance on host biosynthetic machinery make these viruses ideal model systems for the study of plant DNA replication and gene expression. Geminivirus genomes also have considerable potential as vectors for the expression of foreign genes in plants, although this potential has yet to be fully exploited. Because of their significance to plant pathology, plant molecular biology, and plant biotechnology, it is not surprising that geminiviruses have become the subjects of intensive research. As a result of efforts concentrated on a few model viruses, remarkable progress has been made toward understanding the mechanisms of viral replication and pathogenesis in the dozen years or so since the first molecular studies were initiated. Several recent reviews of this progress are available (Stanley 1991; Lazarowitz 1992; Mullineaux et al. 1992; Bisaro 1994). In this review, I focus on what is presently known about geminivirus DNA replication, with emphasis on the sequence requirements for replication and on the roles of viral proteins in this process.

DNA Replication in Eukaryotic Cells

THE GEMINIVIRIDAE

General Features

The geminivirus family derives its name from the unusual twin icosahedral (geminate) capsid structure of its members. Each paired particle encapsidates a single molecule of covalently closed, circular, single-stranded DNA (ssDNA) varying in size from 2.5 kb to 3.0 kb, depending on the virus. The genomic ssDNA is replicated in the nucleus of the host cell by a rolling-circle mechanism utilizing double-stranded DNA (dsDNA) intermediates in a manner similar to the ssDNA-containing bacteriophages. Viral dsDNAs, which include open and covalently closed (supercoiled) circular forms, are organized as minichromosomes apparently complexed with histone proteins (Pilartz and Jeske 1992). The nucleus is also the site of virus assembly, and nuclear inclusions consisting of large numbers of virus particles have been observed in infected cells by transmission electron microscopy (see, e.g., Esau 1977; Kim et al. 1978; Rushing et al. 1987).

Viral Genome Organization

The geminivirus family is diverse and can be divided into at least three subgroups based on genome organization, host range, and type of insect vector (Matthews 1991). Subgroup I comprises viruses that have a single component genome, are transmitted by leafhoppers, and usually infect monocotyledonous plants. Well-characterized subgroup I pathogens include maize streak virus (MSV) and wheat dwarf virus (WDV) (Fig. 1).

The second subgroup until recently contained only a single member, beet curly top virus (BCTV). BCTV also has a monopartite genome and is transmitted by a leafhopper vector, but it infects only dicotyledonous plants. The genome organization of BCTV also differs in significant ways from MSV and its relatives (Fig. 1).

Subgroup III contains viruses with monopartite genomes (~2.8 kb) as well as bipartite viruses that have two genome components, each of similar size (~2.6 kb). In the latter, the two genomic DNAs, designated A and B, differ in sequence except for a common region of 200–250 bp that is nearly identical in the genome components of any given virus, but differs between viruses. Both genome components are required for infectivity (Hamilton et al. 1983; Stanley 1983), although DNA A contains all viral information necessary for replication and encapsidation (Rogers et al. 1986; Townsend et al. 1986; Sunter et al. 1987). The B component provides genes whose functions are required for cell-to-cell and systemic spread of the virus from the inoculation site. In contrast, the movement

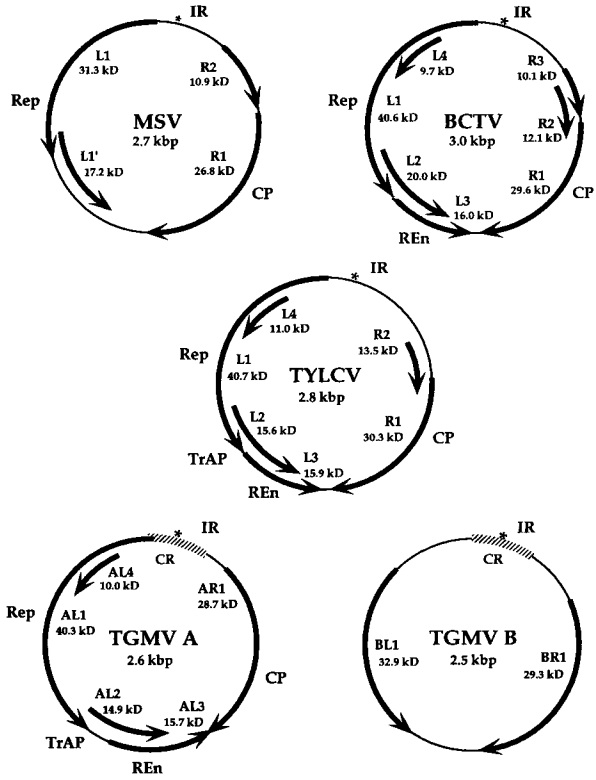

Figure 1 Geminivirus genome organization. The diagrams depict the double-stranded replicative forms of maize streak virus (MSV, subgroup I), beet curly top virus (BCTV, subgroup II), tomato yellow leaf curl virus (TYLCV, monopartite, subgroup III), and tomato golden mosaic virus (TGMV, A and B, bipartite, subgroup III). The solid arrows indicate the positions of viral genes with the approximate molecular mass of each encoded protein given in kilodaltons. Viral genes are designated by number and the direction of transcription: leftward (L, complementary sense) or rightward (R, viral sense). Certain viral genes are also indicated by name, including Rep (replication initiator protein), TrAP (transcriptional activator protein), REn (replication enhancer), and CP (coat or capsid protein). The position of the conserved hairpin is indicated by an asterisk within the intergenic region (IR). The common region (CR), a sequence of ~ 230 bp that is nearly identical in TGMV DNAs A and B, is indicated by a hatched box. (Sequence references cited in Lazarowitz 1992.)

protein genes of viruses with monopartite genomes belonging to subgroups I, II, and III are, of necessity, encoded in the single genome component. All subgroup III viruses are transmitted by a single species of whitefly (*Bemisia tabaci* Genn.) and individually have relatively narrow

host ranges within the dicots. The most intensively investigated bipartite viruses include tomato golden mosaic virus (TGMV), African cassava mosaic virus (ACMV, formerly cassava latent virus), and squash leaf curl virus (SqLCV) (Fig. 1). Tomato yellow leaf curl virus (TYLCV) is the best studied of the monopartite subgroup III viruses.

All geminivirus genome components possess an intergenic region (IR) from which viral genes diverge in both the viral and complementary sense (Fig. 1). The IR contains divergent RNA polymerase II-type promoters responsible for the expression of viral genes and also contains sequence elements necessary for the replication of viral DNA. A striking feature within the IR is a conserved inverted repeat that is capable of forming a hairpin. In the dicot-infecting viruses, this sequence element has the consensus GGCCAT/ACCGNT/AA/TTAATATTACCGGA/TT GGCC (Lazarowitz 1992). The invariant sequence TAATATTAC (underlined), located in the loop of the hairpin, has been found in all geminivirus genomes sequenced to date.

Two systems of gene nomenclature are currently in use. Both designate genes and gene products by number, but one denotes genes as viral or complementary sense (V or C), whereas the other indicates genes as oriented in the rightward (R; viral sense; clockwise) or leftward (L; complementary sense; counterclockwise) direction on the genome map (Fig. 1). Efforts to standardize the nomenclature are in progress, but in the interim, the use of gene and gene product names based on function will serve to reduce confusion. Throughout this review, the R and L system will be employed and functional names will be used where appropriate.

Rolling-circle Replication

A considerable body of evidence supports the idea that geminivirus replication occurs by a rolling-circle mechanism (Saunders et al. 1991; Stenger et al. 1991 and references therein). Rolling-circle replication (RCR) is also employed by the ssDNA-containing coliphages (e.g., ϕX174) (Kornberg and Baker 1992) and certain bacterial plasmids (Koepsel et al. 1985; Gros et al. 1987). The parvoviruses employ an analogous rolling-hairpin mechanism (Im and Muzyczka 1990). A characteristic feature of RCR is the involvement of a replication initiator protein (Rep) with a nicking-closing activity similar to that found in topoisomerases. RCR occurs in three stages. In the first stage (SS→RF synthesis), viral ssDNA (plus strand) enters the cell and is converted into a covalently closed dsDNA replicative form (RF) in a process involving host-directed, RNA-primed synthesis of a complementary (minus)

strand. The RF serves as template for viral transcription as well as a template for further replication. The purpose of the second stage of RCR (RF→RF synthesis) is to generate additional RF DNA. This step is initiated by viral Rep protein, gene A protein (gpA) in the case of ϕX174, whose function is to nick the plus strand at a specific sequence. Following phosphodiester bond cleavage, Rep protein covalently binds to the 5′ terminus via a phosphotyrosine linkage. The 3′-OH terminus is used as a primer for the synthesis of nascent plus strand, which displaces the parental plus strand from the intact minus-strand template. Synthesis again is carried out by host replication proteins. Completion of the nascent plus strand regenerates the origin of replication, which again is nicked by Rep, this time acting as a terminase to release the displaced unit-length plus strand, which is simultaneously ligated to circular form by the closing activity. In the process, Rep is transferred to the newly created 5′ terminus. Early in the replication cycle, the circularized ssDNA is used as template for synthesis of minus-strand DNA, resulting in the amplification of RF. The third stage of RCR (RF→SS synthesis), which occurs late in the replication cycle, is responsible for the accumulation of viral genomes for encapsidation. This stage is similar to RF→RF synthesis, except that priming is prevented and ssDNA is the predominant product.

REPLICATION-ASSOCIATED PROTEINS

Geminiviruses do not encode a gene product with polymerase activity, but instead rely on the machinery of the host to replicate their chromosomes. The host proteins required for geminivirus DNA synthesis have yet to be identified, but presumably DNA polymerase-α and -δ-like activities are involved, as well as replication accessory factors commonly found in other systems (SSB, helicase, etc.). Viral proteins that function in replication serve as specificity and initiation factors, replication enhancers, or apparently as regulators of ssDNA versus dsDNA synthesis and/or accumulation.

AL1 (Rep) Protein

All geminiviruses encode Rep (replication initiator protein), which carries out several distinct functions in viral replication. This highly conserved approximately 40-kD polypeptide is the product of the AL1 gene (also AC1, C1, or L1) in subgroup II and subgroup III viruses. Among the monocot-infecting geminiviruses of subgroup I, Rep is expressed

from a spliced mRNA that fuses the L1 and L1′ ORFs (also C1 and C2), which correspond to its amino- and carboxy-terminal halves, respectively (Fig. 1) (Schalk et al. 1989; Mullineaux et al. 1990). Rep is the only viral protein that is absolutely required for viral DNA replication (Elmer et al. 1988) and, in association with host proteins, Rep is sufficient to support the replication in *trans* of DNA molecules that contain a compatible origin of replication (Hayes and Buck 1989; Hanley-Bowdoin et al. 1990).

On the basis of homologies noted between Rep and the replication initiator proteins of eubacterial plasmids and bacteriophages, it was proposed that Rep initiates RCR by functioning as a site- and strand-specific endonuclease (Ilyina and Koonin 1992; Koonin and Ilyina 1992). Three conserved sequence motifs were identified in the amino-terminal half of Rep (Fig. 2). The functions of motifs I and II are not known, but motif II contains two invariant histidine residues that may bind Mg^{++} or Mn^{++}. Motif III contains a tyrosine residue predicted to participate in phosphodiester bond cleavage and to covalently bind the 5′ terminus exposed by nicking (van Mansfeld et al. 1986; Yasukawa et al. 1991; Noirot-Gros et al. 1994).

Recent studies have confirmed that the AL1 gene product is a replication initiator protein. Rep from TYLCV and WDV, expressed in *Escherichia coli* and partially purified, were shown in vitro to introduce a

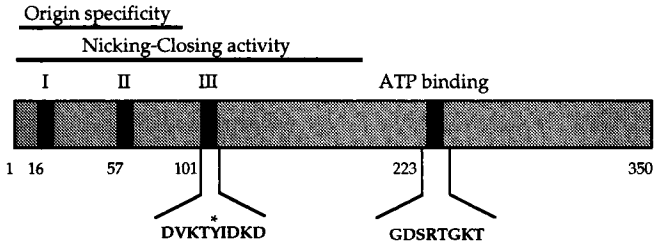

Figure 2 Functional domains and motifs of geminivirus Rep. A diagram of Rep, combining information obtained from TGMV, TYLCV, WDV, and BCTV, is shown. The approximate amino acid coordinates and amino acid sequences given are from TGMV Rep. The positions of motifs I, II, and III, which are conserved between Rep and the rep proteins of plasmids and phage that replicate by RCR, are indicated (Koonin and Ilyina 1992). The sequence of motif III, which contains the putative catalytic tyrosine (*asterisk*), and the sequence of the ATP-binding site (Desbiez et al. 1995) are shown. A region responsible for origin specificity, which likely contains the dsDNA-binding domain, resides near the amino-terminal end of the protein (Choi and Stenger 1995). A region that retains nicking-closing activity is also shown (Heyraud-Nitschke et al. 1995).

specific nick in the plus strand within the invariant sequence TAATATT↓AC (Laufs et al. 1995; Heyraud-Nitschke et al. 1995). This sequence was previously identified by genetic analysis as the replication initiation site (Stenger et al. 1991; Heyraud et al. 1993b). The reaction is dependent on Mg^{++} or Mn^{++} and requires that the substrate be single-stranded. After cleavage, Rep remains covalently attached to the adenine residue at the newly created 5′ terminus. A closing activity capable of ligating upstream and downstream cleavage products was also demonstrated (Laufs et al. 1995). Both the nicking and closing activities reside in the amino-terminal region of the protein (Heyraud-Nitschke et al. 1995).

Because Rep contains a consensus NTP-binding motif, it has been proposed that it might possess an ATPase and associated DNA helicase activity (Gorbalenya and Koonin 1989; Gorbalenya et al. 1990). A putative geminivirus helicase could unwind and displace viral-strand DNA from the minus-strand template in advance of the replication fork, or unwind and expose the origin of replication to proteins of the replication apparatus. Studies with TYLCV Rep revealed an intrinsic, DNA-independent ATPase activity (Desbiez et al. 1995). Mutation of the conserved NTP-binding motif, which resembles a phosphate-binding fold or P-loop, led to either loss or reduction of ATPase activity. The same mutations, when introduced into the TYLCV genome, either abolished or reduced replication in protoplasts (Desbiez et al. 1995). Although Rep ATPase activity is essential for replication, its precise role remains unclear. A helicase activity has yet to be demonstrated (J. Laufs and B. Gronenborn, pers. comm.), and the DNA-independent nature of the Rep ATPase suggests it is unlikely to support one. The ATPase activity also is not required for the nicking-closing reaction (Heyraud-Nitschke et al. 1995). Thus, for the present, Rep ATPase remains an activity in search of a function.

In studies using extracts from transgenic plants or insect cells expressing the TGMV AL1 gene, Rep was shown to be a dsDNA-binding protein that interacts specifically with a 13-bp element located in the IR, 34 bp upstream of the conserved hairpin (Fig. 3) (Fontes et al. 1992, 1994a). The element, referred to as a high-affinity Rep-binding site, contains two 5-bp direct repeats separated by a central core of 3 bp (5′-GGTAGTAAGGTAG). Competitive DNA-binding studies suggested that Rep has a somewhat greater affinity for the 3′ repeat. Mutation of the Rep-binding site confirmed that it is an essential *cis*-acting element required for viral DNA replication (Fontes et al. 1994a). However, the Rep-binding site is not required for specific nicking at the invariant se-

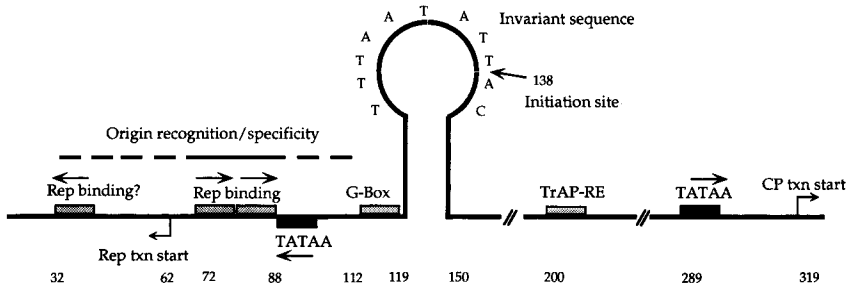

Figure 3 Organization of a geminivirus replication origin. A diagram of the TGMV replication origin is presented. Shown are the relative positions of Rep-binding sites, the invariant sequence (TAATATTAC), and the site where plus-strand synthesis initiates. Sequences involved in origin recognition/specificity are also depicted (Fontes et al. 1994b). The locations of sequence elements that interact with the transcription machinery, including TATAA sequences, Rep and CP transcription start sites (references cited in Lazarowitz 1992), a putative binding site for G-box family transcription factors, and a putative TrAP response element (the conserved late element; Argüello-Astorga et al. 1994) are also indicated. A sequence that appears to be an additional Rep-binding site in inverted orientation has been identified by sequence analysis (Argüello-Astorga et al. 1994), but its function has yet to be determined. Nucleotide coordinates are from TGMV DNA A.

quence (TAATATTAC), as single-stranded oligonucleotides containing the initiation site but lacking the GGTAG repeats are effective substrates for Rep endonuclease (Laufs et al. 1995; Heyraud-Nitschke et al. 1995). In this context, it is interesting to note that in vitro binding studies using purified, *E. coli*-expressed TGMV Rep with double- and single-stranded IR DNAs revealed that Rep has a significantly greater affinity (more than fourfold) for ssDNA than for dsDNA (Thömmes et al. 1993). The ssDNA-binding activity is apparently specific, but it is not known whether specificity is imparted by sequence elements in the IR, or by structure (e.g., the hairpin). The separation of nicking (which may depend on an ssDNA-binding activity) from binding-site recognition in the dsRF points to at least two distinct functions for Rep in replication initiation.

Attempts to construct pseudorecombinants composed of the A and B genome components from different bipartite viruses have only been successful when the heterologous genome components were derived from different strains of the same virus or from very closely related viruses. The inviability of other combinations is a consequence of the inability of Rep to sponsor replication of the heterologous genome component. The

roles of specific *cis*-acting elements (including the Rep-binding site) and *trans*-acting proteins (Rep) in specific origin recognition have been investigated. Rep from TGMV and bean golden mosaic virus (BGMV) interact only with their own binding sites in vitro. However, TGMV and BGMV DNA B mutants in which Rep-binding sites were exchanged by site-directed mutagenesis were not replicated by either TGMV or BGMV DNA A, suggesting that Rep binding at these sites is necessary but not sufficient for specific origin recognition (Fontes et al. 1994b). The additional required sequences were not identified. Studies using chimeric viral genomes composed of sequences from the Logan and CFH strains of BCTV, which exhibit distinct replication specificity, demonstrated that Rep amino acids 3–89 contain the domain responsible for specific origin recognition (Choi and Stenger 1995).

The Rep-binding site lies between the TATA box and the AL1 transcription start site (Fig. 3), suggesting that binding to initiate replication may have the additional consequence of interfering with expression of the Rep gene itself. This is, in fact, the case. In protoplast cotransfection experiments, the expression of a reporter gene replacing the TGMV AL1 ORF in DNA A was reduced or abolished by the addition of a construct constitutively expressing the AL1 ORF (Sunter et al. 1993). Therefore, Rep regulates its own expression, a property it shares with other viral proteins that also are involved in replication initiation (Reed et al. 1976; Hansen et al. 1981). Autoregulation of TGMV Rep expression occurs at the level of transcription, and Rep can repress a heterologous promoter when its binding site is inserted between the TATA box and transcription start site (Eagle et al. 1994). Interestingly, recent work suggests that the TGMV AL4 protein, which is encoded by a small gene that lies entirely within AL1 but in a different reading frame (Fig. 1), also contributes to suppression of AL1 expression (Gröning et al. 1994). However, the in vivo role of AL4 protein is unclear, as some AL4 mutants have no apparent effect on viral replication and pathogenesis (Elmer et al. 1988; Etessami et al. 1991).

Transcriptional repression and replication initiation appear to be distinct activities, because certain Rep mutants that are replication defective can still fully repress transcription. Transcriptional repression also does not require a functional origin of replication (Eagle et al. 1994). The mechanism by which Rep represses its own expression is not known but is likely to involve interference with the assembly of a transcription initiation complex (Buratowski and Sharp 1992). It should perhaps be mentioned here that Rep does not repress expression of the BL1 gene, despite identical AL1 (Rep) and BL1 promoters and binding sites in the common

regions of the A and B genome components (Sunter et al. 1993). Differential regulation may be due to the use of additional downstream BL1 transcription start sites mapped in TGMV DNA B (Sunter and Bisaro 1989). Thus, the bipartite viruses have devised a way to autoregulate an early gene required for replication without repressing a movement function required later in the replication cycle.

Rep from the subgroup I virus WDV was shown to stimulate transcription of the viral capsid protein gene (V1). The studies of Hofer et al. (1992) found that a functional Rep gene is required both for replication and for measurable coat protein promoter activity. Certain IR sequences, including the putative Rep-binding site and the hairpin region, were required for replication and for coat protein expression. Other IR sequences were necessary only for replication or for rightward transcription, suggesting that these are independent, Rep-mediated processes (Hofer et al. 1992). At this time, it is not known whether WDV Rep also regulates its own expression. However, that a single protein might initiate DNA replication as well as regulate early and late gene expression in subgroup I viruses suggests a simple and elegant means of coordinating the viral multiplication cycle. In contrast, the regulatory circuitry is much more complex in the dicot-infecting geminiviruses, where replication and transcription are also influenced by other viral proteins (see below). Relevant to this discussion, it has been shown in at least one dicot geminivirus (TGMV) that Rep has little or no effect on expression of the coat protein gene (Gröning et al. 1994).

To date, several distinct activities have been identified for Rep. Some of these, including the recognition of a specific binding site in the RF, are important for origin recognition, whereas others, such as ssDNA binding, a nicking-closing activity, and an ATPase activity, may be essential for replication initiation or termination. Additional activities, which may prove to be subgroup-specific, include autoregulation (repression) of early gene expression and stimulation of late (coat protein) gene expression. The picture that is beginning to emerge from biochemical and genetic studies is that different domains of Rep are responsible for its various biochemical activities (Fig. 2). How these activities might be temporally regulated during the replication cycle is unknown. If previous experience with other DNA viruses is any guide, a likely mechanism is posttranslational modification, and in particular, phosphorylation (Fanning and Knippers 1992). TYLCV and TGMV Rep are phosphoproteins (cited in Laufs et al. 1995; M.D. Hartitz and D.M. Bisaro, in prep.). Whether phosphorylation is a mechanism by which the functions of Rep are regulated remains to be seen.

AL3 (REn) Protein

The AL3 gene (also AC3, C3, or L3) is found in all subgroup II and III geminiviruses, but is absent from the genomes of viruses belonging to subgroup I. Comparatively little is known about the function of the approximately 15-kD AL3 gene product. Mutational analysis showed that an intact AL3 gene is not required for infectivity, but AL3 mutants elicit delayed and attenuated symptoms in inoculated plants (Elmer et al. 1988). AL3 mutants also accumulate reduced (as much as 50-fold) amounts of viral DNA in plants and protoplasts (Sunter et al. 1990; Etessami et al. 1991; Stanley et al. 1992). Because of this interesting phenotype, the AL3 gene product is often referred to as a replication enhancer (REn). Exactly how the AL3 protein enhances the replication (or the accumulation) of viral DNA is not known, but AL3 activity is not virus-specific (Sunter et al. 1994; Hormuzdi and Bisaro 1995).

A clue to the mechanism by which AL3 protein acts comes from the studies of Fontes et al. (1994a), who observed that certain DNA molecules harboring mutations in the 5′-GGTAG repeat of the Rep-binding site could be induced to replicate only in the presence of both Rep and AL3 protein. One interpretation of this finding is that AL3 functions via an interaction with Rep, perhaps by stabilizing Rep/DNA interactions (Fontes et al. 1994a).

Little is known about the AL3 protein itself, except that its sequence predicts a high degree of hydrophobic character. In infected plants, AL3 protein is found in both the soluble and organelle fractions in amounts comparable to Rep (Pedersen and Hanley-Bowdoin 1994). This suggests that AL3 functions catalytically rather than by acting to stabilize viral DNA, a mechanism that would likely require a large quantity of protein.

AL2 (TrAP) Protein

A key step in the control of viral DNA synthesis follows the production of circular plus-strand DNA by RCR. The nascent ssDNA may either reenter the replication pool with subsequent priming and minus-strand synthesis (RF→RF synthesis), or it may be removed from the replication pool by encapsidation or other means (RF→SS synthesis). In the subgroup III geminiviruses, this step appears to be regulated by transcriptional activator protein, or TrAP (also AL2, AC2, or C2).

All subgroup III geminiviruses encode an allele of the AL2 gene, which is not found in subgroup I virus genomes. An ORF resembling AL2 is found in BCTV (subgroup II), but recent work suggests it has a different function (Hormuzdi and Bisaro 1995). TGMV AL2 mutants are

unable to infect plants, yet they retain the ability to synthesize DNA in transient leaf disc and protoplast replication assays (Elmer et al. 1988). Analysis of transgenic plants expressing the leftward TGMV proteins showed that the AL2 ORF is required for the accumulation of ssDNA (Hayes and Buck 1989). Protoplast studies further showed that TGMV AL2 mutants accumulate reduced amounts of ssDNA and do not accumulate capsid protein (Sunter et al. 1990). These observations suggested that capsid protein is required to stabilize ssDNA, and that one function of TrAP is to activate expression of the capsid protein gene. Activation was subsequently demonstrated (Sunter and Bisaro 1991; Gröning et al. 1994). It was later shown that TrAP is also required to activate the BR1 gene, and that activation occurs at the level of transcription (Sunter and Bisaro 1992). The noninfectious nature of TGMV AL2 mutants is therefore due to insufficient accumulation of BR1 protein, which is necessary for systemic spread. The BR1 protein binds ssDNA and localizes to the nucleus of infected cells (Pascal et al. 1994). Because both capsid protein and BR1 protein are capable of interacting with ssDNA and removing it from the replication pool, placing the expression of these genes under the control of TrAP ensures they are not made prematurely. However, the possibility that TrAP also plays a more direct role in regulating a switch from RF→RF synthesis to RF→SS synthesis cannot be excluded at this time. Among the members of subgroup I, this switch may be under the control of Rep, which is required in these viruses for coat protein expression (see above). In the subgroup II geminiviruses, the available evidence suggests the involvement of yet another protein (R2, see below).

The A genome components of different bipartite geminiviruses can complement a TGMV AL2$^-$ mutant, proving that TrAP function is not virus specific (Sunter et al. 1994). Activation of the capsid protein and BR1 promoters appears to be mediated by a sequence element within the IR that is conserved among most subgroup III geminiviruses (Argüello-Astorga et al. 1994). However, attempts to demonstrate specific TrAP binding to DNA fragments containing this sequence (or any other) have so far been unsuccessful, although TrAP binds both ssDNA and dsDNA in a non-sequence-specific manner (M.D. Hartitz and D.M. Bisaro, in prep.).

R2 Protein

The R2 gene (also V2) of BCTV encodes a protein with a unique function, despite the fact that it shares a common designation with genes

found in some other types of geminivirus genomes. The results of mutational studies suggest that the small product of the BCTV R2 gene (~12 kD) plays a direct role in regulating a switch from RF→RF synthesis to RF→SS synthesis. In infected plants and leaf discs, R2 mutants accumulate reduced amounts of ssDNA and increased amounts of dsDNA relative to wild-type virus (Stanley et al. 1992; Hormuzdi and Bisaro 1993). In protoplasts, the reduction is large (~10-fold) and is accompanied by a similar increase in dsDNA levels (Hormuzdi and Bisaro 1993). Clearly, the absence of active R2 protein results in the overamplification of dsDNA at the expense of ssDNA. The molecular basis of this phenotype is unknown, but an interaction between R2 protein and ssDNA or the replication machinery may prevent minus-strand synthesis. Alternatively, R2 protein may facilitate virion assembly, in which case encapsidation is the principal mechanism for withdrawing ssDNA from the replication pool. However, the distinctly different phenotypes of R2 mutants and capsid protein mutants make this scenario less attractive. Although capsid mutants accumulate reduced amounts of ssDNA, they do not overproduce dsDNA. Additional work is needed to elucidate the function of BCTV R2 protein.

THE ORIGIN OF REPLICATION

The IR is a complex mosaic of controlling elements containing divergent promoters for viral and complementary sense transcription, as well as the plus-strand origin of replication. The replication origin appears to be modular and resembles in some respects those found in mammalian DNA viruses and other simple eukaryotic genomes (DePamphilis 1993), but there are also elements unique to RCR that lend a distinctly prokaryotic flavor to the geminivirus origin core.

Eukaryotic origins frequently contain promoter elements, and the transcription factors that bind to them often function as auxiliary replication factors that enhance replication or impart tissue specificity (DePamphilis 1988). To what extent certain of the geminivirus IR elements are involved in both replication and transcription remains to be determined. It is worth noting that most geminivirus genomes contain a GC-rich element immediately 5′ of the conserved hairpin that, in some cases, resembles a binding site for the G-box family of plant transcription factors (Argüello-Astorga et al. 1994). The GC-rich sequence in MSV stimulates rightward transcription and binds maize nuclear factors (Fenoll et al. 1988, 1990).

The Plus-strand Origin

Because most studies have used subgroup III virus origins, these are discussed in detail. The recent sequence analysis of Argüello-Astorga and colleagues (1994), which identified iterative elements within geminivirus IRs that appear to correspond to Rep-binding sites, indicates that the organization of these elements is similar in most dicot-infecting geminiviruses. Viruses belonging to subgroup I, however, are quite different with respect to the relative locations of their putative Rep-binding sites and the conserved hairpin. Whether this reflects fundamental differences in mechanisms of origin recognition and initiation of DNA synthesis is not known.

Using chimeric DNA molecules containing IR sequences of TGMV and SqLCV, Lazarowitz and coworkers delimited the replication origin to a sequence of about 90 nucleotides encompassing the conserved hairpin and about 50 nucleotides of 5′ upstream sequence, including the Rep-binding site (Fig. 3) (Lazarowitz et al. 1992). This sequence was sufficient to drive the Rep-dependent replication of nonviral sequences to which it was joined, and both ssDNA and dsDNA products were synthesized. Recent experiments with chimeric BCTV Logan and BCTV CFH genomes yielded similar results (Choi and Stenger 1995). The relatively small origin fragments defined by these studies must contain the origin core, the minimal sequence required for replication.

The origin recognition element (ORE) is a critical component of the origin core (DePamphilis 1993 and this volume). This element is responsible for binding specific origin recognition proteins, which are required for replication initiation. In the geminiviruses, Rep appears to function as an origin recognition protein, and the high-affinity Rep-binding site as an ORE. Additional as-yet-undefined sequences are probably also involved in origin recognition (Fontes et al. 1994b).

The consequences of Rep-Rep-binding-site interactions are not yet known, but one can envision several outcomes which are not mutually exclusive. First, Rep may participate in origin unwinding, either by acting as a helicase or by assisting a cellular helicase (Stahl et al. 1986; Dodson et al. 1987; Im and Muzyczka 1990). Second, Rep binding could lead to distortion of the double helix, which in turn might promote localized duplex melting or result in extrusion of the conserved hairpin (Noirot et al. 1990). A third possibility is that Rep recruits cellular replication proteins to the origin. This could occur indirectly as a consequence of duplex melting or unwinding, or, more directly, through specific interactions with cellular replication proteins (Dornreiter et al. 1990). Whatever the precise mechanism, the primary function of

sequence-specific Rep binding may be to prepare the origin for interaction with host replication proteins. It may also prepare the origin for interaction with an additional Rep molecule or complex that recognizes the invariant sequence, either as ssDNA or as part of the single-stranded hairpin loop, as a substrate for cleavage.

The conserved hairpin defines the 3′ boundary of the origin core. Nuclease S1 mapping provided evidence that the hairpin (cruciform in dsDNA) structure exists in duplex DNA (Sunter et al. 1985), although direct evidence that it is present in viral chromosomes in vivo has not yet been obtained. The importance of this sequence for replication, however, is well established (Revington et al. 1989; Lazarowitz et al. 1992). The Rep endonuclease cleavage site was initially mapped in vivo to a 20-bp region containing the conserved hairpin (Stenger et al. 1991) and later to the invariant TAATATTAC sequence in the loop of the hairpin (Heyraud et al. 1993a,b; Stanley 1995). Biochemical studies confirmed that Rep cleavage is between the last T and A of the invariant sequence, thereby defining the site of plus-strand initiation (Heyraud-Nitschke et al. 1995; Laufs et al. 1995).

The sequence requirements for nicking have been investigated only to a limited extent. TYLCV Rep is able to nick, with reduced efficiency, the origin sequence of WDV (and vice versa) even though the hairpin stems of TYLCV and WDV differ considerably in length and sequence (Heyraud-Nitschke et al. 1995; Laufs et al. 1995). TYLCV Rep can also nick, again with reduced efficiency, oligonucleotides containing substitutions in positions immediately flanking the cleavage site in the invariant sequence. A hairpin structure also is not required since oligonucleotides lacking either the 5′ or 3′ portions of the stem are efficiently cleaved (Heyraud-Nitschke et al. 1995; Laufs et al. 1995). Because it appears that neither the hairpin structure nor the exact invariant sequence is required to direct cleavage in vitro, precisely how the cleavage site is defined is not yet clear.

The situation in vivo is somewhat different. Although some point mutations in the stem that disrupt base-pairing appear to be tolerated (Roberts and Stanley 1994), the entire hairpin is necessary for replication (Lazarowitz et al. 1992). These observations suggest that the hairpin structure and not sequence per se is required for replication. There is some natural variation in length and sequence between the hairpin stems of different geminiviruses, but the extent to which these differences might contribute to replication efficiency or specificity has not been examined. In addition, the significance of each individual position in the invariant sequence has not yet been assessed. However, in ACMV, the

third T could be mutated to a C without compromising the ability of the virus to infect plants, although disease development was delayed and attenuated. In contrast, alteration of the second A residue was not tolerated (Roberts and Stanley 1994). Limited natural sequence variation exists in the few nucleotides of the loop that are not part of the invariant sequence. The identity of these nucleotides does not appear to contribute in a significant way to replication efficiency or specificity (Fontes et al. 1994b).

A WDV mutant which is replication competent, but unable to process unit-length genomes from replicative intermediates, was constructed by deletion of the conserved hairpin. This mutant accumulated only high-molecular-weight, concatemeric forms of the viral genome in transfected protoplasts (Kammann et al. 1991). Replication initiation in this case apparently occurred at an alternate site in the IR that is highly homologous to the right half of the conserved hairpin (Heyraud et al. 1993b). The behavior of this unusual deletion mutant indicates that the requirements for initiation differ from those of termination, with the latter being somewhat more stringent. This is supported by in vitro experiments which suggest that Rep-catalyzed joining, but not nicking, is stimulated when substrate molecules contain sequences capable of base-pairing to form a hairpin (Heyraud-Nitschke et al. 1995).

The Minus-strand Origin

That viral ssDNA (plus strand) purified from virions is infectious is taken as evidence that RNA priming and minus-strand synthesis can be accomplished entirely by host enzymes (Goodman 1977; Hamilton et al. 1981). Interestingly, the encapsidated genomic ssDNA of subgroup I viruses is associated with a small, complementary (minus strand) ssDNA about 80 nucleotides long that contains several ribonucleotides covalently linked to the 5′ terminus (Donson et al. 1984, 1987; Hayes et al. 1988). These complementary DNAs are capable of priming minus-strand DNA synthesis in vitro, and the ribonucleotides are believed to represent remnants of the authentic in vivo primer. In MSV, WDV, and Digitaria streak virus, the complementary DNAs are annealed to sequences located in a small intergenic region opposite the larger IR on the circular genome map (Fig. 1). Similar complementary DNAs are not associated with the genomic ssDNA of subgroup II and subgroup III geminiviruses. However, analysis of replicative intermediates in ACMV-infected cells identified a heterogeneous population of complementary DNAs that also appear to contain ribonucleotides. Hybridization analysis suggested that the

putative RNA primer initiates within the IR (Saunders et al. 1992). This is in agreement with the observation that the minimal origin described for subgroup III viruses by Lazarowitz et al. (1992) supports the synthesis of both single- and double-stranded DNA.

FUTURE DIRECTIONS

This is an exciting time for geminivirus research. Past work has taught us much about the mechanism of viral DNA replication and about the general roles of viral proteins in this process and has paved the way for new and important questions. What are the precise functions and activities of viral proteins involved in replication? How are these activities regulated in multifunctional viral proteins? How do viral proteins interact with host proteins during replication, and how do these interactions subvert the host replication machinery for the purpose of viral DNA replication? Geminivirus replication, as might be expected, appears to occur preferentially in cells that are actively synthesizing cellular DNA (Accotto et al. 1993). Recent evidence suggests that Rep can induce the accumulation of proliferating cell nuclear antigen (PCNA, a polymerase-δ processivity factor) in transgenic tobacco plants (Nagar et al. 1995). Do geminiviruses, like the mammalian DNA tumor viruses, possess the ability to prepare the host for viral DNA replication by stimulating normally quiescent cells to enter S phase? If so, what other viral and host proteins are involved? These and other questions are currently under investigation in a number of laboratories. What is learned during the next few years promises to teach us a great deal more about the mechanisms of viral DNA replication and pathogenesis and will provide new insights into host-pathogen interactions and the fundamental mechanisms of plant DNA replication and its control.

ACKNOWLEDGMENTS

I thank Bruno Gronenborn, Linda Hanley-Bowdoin, Jürgen Laufs, John Stanley, and Drake Stenger for preprints and information prior to publication. I also thank Judith Brown, Linda Hanley-Bowdoin, and Garry Sunter for critical reading of the manuscript and suggestions for its improvement. Financial support from the U.S. Department of Agriculture/National Research Initiative Competitive Grants Program (grant 93-37301-9076) is gratefully acknowledged.

REFERENCES

Accotto, G.-P., P.M. Mullineaux, S.C. Brown, and D. Marie. 1993. Digitaria streak geminivirus replicative forms are abundant in S-phase nuclei of infected cells. *Virology* **195:** 257–259.

Argüello-Astorga, G.R., R.G. Guevara-Gonzalez, L.R. Herrera-Estrella, and R.F. Rivera-Bustamante. 1994. Geminivirus replication origins have a group-specific organization of iterative elements: A model for replication. *Virology* **203:** 90–100.

Bisaro, D.M. 1994. Recombination in geminiviruses: Mechanisms for maintaining genome size and generating genomic diversity. In *Homologous recombination and gene silencing in plants* (ed. J. Paszkowski), pp. 39–60. Kluwer, Dordrecht.

Brown, J.K., D.R. Frohlich, and R. Rosell. 1995. The sweetpotato/silverleaf whiteflies: Biotypes of *Bemisia tabaci* Genn., or a species complex? *Annu. Rev. Entomol.* **40:** 511–534.

Buratowski, S., and P.A. Sharp. 1992. Initiation of transcription by RNA polymerase II. In *Transcriptional regulation* (ed. S.L. McKnight), pp. 227–246. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.

Choi, I.-R. and D.C. Stenger. 1995. Strain-specific determinants of beet curly top geminivirus DNA replication. *Virology* **206:** 904–912.

DePamphilis, M.L. 1988. Transcriptional elements as components of eukaryotic origins of replication. *Cell* **52:** 635–638.

———. 1993. Eukaryotic DNA replication: Anatomy of an origin. *Annu. Rev. Biochem.* **62:** 29–63.

Desbiez, C., C. David, A. Mettouchi, J. Laufs, and B. Gronenborn. 1995. Rep protein of tomato yellow leaf curl geminivirus (TYLCV) has an ATPase activity required for viral DNA replication. *Proc. Natl. Acad. Sci.* **92:** 5640–5644.

Dodson, M., F.B. Dean, P. Bullock, H. Echols, and J. Hurwitz. 1987. Unwinding of duplex DNA from the SV40 origin of replication by T antigen. *Science* **238:** 964–967.

Donson, J., G.P. Accotto, M.I. Boulton, P.M. Mullineaux, and J.W. Davies. 1987. The nucleotide sequence of a geminivirus from *Digitaria sanguinalis*. *Virology* **161:** 160–169.

Donson, J., B.A.M. Morris-Krsinich, P.M. Mullineaux, M. Boulton, and J.W. Davies. 1984. A putative primer for second-strand synthesis of maize streak virus is virion-associated. *EMBO J.* **3:** 3069–3073.

Dornreiter, I., A. Hoss, A.K. Arthur, and E. Fanning. 1990. SV40 T antigen binds directly to the large subunit of purified DNA polymerase alpha. *EMBO J.* **9:** 3329–3336.

Eagle, P.A., B.M. Orozco, and L. Hanley-Bowdoin. 1994. A DNA sequence required for geminivirus replication also mediates transcriptional regulation. *Plant Cell* **6:** 1157–1170.

Elmer, J.S., L. Brand, G. Sunter, W.E. Gardiner, D.M. Bisaro, and S.G. Rogers. 1988. Genetic analysis of tomato golden mosaic virus. II. The product of the AL1 coding sequence is required for replication. *Nucleic Acids Res.* **16:** 7043–7060.

Esau, K. 1977. Virus-like particles in nuclei of phloem cells in spinach leaves infected with the curly top virus. *J. Ultrastruct Res.* **61:** 78–88.

Etessami, P., K. Saunders, J. Watts, and J. Stanley. 1991. Mutational analysis of complementary-sense genes of African cassava mosaic virus DNA A. *J. Gen. Virol.* **72:** 1005–1012.

Fanning, E. and R. Knippers. 1992. Structure and function of simian virus 40 large tumor antigen. *Annu. Rev. Biochem.* **61:** 55–85.

Fenoll, C., D.M. Black, and S.H, Howell. 1988. The intergenic region of maize streak virus contains promoter elements involved in rightward transcription of the viral genome. *EMBO J.* **7:** 1589–1596.

Fenoll, C., J.J. Schwartz, D.M. Black, M. Schneider, and S.H. Howell. 1990. The intergenic region of maize streak virus contains a GC-rich element that activates rightward transcription and binds maize nuclear factors. *Plant Mol. Biol.* **15:** 865–877.

Fontes, E.P.B., V.A. Luckow, and L. Hanley-Bowdoin. 1992. A geminivirus replication protein is a sequence-specific DNA binding protein. *Plant Cell* **4:** 597–608.

Fontes, E.P.B., P.A. Eagle, P.S. Sipe, V.A. Luckow, and L. Hanley-Bowdoin. 1994a. Interaction between a geminivirus replication protein and origin DNA is essential for viral replication. *J. Biol. Chem.* **269:** 8459–8465.

Fontes, E.P.B., H.J. Gladfelter, R.L. Schaffer, I.T.D. Petty, and L. Hanley-Bowdoin. 1994b. Geminivirus replication origins have a modular organization. *Plant Cell* **6:** 405–416.

Goodman, R.M. 1977. Infectious DNA from a whitefly-transmitted virus of *Phaseolus vulgaris*. *Nature* **266:** 54–55.

Gorbalenya, A.E. and E.V. Koonin. 1989. Viral proteins containing the purine NTP-binding sequence pattern. *Nucleic Acids Res.* **17:** 8413–8440.

Gorbalenya, A.E., E.V. Koonin, and Y.I. Wolf. 1990. A new superfamily of putative NTP-binding domains encoded by genomes of small DNA and RNA viruses. *FEBS Lett.* **262:** 145–148.

Gröning, B.R., R.J. Hayes, and K.W. Buck. 1994. Simultaneous regulation of tomato golden mosaic virus coat protein and AL1 gene expression: Expression of the AL4 gene may contribute to suppression of the AL1 gene. *J. Gen. Virol.* **75:** 721–726.

Gros, M.F., H. te Riele, and S.D. Ehrlich. 1987. Rolling circle replication of single-stranded DNA plasmid pC194. *EMBO J.* **6:** 3863–3869.

Hamilton, W.D.O., D.M. Bisaro, R.H.A. Coutts, and K.W. Buck. 1983. Demonstration of the bipartite nature of the genome of a single-stranded DNA plant virus by infection with the cloned DNA components. *Nucleic Acids Res.* **11:** 7387–7396.

Hamilton, W.D.O., R.C. Sanders, R.H.A. Coutts, and K.W. Buck. 1981. Characterisation of tomato golden mosaic virus as a geminivirus. *FEMS Microbiol. Lett.* **11:** 263–267.

Hanley-Bowdoin, L., J.S. Elmer, and S.G. Rogers. 1990. Expression of functional replication protein from tomato golden mosaic virus in transgenic tobacco. *Proc. Natl. Acad. Sci.* **87:** 1446–1450.

Hansen, U., D. Tenen, D.M. Livingston, and P.A. Sharp. 1981. T antigen repression of SV40 early transcription from two promoters. *Cell* **27:** 603–612.

Hayes, R.J. and K.W. Buck. 1989. Replication of tomato golden mosaic virus DNA B in transgenic plants expressing open reading frames (ORFs) of DNA A: Requirement of ORF AL2 for production of single-stranded DNA. *Nucleic Acids Res.* **17:** 10213–10222.

Hayes, R.J., H. Macdonald, R.H.A. Coutts, and K.W. Buck. 1988. Priming of complementary DNA synthesis *in vitro* by small DNA molecules tightly bound to virion DNA of wheat dwarf virus. *J. Gen. Virol.* **69:** 1345–1350.

Heyraud, F., V. Matzeit, S. Schaefer, J. Schell, and B. Gronenborn. 1993a. The conserved nonanucleotide motif of the geminivirus stem-loop sequence promotes replicational release of virus molecules from redundant copies. *Biochimie* **75:** 605–615.

Heyraud, F., V. Matzeit, M. Kammann, S. Schaefer, J. Schell, and B. Gronenborn. 1993b. Identification of the initiation sequence for viral-strand DNA synthesis of wheat dwarf

virus. *EMBO J.* **12:** 4445–4452.

Heyraud-Nitschke, F., S. Schumacher, J. Laufs, S. Schaefer, J. Schell, and B. Gronenborn. 1995. Determination of the origin cleavage and joining domain of geminivirus rep proteins. *Nucleic Acids Res.* **23:** 910–916.

Hofer, J.M.I., E.L. Dekker, H.V. Reynolds, C.J. Woolston, B.S. Cox, and P.M. Mullineaux. 1992. Coordinate regulation of replication and virion sense gene expression in wheat dwarf virus. *Plant Cell* **4:** 213–223.

Hormuzdi, S.G. and D.M. Bisaro. 1993. Genetic analysis of beet curly top virus: Evidence for three virion sense genes involved in movement and regulation of single-and double-stranded DNA levels. *Virology* **193:** 900–909.

———. 1995. Genetic analysis of beet curly top virus: Examination of the roles of L2 and L3 genes in viral pathogenesis. *Virology* **206:** 1044–1054.

Ilyina, T.V. and E.V. Koonin. 1992. Conserved sequence motifs in the initiator proteins for rolling circle DNA replication encoded by diverse replicons from eubacteria, eucaryotes, and archaebacteria. *Nucleic Acids Res.* **20:** 3279–3285.

Im, D.-S. and N. Muzyczka. 1990. The AAV origin binding protein Rep68 is an ATP-dependent site-specific endonuclease with DNA helicase activity. *Cell* **61:** 447–457.

Kammann, M., H.-J. Schalk, V. Matzeit, D. Schaefer, J. Schell, and B. Gronenborn. 1991. DNA replication of wheat dwarf virus, a geminivirus, requires two *cis*-acting signals. *Virology* **184:** 786–790.

Kim, K.S., T.L. Shock, and R.M. Goodman. 1978. Infection of *Phaseolus vulgaris* by bean golden mosaic virus: Ultrastructural aspects. *Virology* **89:** 22–33.

Koepsel, R.R., R.W. Murray, W.D. Rosenblum, and S.A. Khan. 1985. The replication initiator protein of plasmid pT181 has sequence-specific endonuclease and topoisomerase activity. *Proc. Natl. Acad. Sci.* **82:** 6845–6849.

Koonin, E.V. and T.V. Ilyina. 1992. Geminivirus replication proteins are related to prokaryotic plasmid rolling circle DNA replication initiator proteins. *J. Gen. Virol.* **73:** 2763–2766.

Kornberg, A. and T.A. Baker. 1992. *DNA replication.* W.H. Freeman, San Francisco.

Laufs, J., W. Traut, F. Heyraud, V. Matzeit, S.G. Rogers, J. Schell, and B. Gronenborn. 1995. *In vitro* cleavage and ligation at the viral origin of replication by the replication protein of tomato yellow leaf curl virus. *Proc. Natl. Acad. Sci.* **92:** 3879–3883.

Lazarowitz, S.G. 1992. Geminiviruses: Genome structure and function. *Crit. Rev. Plant Sci.* **11:** 327–349.

Lazarowitz, S.G., L.C. Wu, S.G. Rogers, and J.S. Elmer. 1992. Sequence-specific interaction with the viral AL1 protein identifies a geminivirus DNA replication origin. *Plant Cell* **4:** 799–809.

Matthews, R.E.F. 1991. *Plant virology.* Academic Press, San Diego.

Mullineaux, P.M., J.W. Davies, and C.J. Woolston. 1992. Geminiviruses as gene vectors. In *Genetic engineering with plant viruses* (ed. T.M.A. Wilson and J.W. Davies), pp. 187–215. CRC Press, Boca Raton, Florida.

Mullineaux, P.M., F. Guerineau, and G.P. Accotto. 1990. Processing of complementary sense RNAs of Digitaria streak virus in its host and in transgenic tobacco. *Nucleic Acids Res.* **18:** 7259–7265.

Nagar, S., T.J. Pedersen, K. Carrick, L. Hanley-Bowdoin, and D. Robertson. 1995. A geminivirus induces expression of a host DNA synthesis protein in terminally differentiated plant cells. *Plant Cell* **7:** 705–719.

Noirot, P., J. Bagonetti, and R. Novick. 1990. Initiation of rolling-circle replication in

pT181 plasmid: Initiator protein enhances cruciform extrusion at the origin. *Proc. Natl. Acad. Sci.* **87:** 8560–8564.

Noirot-Gros, M.F., V. Bidnenko, and S.D. Ehrlich. 1994. Active site of the replication protein of the rolling circle plasmid pC194. *EMBO J.* **13:** 4412–4420.

Pascal, E., A.A. Sanderfoot, B. Ward, R. Medville, R. Turgeon, and S.G. Lazarowitz. 1994. The geminivirus BR1 movement protein binds single-stranded DNA and localizes to the cell nucleus. *Plant Cell* **6:** 995–1006.

Pedersen, T.J. and L. Hanley-Bowdoin. 1994. Molecular characterization of the AL3 protein encoded by a bipartite geminivirus. *Virology* **202:** 1070–1075.

Pilartz, M. and H. Jeske. 1992. Abutilon mosaic virus double-stranded DNA is packed into minichromosomes. *Virology* **189:** 800–802.

Reed, S.I., G.R. Stark, and J.C. Alwine. 1976. Autoregulation of SV40 gene A by T antigen. *Proc. Natl. Acad. Sci.* **73:** 3083–3087.

Revington, G.N., G. Sunter, and D.M. Bisaro. 1989. DNA sequences essential for replication of the B genome component of tomato golden mosaic virus. *Plant Cell* **1:** 985–992.

Roberts, S. and J. Stanley. 1994. Lethal mutations within the conserved stem-loop of African cassava mosaic virus DNA are rapidly corrected by genomic recombination. *J. Gen. Virol.* **75:** 3203–3209.

Rogers, S.G., D.M. Bisaro, R.B. Horsch, R.T. Fraley, N.L. Hoffmann, L. Brand, J.S. Elmer, and A.M. Lloyd. 1986. Tomato golden mosaic virus A component DNA replicates autonomously in transgenic plants. *Cell* **45:** 593–600.

Rushing, A.E., G. Sunter, W.E. Gardiner, R.R. Dute, and D.M. Bisaro. 1987. Ultrastructural aspects of tomato golden mosaic virus infection in tobacco. *Phytopathology* **77:** 1231–1236.

Saunders, K., A. Lucy, and J. Stanley. 1991. DNA forms of the geminivirus African cassava mosaic virus consistent with a rolling circle mechanism of replication. *Nucleic Acids Res.* **19:** 2325–2330.

———. 1992. RNA-primed complementary-sense DNA synthesis of the geminivirus African cassava mosaic virus. *Nucleic Acids Res.* **20:** 6311–6315.

Schalk, H.-J., V. Matzeit, B. Schiller, J. Schell, and B. Gronenborn. 1989. Wheat dwarf virus, a geminivirus of graminaceous plants, needs splicing for replication. *EMBO J.* **8:** 359–364.

Stahl, H., P. Droge, and R. Knippers. 1986. DNA helicase activity of SV40 large tumor antigen. *EMBO J.* **5:** 1939–1944.

Stanley, J. 1983. Infectivity of the cloned geminivirus genome requires sequences from both DNAs. *Nature* **305:** 643–645.

———. 1991. The molecular determinants of geminivirus pathogenesis. *Semin. Virol.* **2:** 139–149.

———. 1995. Analysis of African cassava mosaic virus recombinants suggests strand nicking occurs within the conserved nonanucleotide motif during the initiation of rolling circle DNA replication. *Virology* **206:** 707–712.

Stanley, J., J. Latham, M.S. Pinner, I. Bedford, and P.G. Markham. 1992. Mutational analysis of the monopartite geminivirus beet curly top virus. *Virology* **191:** 396–405.

Stenger, D.C., G.N. Revington, M.C. Stevenson, and D.M. Bisaro. 1991. Replicational release of geminivirus genomes from tandemly repeated copies: Evidence for rolling circle replication of a plant viral DNA. *Proc. Natl. Acad. Sci.* **88:** 8029–8033.

Sunter, G. and D.M. Bisaro. 1989. Transcription map of the B genome component of

tomato golden mosaic virus and comparison with A component transcripts. *Virology* **173:** 647–655.

———. 1991. Transactivation in a geminivirus: AL2 gene product is needed for coat protein expression. *Virology* **180:** 416–419.

———. 1992. Transactivation of geminivirus AR1 and BR1 gene expression by the viral AL2 gene product occurs at the level of transcription. *Plant Cell* **4:** 1321–1331.

Sunter, G., K.W. Buck, and R.H.A. Coutts. 1985. S1-sensitive sites in the supercoiled double-stranded form of tomato golden mosaic virus DNA component B: Identification of regions of potential alternative secondary structure and regulatory function. *Nucleic Acids Res.* **13:** 4645–4659.

Sunter, G., M.D. Hartitz, and D.M. Bisaro. 1993. Tomato golden mosaic virus leftward gene expression: Autoregulation of geminivirus replication protein. *Virology* **195:** 275–280.

Sunter, G., D.C. Stenger, and D.M. Bisaro. 1994. Heterologous complementation by geminivirus AL2 and AL3 genes. *Virology* **203:** 203–210.

Sunter, G., W.E. Gardiner, A.E. Rushing, S.G. Rogers, and D.M. Bisaro. 1987. Independent encapsidation of tomato golden mosaic virus A component DNA in transgenic plants. *Plant Mol. Biol.* **8:** 477–484.

Sunter, G., M.D. Hartitz, S.G. Hormuzdi, C.L. Brough, and D.M. Bisaro. 1990. Genetic analysis of tomato golden mosaic virus. ORF AL2 is required for coat protein accumulation while ORF AL3 is necessary for efficient DNA replication. *Virology* **179:** 69–77.

Thömmes, P., T.A.M. Osman, R.J. Hayes, and K.W. Buck. 1993. TGMV replication protein AL1 preferentially binds to single-stranded DNA from the common region. *FEBS Letters* **319:** 95–99.

Townsend, R., J. Watts, and J. Stanley. 1986. Synthesis of viral DNA forms in *Nicotiana plumbaginafolia* protoplasts inoculated with cassava latent virus (CLV); evidence for independent replication of one component of the CLV genome. *Nucleic Acids Res.* **14:** 1253–1265.

van Mansfeld, A.D.M., H.A.A.M. Teeffelen, P.D. Baas, and H.S. Jansz. 1986. Two juxtaposed tyrosyl-OH groups participate in ϕX174 gene A protein catalysed cleavage and ligation of DNA. *Nucleic Acids Res.* **14:** 4229–4238.

Yasukawa, H., T. Hase, A. Sakai, and Y. Masamune. 1991. Rolling-circle replication of the plasmid pKYM isolated from a gram-negative bacterium. *Proc. Natl. Acad. Sci.* **88:** 10282–10286.