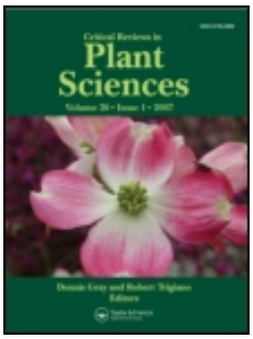

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Geminiviruses: Models for Plant DNA Replication, Transcription, and Cell Cycle Regulation

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ABSTRACT: Geminiviruses have small, single-stranded DNA genomes that replicate through double-stranded intermediates in the nuclei of infected plant cells. Viral double-stranded DNA also assembles into minichromosomes and is transcribed in infected cells. Geminiviruses encode only a few proteins for their replication and transcription and rely on host enzymes for these processes. However, most plant cells, which have exited the cell cycle and undergone differentiation, do not contain the replicative enzymes necessary for viral DNA synthesis. To overcome this barrier, geminiviruses induce the accumulation of DNA replication machinery in mature plant cells, most likely by modifying cell cycle and transcriptional controls. In animals, several DNA viruses depend on host replication and transcription machinery and can alter their hosts to create an environment that facilitates efficient viral replication. Analysis of these viruses and their proteins has contributed significantly to our understanding of DNA replication, transcription, and cell cycle regulation in mammalian cells. Geminiviruses have the same potential for plant systems. Plants offer many advantages for these types of studies, including ease of transformation, well-defined cell populations and developmental programs, and greater tolerance of cell cycle perturbation and polyploidy. Our knowledge of the molecular and cellular events that mediate geminivirus infection has increased significantly during recent years. The goal of this review is to summarize recent research addressing geminivirus DNA replication and its integration with transcriptional and cell cycle regulatory processes.

KEY WORDS: geminiviruses, plant DNA replication, plant DNA transcription, cell cycle regulation, DNA genomes.

I. INTRODUCTION

Geminiviruses are a large, diverse family of plant viruses that infect a broad variety of plants and cause significant crop losses worldwide. They are characterized by twin icosahedral capsids and circular single-stranded DNA (ssDNA) genomes that replicate through double-stranded DNA (dsDNA) intermediates in infected cells. Geminiviruses contribute only a few factors for their replication and transcription and are dependent on the nuclear DNA and RNA polymerases of their plant hosts. These properties are unusual among plant viruses, most of which are RNA viruses or replicate through RNA intermediates using virus-encoded replicases. Because of their importance as plant pathogens and their differences from other plant-infecting viruses, there has been considerable interest in basic and applied aspects of geminivirus biology in recent years, and several excellent reviews describing these viruses have been written (Harrison, 1985; Davies et al., 1989; Bisaro et al., 1990; Stanley, 1991; Brown et al., 1992; Lazarowitz, 1992;

Timmermans et al., 1994; Laufs et al., 1995a; Bisaro, 1996; Palmer and Rybicki, 1998).

The *Geminiviridae* family consists of three subgroups that differ with respect to insect vector, host range, and genome structure (Rybicki, 1994; Briddon et al., 1995; Padidam et al., 1995a). Subgroup I includes leafhopper-transmitted viruses that generally infect monocot plants and have single-component genomes. Subgroup III includes whitefly-transmitted viruses that infect dicot plants and most commonly have bipartite genomes. Subgroup II viruses are transmitted by leafhoppers and have single-component genomes like Subgroup I, but infect dicot plants like Subgroup III. Members of the three subgroups use similar replication and transcription strategies, but there are differences, in particular between Subgroup I vs. Subgroup II and III geminiviruses. The replication and transcription processes are best characterized for Subgroup III viruses, and our review focuses primarily on this subgroup.

II. GENETIC STUDIES OF REPLICATION AND TRANSCRIPTION

Geminiviruses have small genomes consisting of either one or two circular ssDNA molecules ranging from 2.5 to 3 x 10^3 nucleotides in size (Figure 1). The genomic DNAs contain divergent coding sequences separated by 5′ intergenic regions. The coding capacity of the genomes varies among the different subgroups. Subgroup I viruses specify four open reading frames for polypeptides greater than 10 kDa, whereas Subgroup II and III viruses encode six to seven open reading frames. There are currently two nomenclatures for geminivirus genes (Table 1). The first identifies viral genes as to whether they are specified by the virion (V) or complementary (C) sense DNA strands, whereas the second designates genes with respect to the left (L) or right (R) of the 5′ intergenic region. The V and L designations are equivalent, as are C and R. In this review, the protein homologues corresponding to the C1:C2, C1, and AL1 genes of the three subgroups are designated as Rep, whereas the protein for the C1 open reading of Subgroup I geminiviruses is called RepA. All other viral proteins are identified by their gene names, that is, AL3 is encoded by the AL3 gene.

This section describes genetic experiments that address the functions of the various viral proteins. Genetic approaches have also been used to identify the *cis*-elements that mediate geminivirus replication and transcription. The reader is referred to Section IV for a description of these studies.

A. Bipartite Subgroup III Geminiviruses

The genomes of Subgroup III geminiviruses typically consist of two DNA components, designated A and B (Stanley et al., 1983; Howarth et al., 1985; Frischmuth et al., 1990), as shown for tomato golden mosaic virus (TGMV) in Figure 1A (Hamilton et al., 1984). Both components are required for efficient infection (Hamilton et al., 1983; Klinkenberg et al., 1990; Evans et al., 1993; Padidam et al., 1995b; Sung et al., 1995a). *Agrobacterium*-mediated transformation was used to create plants with stably integrated copies of TGMV A or B in their genomes. The presence of freely replicating viral DNA and particles in transgenic plants containing partial tandem copies of TGMV A demonstrated that the A component encodes all of the information necessary for viral replication and encapsidation (Rogers et al., 1986; Sunter et al., 1987). In contrast, the B component cannot replicate in the absence of A DNA but is required for systemic movement and symptom production (Brough et al., 1988; Pascal et al., 1993; Gilbertson et al., 1996; Sanderfoot et al., 1996a). Transient replication studies in leaf discs or protoplasts also showed that the A component can replicate autonomously, while B DNA cannot (Townsend et al., 1986; Elmer et al., 1988b; Lazarowitz et al., 1991).

The A component contains five open reading frames, four (AL1, AL2, AL3, and AL4) specified by overlapping sequences on the complementary strand and one (AR1) encoded on the virion strand (Figure 1A). The B DNA encodes two genes, one (BL1) on the complementary strand and the other (BR1) on the virion strand. Mutations in the AL1, AL2, BL1, and BR1 genes blocked infectivity (Elmer et al., 1988a; Etessami

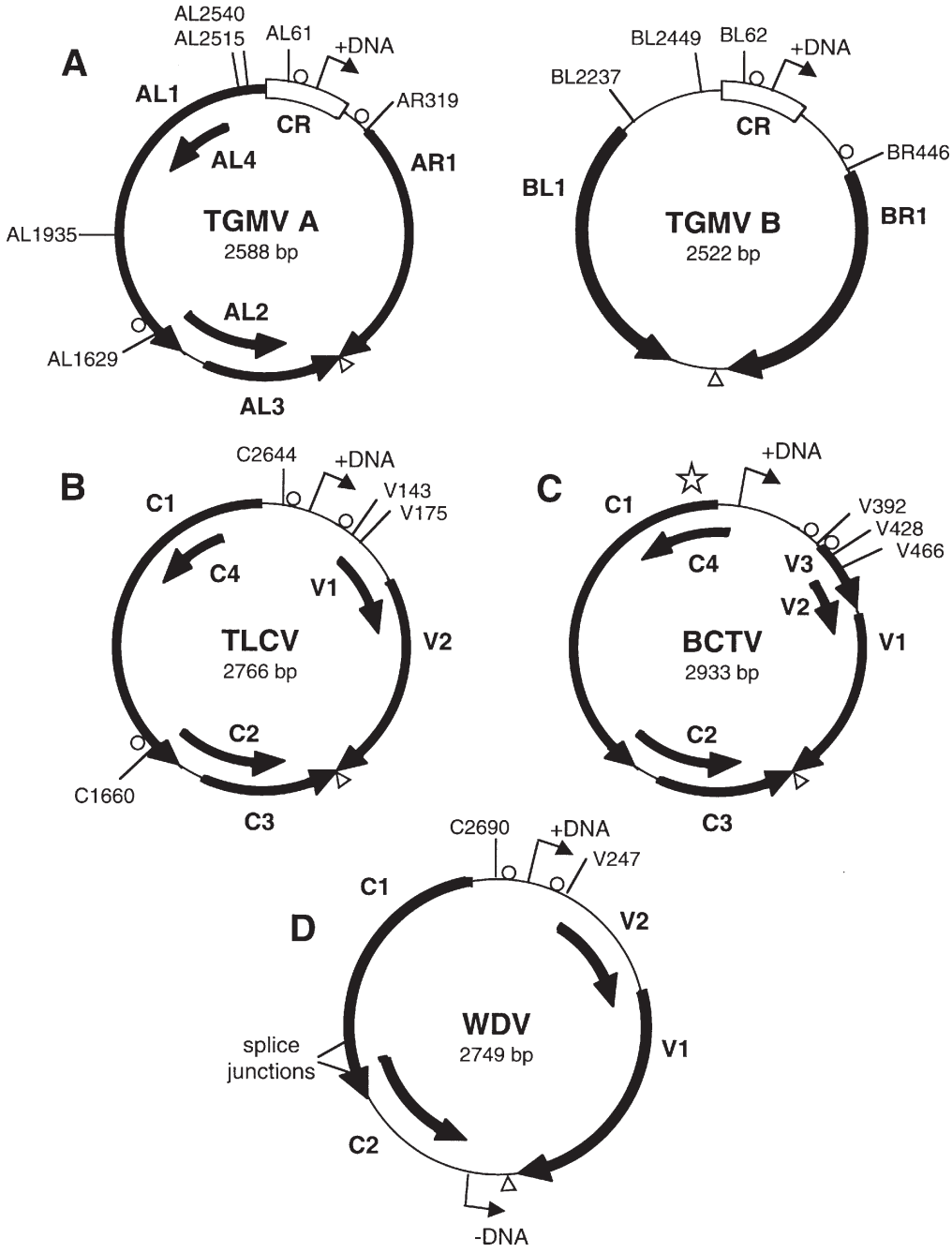

FIGURE 1. Geminivirus genome organization. Panels A and B show the genomic organization of two Subgroup III geminiviruses. The two genome components of TGMV are shown in **A**, whereas the single genomic DNA of TLCV is drawn in **B**. The genome of a Subgroup II virus, BCTV, is depicted in **C**. The genome of a Subgroup I virus, WDV, is diagrammed in **D**. The size of each DNA is given in base pairs. The open boxes in **A** correspond the common region (CR) or 5′ intergenic sequences that are conserved among the A and B DNAs of TGMV. The dark arrows indicate viral open reading frames and their directions of transcription. The name of each open reading frame is shown in bold type. The 5′ end of viral mRNAs are labeled with respect to genome position and polarity. The star in panel **C** indicates that the 5′ ends of the complementary transcripts for BCTV are not known. The open circles indicate TATA box motifs, while the triangles designate bidirectional polyadenylation sites. The splice sites in WDV C1 and C2 are also marked in **D**. The DNA cleavage site for initiation of plus-strand DNA replication is shown for each virus. The position of a primer for WDV minus-strand synthesis is also given in **D**.

TABLE 1
Geminivirus Function and Nomenclature[a]

	Subgroup I	Subgroup II	Subgroup III
Replication			
• Plus-strand origin	Long intergenic region (LIR)	5′ intergenic region (IR)	5′ intergenic region (IR) or common region (CR)
• Minus-strand origin	Short intergenic region (SIR)	Unknown	Unknown but may be in common region
• Essential factor	C1:2 (Rep)	C1 (Rep)	AL1 (C1, Rep)
• Accessory factor	C1′ (RepA)	C3 and V3 (?)	AL3 (C3)
Transcription			
• Promoters	Long intergenic region (LIR)	5′ intergenic region (IR)	5′ intergenic region (IR) or common region (CR)
• Activators	C1:2 (Rep)	C2 (TrAP)	AL2 (C2, TrAP)
• Repressors			AL1 (C1, Rep) and AL4 (C4)
Host activation	C1′ (RepA)	C4	AL1 (C1, Rep)
Encapsidation	V2	V2	Double component: AR1 (V1) Single component: V2
Movement	V1 and V2	V1 and V2	Double component: BL1 (BC1) and BR1 (BV1) Single component: V1 and V2
Symptoms		C4	BL1 (BC1)

[a] Labeling corresponds to Figure 1. Alternative nomenclature is indicated in parentheses.

et al., 1988 and 1991; Morris et al., 1991; Sung and Coutts, 1995a), establishing that their products are essential for virus function. Mutations in the AL3 gene resulted in severely delayed and attentuated symptoms (Elmer et al., 1988a; Etessami et al., 1991; Morris et al., 1991; Sung and Coutts, 1995a), indicating that its polypeptide is necessary for efficient infection. The AR1 gene product was dispensable when mechanical or agroinoculation was used to infect well-adapted host plants but was required if transmission was through the whitefly vector or in a suboptimal host (Stanley et al., 1986b; Gardiner et al., 1988; Briddon et al., 1990; Pooma et al., 1996a; Hofer et al., 1997). No phenotype was detected for AL4 mutants under a variety of conditions (Sung and Coutts, 1995a; Pooma et al., 1996b).

Infectivity studies reveal if a sequence contributes to some aspect of the viral life cycle but do not provide information regarding what viral processes are involved. Consequently, assays based on transfected protoplasts and agro-inoculated leaf discs were developed to specifically examine geminivirus replication (Townsend et al., 1986; Elmer et al., 1988b). Mutation of the AL1 open reading frame blocked viral replication, whereas an AL3 mutant displayed greatly reduced DNA levels (Elmer et al., 1988a; Sunter et al., 1990; Sung and Coutts, 1995a), indicating that both gene products are involved in replication. In addition, transgenic plants that contained the AL1 gene and constitutively expressed the Rep protein in the absence of AL3 supported replication of DNA B, demonstrating that Rep is sufficient for replication in the presence of host factors (Hayes et al., 1989; Hanley-Bowdoin et al., 1990). Mutations in AR1 and BR1 impacted ssDNA levels under some conditions (Jeffrey et al., 1996; Padidam et al., 1996), suggesting that these proteins may sequester ssDNA. This hypothesis is consistent with the functions of these proteins. AR1 is the coat protein (Kallender et al., 1988), whereas BR1 is a movement protein that binds and transports ssDNA across the nuclear envelope (Pascal et al., 1994; Sanderfoot et al., 1996a). AL2 also impacted ssDNA accumulation, most likely reflecting its transactivation of AR1 and BR1 expression (Sunter et al., 1991). In contrast, BL1 mutations had no apparent effect on viral DNA accumulation in transient assays, compatible with its role in cell-to-cell movement (Noueiry et al., 1994; Ward et al., 1997). AL4 also had no detectable effect on viral replication (Elmer et al., 1988a).

Transient systems were also used to identify viral proteins involved in transcription. The 5′ intergenic region that contains geminivirus promoter sequences was fused upstream of β-glucuronidase or luciferase coding sequences, and reporter activity was assayed in the presence of mutant viral DNA components or plant expression cassettes corresponding to various viral proteins. These experiments showed that the AL2 protein specifically enhances the activity of reporter constructs containing the AR1 or BR1 promoter region (Sunter and Bisaro, 1991). Experiments in intact plants showed that an AL2 mutation can be overcome by transient expression of BR1 (Jeffrey et al., 1996), thereby establishing that the infectivity requirement for AL2 reflects its activation of the BR1 promoter. Transient transcription assays also revealed that Rep strongly suppresses the activity of its own promoter (Sunter et al., 1993; Eagle et al., 1994; Groning et al., 1994; Hong et al., 1995). Similar results were also seen in transgenic plants containing AL1 promoter-reporter fusions (Haley et al., 1992). Thus, Rep has a dual role in the viral life cycle, acting both in replication and transcription. In analogous experiments, AL4 also weakly regulated the AL1 promoter (Groning et al., 1994; Eagle et al., 1997), but the significance of these results is unclear because of the failure to detect a role for AL4 during infection (Pooma and Petty, 1996b).

B. Single-Component Subgroup II and III Geminiviruses

The genomes of some Subgroup III geminiviruses consist of a single DNA component (Kheyr-Pour et al., 1991; Navot et al., 1991; Dry et al., 1993; Noris et al., 1994). In these viruses, the arrangement of the complementary genes (C1, C2, C3, and C4) is identical to those on the A component of bipartite viruses, as shown for tomato leaf curl virus (TLCV) in Figure 1B. The organization of the complementary genes in the

single-component genome of beet curly top virus (BCTV), a Subgroup II geminivirus (Figure 1C), is also very similar (Stanley et al., 1986a). Genetic studies demonstrated that the Rep and C3 proteins of tomato yellow leaf curl virus (TYLCV), which resembles TLCV, and of BCTV function during replication (Stanley et al., 1992b; Hormuzdi et al., 1995; Jupin et al., 1995). Analogous to bipartite geminiviruses, Rep is essential for replication and C3 enhances viral DNA accumulation. Like the AL2 protein, C2 was required for TYLCV infectivity, but it is not known if this protein acts as a transactivator of viral gene expression (Wartig et al., 1997). In contrast, no phenotype was detected for a BCTV C2 mutant, suggesting that this open reading frame is dispensable for Subgroup II geminiviruses (Stanley et al., 1992b; Hormuzdi and Bisaro, 1995). Mutation of the C4 open reading frames of both viruses attenuated symptoms in a host-dependent fashion, possibly by impacting viral movement or the ability to induce host replication machinery (Stanley et al., 1992a; Jupin et al., 1994; Rigden et al., 1994; Latham et al., 1997). The C4 proteins of TYLCV and BCTV do not resemble the predicted AL4 polypeptides of bipartite geminiviruses, and it is not surprising that they display distinctive phenotypes.

The virion strands of the single-component geminiviruses are more complex than their bipartite counterparts. TLCV specifies two overlapping coding regions (V1 and V2) on its virion strand (Figure 1B), whereas the virion strand of BCTV encodes three polypeptides (V1, V2, and V3; Figure 1C). Mutations in the TYLCV V1 coding sequence and the V2 coat protein gene had no effect on replication but blocked infectivity, most likely by inhibiting movement (Rigden et al., 1993; Padidam et al., 1996). Interestingly, TYLCV V1 and V2 mutants accumulated lower levels of ssDNA (Wartig et al., 1997), suggesting that these proteins sequester viral DNA like the AR1 and BR1 proteins of bipartite geminiviruses. Likewise, the BCTV V1 coat protein and V3 polypeptide were dispensable for replication but were required for infection, implicating both proteins in viral movement (Briddon et al., 1989; Hormuzdi et al., 1993). Genetic experiments also uncovered a role for the BCTV coat protein in insect transmission (Briddon et al., 1990). In contrast, the phenotypes of BCTV V2 mutants displayed host dependency, that is, there was no effect on infection of the permissive host *Nicotiana benthamiana*, but infection of the natural host *Beta vulgaris* was blocked (Stanley et al., 1992b; Hormuzdi and Bisaro, 1993). BCTV V2 mutants displayed reduced ssDNA and higher dsDNA levels (Hormuzdi and Bisaro, 1993), suggesting V2 may modulate the conversion of dsDNA to ssDNA.

C. Subgroup I Geminiviruses

The single-component genomes of Subgroup I geminiviruses have a unique organization (MacDowell et al., 1985; Donson et al., 1987; Lazarowitz, 1988). As shown for wheat dwarf virus (WDV, Figure 1D), the complementary strand encodes two overlapping open reading frames (C1 and C2). WDV mutants with deletions in either the C1 or C2 gene were unable to replicate, indicating that both complementary open reading frames are required for viral replication (Schalk et al., 1989). In contrast, WDV mutants that included a frameshift in the overlapping C1:C2 region or an in-frame C1:C2 fusion replicated efficiently. These results, in combination with transcript mapping studies (Schalk et al., 1989; Mullineaux et al., 1990; Dekker et al., 1991), indicated that the Rep protein of Subgroup I geminiviruses is translated from spliced mRNA and is highly homologous to Rep proteins of Subgroup II and III viruses. The importance of splicing was further demonstrated by the inability of MSV mutants with altered C1:C2 splice site junctions to replicate (Wright et al., 1997). Transient assays uncovered roles for Rep in activation of virion-sense transcription of Subgroup I geminiviruses (Hofer et al., 1992; Zhan et al., 1993; Collin et al., 1996). No replication enhancer protein analogous to AL3/C3 has been identified in Subgroup I viruses.

Like other single-component geminiviruses, the Subgroup I genomes specify multiple overlapping open reading frames (V1 and V2) on their virion strands (Figure 1D). The V2 gene encodes the coat protein, which in combination with V1 mediates viral movement (Morris-Krisinich et al.,

1985; Boulton et al., 1993). Mutations in both open reading frames blocked infectivity and reduced ssDNA accumulation (Lazarowitz et al., 1989; Woolston et al., 1989; Boulton et al., 1993). There is evidence that all of the viral open readings frames of Subgroup I geminiviruses affect host range and symptomology (Boulton et al., 1991a,b)

III. GENERAL REPLICATION AND TRANSCRIPTION STRATEGIES

All geminiviruses employ the same general strategies to duplicate and express their genomes. They use a rolling circle replication system to amplify their ssDNA genomes and to produce dsDNAs that then serve as replicative and transcriptional templates. The double-stranded form is divergently transcribed from a 5′ intergenic region that also includes the plus-strand origin of replication. This section describes these basic mechanisms and discusses their relationships to other biological systems.

A. Rolling Circle Replication

Rolling circle replication is a two-step process in which leading and lagging-strand DNA synthesis are separate events (Kornberg et al., 1992). In the first phase, the single-stranded "plus" strand is used as a template for the synthesis of the "minus" strand to generate a double-stranded, replicative form (RF). During the second step, the replicative form serves as a template for plus-strand synthesis to generate free ssDNA. A hallmark of all rolling circle systems is the use of a site-specific nick to prime plus-strand DNA synthesis. Minus-strand synthesis is primed by an RNA molecule that is generated either through RNA polymerase or DNA primase activity. (In geminivirus replication, the plus strand corresponds to the virion strand found in both ssDNA and dsDNA, whereas the minus strand is the complementary strand found only in dsDNA.)

Several experimental approaches established that geminiviruses use a rolling circle mechanism for their replication. The earliest experiments used two-dimensional electrophoresis to examine the replicative intermediates generated during infection by the bipartite geminivirus, African cassava mosaic virus (ACMV; Saunders et al., 1991). These studies detected five putative replication intermediates. One consisted of heterogeneous, subgenomic complementary DNA associated with genome-length virion DNA as predicted for intermediates generated during minus-strand synthesis. Another contained unit-length complementary DNA and virion DNA ranging from one to two genome lengths as expected for strand-displacement intermediates formed during plus-strand synthesis. The other intermediates comprised concatemeric virion ssDNA, dsDNA, or partially ssDNA, all of which are consistent with a rolling circle replication mechanism.

Later studies established that geminivirus plus-strand DNA synthesis initiates through a DNA cleavage event at a specific site *in vivo*, thereby fulfilling a key requirement for rolling circle replication (Stenger et al., 1991; Heyraud et al., 1993a). Stenger et al. (1991) infected plants with plasmids containing tandem genome repeats from different BCTV strains and recovered two predominant progeny genotypes dependent on the arrangement of the repeated parental genomes in the inocula. The DNA sequences of the progeny were consistent with rolling circle replication that initiates and/or terminates plus-strand DNA synthesis within a conserved hairpin sequence located in the 5′ intergenic region. Similar results were observed for WDV (Heyraud et al., 1993a,b) and ACMV (Stanley, 1995) heterodimers. The initiation site was precisely mapped *in vivo* to the conserved nonanucleotide sequence (TAATATT↓AC) in the loop of the hairpin (Stanley, 1995). Biochemical studies showed that the geminivirus Rep protein is a site-specific endonuclease that nicks plus-strand viral DNA at the same position *in vitro* (Laufs et al., 1995c; Orozco et al., 1996). Together these studies demonstrated that plus-strand DNA synthesis initiates within a conserved hairpin motif through the endonucleolytic action of Rep.

The subsequent events involved in plus-strand DNA synthesis and the enzymes that mediate them are not well characterized. Because geminiviruses do not encode a protein with de-

tectable homology to known DNA polymerases, plant nuclear replication machinery is most likely responsible for the elongation phase of plus-strand DNA synthesis. One exception may be the source of the DNA helicase that catalyzes unwinding of the parental plus-strand DNA. The viral Rep protein, which is an ATPase (Desbiez et al., 1995; Orozco et al., 1997) and displays limited sequence identity to known DNA helicases (Gorbalenya et al., 1993), may catalyze DNA unwinding. However, no helicase activity has been demonstrated for any Rep protein to date. In contrast, it is well established that the DNA cleavage/ligation activities of Rep mediate termination of plus-strand replication and resolution of concatameric DNAs (Heyraud-Nitschke et al., 1995; Laufs et al., 1995c).

Even less is known about the proteins and events involved in geminivirus minus-strand synthesis. It is generally believed that minus-strand DNA synthesis is mediated entirely by host factors because only the coat protein, which is not required for viral replication (Elmer et al., 1988a; Woolston et al., 1989), has been detected in virions. Thus, none of the other viral proteins are thought to be present or required for minus-strand synthesis during the initial conversion of virion DNA to RF. However, the possibility that other viral proteins are packaged at low copy number into virions and present throughout the viral replication cycle has not been rigorously eliminated. It is also unclear what mechanism is used to prime minus-strand synthesis. A small oligonucleotide complementary to the 3′ intergenic region has been isolated from Subgroup I virions (Donson et al., 1984; Hayes et al., 1988; Morris et al., 1992). This oligonucleotide can be extended by DNA polymerase *in vitro* and may prime minus-strand synthesis *in vivo*. Interestingly, sequences located in the 3′-intergenic region of WDV have been implicated in replication and may constitute the minus-strand origin (MacDonald et al., 1988; Kammann et al., 1991). Analogous oligonucleotides have not been detected in virions of Subgroup II and III geminiviruses, suggesting that these viruses use a different mechanism to prime minus-strand synthesis. This idea is consistent with two-dimensional electrophoretic analyses of ACMV replication intermediates, which indicated that minus-strand synthesis is primed within the 5′ intergenic region (Saunders et al., 1992). The primers found in Subgroup I virions and the ACMV replication intermediates contain ribonucleotides (Donson et al., 1984; Hayes et al., 1988; Saunders et al., 1992), further supporting their involvement in priming minus-strand synthesis.

Rolling circle replication is most common in bacterial systems, where it is used frequently for viral and plasmid DNA replication (Eisenberg et al., 1979; Meyer et al., 1979; Koepsel et al., 1985; de la Campa et al., 1990). Recent experiments showed that TLCV can replicate in *Agrobacterium tumefaciens* dependent on an intact C1 coding sequence (Rigden et al., 1996), suggesting that geminiviruses may have evolved from bacterial replicons. There are fewer examples of rolling circle replication in eukaryotes. Circoviruses, a family of eukaryotic single-stranded DNA viruses with plant and animal hosts, are thought to replicate through a rolling circle mechanism (Rhode et al., 1990; Meehan et al., 1997). Although these viruses are not well characterized, their putative origins and replication proteins resemble their geminivirus counterparts (Boevink et al., 1995; Hafner et al., 1997; Mankertz et al., 1997). Parvoviruses, which infect mammals, replicate through a related rolling hairpin mechanism (Snyder et al., 1990; Ni et al., 1994).

B. Bidirectional Transcription

The viral messenger RNAs have been characterized for several geminiviruses (Townsend et al., 1985; Petty et al., 1988; Hanley-Bowdoin et al., 1989; Sunter and Bisaro, 1989a; Sunter et al., 1989b; Frischmuth et al., 1991; Mullineaux et al., 1993). These studies established that geminiviruses genomes are transcribed in a bidirectional manner resulting in mRNAs that correspond to both the virion and complementary-sense open reading frames. The viral RNAs are polyadenylated and initiate downstream of either consensus TATA box motifs or initiator elements, indicating that they are transcribed by host RNA polymerase II. Geminivirus transcription is complex, frequently leading to multiple overlapping RNA species,

many of which are polycistronic. There are differences in the expression strategies used by different Subgroups, particularly with respect to RNA processing.

The transcription profile of the bipartite Subgroup III geminivirus, TGMV, has been characterized in detail (Petty et al., 1988; Hanley-Bowdoin et al., 1989; Sunter and Bisaro, 1989a; Sunter et al., 1989b). TGMV A acts as a template for six RNAs, whereas the B DNA is transcribed to give four RNAs (Figure 1A). A single virion-sense RNA is transcribed from each genome component, which is translated to give either the coat protein or BR1. In contrast, complementary-sense transcription is more complex, leading to multiple overlapping RNAs with different 5′ ends and a common 3′ end. The polyadenylation sites for complementary and virion-sense RNAs overlap such that they share a few nucleotides at their 3′ ends. All of the complementary-sense RNAs generated from TGMV B specify BL1, whereas the complementary-sense RNAs from TGMV A have different coding capacities. The largest transcript (AL61) encodes the entire left side of TGMV A and is the only RNA that can be translated to produce full-length Rep protein. (The transcripts are designated according to their 5′ ends.) Two of the RNAs (AL2540 and AL2515) may be translated to give AL4, which is represented by the first of three open reading frames on these RNAs. The smallest RNAs (AL1935 and AL1629) specify AL2 from their first open reading and AL3 from a second coding region. There is no RNA that encodes AL3 as its first open reading frame, indicating that AL3 is expressed from a polycistronic mRNA. The polycistronic character and predicted translation properties of the various TGMV A complementary-sense RNAs have been confirmed by translation *in vitro* (Thommes et al., 1994). Other Subgroup II and III geminiviruses display similar complementary-sense transcription patterns, independent of the number of genome components (Frischmuth et al., 1991 and 1993; Mullineaux et al., 1993). Unlike TGMV, multiple virion-sense mRNAs have been documented for the single-component viruses, BCTV and TLCV, most likely reflecting the greater complexity of their virion-sense genes (Frischmuth et al., 1993; Mullineaux et al., 1993).

The mRNAs of Subgroup I geminiviruses are also transcribed bidirectionally from multiple initiation sites and terminate at overlapping polyadenylation signals (Morris-Krisinich et al., 1985; Accotto et al., 1989; Dekker et al., 1991; Wright et al., 1997). However, unlike Subgroup II and III viruses, RNA processing is an essential component of the expression strategy of Subgroup I geminiviruses. The complementary strands of Subgroup I genomes encode two open reading frames, C1 and C2, which together specify the essential viral replication protein — Rep. RNA mapping studies of WDV, MSV, *Digitaria* streak virus (DSV), and tobacco yellow dwarf virus (TYDV) uncovered a spliced mRNA with fused C1 and C2 sequences (Accotto et al., 1989; Schalk et al., 1989; Mullineaux et al., 1990; Dekker et al., 1991; Morris et al., 1992; Wright et al., 1997). The excised sequences, which are AT rich and contain a potential branch point and consensus splicing signals, are typical of plant introns, thereby indicating that the RNA is processed by host machinery. Interestingly, the complementary-sense RNA is only partially processed. The C1 open reading frame of the unspliced RNA may be translated to give the RepA polypeptide, but there is currently no genetic or biochemical evidence supporting the synthesis of RepA during infection. In contrast, the replication properties of mutant viral genomes clearly established that the spliced C1:C2 mRNA and its resulting Rep translation product are sufficient and required for replication (Schalk et al., 1989; Wright et al., 1997). Recently, an intron was identified in the V1 coding region of MSV and DSV, and it has been proposed that differential RNA processing may have a regulatory role in virion-sense expression of some Subgroup I geminiviruses (Wright et al., 1997).

IV. ORIGINS OF REPLICATION AND PROMOTERS

Geminivirus replication requires two origins — one for plus-strand replication and another for minus-strand synthesis. The plus-strand origin of viruses from all three subgroups has been mapped to the 5′ intergenic region, which

also contains the promoters for virion and complementary-sense transcription. The *cis* elements that mediate viral replication and transcription are best characterized for the Subgroup III geminivirus TGMV. This section describes the TGMV plus-strand origin and promoters and their relationships to sequences in other geminivirus genomes. The reader is referred to Section III.A for a description of what is known about minus-strand synthesis.

A. Plus-Strand Origin

The TGMV plus-strand origin was mapped to the common region by two different approaches. (The common region refers to a ca. 200-bp sequence in the 5′ intergenic region that is conserved between the two genome components of a bipartite geminivirus.) Lazarowitz and co-workers (1992) showed that a 303-bp fragment that includes the common region can support episomal replication in leaf discs agro-inoculated with a tandem copy replicon. Orozco et al. (1998) defined the minimal origin to an 89-bp common region sequence in protoplast assays that examined the capacities of various TGMV DNA sequences to support direct replication of a bacterial plasmid when the viral replication proteins were supplied in *trans*. The DNA sequence of the 89-bp fragment is conserved at all but one position between TGMV A and B, thereby establishing the identity of the plus-strand origins on both genome components. Origins for plus-strand DNA synthesis have also been located to the 5′ intergenic regions of ACMV, squash leaf curl virus (SqLCV), WDV, BCTV, and TYLCV (Saunders et al., 1991; Frischmuth et al., 1992; Lazarowitz et al., 1992; Stenger et al., 1992; Heyraud et al., 1993a; Choi et al., 1995; Jupin et al., 1995).

The TGMV plus-strand origin is located on the left side of the common region between TGMV A positions 64 to 153 (Figure 2; Orozco et al., 1998). The origin overlaps the complementary-sense promoter that is responsible for AL61 mRNA transcription. Site-directed mutagenesis identified six *cis* elements that contribute to TGMV plus-strand origin function. Comparison of the sequences of the TGMV plus-strand origin and the 5′-intergenic regions of other geminiviruses uncovered similar elements in many Subgroup II and III viruses. In contrast, only one of the elements is conserved in Subgroup I viruses, suggesting that their plus-strand origins may be significantly different.

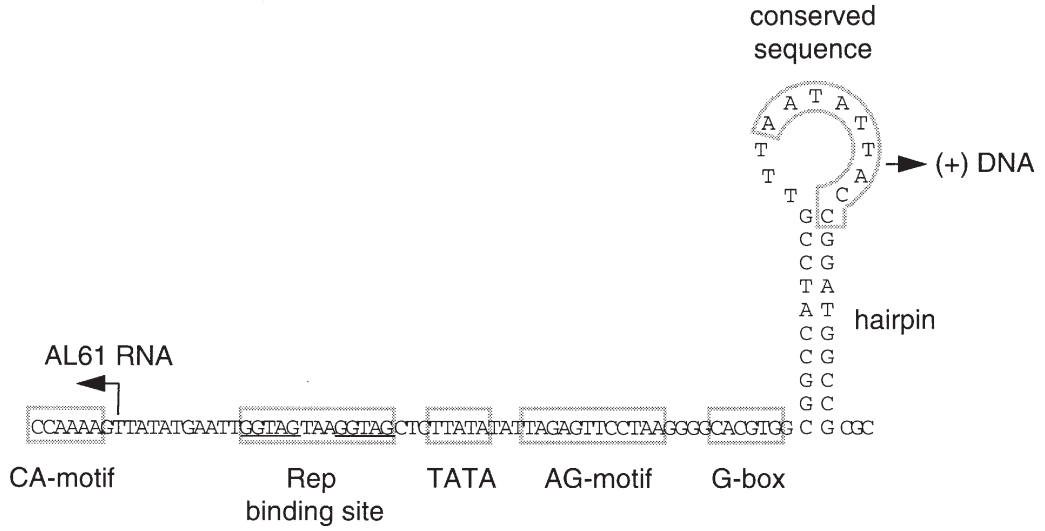

FIGURE 2. The TGMV plus-strand origin of replication and AL61 promoter. The DNA sequence corresponding to TGMV A positions 54 to 153 is shown. Only the top strand of the duplex DNA is given. The initiation sites and directions of synthesis for plus-strand DNA replication and AL61 transcription are indicated. Other functional elements are boxed. The hairpin structure is drawn and the conserved nonanucleotide loop sequence is marked.

The origin element that is common to all geminivirus genomes is a hairpin motif (Arguello-Astorga et al., 1994b). In TGMV, the hairpin is located at the 3′ edge of the minimal origin and contains a GC-rich stem and an AT-rich loop. Analysis of mutants that disrupted this motif or changed the sequence of the stem but maintained base pairing established the importance of the hairpin for origin function and revealed that its structure is essential for activity (Revington et al., 1989; Lazarowitz et al., 1992; Orozco and Hanley-Bowdoin, 1996). The loop contains a nonanucleotide sequence, 5′-TAATATTAC, that is conserved among all geminivirus genomes and is found in the plus-strand origins of other rolling circle systems (Rogers et al., 1986; Baas, 1987). The plus-strand cleavage site has been mapped to same position in the conserved sequence in all three geminivirus subgroups (Heyraud et al., 1993b; Laufs et al., 1995c; Orozco and Hanley-Bowdoin, 1996). Mutations in the nonanucleotide sequence inhibited geminivirus replication *in vivo* and Rep-mediated DNA cleavage *in vitro* (Laufs et al., 1995c; Stanley, 1995; Orozco and Hanley-Bowdoin, 1996). However, there is some sequence flexibility in the loop element (Schneider et al., 1992; Stanley, 1995) as well as at degenerate nucleotide positions in the 5′ end of loop (Fontes et al., 1994b). The sequence of the stem also displays flexibility if structure is maintained (Orozco and Hanley-Bowdoin, 1996). However, competitive replication assays revealed that stem variants replicate less efficiently than wild type. This result is consistent with the observation that the stem sequences of Subgroup II and III geminiviruses are highly conserved. In contrast, the stems of Subgroup I viruses are much longer and more degenerate, suggesting that they may have different or additional roles in replication.

A second key element in the TGMV plus-strand origin is the binding site for Rep. *In vitro* studies showed that TGMV Rep is a sequence-specific DNA binding protein that recognizes a 13-bp directly repeated motif, 5′-*GGTAG*TAA*GGTAG* (Figure 2; Fontes et al., 1992 and 1994a). Mutations that impaired Rep/DNA binding *in vitro* also interfered with replication *in vivo* (Fontes et al., 1994a; Orozco et al., 1998), establishing the importance of this sequence in plus-strand origin function. The TGMV Rep binding site is located upstream of the hairpin and between the TATA box and transcription start site of the AL61 promoter. Related but not identical motifs are positioned similarly in the 5′ intergenic regions of other Subgroup II and III geminiviruses (Arguello-Astorga et al., 1994a,b; Fontes et al., 1994b). *In vitro* experiments showed that the Rep protein of bean golden mosaic virus (BGMV) specifically recognizes the sequence, 5′-*GGAG*ACT*GGAG* (Fontes et al., 1994b). However, TGMV Rep was unable to bind the BGMV sequence and BGMV Rep could not bind the TGMV sequence *in vitro*. TGMV and BGMV Rep also displayed tight specificity for replication of their cognate genomes. Together, these results showed that the Rep binding sites of TGMV and BGMV act as origin recognition elements to confer virus-specific replication.

There is considerable evidence that the tight specificity of Rep for its origin binding site is a general feature of Subgroup II and III geminiviruses. An integrated, subgenomic copy of BCTV DNA was mobilized and amplified only in transgenic plants infected with the cognate wild-type virus and not with other BCTV strains (Stenger, 1994). Pseudorecombinants formed by mixing A and B genome components from different viruses were not infectious unless derived from related strains (Stanley et al., 1985; Gilbertson et al., 1993; Sung et al., 1995b). Coinoculation of the A component of tomato mottle virus (ToMV) and the B DNA of bean dwarf mosaic virus (BDMV) resulted in a recombinant virus with only ToMV common region sequences (Hou et al., 1996). Similarly, replication experiments with SqLCV and TGMV pseudorecombinants revealed that the lack of infectivity of heterologous DNA components can be due to an incompatibility between the B component replication origin and *trans*-acting replication factors encoded by the A component (Lazarowitz et al., 1992). These studies showed that the SqLCV Rep protein interacts with a 90-bp common region fragment, which includes the hairpin motif and its predicted Rep binding site, to confer virus-specific replication *in vivo*. Analysis of chimeric viruses between different strains of BCTV or TYLCV suggested that the N-termini

of their Rep proteins specifically interact with the homologous 5′ intergenic region to support viral replication (Choi and Stenger, 1995; Jupin et al., 1995). Chimeric BCTV origins with exchanged Rep binding motifs displayed altered replication specificity, establishing that these sequences mediate origin recognition for the closely related viral strains used in this study (Choi et al., 1996). However, TGMV and BGMV chimeric B components with exchanged Rep binding sites failed to replicate when either TGMV or BGMV Rep was supplied in *trans* (Fontes et al., 1994b; Gladfelter et al., 1997). Thus, the Rep binding site is necessary but not sufficient to confer virus-specific origin recognition between these more distantly related viruses. Origin recognition may involve a second *cis* element or may be influenced by protein/protein interactions in a virus-specific manner, as discussed in Section IV. It should also be noted that there is one example of a satellite DNA associated with TLCV infection that does not display tight specificity in its interactions between Rep and the plus-strand origin (Dry et al., 1997).

Although the Rep binding sites of Subgroup II and III geminiviruses act as virus-specific origin elements (Fontes et al., 1994b; Choi and Stenger, 1996; Gladfelter et al., 1997), they have related sequences that typically match the consensus, 5′-*GG-AG*TAYY*GG-AG*. Site-directed mutagenesis of the TGMV Rep binding site (5′-*GGTAG*TAA*GGTAG*) revealed that the sequence requirements of the two repeats are equivalent (Orozco et al., 1998). The conserved GG and AG dinucleotides are essential, whereas the T residue at the variable position is not. However, when the T residue was deleted such that the TGMV repeats resembled those of other geminiviruses like BGMV (5′-*GGAG*ACT*GGAG*) and abuliton mosaic virus (ABMV, 5′-*GGAG*TATT*GGAG*), the binding site was not functional. Similarily, changing the TGMV sequence between the two repeats to resemble BGMV did not interfere with function, whereas the addition of one residue between the repeats of BGMV abolished replication. Together, these results showed that spacing within and between the repeats is important for Rep/DNA binding and origin function. Choi and Stenger (1996) showed that spacing and sequence of the BCTV Rep binding sites are also important for strain-specific recognition. However, BCTV replication only required the center residues and right repeat of the consensus motif, whereas TGMV replication required both repeats.

The genomes of Subgroup II and III geminiviruses contain additional full or partial copies of their respective Rep binding sites that might act during viral replication and/or transcription (Arguello-Astorga et al., 1994b). Recent DNase I footprinting studies showed that the TLCV Rep protein binds to two related sequences in its 5′ intergenic region (Behjatnia et al., 1998). A partial copy of the TGMV Rep binding site also displays *in vitro* binding activity, but deletion or mutation of the site had no detectable effect on replication and only a minimal effect on transcription *in vivo* (Orozco et al., 1998). These results established that the TGMV partial site does not facilitate recruitment of Rep during replication and only makes a limited contribution to transcription. However, given the conservation of the reiterated Rep binding sites among Subgroup II and III genomes, it is possible that they have a role in some other aspect of the geminivirus life cycle.

Less is known about the mechanism whereby the Rep proteins of Subgroup I geminiviruses recognize their origins. Sequences analogous to the Rep binding sites of Subgroup II and III viruses have not been found in Subgroup I genomes. In addition, there is evidence suggesting that origin recognition by Subgroup I geminiviruses may not display the same degree of specificity as Subgroup II and III viruses (Heyraud et al., 1993b; Palmer and Rybicki, 1998). Based on sequence comparisons of eight Subgroup I geminiviruses, Arguello-Astorga et al. (1994b) proposed that Subgroup I Rep proteins bind to reiterated sequences in the stem of the hairpin and in the complementary-sense promoter of their 5′ intergenic regions. However, recent experiments visualizing WDV Rep/DNA complexes by electron microscopy indicated that like TGMV Rep, the WDV Rep protein binds to a sequence located upstream of the hairpin and between the TATA box and transcription start site of the complementary-sense promoter (Sanz-Burgos and Gutierrez, 1998). These results suggest that plus-stand ori-

gins of all three subgroups are configured similarly and that the Rep proteins of Subgroup I viruses may also autoregulate their own expression. Interestingly, the electron micrographs showed that WDV Rep binds DNA as a large protein complex to induce ca. a 90° bend in the DNA (Sanz-Burgos and Gutierrez, 1998). The apparent large size of the WDV Rep/DNA complex is consistent with the observation that TGMV Rep oligomerizes in solution to form octameric complexes (Settlage et al., 1996; Orozco et al. 1997).

The TGMV plus-strand origin overlaps the complementary-sense AL61 promoter, and they share three elements (Figure 2). In addition to its role in origin recognition, the Rep binding site is involved in negative regulation of transcription (Eagle et al., 1994). The TGMV plus-strand origin also contains two host transcription factor binding sites — a TATA box element located immediately upstream of the Rep binding site and a G-box motif at the base of the hairpin (Eagle and Hanley-Bowdoin, 1997). Similar motifs have been identified at the same positions in the 5′ intergenic regions of other geminiviruses and may have roles in viral replication as well as transcription (Fenoll et al., 1990; Arguello-Astorga et al., 1994b). Replication analysis of mutant TGMV origins revealed that neither host transcription factor binding site is essential for viral replication (Eagle and Hanley-Bowdoin, 1997). However, competition assays in the presence of wild-type origins demonstrated that both motifs contribute to efficient origin utilization. TATA box sequences and TATA-binding protein have been implicated in origin function in yeast (Lue et al., 1993) and may also be important for geminivirus replication. However, eukaryotic origins typically include AT-rich elements that facilitate duplex DNA unwinding during the initiation of replication (DePamphilis, 1993). The TATA box mutations in the TGMV AL61 promoter altered a region of high A/T content and may have impacted origin structure and function independently of the ability to recruit the TATA-binding protein. In contrast, the effect of the G-box mutation cannot be attributed to changing an origin structural element and, instead, is likely to reflect reduced affinity for a putative G-box factor. A G-box factor might facilitate Rep recruitment and binding to the origin, modulate chromatin assembly and origin accessibility, or stabilize an origin conformation required for efficient replication.

Two additional elements have been identified in the TGMV plus-strand origin (Figure 2). One element, the AG-motif, is between the Rep binding site and hairpin and is essential for origin function (Orozco et al., 1998). The importance of this motif is also supported by the replication characteristics of two closely related SqLCV strains (Lazarowitz, 1991). One contains a sequence homologous to the AG-motif and replicates efficiently in a variety of host species, whereas the other lacks the motif and displays restricted replication. Even though the AG-motif separates the TATA and G-box elements of the AL61 promoter, it has no detectable role in transcription. The second element, the CA-motif, is located outside of the minimal origin immediately upstream of the Rep binding site. Deletion of this sequence resulted in a 20-fold reduction of TGMV replication. Site-directed mutants that modified the conserved 5′-CCAAAA bps in this region replicated to high levels in the absence but not in the presence of a wild-type origin, suggesting that the CA-motif acts as an efficiency element. Recent studies suggested that the WDV plus-strand origin also consists of a minimal core sequence and flanking auxiliary sequences that contribute to efficient replication (Sanz-Burgos and Gutierrez, 1998). The roles of these various elements in either the TGMV or WDV origins are not known, but one possibility is that they bind host factors that facilitate initiation of plus-strand DNA replication. This hypothesis is consistent with the observations that like many protein binding sites, the TGMV AG-motif is palindromic in character.

The six elements in the TGMV plus-strand origin are closely spaced (Figure 2), suggesting that they may interact with each other during initiation of replication. This idea is supported by the observation that 5′ intergenic regions of other Subgroup III geminiviruses contain related sequences that are arranged similarly with the exception of a variable length sequence between the AG- and G-box motifs. In addition, TGMV origin activity is sensitive to changes in spacing and

phasing (Orozco et al., 1998), suggesting that there are interactions between two or more *cis* elements, most likely through the proteins that bind to them. To date, only Rep has been shown to bind the TGMV plus-strand origin and to be necessary for initiation of replication (Elmer et al., 1988a; Fontes et al., 1992). However, the complexity of the TGMV origin strongly supports the existence of other proteins that act with Rep to direct initiation of DNA replication.

B. Complementary- and Virion-Sense Promoters

The TGMV mRNAs have upstream sequences typical of eukaryotic RNA polymerase II promoters (Sunter and Bisaro, 1989a; Sunter et al., 1989b). Four initiate 20 to 30 bp downstream of TATA box motifs, whereas the remainder have sequences that resemble initiator elements overlapping their 5′ ends. The capacity of these upstream sequences to direct transcription has been confirmed for four RNAs — the complementary-sense AL61 and AL1629 mRNAs and the virion-sense AR1 and BR1 RNAs.

The AL61 RNA is the only transcript that specifies the full-length AL1 open reading frame. Its promoter was mapped to the TGMV A common region using reporter gene fusions and transient protoplast assays (Eagle et al., 1994). Equivalent sequences from ACMV also supported reporter expression in transgenic plants (Zhan et al., 1991). The abilities of the complementary-sense promoters from TGMV and ACMV to direct transcription in the absence of viral proteins further substantiated the role of host RNA polymerases in geminivirus transcription. Like many viral promoters, the TGMV AL61 promoter supports high levels of transcription. Deletion studies showed that most of its activity can be attributed to the 60 bp immediately preceding the AL61 transcription start site (Eagle and Hanley-Bowdoin, 1997; Settlage and Hanley-Bowdoin, unpublished results). This region, which fully overlaps the plus-strand origin, contains TATA-box and G-box motifs (Figure 2). Mutations in both host factor binding sequences were detrimental to promoter function (Eagle and Hanley-Bowdoin, 1997). In particular, G-box mutants displayed very low activity, indicating that this element is the primary transcriptional activating sequence. As discussed above, TATA and G-box mutations also influenced origin activity, indicating that they have dual roles in viral transcription and replication.

The TGMV AL61 promoter also includes the Rep binding site between the transcription start site and the TATA box. Analysis of transcription in the presence of Rep revealed that the AL61 promoter is autoregulated through the Rep binding site (Sunter et al., 1993; Eagle et al., 1994; Groning et al., 1994). Like replication, transcriptional repression is specific for the homologous Rep protein (Gladfelter et al., 1997). However, analysis of origin and Rep mutants demonstrated that the AL61 promoter is fully functional in the absence of replication (Eagle et al., 1994). Several features of Rep-mediated repression, including its dependency on the position and orientation of the Rep binding site, an intact G-box and specific host interactions, suggested that it occurs through active interference with the transcription apparatus and not by steric hindrance (Eagle et al., 1994; Eagle and Hanley-Bowdoin, 1997). The AL61 promoter is also negatively regulated by the AL4 protein, whose coding region overlaps the AL1 gene (Groning et al., 1994). The *cis* element involved in AL4 repression is distinct from the Rep binding site and located upstream of the G-box motif in the AL61 promoter (Eagle and Hanley-Bowdoin, 1997).

The complementary-sense promoters of many Subgroup II and III geminiviruses are configured like the TGMV AL61 promoter and are probably regulated through similar mechanisms. Haley et al. (1992) showed that the equivalent AC1 promoter of ACMV is negatively regulated by its Rep protein. However, there are several important differences between the ACMV and TGMV promoters (Hong and Stanley, 1995). Unlike TGMV, the ACMV AC1 promoter does not contain a G-box motif, and its activation appears to involve multiple *cis* elements. In addition, the AC1 promoter is not sensitive to negative regulation by its C4 protein. The putative Rep binding site of ACMV lacks repeated motifs, suggesting that its Rep protein contacts the promoter differ-

ently than TGMV Rep. This difference may account for the ability of ACMV but not TGMV Rep to repress a truncated promoter containing only the TATA box and the transcription start site. Nonetheless, like TGMV, the ACMV Rep protein cannot fully silence its promoter, suggesting that it also targets activated transcription.

The TGMV B component contains sequences that are homologous to the AL61 promoter, but the 5′ end of the BL1 mRNA does not correspond to the AL61 transcription start site (Sunter and Bisaro, 1989a). In addition, BL1 mRNA synthesis is not regulated by Rep (Sunter et al., 1993). Together, these results indicated that BL1 mRNA transcription utilizes different promoter sequences, most likely to bypass Rep inhibition. In contrast, virion-sense expression is regulated similarly for both genomic components.

The virion-sense promoters of TGMV A and B are not as well characterized as their complementary-sense counterparts. Transient reporter assays showed that common region DNA and downstream sequences, which contain the putative TATA box and transcription start site, are sufficient to support virion-sense transcription when the viral protein AL2 is supplied in *trans* (Groning et al., 1994; Sunter et al., 1997). Similar sequences from ACMV also direct transcription in an AC2-dependent manner (Haley et al., 1992). Like Rep-mediated repression of the AL61 promoter, AL2 activation of the AR1 and BR1 promoters is independent of replication. There is no information regarding the identities of the *cis* elements, including the AL2-responsive element, that contribute to AR1 or BR1 promoter activity. Recent studies in transgenic plants revealed that regulation of virion-sense transcription is complex (Sunter and Bisaro, 1997). An AR1 promoter fragment containing common region DNA and adjacent sequences was subject to AL2-mediated regulation in mesophyll cells. However, the same reporter construct was constitutively expressed in vascular tissue, suggesting that a regulatory element was missing. This element, which was mapped to sequences downstream of the AR1 gene, negatively regulated expression and conferred AL2 dependence in vascular tissue. Thus, virion-sense expression is controlled differently in different tissues. Similar experiments have not been performed with the AL61 promoter, but it is interesting that AL1-mediated repression is specific for certain cultured cell lines (Eagle et al., 1994).

The other TGMV TATA box-containing promoter is located upstream of the AL2 gene. Sequences upstream of the AL1629 transcription start site and overlapping the AL1 coding region directed reporter expression in tobacco protoplasts (Pedersen and Hanley-Bowdoin, unpublished results). Similar sequences from ACMV also displayed promoter activity (Zhan et al., 1991). Neither the TGMV nor ACMV promoter was responsive to Rep, AL2, or AL3 (Haley et al., 1992; Pedersen and Hanley-Bowdoin, unpublished results), and it is unclear how the AL2 promoter is regulated. One possibility is that the upstream AL61 promoter negatively influences the AL2 promoter, thereby providing for temporal regulation of geminivirus transcription. According to this scenario, the AL61 promoter and its B component homologue are active early in the viral life cycle. When sufficient Rep and/or AL4 protein accumulates, these promoters are repressed such that they no longer interfere with the function of downstream promoters. Thus, the AL2 and BL1 promoters are activated midway in the virus life cycle, whereas the AR1 and BR1 promoters are activated late after synthesis of the AL2 protein and possibly after viral replication. This expression strategy was first proposed by Howarth et al. (1985), but only one study has addressed the temporal aspects of the geminivirus life cycle. Brough et al. (1992b) showed that double-stranded TGMV DNA is present in tobacco protoplasts within 18 h, whereas the single-stranded form accumulates between 18 to 24 h posttransfection. They found that AR1 promoter activity was not dependent on viral replication but was 60- to 90-fold higher for a replicating vs. a nonreplicating template. These studies suggested that geminivirus replication and transcription are coordinated, but further experiments are required to define the temporal steps during infection.

There is limited information about the promoters of Subgroup I geminiviruses. Three TATA consensus sequences in the MSV genome may be involved in complementary-sense transcription (Boulton et al., 1991a). Mutation of one of the

TATA boxes correlated with reduced symptoms and restricted host range, possibly by influencing the synthesis of Rep and indirectly viral replication. In contrast, a single TATA box motif is located upstream of the most abundant MSV virion mRNA. Fenoll et al. (1988) showed that the MSV 5′ intergenic region can support virion-sense transcription in maize protoplasts and identified an upstream activating element in this sequence. The activating element was mapped to two GC-rich boxes on the distal side of the origin hairpin motif relative to the transcription start site (Fenoll et al., 1990). DNA binding activity specific for the MSV GC-rich boxes was detected in maize nuclear extracts. This activity most likely corresponded to a host transcription factor that activates virion-sense expression. Interestingly, the MSV GC-rich boxes and the TGMV G-box element are positioned similarly with respect to their hairpins, implying that the MSV transcription element and its protein factor may also have roles in replication (Arguello-Astorga et al., 1994b). The virion promoters of WDV and chloris striate mosaic virus (CSMV) were also enhanced in the presence of an intact C2 open reading frame or a Rep expression cassette (Hofer et al., 1992; Zhan et al., 1993). Thus, Subgroup I geminiviruses may encode a transactivator of virion-sense transcription analogous to the AL2/C2 proteins of Subgroup III viruses. Recent experiments identified an intrinsically bent DNA sequence in the virion-sense promoter of WDV that might influence its activity (Suarez-Lopez et al., 1995). Another key regulatory component of Subgroup I expression is likely to be alternative splicing of both complementary and virion-sense RNAs (Wright et al., 1997), as discussed in Section III.B.

Bidirectional transcription and alternative splicing are used by other DNA viruses to express their genomes, with the best-characterized example being the papovavirus, simian virus 40 (SV40; for review see DePamphilis, 1987). The SV40 genome has two expression units that are divergently transcribed. One unit encodes the "early" proteins involved in replication and gene expression, whereas the other unit specifies "late" proteins that are structural components of the virus. The early genes are expressed prior to viral replication, whereas the late genes are transcribed after replication. SV40 also uses alternative splicing to expand its repertoire of viral proteins. Differential splicing is also a regulatory feature of other plant viral and host genes (Nash et al., 1992; Kiss-Laszlo et al., 1996).

V. VIRAL REPLICATION AND TRANSCRIPTION FACTORS

Genetic studies described in Section II established that the Rep and AL3/C3 proteins are involved in geminivirus replication and that Rep and AL2/C2 play important roles in regulating viral gene expression. This section discusses biochemical characterization of these viral replication and transcription factors.

A. The AL1/C1/Rep Protein

The Rep protein localizes to nuclei of infected plant cells (Nagar et al., 1995), where it plays key roles in geminivirus DNA replication and transcription (Laufs et al., 1995a). Rep confers virus-specific recognition of its cognate origin of replication (Choi and Stenger, 1995; Jupin et al., 1995; Gladfelter et al., 1997) and initiates plus-strand DNA replication (Heyraud-Nitschke et al., 1995; Laufs et al., 1995c; Orozco and Hanley-Bowdoin, 1996). It represses its own expression at the level of transcription (Sunter et al., 1993; Eagle et al., 1994) and may activate virion-sense transcription of some geminiviruses (Hofer et al., 1992; Zhan et al., 1993). In addition, Rep induces the expression of a host DNA synthesis protein, proliferating cell nuclear antigen, in non-dividing plant cells (Nagar et al., 1995), possibly through interactions with the plant cell cycle machinery.

DNAs that replicate through rolling circle mechanisms encode a protein that catalyzes initiation and termination of plus-strand DNA synthesis. Geminivirus Rep proteins contain three conserved amino acid sequences that are related to motifs found in initiator proteins of other rolling circle systems (Figure 3; Ilyina et al., 1992; Koonin et al., 1992). Laufs et al. (1995c) demonstrated that TYLCV Rep specifically cleaves and

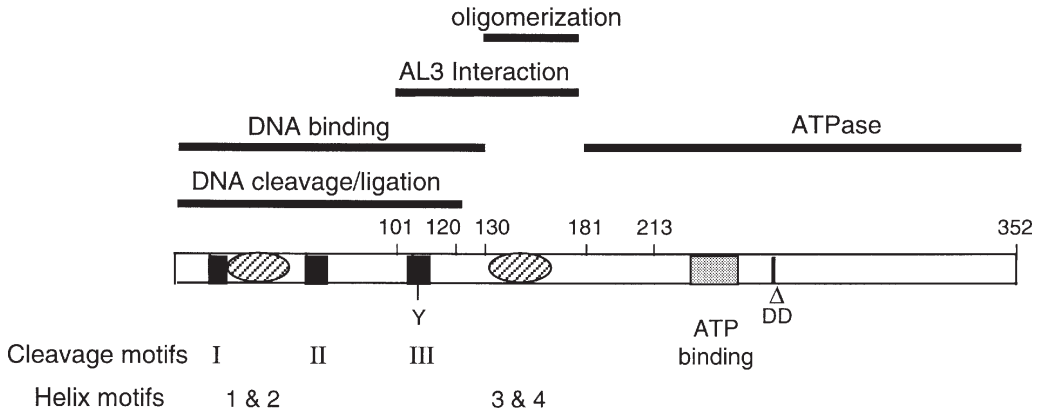

FIGURE 3. TGMV Rep domains and predicted motifs. Solid boxes mark the locations of the three conserved DNA cleavage motifs in the Rep protein. The active site tyrosine residue is shown in Motif III. The stippled box shows the location of the ATP binding motif which in combination with the indicated conserved aspartic acid residues are related to DNA helicases. 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, AL3 interaction, DNA binding, and DNA cleavage/ligation. The numbers correspond to amino acid positions in Rep.

ligates viral plus-strand DNA within the loop of the hairpin motif. DNA cleavage/ligation activity has also been detected for WDV and TGMV Rep (Heyraud-Nitschke et al., 1995; Orozco and Hanley-Bowdoin, 1996 and 1998). Several lines of evidence established the importance of this activity for geminivirus replication *in vivo*. First, the initiation site for rolling circle replication has been mapped to a conserved loop in several geminivirus genomes *in vivo* (Stenger et al., 1991; Heyraud et al., 1993a; Heyraud et al., 1993b). Second, the initiation site for replication and the *in vitro* cleavage site are at the same positions (Stanley, 1995). Third, mutations in the loop that block cleavage *in vitro* also interfere with replication *in vivo* (Orozco and Hanley-Bowdoin, 1996). Similarly, mutations in Rep that inhibit cleavage *in vitro* also block replication *in vivo* (Laufs et al., 1995b; Hoogstraten et al., 1996; Orozco and Hanley-Bowdoin, 1998).

DNA cleavage activity of Rep has only been detected using single-stranded oligonucleotide substrates and not when the same oligonucleotides were paired with their complements (Laufs et al., 1995c; Orozco and Hanley-Bowdoin, 1996). This suggests that the cleavage substrate is the ssDNA loop region of the conserved hairpin. Genetic experiments established that the hairpin structure is required for TGMV origin function (Orozco and Hanley-Bowdoin, 1996). However, high-molecular-weight genomic concatemers were observed for WDV when its hairpin was deleted (Kammann et al., 1991). The initiation site for plus-strand synthesis in the WDV deletion mutant mapped to a cryptic site that contained a partial copy of the hairpin motif that is unable to form a cruciform structure (Heyraud et al., 1993a). Thus, in TGMV replication, the hairpin structure appears to be required for cleavage during initiation and termination of plus-strand DNA synthesis, whereas the structure may only be required for termination of WDV plus-strand replication. There is precedence for the involvement of cruciform structures in initiation of plus-strand DNA synthesis of some bacterial plasmids and in origin function of some eukaryotic viruses (Lefebvre et al., 1984; Gros et al., 1987; Williams et al., 1993). Interestingly, the pT181 RepC protein only cleaves single-stranded DNA that results from extrusion of a cruciform in the plus-strand origin of the bacterial plasmid (Jin et al., 1997).

Several other biochemical activities have been demonstrated for Rep that are likely to contribute to its function *in vivo*. Fontes et al. (1992 and 1994a,b) showed that the Rep proteins of TGMV and BGMV bind dsDNA in a sequence-specific manner. The functional importance of the dsDNA binding activity was demonstrated by the nega-

tive impact of mutations in the TGMV Rep recognition site on binding *in vitro* and on viral replication and transcriptional repression *in vivo* (Eagle et al., 1994; Fontes et al., 1994a). Conversely, TGMV Rep mutants that are impaired for DNA binding *in vitro* are not functional *in vivo* (Orozco and Hanley-Bowdoin, 1998). TGMV and BGMV Rep display tight binding specificity for their cognate binding motifs *in vitro* (Fontes et al., 1994b). This specificity is consistent with studies showing that TGMV but not BGMV Rep supports replication of TGMV B and repression of the TGMV AL61 promoter, whereas the opposite is true for BGMV B and the BGMV AL1 promoter (Gladfelter et al., 1997). Mutant TGMV and BGMV origins with exchanged Rep binding sites further established the requirement for specific Rep/DNA binding during replication *in vivo*. The putative Rep binding motifs of two BCTV strains have also been implicated in Rep-specific replication *in vivo* (Choi and Stenger, 1996). Together, these results strongly support a role for Rep/dsDNA binding during origin recognition and transcriptional repression. TGMV Rep also binds single-stranded DNA with high affinity in a sequence-specific manner (Thommes et al., 1993). The ssDNA binding activity might be involved in recruiting Rep to the hairpin loop for DNA cleavage. This hypothesis is supported by the observations that DNA cleavage is not dependent on the origin recognition site such that Rep must also specifically recognize and interact with its cleavage site (Orozco and Hanley-Bowdoin, 1996) and that the DNA cleavage and binding activities of TGMV Rep display nearly identical amino acid sequence requirements (Orozco and Hanley-Bowdoin, 1998).

All geminivirus Rep proteins contain a sequence that is related to the consensus nucleotide triphosphate binding motif and show weak identity to DNA helicases (Figure 3; Gorbalenya et al., 1989; Gorbalenya and Koonin, 1993). ATPase activity has been detected for TYLCV and TGMV Rep (Desbiez et al., 1995; Orozco et al., 1997), whereas no helicase activity has been reported for a geminivirus replication protein. In addition, the DNA-independent nature of the ATPase activity is not consistent with Rep proteins acting as DNA helicases. One possibility is that ATP hydrolysis mediates a conformational change that is necessary for initiation of plus-strand synthesis. This idea is compatible with the findings that mutation of the NTP binding motif inhibits the ability of Rep to support viral replication but has no impact on Rep-mediated transcriptional repression (Eagle et al., 1994; Desbiez et al., 1995; Hanson et al., 1995) and that the specific Rep/DNA contacts necessary for transcriptional repression are a subset of those required for replication (Gladfelter et al., 1997). One possible scenario is that the initial contacts between Rep and its binding site are conserved between replication and transcription. The initial Rep/DNA complex is able to repress transcription but must undergo an ATP-dependent conformational change and form additional virus-specific contacts prior to replication. These contacts could reflect protein/DNA or protein/protein interactions. Protein binding followed by rearrangement of the origin complex and alteration of the initial contacts during replication initiation has been described for other small DNA viruses (Frattini et al., 1994; SenGupta et al., 1994).

Rep proteins are also involved in multiple protein/protein interactions. Settlage et al. (1996) showed that TGMV and BGMV Rep both form oligomers in a virus-nonspecific manner. Size exclusion chromatography indicated that TGMV Rep protein complexes are large, containing approx. eight subunits (Orozco et al., 1997). Rep oligomerization could contribute to replication and transcription activity in several ways. During cleavage, a single tyrosine residue in the active site is covalently crosslinked to the 5′ end of the cleaved DNA (Laufs et al., 1995b) and is not available for the second cleavage event that resolves the DNA concatemer. Rep dimerization could provide a second active site tyrosine for termination of rolling circle replication. There is precedence for dimerization of replication initiator proteins in other rolling circle systems (Wang et al., 1993). However, recent experiments using a coupled cleavage/ligation assay suggested that oligomerization is not necessary for these activities *in vitro* (Orozco and Hanley-Bowdoin, 1998). In contrast, the DNA binding activity of Rep is dependent on multimerization. This dependency was established by experiments showing that the

DNA binding domain of TGMV Rep is functional only when fused to either the homologous or a heterologous protein interaction domain (Orozco and Hanley-Bowdoin, 1998). TGMV Rep also interacts with the viral replication accessory factor AL3 and with a plant retinoblastoma factor (Settlage et al., 1996; Ach et al., 1997). These interactions are discussed in Sections V.B and VI.

Recent experiments have begun to identify the domains and amino acids that mediate the different Rep functions (Figure 3). The N-termini of the Rep proteins from WDV, TYLCV, and TGMV are sufficient to support DNA cleavage and ligation *in vitro* (Heyraud-Nitschke et al., 1995; Orozco et al., 1997). The smallest peptide with cleavage/ligation activity includes amino acids 1 to 120 from TGMV Rep (Orozco et al., 1997). This region contains the conserved Motifs I, II, and III that are associated with the initiator proteins of other rolling circle replication systems (Ilyina and Koonin, 1992). Motif III ($YXXK_{107}$) corresponds to the active site for DNA cleavage. The conserved Y_{104} residue is involved in nucleophilic attack of the phosphodiester DNA backbone and is covalently linked to the 5′ end of the cleaved DNA (Laufs et al., 1995a,b). In contrast, the function of K_{107} is not known. Mutation of Y_{104} and K_{107} blocked DNA cleavage and replication by TYLCV and TGMV Rep proteins (Laufs et al., 1995b; Orozco and Hanley-Bowdoin, 1998). Mutation of Y_{104} and K_{107} also inhibited the replication activity of BGMV Rep (Hoogstraten et al., 1996). Deletion and site-directed mutagenesis of Motifs I ($FLTY_{18}$) and II (HLH_{60}) of TGMV Rep also blocked DNA cleavage and replication, but the precise roles of these motifs are not known (Orozco and Hanley-Bowdoin, 1998). Mutations in the three motifs also interfered with Rep/dsDNA binding, suggesting that DNA cleavage and binding are closely associated in TGMV Rep. This tight association is underscored by the observation that a Y104A mutation in TGMV Rep interfered with DNA cleavage and binding, whereas a Y104F mutation only blocked cleavage (Orozco and Hanley-Bowdoin, 1998). Thus, the aromatic ring of Y104 is essential for DNA binding, while the hydroxyl group is required for DNA cleavage.

The dsDNA binding, oligomerization, and AL3 interaction domains of TGMV Rep also map to the N-terminal half of the protein (Orozco et al., 1997; Settlage and Hanley-Bowdoin, in preparation). Amino acids 1 to 130 are sufficient for DNA binding, amino acids 134 to 181 confer oligomerization activity, and amino acids 101 to 181 are necessary for AL3 interaction (Figure 3). The overlap between the AL3 interaction and Rep oligomerization domains may indicate that they are mutually exclusive events. Alternatively, AL3/Rep interactions may require Rep oligomers. The ability of AL3 to form oligomers and the relatively weak nature of AL3/Rep interactions are consistent with this idea (Settlage et al., 1996). Recent results demonstrated that Rep oligomerization is a prerequisite for DNA binding *in vitro* (Orozco and Hanley-Bowdoin, 1998). Interestingly, *in vivo* studies that used chimeric proteins and origins to map the origin recognition domains implicated both the DNA binding and oligomerization domains of Rep in origin recognition. The first 116 amino acids of TGMV and BGMV Rep interacted with their respective origin binding sites in replication assays (Figure 3; Gladfelter et al., 1997), most likely reflecting specific protein/DNA contacts. Similarly, the first 89 and 116 amino acids of BCTV and TYLCV Rep, respectively, conferred virus-specific replication (Choi and Stenger, 1995; Jupin et al., 1995; Choi and Stenger, 1996). However, analysis of TGMV and BGMV chimeras revealed that amino acids 121 and 208 also play a role in origin recognition (Figure 3; Gladfelter et al., 1997). Although this region might interact with an unknown *cis*-element in the origin, a more likely explanation is that the second determinant corresponds to the Rep oligomerization domain. Rep oligomerization could influence origin recognition specificity by altering the spatial arrangement of the Rep protein complex. Small changes in the three-dimensional structure of the Rep complex might affect origin recognition by modifying the way bound Rep interacts with its hairpin cleavage site or with other origin binding proteins dependent on the spacing of the corresponding *cis* elements. Architectural constraints imposed by protein interactions and DNA structure are important during recombination (DeVargas et al., 1989) and for enhancer function during transcription (Falvo et al., 1995; Giese et al., 1995).

The N-termini of Rep proteins from dicot-infecting geminiviruses contain two sets of highly predicted, conserved α-helices (Orozco et al., 1997). Helices 1 and 2 between TGMV Rep amino acids 25 to 52 overlap the DNA binding and cleavage domains (Figure 3). The sequences of both helices show a high degree of homology among different geminiviruses, especially helix 2, which is conserved at 9 of 11 positions and displays a strong amphipathic character (Orozco et al., 1997). Mutations in either helix inhibited DNA binding and cleavage *in vitro* and viral replication *in vivo*, thereby demonstrating the importance of these motifs for Rep function and further supporting the close relationship between DNA cleavage and binding (Orozco and Hanley-Bowdoin, 1998). Helical motifs have not been described in other rolling circle initiator proteins, but most DNA binding motifs include α-helical regions that recognize and contact DNA (Pabo et al., 1992). However, the Rep N-terminus shows no obvious homology to the α-helical motifs of basic/helix-loop-helix, ets, homeodomain, zinc finger, or basic/leucine zipper proteins (for review see Harrison, 1991; Pabo and Sauer, 1992; Nelson, 1995). AL1 helices 1 and 2, which are separated by a 5 amino acid loop, most resemble the helix-turn-helix motif, but sequence analyses failed to uncover a nearby third helix characteristic of most helix-turn-helix DNA binding domains (Wintjens et al., 1996). The second set of predicted α-helices is located between TGMV Rep amino acids 131 to 152 in the Rep oligomerization domain (Figure 3). Several classes of DNA binding proteins, including members of the basic/helix-loop-helix, homeodomain, and basic/leucine zipper families, use α-helices for dimerization (Murre et al., 1989; Pabo and Sauer, 1992; Gehring et al., 1994). However, mutational studies indicated that although the second set of helices in TGMV Rep may contribute to complex formation, sequences downstream form the core oligomerizaton domain (Orozco, Ling, Elledge, and Hanley-Bowdoin, in preparation).

Very little is known about the C-terminus of the Rep protein. To date, the only biochemical activity that has been attributed to the C-terminus is ATP hydrolysis (Desbiez et al., 1995). The ATPase domain of TGMV Rep was mapped between amino acids 182 to 352 (Orozco et al., 1997). This region contains a conserved motif ($GXXXXGKT_{230}$) that corresponds to the P loop of the phosphate binding site of many proteins that bind or hydrolyze NTPs (Walker et al., 1982; Gorbalenya and Koonin, 1989). Site-directed mutations that altered the lysine residue in the P loop of TYLCV Rep impaired ATP hydrolysis *in vitro* and replication *in vivo* (Desbiez et al., 1995), establishing the functional significance of this motif. Mutations in the P-loop sequence of BGMV Rep also interfered with virus infectivity and replication, with some of the mutants displaying temperature sensitive phenotypes (Hanson et al., 1995). Interestingly, P-loop mutations only blocked the replication activity of TGMV Rep and had no effect on its ability to repress transcription (Eagle et al., 1994). It is likely that other, yet-to-be-identified activities are also associated with the Rep C-terminus. Deletion of only 39 amino acids from the TGMV Rep C-terminus abolished DNA replication and repression (Eagle, Orozco and Hanley-Bowdoin, unpublished results), indicating that this region is required for protein function *in vivo*. Deletion of the C-terminal 139 amino acids of TGMV Rep also enhanced dsDNA binding activity, suggesting that a negative effector of DNA binding may be located in this region. Many transcription factors contain regions that inhibit their DNA binding activity unless complexed with other proteins or cofactors (Janknecht et al., 1993; Bardwell et al., 1994). The Rep C-terminus may also mediate other protein interactions, ssDNA binding, nuclear localization, and/or attachment to the nuclear matrix.

B. The AL3/C3 Protein

The AL3/C3 protein greatly enhances viral DNA accumulation of Subgroup II and III geminiviruses through an unknown mechanism (Elmer et al., 1988a; Sunter et al., 1990). TGMV AL3 is located in nuclei of infected plant cells at levels similar to the Rep protein (Pedersen et al., 1994; Nagar et al., 1995), suggesting that it might act with AL1 during initiation of viral DNA replication. There is only limited information regard-

ing the biochemical properties of AL3. Two protein interactions have been demonstrated for TGMV and BGMV AL3 — oligomerization and interaction with Rep (Settlage et al., 1996). Neither of these interactions displays virus specificity, consistent with the ability of AL3/C3 proteins from different geminiviruses to functionally substitute for each other in replication assays (Sunter et al., 1994; Gladfelter et al., 1997).

The mechanism by which AL3 enhances viral DNA accumulation may reside in its ability to interact with Rep. The AL3 protein sequence shows no homology to any known enzymatic motifs. Thus, it is more likely that the structure of the AL3/Rep complex is important for replication rather than a catalytic activity of AL3 that affects Rep. Two experimental observations suggested that AL3 might increase the affinity of Rep for the origin, analogous to the effect of E2 on E1 during papillomavirus replication (Mohr et al., 1990; Li et al., 1994). TGMV Rep overcame a detrimental origin binding site mutation in the presence of AL3 (Fontes et al., 1994a), and BGMV Rep only supported replication from a chimeric origin in the presence of its homologous AL3 (Gladfelter et al., 1997). Another possibility is that AL3 directs Rep to its cleavage site in the origin during replication of Subgroup II and III geminiviruses. In these viruses, the Rep/DNA binding site is located upstream and distal from the hairpin where Rep catalyzes DNA cleavage. This separation is likely to have an adverse affect on replication unless a mechanism exists to direct bound Rep to its cleavage site. AL3 might enhance replication by performing this function. Although Subgroup I geminiviruses do not encode an AL3 homologue, it is possible that their unique RepA protein translated from the unspliced C1 intron might serve a similar function. AL3/Rep complex formation might also enhance the DNA cleavage/ligation (Laufs et al., 1995c; Orozco and Hanley-Bowdoin, 1996) or putative helicase (Koonin et al., 1991) activities of Rep.

Alternatively, interaction with Rep may only be necessary to recruit AL3 to the origin where it then influences some other aspect of initiation. This idea is consistent with lack of detectable AL3/DNA binding activity. AL3 might be analogous to transcription factors that enhance replication by stimulating the assembly or activity of the initiation complex on the origin or by overcoming chromatin-mediated repression of initiation (DePamphilis, 1988). Interestingly, the acidic N-terminus of the AL3 protein resembles some transcriptional activation domains (Cress, 1991).

C. The AL2/C2 Protein

The AL2/C2 protein is a nuclear protein (Sanderfoot et al., 1995) that transactivates virion-sense gene expression of Subgroup III geminiviruses in a virus-nonspecific manner (Sunter and Bisaro, 1991; Sunter et al., 1994; Saunders et al., 1995). Nuclear run-on experiments established that activation is at the level of transcription (Sunter et al., 1992). Sunter and Bisaro (1997) showed that TGMV AL2 regulates virion-sense expression through two distinct mechanisms. In transgenic plants containing AR1 promoter-reporter fusions, AL2 activated the promoter in mesophyll cells and derepressed it in phloem tissue. Hong et al. (1996) also showed that ACMV infection activates a transgene that is under the control of the AV1 promoter. Together these experiments showed that AL2 can activate a gene in a chromosomal context and might transactivate host genes during the infection process.

Little is known about the mechanisms of AL2-mediated transcriptional activation. DNA binding studies showed that recombinant TYLCV C2 and potato yellow mosaic virus (PYMV) AC2 proteins bind ssDNA with high affinity and dsDNA with low affinity (Noris et al., 1996; Sung et al., 1996). Competition assays revealed that both DNA binding activities is sequence independent. The DNA binding domain was mapped to amino acids 33 to 103 in the center of TYLCV C2 (Noris et al., 1996). This region of AL2/C2, which is rich in cysteines and histidines, might form a zinc finger to mediate DNA binding (Coleman, 1992; Sunter and Bisaro, 1992). The AL2/C2 protein also includes a basic N-terminus and an acidic C-terminus, and its organization is similar to that of the T4 phage gene 32 protein, a single-stranded DNA binding protein that is also involved in viral replication (Noris et al., 1996). However, no role has been detected for AL2/C2 in geminivirus replication, and similarly charged domains are found in many eukaryotic transcription factors (Cress, 1991; Pabo and Sauer, 1992).

The lack of sequence-specific DNA binding by AL2/C2 is not consistent with its ability to specifically activate virion-sense transcription. Noris et al. (1996) suggested that the lack of specificity may reflect a deficiency in the recombinant proteins used for binding assays. It is possible that specific DNA binding requires a post-translational modification or a cofactor that is absent from *E. coli*-produced proteins. Alternatively, the DNA binding activity of AL2/C2 may be unrelated to its transactivation activity. Herpes simplex virus VP16 and adenovirus E1A proteins are potent transcriptional activators that do not bind DNA. Instead, they are recruited through protein interactions to promoters where they interact with the basal transcriptional apparatus to activate RNA synthesis (Liu et al., 1994; Misra et al., 1996; Ptashne et al., 1997). Geminivirus AL2/C2 proteins may use a similar mechanism to activate virion-sense transcription. The ability of the acidic region in the AL2 C-terminus to confer transcriptional activation to a heterologous DNA binding domain is consistent with this hypothesis (D. Bisaro, personal communication).

VI. GEMINIVIRUS/HOST INTERACTIONS

Geminiviruses depend on host DNA replication and transcription machinery. However, the host factors required for these viral processes and the mechanism whereby geminiviruses become established in plant nuclei are not known. Recent studies have begun to provide insight into how gemini-viruses interact with and modify their hosts during infection. This section discusses these interactions as they relate to replication and expression of both viral and host genomes.

A. DNA Replication

During development, plant cells leave the cell division cycle and lack detectable levels of DNA replication enzymes after differentiation (Coello et al., 1992; Daidoji et al., 1992; Nagar et al., 1995). DNA replication and cell division are confined to apical meristems, developing leaves and the cambium of mature plants (Martinez et al., 1992; Staiger et al., 1993). Hence, viral replication may be restricted to meristematic cells or geminiviruses may modify differentiated cells to induce the synthesis of replication enzymes. Some geminiviruses, like BCTV, AbMV, and SqLC, are restricted to the phloem (Esau, 1977; Abouzid et al., 1988; Horns et al., 1991; Sanderfoot et al., 1996b) and may replicate in procambial cells using preexisting plant machinery. However, other geminiviruses are not confined to vascular tissue and, instead, are found in a variety of tissues. TGMV particles, DNA, and replication proteins are in nuclei of differentiated cells throughout the leaf, stem, and root of infected *N. benthamiana* plants (Rushing et al., 1987; Nagar et al., 1995; Nagar, Bass, Hanley-Bowdoin and Robertson, in preparation). MSV DNA is in vascular and mesophyll cells of mature corn leaves (Lucy et al., 1996). Interestingly, neither virus was detected in meristematic cells. Analysis of a BDMV variant expressing green fluorescent protein showed that the virus localizes to vascular tissue in stem and root but is found throughout the leaf (Sudarshana et al., 1998). Together these results suggested that geminiviruses can replicate in differentiated plant cells after induction of host machinery. This hypothesis was strongly supported by immunohistochemical data showing that proliferating cell nuclear antigen (PCNA), the processivity factor of host DNA polymerase δ (Brown et al., 1993), accumulates in differentiated cells of TGMV-infected plants but not in equivalent healthy cells (Nagar et al., 1995). PCNA was also detected in differentiated cells of transgenic plants expressing TGMV Rep, thereby establishing that the Rep protein is sufficient for host induction.

Geminiviruses are analogous to some animal DNA viruses in their reliance on host replication machinery and their ability to replicate in differentiated cells. Mammalian DNA viruses use a combination of mechanisms to induce replication proteins in their hosts. Adenovirus E1A and SV40 large T antigen directly activate transcription of some host genes through interactions with host transcription factors (Lee et al., 1991; Gruda et al., 1993; Labrie et al., 1995; Damania et al., 1996; Eckner et al., 1996). Recent experiments showed that TGMV infection modifies PCNA transcriptional controls to induce mRNA and pro-

tein accumulation in differentiated plant cells (Egelkrout, Robertson, and Hanley-Bowdoin, in preparation). By analogy with mammalian DNA tumor antigens, TGMV Rep might activate PCNA transcription directly. The sequence-specific DNA binding (Fontes et al., 1992) and the transcriptional regulatory properties of TGMV Rep (Eagle and Hanley-Bowdoin, 1997) are consistent with a direct role in transcriptional activation.

Mammalian viral proteins are also involved in protein interactions that modify cell cycle controls. SV40 large T-antigen, adenovirus E1A, and human papillomavirus E7 bind the retinoblastoma proteins — pRB, p107 and p130 — and cause the release of E2F transcription factors (for review see Hamel et al., 1992; Nevins, 1992; Lam et al., 1994). E2F factors in turn induce transcription of genes encoding proteins required for DNA replication and cell cycle progression. Several lines of evidence suggested that geminiviruses may also induce replication competency in their hosts through interactions with plant cell cycle regulatory components. First, the Rep proteins of several geminiviruses have proven recalcitrant to stable constitutive expression in transgenic plants, indicating that they interfere with a fundamental process like cell division. Second, some of the morphological changes associated with plant cell dedifferentiation, that is, nuclear rounding and migration to the cell center, are observed in TGMV-infected and AL1-transformed plant cells (Nagar et al., 1995), suggesting that Rep can initiate the dedifferentiation process. Third, cDNAs corresponding to plant pRB homologues have been isolated from maize (Grafi et al., 1996; Xie et al., 1996; Ach et al., 1997; Murray, 1997). There is no data demonstrating that these plant "tumor suppressor" proteins function in cell cycle regulation, but the maize pRB proteins resemble their mammalian counterparts in several key ways. They contain A and B pocket domains, which are involved in many of the protein interactions of mammalian pRB proteins (for review see Wang et al., 1994). The maize proteins also interact with SV40 large T antigen, papillomavirus E7, and plant cyclin δ through LXCXE motifs (Grafi et al., 1996; Ach et al., 1997). These motifs are found in many mammalian proteins that bind pRB (Ewen et al., 1993). Fourth, WDV RepA and TGMV Rep interact with pRB proteins from human (Xie et al., 1995; Collin et al., 1996) and maize (Grafi et al., 1996; Xie et al., 1996; Ach et al., 1997). Unlike TGMV Rep, no interaction between the full-length WDV Rep and pRB has been detected (C. Gutierrez, personal communication). WDV RepA interacts with the hypophosphorylated form of maize pRB through a canonical LXCXE motif (Grafi et al., 1996; Xie et al., 1996). However, none of the Rep proteins of Subgroup II and III geminiviruses, including TGMV, contain the LXCXE consensus and, thus, must interact with pRB through a different amino acid motif. The interactions between pRB and the Rep or RepA proteins are intriguing, but to date there is no direct evidence that these protein complexes form during infection and are necessary for host induction.

Other geminivirus proteins may also be involved in reprogramming plant cells. One of the characteristics of BCTV infection is vein swelling in systemically infected leaves. Microscopic studies revealed that the enations are due to cell division and expansion of phloem parenchyma cells (Esau et al., 1978). Mutation of the BCTV C4 gene resulted in an infectious but asymptomatic virus, suggesting that there was a link between C4 and the changes in phloem morphology. Recent studies (Latham et al., 1997) showed that transgenic *N. benthamiana* plants that ectopically express BCTV C4 develop abnormally and produce tumors. Thus, BCTV C4 can cause cell division in plants in the absence of other viral proteins. However, because C4 mutants are infectious, another viral protein must be the primary determinant of host induction. The most likely candidate is BCTV Rep, which could not be expressed constitutively in transgenic plants (Latham et al., 1997).

If geminiviruses cause mature plant cells to reenter the cell division cycle and progress into S phase, an important question is what happens to host chromosomal DNA. *In situ* hybridization studies revealed that nuclear architecture is altered in TGMV-infected cells (Nagar, Bass, Hanley-Bowdoin, and Robertson, in preparation). Viral DNA occurs as small and large inclusions in the interior of the nucleus, whereas host DNA is located predominantly at the periphery (Figure 4).

FIGURE 4. Tomato golden mosaic virus DNA localizes to distinct areas in the nucleus. Fixed sections from TGMV-infected *N. benthamiana* plants 9 days postinoculation were hybridized with Texas Red®-labeled oligonucleotides complementary to the plus strand of TGMV A and then incubated with anti-AL1 primary antibodies and Marina Blue®-labeled secondary antibodies to detect AL1. Some cells, such as this stem pith cell, naturally contain two nuclei that exhibit asynchronous stages of infection. TGMV DNA accumulates in discrete areas of the nucleus (right panel), while AL1 shows a diffuse nuclear staining pattern (left panel). The image on the left was photographed using UV fluorescence and shows autofluorescence that is distinct from AL1 signal in a color image. Both nuclei contain AL1 (left panel), but only one of them (right, arrow) has discrete areas of TGMV DNA accumulation.

This compartmentalization may account for the apparent rarity of geminivirus DNA integration into plant chromosomes (Bejarano et al., 1996). In some infected cells, chromosomal DNA is condensed, suggesting that it is not actively replicated or transcribed. However, TGMV-mediated induction of the PCNA promoter (Egelkrout, Robertson, and Hanley-Bowdoin, in preparation) and constitutive expression of the EF-4A and histone 2A genes in infected cells (Sampson, Thompson. and Robertson, unpublished results) established that at least some chromosomal genes are accessible to the transcription apparatus. Similarly, in bromodeoxyuridine (BrdU) incorporation experiments, chromosomal as well as viral DNA was efficiently labeled by the nucleotide analog (Nagar, Hanley-Bowdoin and Robertson, in preparation), suggesting that chromosomal DNA also replicates in infected cells. These results strongly support the idea that geminiviruses modify plant cell cycle controls to create an S phase-like state that can sustain high levels of DNA replication.

Much of the geminivirus infection cycle, including viral replication and transcription, occurs in the nucleus. However, there is limited evidence that geminiviruses are also in other cellular compartments. AbMV DNA was detected in intact, purified chloroplasts (Groning et al., 1987; Groning et al., 1990). The detection of both single- and double-stranded forms implied that AbMV DNA can replicate in plastids, which are prokaryotic in character. Recently, Rigden et al. (1996) showed that TLCV replicates in *Agrobacterium tumefaciens* in a Rep-dependent fashion. These results suggest that geminiviruses may have descended from bacterial episomes and that their genomes have retained the capacity to be replicated by prokaryotic enzymes.

B. Gene Expression

Several host factors have been implicated in the transcription of geminivirus genes. Many viral promoters have TATA box elements upstream of their transcription start site (Boulton et al., 1991a; Eagle and Hanley-Bowdoin, 1997), indicating that the plant basal transcription apparatus is necessary for geminivirus expression. The complementary-sense promoter for Rep expression of many dicot-infecting viruses contains a G-box element that is essential for activity (Arguello-Astorga et al., 1994b; Eagle and Hanley-Bowdoin, 1997). The G-box element is found in a variety of plant promoters (for review see de Vetten et al., 1994), and several plant proteins specifically bind the G-box motifs and activate transcription. These proteins typically include a basic/leucine zipper domain that mediates protein dimerization and DNA binding as well as a proline-rich region that confers transcriptional activation (Schindler et al., 1992). However, there is considerable diversity among G-box factors, and the factor involved in geminivirus gene expression has not been identified.

Host factors also mediate AL2-independent expression of the TGMV AR1 gene in the phloem (Sunter and Bisaro, 1997). The sequences required for phloem expression are between positions -163 and -107 relative to the AR1 transcription start site and do not include the G-box element located at position -200 (Meyer, Sunter, and Bisaro, in preparation). A tissue-specific, negative regulatory element has also been identified downstream of the TGMV AR1 coding region (Sunter and Bisaro, 1997). There is no information regarding the host factors that regulate TGMV AR1 expression through either element. Fenoll et al. (1988, 1990) showed that the virion-sense promoter of MSV includes a GC-rich element that is required for activation and specifically binds plant nuclear proteins *in vitro*.

Host transcription factors also play a role in geminivirus replication. The TGMV complementary-sense promoter and plus-strand origin overlap (Figure 2). Mutations in the TATA box and G-box motifs impaired origin efficiency as well as promoter function (Eagle and Hanley-Bowdoin, 1997). The G-box element is of particular interest because of its conserved location at the base of the hairpin in many geminivirus genomes (Arguello-Astorga et al., 1994b). The corresponding G-box factor represents the first example of a plant transcription factor that also acts as a replication factor. Initiation of DNA replication and transcription both require the recruitment and assembly of large protein complexes on DNA. These common requirements have resulted in proteins that function in both processes, and many

systems achieve these goals through similar mechanisms. Transcription factors that enhance viral replication often interact directly with replication initiator proteins. This interaction can facilitate recruitment of the replication protein, stabilize it on the origin, or enhance its activity. Bovine papillomavirus encodes a transcription factor, E2, that interacts with the viral replication initiator protein E1 and recruits it to the origin (Li and Botchan, 1994). The E2 activation domain also facilitates replication by disrupting nucleosomes. The mammalian transcription factor NF1 interacts with the adenovirus DNA polymerase (Cleat et al., 1989; Armentero et al., 1994) and stabilizes the preinitiation complex on the origin (Mul et al., 1992). The AP1 components c-Jun and c-Fos interact with the SV40 initiator protein large T antigen and stimulate its helicase activity (Guo et al., 1996; Ito et al., 1996). Likewise, a G-box factor might increase the efficiency of geminivirus replication by facilitating binding of Rep or another essential replication factor to the origin, enhancing Rep catalytic activity or altering origin structure.

Geminiviruses also impact the expression of host genes. The ability of TGMV infection and the Rep protein to activate the PCNA promoter in differentiated plant cells (Egelkrout, Robertson, and Hanley-Bowdoin, in preparation) is the first example of a host gene corresponding to a DNA replication factor that is up-regulated. However, the DNA replication apparatus is complex and requires the action of many proteins. Thus, it is likely that many plant genes are activated in response to geminivirus infection through mechanisms like those described in the previous section.

Recent experiments provided evidence that geminivirus infection can also repress endogenous host genes (Kjemtrup et al., 1998). A TGMV-derived episome carrying DNA fragments from a plant gene encoding one of three subunits of magnesium chelatase, a chloroplast enzyme required for chlorophyll synthesis, silenced the endogenous copy of the gene. The infected plants displayed yellow and light green areas that failed to accumulate normal levels of chlorophyll. Similarly, TGMV-based episomes containing either luciferase or green fluorescent protein sequences repressed the homologous, chromosomal transgene, but had no effect on the heterologous transgene. Once induced, gene silencing was stably maintained but was not transmitted through seed, indicative of an epigenetic event. Viral DNA levels were also suppressed in silenced tissue. The lack of viral DNA in the majority of cells, that showed silencing suggested that a diffusible signal may be transmitted to healthy cells which causes gene silencing in the absence of the virus. There is precedence for diffusible factors causing silencing in other systems (Voinnet et al., 1997).

Gene silencing is best understood for some of the plant RNA viruses, which appear to inhibit gene expression primarily through post-transcriptional mechanisms (Baulcombe, 1996). The mechanism of geminivirus-mediated gene silencing is not known, but it is likely to involve transcriptional as well as posttranscriptional components. Geminivirus genomes contain few target sites for eukaryotic DNA methylation. This probably reflects evolutionary selection for undermethylated DNA, because geminivirus promoters and origins are both negatively influenced by DNA methylation (Ermak et al., 1993). It may also be due to the lack of a mechanism to propagate methylation during replication (Brough et al., 1992a). Thus, geminiviruses carrying foreign DNA may be methylated, and this methylation then propagated to the chromosomal copy of the same sequence, thereby inhibiting both chromosomal gene expression and geminivirus replication. Independent of the mechanism, these experiments established the usefulness of geminiviruses as episomal vectors in reverse genetic experiments.

VII. FUTURE DIRECTIONS

Many aspects of geminivirus replication and transcription are not well understood, and little is known about the way these viruses interface with their hosts. A better understanding of these processes will increase our knowledge of these important plant pathogens and provide valuable insight in plant nuclear processes and cell cycle regulation.

A major area of interest is the mechanisms whereby geminiviruses modify their hosts to render quiescent plant cells competent for viral DNA

replication. Does the Rep protein modify plant cell cycle controls and/or activate transcription of host DNA synthesis genes directly? Studies of the impact of Rep on PCNA promoter activity and of Rep/pRB interactions on host induction will begin to address this question. It will also be important to identify other plant proteins that are induced or targeted by Rep during host activation. It is not known if all geminiviruses use a common set of mechanisms to induce their hosts. Are the dissimilar tissue distributions displayed by some viruses due to differences in their abilities to activate their hosts or do they reflect constraints on viral movement? Why are some but not all geminivirus infections associated with plant cell proliferation? Another important question is whether other viral proteins are involved in the host induction process. It has been proposed that AL2, AL3, and C4 contribute to induction by Subgroup II/III geminiviruses, but it is not known what roles these protein might play.

Another area of future research is the biochemistry of geminivirus DNA replication. A key question is what are the events that lead to plus-strand DNA synthesis. The main player in this process is the Rep protein, and a full understanding of its activities and structure is critical to answering this question. It will also be crucial to determine how other viral and host proteins interface with Rep during initiation. These studies will provide insight into the mechanisms whereby AL3 enhances geminivirus replication and host transcription factors impact origin activity. It is also not known what host replication factors are required for geminivirus DNA synthesis and how they function during viral replication. It is likely that some of the host replication components interact with Rep and/or AL3, and it may be possible to exploit these interactions to begin characterizing relevant plant proteins. These types of studies will serve as the basis of future experiments that reconstitute viral replication *in vitro* with purified components, as has been accomplished for SV40 (Waga et al., 1994). They will also yield valuable tools for the analysis of nuclear DNA replication complexes in both healthy and infected plants.

There is little information regarding minus-strand DNA synthesis, its priming, or the host proteins that mediate the process. How does the initial single to double-stranded conversion occur in differentiated plant cells lacking DNA replication machinery? Double-stranded DNA is the transcriptionally active form and is necessary for production of the Rep protein, which in turn induces the accumulation of host replication enzymes. Do virions contain a few molecules of Rep that could start the induction process? Alternatively, are DNA repair enzymes, which are presumably present in all differentiated cells, able to support sufficient minus-strand synthesis to begin the infection process, with plant DNA replication machinery responsible for amplification of viral DNA to high levels? Another possibility is that geminivirus replication is confined initially to meristematic cells with their full complement of replication enzymes such that differentiated cells only support replication at a later stage in the infection process after viral induction. This model predicts that either the double-stranded form of viral DNA moves cell to cell or that the Rep protein translocates across plasmodesmata.

Several aspects of geminivirus gene expression also have the potential to provide insight into viral and host processes. Studies of AL2 and its mechanism of action may yield information about transcriptional coactivators and their targets in plants. Because of its simplicity and both positive and negative regulation, the TGMV AL61 promoter may be useful for *in vitro* transcription studies using plant components. Analysis of RNA processing by Subgroup I geminiviruses may increase our understanding of how differential splicing is regulated in plants. Geminiviruses also afford a unique opportunity to study the relationship between DNA replication and transcription in plants. Finally, our understanding of gene silencing in plants may be greatly facilitated by analysis of the mechanisms whereby recombinant geminiviruses inhibit the expression of chromosomal genes.

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