Geminivirus Replication Origins Have a Modular Organization


Tomato golden mosaic virus (TGMV) and bean golden mosaic virus (BGMV) are closely related geminiviruses with bipartite genomes. The A and B DNA components of each virus have cis-acting sequences necessary for replication, and their A components encode trans-acting factors required for this process. We showed that virus-specific interactions between the cis- and trans-acting functions are required for TGMV and BGMV replication in tobacco protoplasts. We also demonstrated that, similar to the essential TGMV AL1 replication protein, BGMV AL1 binds specifically to its origin in vitro and that neither TGMV nor BGMV AL1 proteins bind to the heterologous origin. The in vitro AL1 binding specificities of the B components were exchanged by site-directed mutagenesis, but the resulting mutants were not replicated by either A component. These results showed that the high-affinity AL1 binding site is necessary but not sufficient for virus-specific origin activity in vivo. Geminivirus genomes also contain a stem-loop sequence that is required for origin function. A BGMV B mutant with the TGMV stem-loop sequence was replicated by BGMV A, indicating that BGMV AL1 does not discriminate between the two sequences. A BGMV B double mutant, with the TGMV AL1 binding site and stem-loop sequences, was not replicated by either A component, indicating that an additional element in the TGMV origin is required for productive interaction with TGMV AL1. These results suggested that geminivirus replication origins are composed of at least three functional modules: (1) a putative stem-loop structure that is required for replication but does not contribute to virus-specific recognition of the origin, (2) a specific high-affinity binding site for the AL1 protein, and (3) at least one additional element that contributes to specific origin recognition by viral trans-acting factors.

INTRODUCTION

The geminiviruses are a large and diverse family of plant-infecting viruses that share a unique particle structure of fused incomplete icosahedra and have genomes comprised of circular, single-stranded DNA (reviewed by Stanley, 1991; Lazarowitz, 1992). Their genomes replicate via double-stranded DNA intermediates that are transcribed in the nuclei of infected plant cells. Geminiviruses encode only a few proteins necessary for their replication and transcription and depend largely on host factors to mediate these processes. Consequently, geminiviruses are excellent model systems for investigating both DNA replication and transcription in plant cells.

The geminivirus family can be divided into four subgroups defined by their biological properties (Harrison, 1985) and molecular genetics. One of the largest subgroups comprises the whitefly-transmitted geminiviruses that infect dicotyledonous plants and have bipartite genomes (Haber et al., 1981). Their genomes are composed of two ~2.6-kb DNA components, designated A and B, that are dissimilar in sequence except for a highly conserved common region of approximately 200 nucleotides. The common region is located in a 5' Intergenic region on both DNA components and includes replication (Revington et al., 1989; Lazarowitz et al., 1992) and transcription (Townsend et al., 1985; Hanley-Bowdoin et al., 1988; Sunter et al., 1989) signals. The common region also contains a GC-rich inverted repeat that could form a cruciform or stem-loop structure. (In this study, "stem-loop" refers to the sequence and potential secondary structure, whose existence has not yet been demonstrated.) An invariant AT-rich sequence, 5'-TAA-TATTAC, in the loop is found in all geminivirus genomes. Mutations that either disrupt the invariant sequence or partially delete the GC-rich stem sequence prevent replication in vivo (Revington et al., 1989; Lazarowitz et al., 1992).

The A component of bipartite genome geminiviruses encodes all of the viral proteins required for replication and encapsidation (Rogers et al., 1986; Townsend et al., 1986; Sunter et al., 1987), whereas the B component contributes functions necessary for the spread of infection in plants (Brough et al., 1988; Etessami et al., 1988). Two genes, AL1 and AL3,
on the A component encode viral proteins involved in replication. The only viral protein essential for replication is AL1 (Elmer et al., 1988; Hayes and Buck, 1989; Hanley-Bowdoin et al., 1990), but AL3 is necessary for efficient accumulation of viral DNA in plants (Elmer et al., 1988) and in protoplasts (Sunter et al., 1990). There is strong amino acid sequence and functional conservation among the AL1 and AL3 proteins encoded by geminiviruses with bipartite genomes (Howarth and Vandemark, 1989; Ettessami et al., 1991; Lazarowitz et al., 1992). This conservation also extends to the AL1 homologs encoded by geminiviruses with a single genome component (Mullineaux et al., 1985; Accotto et al., 1989; Lazarowitz et al., 1989; Schalk et al., 1989; Stanley et al., 1992).

Three independent lines of evidence show that geminiviruses reproduce their circular, single-stranded DNA genomes by a rolling circle replication mechanism analogous to some prokaryotic viruses and plasmids. First, the nucleotide sequences of beet curly top virus genomes generated from recombinant heterodimers are consistent with rolling circle replication that initiates/terminates (+) strand DNA synthesis within the stem-loop sequence (Stenger et al., 1991). This possibility is further supported by studies of African cassava mosaic virus (ACMV) and wheat dwarf virus (Ettessami et al., 1989; Heyraud et al., 1993). Second, the intracellular DNA forms that accumulate during ACMV infection correspond to rolling circle replication intermediates (Saunders et al., 1991). Third, geminivirus AL1 proteins contain three amino acid sequence motifs that are related to consensus motifs found in the replication initiator proteins of two bacterial plasmid families (Koonin and Ilyina, 1992) and in the gene A protein of bacteriophage ϕX174 (Ilyina and Koonin, 1992). These prokaryotic proteins introduce a sequence-specific nick in the (+) strand to initiate/terminate rolling circle replication. The AL1 protein may serve the same function in geminivirus replication. AL3 does not show homology to any proteins involved in rolling circle replication, and the mechanism whereby AL3 acts as a geminivirus replication accessory factor is unknown.

In spite of the strong functional conservation between the trans-acting replication factors encoded by bipartite genome geminiviruses, these proteins can show specificity for replication of their cognate genomes. The A and B genome components are usually only infectious on plants when both are derived from the same geminivirus. Studies carried out with squash leaf curl virus (SqLCV) and tomato golden mosaic virus (TGMV) revealed that incompatibility between the B component replication origin and trans-acting replication factors encoded by the A component can be responsible for the failure of heterologous DNA components to produce a viable infection (Lazarowitz et al., 1992). These studies also showed that an approximately 90-nucleotide fragment, which includes the stem-loop sequence and 60 nucleotides from the AL1-proximal (left) side of the SqLCV common region, contains the replication origin and the determinant(s) required for specific recognition of the origin by AL1 in vivo. An analogous fragment of the TGMV common region contains the minimal replication origin for this virus (H.J. Gladfelter and L. Hanley-Bowdoin, manuscript in preparation). Thus, SqLCV and TGMV display exclusive origin recognition, but the region(s) of the replication origins that mediates specific recognition and the mechanisms by which specificity is achieved have not been elucidated. Other studies that established that TGMV AL1 binds specifically to a directly repeated sequence on the left side of the TGMV common region in vitro (Fontes et al., 1992, 1994) suggest a possible mechanism for virus-specific, replication origin recognition.

In this study, we showed that TGMV and another closely related geminivirus, bean golden mosaic virus (BGMV), also exhibit strict specificity in the interactions between the cis- and trans-acting factors required for their replication in vivo. Using this system, we examined the role that AL1 plays in specific recognition of the origin, both in vitro and in vivo. The results of this investigation suggested that geminivirus replication origins consist of at least three functional modules.

RESULTS

TGMV and BGMV DNA B Components Are Replicated Selectively by Their Homologous A Components in Tobacco Cells

To establish an experimental system for investigating the determinants of geminivirus replication specificity, it was first necessary to identify two closely related viruses that show selective replication of their homologous origins. Comparison of the nucleotide sequences of TGMV and BGMV revealed that these viruses are closely related (Howarth et al., 1985). However, it was not known whether TGMV and BGMV display selectivity in the interaction between the cis- and trans-acting factors required for their replication. It was also not known if BGMV can replicate in the tobacco cell lines used in TGMV transfection systems (Sunter et al., 1990; Brough et al., 1992; H.J. Gladfelter and L. Hanley-Bowdoin, manuscript in preparation).

To address these questions, partial tandem copies of TGMV A and B or BGMV A and B were introduced by electroporation into protoplasts derived from tobacco suspension cells and assayed for their capacity to replicate autonomously (A components) or in combination with the homologous component (B components), as shown in Figure 1. Recombinant DNAs containing partial tandem repeats of geminivirus genomes support the release and replication of unit-length, circular viral DNA in plant cells. Total DNA was isolated after a 48-hr culture period and analyzed by DNA gel blot hybridization by sequentially probing with virus-specific radiolabeled DNAs under conditions that prevented cross-hybridization between TGMV and BGMV DNA sequences. Cross-hybridization between the A and B components of the same virus was prevented by excluding common region sequences from the probes. Prior to DNA gel blot analysis, replicon DNA was linearized, and the samples were treated with DpnI, which
Figure 1. Virus-Specific Interactions between the cis- and trans-Acting Functions Required for TGMV and BGMV Replication.

Plasmids containing partial tandem copies of TGMV A, TGMV B, BGMV A, and BGMV B were electroporated into tobacco protoplasts in the combinations indicated at the top. Transfection of a given DNA is only digests dam-methylated, input plasmid DNA. Newly synthesized, double-stranded DNA was identified by its resistance to Dpnl digestion and by length (2.6 versus 6 kb for input DNA). The identity of newly synthesized, single-stranded DNA was confirmed by its sensitivity to mung bean nuclease digestion (data not shown). Double- and single-stranded forms of replicated viral DNA were readily detected for TGMV A (Figure 1, lanes 1, 3, and 4) and BGMV A (lanes 5, 7, and 8) either in the presence or the absence of the homologous B component. In contrast, TGMV B only replicated in the presence of TGMV A (Figure 1, cf. lanes 2 and 3), while BGMV B only replicated in the presence of BGMV A (cf. lanes 6 and 7). These results established that BGMV is able to replicate efficiently in tobacco cells and that, similar to TGMV A (Rogers et al., 1986; Hayes and Buck, 1989), BGMV A provides all of the viral trans-acting factors required for replication.

The capacity of TGMV and BGMV A components to replicate the heterologous B components was examined in cotransfection experiments shown in Figure 1. No replication of BGMV B was detected in the presence of TGMV A (lane 4) or of TGMV B in the presence of BGMV A (lane 8). Replication of the heterologous A components in the same samples (Figure 1, lanes 4 and 8) and of the B components in the presence of their homologous A component (lanes 3 and 7) indicated that the protoplasts were competent for viral replication. Thus, the TGMV- and BGMV-encoded replication factors displayed a high degree of specificity for their respective origins, making these two closely related viruses an ideal system for investigating the molecular basis of replication specificity in common host cells.

TGMV and BGMV AL1 Proteins Bind Specifically to Their Cognate Origins of Replication in Vitro

We demonstrated previously that TGMV AL1 specifically binds to a DNA sequence on the left side of the TGMV common region (Fontes et al., 1992) and more recently that this high-affinity binding site is essential for TGMV replication in tobacco cells (Fontes et al., 1994). The TGMV and BGMV AL1 proteins are functionally equivalent, suggesting that BGMV AL1 may specifically bind to sequences in the BGMV common region. This hypothesis was tested using an in vitro immunoprecipitation DNA binding assay devised for TGMV AL1 (Fontes et al., 1992).
Prior to the binding studies, it was necessary to confirm that a monoclonal antibody raised against TGMV AL1 was able to cross-react efficiently with BGMV AL1. Protein extracts from leaves of healthy and BGMV-infected bean plants were immunoprecipitated using a TGMV AL1–specific monoclonal antibody. The precipitated proteins were resolved by SDS-PAGE and detected by immunoblotting using a polyclonal antiserum raised against TGMV AL1, as shown in Figure 2A. Both the monoclonal and polyclonal antibodies cross-reacted with a protein from BGMV-infected plants (Figure 2A, lane 3). Three lines of evidence indicated that this protein is BGMV AL1. First, the protein is approximately 40 kD, which is the size predicted by the BGMV AL1 open reading frame. Second, the BGMV protein comigrated with the TGMV AL1 protein produced in recombinant baculovirus-infected insect cells (Figure 2A, lane 1). Third, no cross-reacting material was detected in protein extracts from healthy beans (lane 2), indicating that the protein is specific to BGMV-infected plants.

The TGMV AL1 monoclonal antibody was then used to immunoprecipitate TGMV AL1 from recombinant baculovirus-infected insect cell extracts and BGMV AL1 from infected bean extracts. The immunocomplexes were incubated with 3’ radiolabeled fragments containing either the TGMV B or the BGMV B common region (Figure 2B, lane 1), and the bound DNA was eluted and resolved on denaturing gels. TGMV AL1 bound to the TGMV common region probe (TGMV; Figure 2B, lane 2), and binding was prevented by competition with a 50-fold molar excess of the unlabeled homologous probe (TGMV; lane 3). Similarly, BGMV AL1 bound to the BGMV common region probe (BGMV; Figure 2B, lane 2), and binding was prevented by competition with a 50-fold molar excess of the unlabeled homologous probe (BGMV; lane 4). In contrast, neither TGMV AL1 binding to the TGMV common region (TGMV; lane 4) nor BGMV AL1 binding to the BGMV common region (BGMV; lane 3) was prevented by competition with a 50-fold molar excess of the heterologous common region DNA. Control extracts lacking the AL1 proteins from recombinant baculovirus-infected insect cells expressing β-galactosidase (TGMV; Figure 2B, lane 7) or from healthy beans (BGMV; lane 7) did not bind to the common region probes. These results demonstrated that the two AL1 proteins bind specifically to their respective common regions and are unable to interact with the heterologous common region in vitro. The conditions used to wash the immunocomplexes after DNA binding were stringent (Fontes et al., 1992), indicating that both AL1 proteins bind to their recognition sequences with high affinity.

To define the BGMV AL1 recognition sequence, site-directed mutagenesis was used to exchange the known TGMV AL1 binding site with the BGMV AL1 binding site predicted by nucleotide sequence alignment and vice versa, as shown in Figure 3A. In the mutant TGMV B-B1, the TGMV B common region was in lanes 7. The TGMV control extract was from recombinant baculovirus-infected insect cells expressing β-galactosidase, and the BGMV control extract was from healthy bean leaves.
modified at five positions to change the TGMV AL1 binding site to the predicted BGMV AL1 binding site. In mutant BGMV BT1, the BGMV B common region was also altered at five positions to change the predicted BGMV AL1 binding site to that for TGMV AL1. Restriction fragments containing these mutated common regions were used in 50-fold molar excess as competitors for AL1 binding to wild-type common region probes in vitro (Figure 2B). The TGMV B-B1 mutant did not compete for TGMV AL1 binding to the wild-type TGMV common region (TGMV; Figure 2B, lane 5), while BGMV B-T1 DNA was an efficient competitor for TGMV AL1 binding (TGMV; lane 6). Conversely, the BGMV B-T1 mutant was unable to compete for BGMV AL1 binding to the wild-type BGMV common region (BGMV; Figure 2B, lane 6), while TGMV B-B1 DNA was an efficient competitor for BGMV AL1 binding (BGMV; lane 5). Thus, the introduced mutations altered the in vitro binding specificities of the common region fragments such that TGMV B-B1 is bound by BGMV AL1 and BGMV B-T1 is bound by TGMV AL1. These data demonstrated that the recognition sequence required for high-affinity binding of BGMV AL1 to the BGMV common region contains the repeated motif 5'-TGGAGAC-TGGAG, which is analogous to the TGMV AL1 binding site 5'-GGTATAAGGTAG (Fontes et al., 1994). It is possible that the BGMV AL1 recognition sequence includes additional 5' sequences, because the BGMV and TGMV B common regions are identical at seven of eight positions immediately upstream of the repeated motif (Figure 3A).

The High-Affinity AL1 Binding Site Is Necessary but Not Sufficient for Specific Geminivirus Origin Recognition

The in vitro DNA binding experiments established that the TGMV and BGMV AL1 proteins recognize related, but distinct, high-affinity binding sites. The failure of either AL1 protein to support the replication of a heterologous B component may be due to the inability of AL1 to bind to the heterologous

Figure 3. Comparison of the TGMV and BGMV AL1 Binding Sites with Other New World Geminivirus DNA Sequences

(A) The DNA sequences from TGMV B, BGMV B, or their mutant derivatives are shown. The AL1 binding site, putative stem-loop, and intervening sequence of TGMV B are compared to the equivalent region of BGMV B. The colons mark nucleotides that are conserved between the TGMV B (nucleotide positions 64 to 157) and BGMV B (nucleotide positions 77 to 188) sequences. The repeated elements of the TGMV AL1 binding site and the equivalent sequences for BGMV are underlined. The inverted repeats that form the putative stem-loop structure are marked by bars above the sequences. Mutations that alter the TGMV AL1 binding site to match BGMV (Bl) or the predicted BGMV AL1 binding site to match TGMV (TI) are shown by lowercase letters or by a dash for deleted bases. Mutations that alter the BGMV loop sequence to the TGMV loop sequence (T4) are also shown in lowercase letters. In BGMV BT14, the mutations of the BGMV AL1 binding site and loop sequence to TGMV sequences are combined. Gaps introduced to maximize the sequence alignments are marked by dots.

(B) Sequences from the TGMV and BGMV B common regions were compared to sequences on the left of the common regions of other bipartite geminiviruses from the New World. The sequences were aligned using the predicted TATA box sequences (long bar) for transcription to the left. Directly repeated elements are underlined, and conserved GG motifs are marked by short bars. The numbers on the right are the distance in nucleotides to the stem-loop sequence for each virus. The viruses and their GenBank accession numbers are TGMV (K02030), BGMV (M91605), abutilon mosaic virus (AbMV; X15984), bean dwarf mosaic virus (BDMV; M88180), BGMV-Brazil (-BZ; M88687), potato yellow mosaic virus (PYMV; D00941), SqLCV (M63158), and tomato mottle virus (TmMoV; M90494).
common region, resulting in the replication specificity observed in vivo. To test this hypothesis, we took advantage of the switch in AL1 binding specificity exhibited by the TGMV B-B1 and BGMV B-T1 mutants. Plasmids containing partial tandem copies of the mutant B components were cotransfected with each of the wild-type A components into tobacco protoplasts and assayed for replication by DNA hybridization, as shown in Figure 4. Consistent with their altered AL1 binding specificities in vitro, TGMV B-B1 failed to replicate in the presence TGMV A (Figure 4, lane 3; TGMV B probe), and no replication of BGMV B-T1 was detected in the presence of BGMV A (lane 8; BGMV B probe). However, TGMV B-B1 also failed to replicate in the presence of BGMV A (lane 7; TGMV B probe), even though BGMV AL1 binds to TGMV B-B1 in vitro. Similarly, BGMV B-T1 did not replicate in the presence of TGMV A (Figure 4, lane 4; BGMV B probe), even though TGMV AL1 binds to BGMV B-T1 in vitro. Both A components retained the capacity to replicate their homologous, wild-type B components (Figure 4, lane 1, TGMV B probe; lane 6, BGMV B probe). In addition, accumulation of TGMV A (lanes 1 to 4) or BGMV A (lanes 5 to 8) was detected in all samples, indicating that the protoplasts were competent for geminivirus replication.

These results showed that mutation of the high-affinity AL1 binding site prevented replication by the homologous AL1 protein, confirming the importance of this site. However, transfer of the in vitro AL1 binding specificity was not sufficient to create a functional replication origin for the nonhomologous AL1 protein. These results established that specific binding of AL1 to its high-affinity site in the common region is necessary for the replication of two different geminiviruses and, thus, contributes to the specificity of geminivirus origin recognition. Nevertheless, because transfer of the high-affinity AL1 binding site alone does not result in productive interaction between AL1 and the mutant origin, additional determinants of specificity must be required for geminivirus origin function.

**Nonconserved Nucleotides in the Putative Stem-Loop Do Not Mediate the Specific Interaction between trans-Acting Replication Factors and the Origin**

Lazarowicz et al. (1992) suggested that nonconserved nucleotides in the loop sequence of the stem-loop might be responsible for the specificity of interaction between AL1 and its cognate replication origin. The stem sequences of TGMV and BGMV are identical, whereas their loop sequences differ at only two nucleotide positions (Figure 3A). To directly assess the role of these variant nucleotides in replication, site-directed mutagenesis was used to alter the loop sequence of wild-type BGMV B to that found in TGMV B, resulting in a mutant designated BGMV B-T4. The loop mutations were also introduced into the BGMV B-T1 mutant, which contains the high-affinity binding site for TGMV AL1, to generate a double mutant designated BGMV B-T14. Plasmids containing partial tandem copies of these mutant B components were cotransfected into tobacco cells with each of the wild-type A components and assayed for replication by DNA gel blot analysis, as shown in Figure 5. The loop mutant BGMV B-T4 was replicated by BGMV A (Figure 5, lane 7; BGMV B probe), but not by TGMV A (lane 3; BGMV B probe), as was wild-type BGMV B (lanes 2 and 6; BGMV B probe). In contrast, no replication of the double mutant BGMV B-T14 was detected in the presence of both TGMV A (Figure 5, lane 4) or BGMV A (lane 8). DNA gel blot analysis of TGMV A (Figure 5, lanes 1 to 4; TGMV A probe), BGMV A (lanes 5 to 8; BGMV A probe), and TGMV B (lanes 1 and 4; TGMV B probe) verified that the protoplasts were competent for geminivirus replication. These results demonstrated

![Figure 4](image-url)
that the loop sequence does not contribute significantly to the ability of BGMV AL1 to specifically recognize the BGMV origin of replication. In addition, because the double mutant BGMV B-T14 does not replicate in the presence of TGMV AL1, the combination of the cognate high-affinity AL1 binding site and the wild-type TGMV loop sequence was not sufficient to confer TGMV replication specificity on the BGMV origin.

**DISCUSSION**

Although the geminiviruses TGMV and BGMV are closely related, we showed that they exhibit strict specificity in the interactions between the cis- and trans-acting factors necessary for their replication in vivo. One mechanism that might mediate this specificity is sequence-specific binding of AL1 to its origin. This type of interaction governs origin recognition of the rolling circle replicons in the pT181 plasmid family (Wang et al., 1993) and probably the filamentous bacteriophages M13 and IKe (Peeters et al., 1986). We demonstrated previously that TGMV AL1 binds with high affinity to a site in the TGMV common region (Fontes et al., 1992) and recently located this binding site on a directly repeated motif near the left edge of the minimal origin of replication (Fontes et al., 1994). In this study, we showed that BGMV AL1 also binds specifically to its common region in vitro. Furthermore, BGMV AL1 did not bind to the TGMV common region, and TGMV AL1 did not recognize the BGMV common region.

Alignment of the common region sequences from various bipartite genome geminiviruses revealed that they frequently contain a directly repeated motif with conserved GG dinucleotides in an analogous position to the TGMV AL1 high-affinity binding site (see Figure 3B). We used this alignment to identify the likely binding site for BGMV AL1 in the TGMV common region and constructed two B component mutants in which the binding site sequences were exchanged between BGMV and TGMV. The BGMV B-T1 mutant contains the TGMV AL1 high-affinity binding site in a wild-type BGMV common region background, and the TGMV B-B1 mutant contains the predicted BGMV AL1 high-affinity binding site in a wild-type TGMV common region background. We found that the in vitro AL1 binding specificities of the mutant common regions were reversed relative to their wild-type parents, i.e., BGMV B-T1 bound specifically to TGMV AL1, and TGMV B-B1 bound specifically to BGMV AL1. These results confirmed that the TGMV sequence 5'-GGTAGTAAGGTAG and the analogous BGMV sequence 5'-TGGAGACTGGAG determine the binding specificity for the cognate AL1 protein.

The success of our nucleotide sequence alignment in predicting the AL1 recognition motif for BGMV AL1 suggested that the related, directly repeated sequences observed in other geminivirus genomes also constitute high-affinity binding sites for their respective AL1 proteins (Figure 3B). The sequence heterogeneity of the predicted binding sites in different geminiviruses makes it likely that AL1 proteins recognize their origins in a highly specific manner. This suggestion is consistent with recent findings that the TGMV and abutilon mosaic virus A components are also unable to support replication of the heterologous B component (Frischmuth et al., 1993). Similar results were also obtained with TGMV and the Old World geminivirus ACMV (Frischmuth et al., 1993).

We have shown previously by site-directed mutagenesis that the high-affinity AL1 binding site forms an essential element of the TGMV replication origin (Fontes et al., 1994). This observation raised the possibility that the AL1 recognition motif is the sole determinant of origin recognition specificity. To test this hypothesis, B component mutants, which have their in vitro AL1 binding specificities exchanged (TGMV B-B1 and BGMV B-T11), were analyzed for their ability to undergo DNA replication in tobacco protoplasts. Neither of the mutant B components
was replicated in the presence of either TGMV A or BGMV A. This lack of replication cannot be attributed to a nonspecific loss of replication competence by the mutant B components, because both mutants can replicate in the presence of chimeric AL1 proteins (E.P.B. Fontes, H.J. Gladfelter, I.T.D. Petty, and L. Hanley-Bowdoin, manuscript in preparation). Thus, as previously shown for TGMV, the BGMV high-affinity AL1 binding site is an essential element of its replication origin (BGMV B-T1 is not replicated by BGMV A). However, because neither of the mutant origins was replicated by the AL1 protein to which it bound efficiently in vitro, additional elements of the replication origin must be involved in specific interaction(s) with viral trans-acting factors in vivo.

Lazarowitz et al. (1992) suggested that nonconserved nucleotides in the loop of the stem-loop sequence in the common region may be responsible for discrimination of the TGMV and SqLCV origins by their respective AL1 proteins. The loop sequences of the TGMV and BGMV B components are also distinct (Figure 3A) and therefore might have a role in origin recognition specificity in vivo. To test this hypothesis, we used site-directed mutagenesis to substitute the TGMV loop sequence into a wild-type BGMV common region background. The resulting mutant, BGMV B-T4, was able to replicate in the presence of BGMV A, but not in the presence of TGMV A. Thus, variation in the putative loop sequence did not contribute to the ability of BGMV AL1 to discriminate between the BGMV and TGMV replication origins. The TGMV loop sequence was also substituted into the mutant BGMV B-T1, which already contains the TGMV AL1 high-affinity binding site in the BGMV common region. The resulting double mutant, BGMV B-T14, had the same phenotype as its BGMV B-T1 parent; i.e., it was unable to replicate in the presence of either the BGMV or TGMV A component. Because the high-affinity AL1 binding site and stem-loop essentially delimit the TGMV minimal origin of replication (H.J. Gladfelter and L. Hanley-Bowdoin, manuscript in preparation), it is likely that additional determinants required for replication of the origin by TGMV-encoded, trans-acting factors are located between these two elements.

The replication assays with BGMV, TGMV, and their mutant derivatives were all performed in tobacco cells so that the specificity of origin recognition could not be mediated by host factors. However, because the source of viral trans-acting factors in these experiments was always a wild-type A component, a possible contribution of the accessory replication protein AL3 to origin recognition specificity cannot formally be excluded. Because the mutants with reversed in vitro AL1 binding specificities cannot be replicated by either of the A components in vivo, we can infer that if AL3 has any role in specific origin recognition, it is unable to act in the absence of the appropriate AL1 binding site. Although AL3 is not essential for TGMV replication, viral DNA accumulation is reduced ~50-fold in its absence (Sunter et al., 1990). Consequently, if a specific interaction between AL3 and the origin is disrupted in the mutants, viral DNA accumulation might fall below detectable levels. Two other factors could exacerbate this problem. First, TGMV AL1 negatively regulates its own synthesis (Sunter et al., 1993), and DNA replication may be limited by the supply of AL1. Second, the replication origin of the A component that supplies the trans-acting factors is wild type in character, and it is in direct competition for AL1 with the mutant B component origin being assayed. To address these potential limitations of the replication assay, we are currently pursuing experiments in which the viral trans-acting factors are supplied from non-replicating expression cassettes.

The results presented here extend our understanding of the structure and function of geminivirus replication origins and represent an important step toward using these viruses to characterize the nuclear DNA replication processes of higher plants. By combining our results with those of previous studies, we can now propose that the replication origins of TGMV, BGMV, and related geminiviruses are modular in structure. Modular organization is also associated with the (+) strand origins of bacterial plasmids (de la Campa et al., 1990) and paroviruses (Snyder et al., 1993; Tam and Astell, 1993) and with the chromosomal origins of yeast (Marahrens and Stillman, 1992). The geminivirus minimal origin contains at least three functional elements, and, by analogy with other viral and plasmid systems, there may also be as yet unidentified replication enhancer elements that lie outside the minimal origin (DePamphilis, 1988; Gennaro, 1993). The three elements of the geminivirus minimal origin, shown schematically in Figure 6, are (1) a high-

Figure 6. Modular Organization of a Geminivirus Origin of Replication.

A schematic drawing of the functional domains of a geminivirus origin of replication is shown. The relative locations of the AL1 binding site (hatched box) and the stem-loop motif in the origin are indicated. The invariant sequence and the AT-rich spacer motif in the loop are marked. The limits of the DNA sequence that contains the probable nick site for initiation of rolling circle replication mapped by Etessami et al. (1989), Stenger et al. (1991), and Heyraud et al. (1993) are shown (▲). Other sites in the origin that may be involved in additional interactions with viral replication proteins are illustrated by the open boxes. Sites that may function in a sequence-specific manner are marked by the large open rectangle, whereas specific interactions that may be mediated by differential spacing are indicated by the small open boxes.
affinity binding site for the AL1 protein that is located on the
left side of the origin, (2) the putative stem-loop structure that
delimits the right side of the origin and contains the invariant
sequence 5'-TAATATTAC, and (3) an intervening sequence that
delimits the right side of the origin and contains the invariant
affinity binding site for the AL1 protein that is located on the
TATTAC sequence in the loop apparently do not contribute to
the (+) strand origin of bacteriophage (pX174 in which nucleo-
tide substitutions can be tolerated without compromising
replication (Heidekamp et al., 1981). The existence of an addi-
tional element that contributes to origin recognition was inferred
from the inability of the TGMV A component to replicate a
BGMV mutant that carries both the high-affinity AL1 binding
site and the stem-loop sequence of TGMV. The sequences of
the two origins differ in the region between these elements,
and the BGMV origin contains 19 additional nucleotides com-
pared to that of TGMV (Figure 3A). Consequently, the additional
interactions with viral proteins required for origin function may
be mediated by sequence specificity, differential spacing, or
some combination of both, as has been observed for the (+)
strand origins of the pT181 plasmid family (Wang et al., 1993).
Experiments designed to distinguish between these possibili-

ties for the geminivirus origins are currently in progress.

METHODS

Plasmid Constructs and Site-Directed Mutagenesis

The position numbers used to describe the following clones refer to
the nucleotide coordinates of the tomato golden mosaic virus (TGMV)
sequence determined by Hamilton et al. (1984), as corrected by
MacDowell et al. (1986), von Arnim and Stanley (1992), and Schaffer
(1992). The corrected size of full-length TGMV DNA B is 2525 bp. The
coordinates for bean golden mosaic virus (BGMV-GA) refer to the sequence
of the Guatemalan isolate (BGMV-GA) determined by J.C. Faria, R.L.
Gilbertson, F.J. Morales, P. Ahlquist, and D.P. Maxwell (personal com-
munication, University of Wisconsin, Madison; GenBank accession
number M91605). In these numbering schemes, the common regions
of both viruses are delimited by positions 1 to 210.

The construction of recombinant plasmids containing partial tan-
dem repeats of the A or B components of TGMV and BGMV has been
described previously by Schaffer (1992). Briefly, plasmids pH401
(Bisaro et al., 1982) and pBH604 (Hamilton et al., 1983), which con-
tain single copies of TGMV A or B DNA, respectively, were used to
subclone fragments that included the TGMV A or B common region
into pZ19U (Mead et al., 1986). The plasmid pTG0.3A contains the 736-
bp EcoRI-Xhol fragment from pBH401, while pTG0.4B contains the 939-
bp BgIII-ClaI fragment from pBH604. Partial tandem copies of TGMV
A and B were generated by insertion of the 2588-bp EcoRI fragment
from pBH401 into EcoRI-digested pTG0.3A and of the 2525-bp ClaI
fragment from pBH604 into ClaI-digested pTG0.4B. The resulting plas-
mids pTG1.3A and pTG1.4B contained 1.3 copies of TGMV DNA A or
1.4 copies of TGMV DNA B, respectively, and had duplicated common
regions.

The plasmids pGA1A and pGB1B, which contain a single copy of
the A or B genome components of BGMV-GA, respectively, have been
described previously by Gilbertson et al. (1991). The 634-bp BgIII-HindII
fragment from pGA1A was subcloned into pZ19U to give pGAI.2A, which
contains the BGMV A common region and flanking sequences.
Similarly, the 489-bp BamHI-HindII fragment from pGB1B was subcloned
into pZ19U to give pGB1.2B, which contains the BGMV B common region and flanking sequences. A partial tandem copy of
BGMV A was constructed by releasing the 2647-bp EcoRI fragment
from pGAI.1A, circularizing it in vitro, followed by linearization with SpeI,
and inserting it into the SpeI site of pGAI.2A. A partial tandem copy
of BGMV B was generated by inserting the 2566-bp BamHI fragment
of pGB1B into BamHIdigested pGB1.2B. The resulting plasmids
pGAI.1A.2A and pGA1.2B contained 1.2 copies of BGMV DNA A or 1.2
copies of BGMV DNA B, respectively, and had duplicated common
regions.

Mutations were introduced into the common regions of plasmids
containing a single copy of TGMV B (pGB) or BGMV B (pGB) using dU-containing, single-stranded DNA templates (Kunkel, 1985), as de-
scribed previously (Petty et al., 1989). The plasmid pGB was made
by inserting the 2525-bp ClaI fragment from pBH604 into the ClaI site
of pBlueScript II KS+ (Stratagene). The TGMV B common region was
modified at TGMV B positions 74, 77 to 79, and 82 using the primer
5'-GTTATCGAGCTGACATTTGTAATACG-3' to create
pTGB-B1 (TGMV B-B1, Figure 3A). The BGMV B common region was
modified at positions 87, 90 to 92, and 94 using the primer 5'-
CGACGCTAGCTGTACGACGCTAGCTACG-3' to create
pTGB-B1 (BGMV B-B1, Figure 3A), and at positions 161 and 163 using the
primer 5'-CACGTGGACGCCCATCCCTATATATAT-3' to create
pTGB-B1 (BGMV B-B1, Figure 3A), and at positions 161 and 163 using the
primer 5'-CACGTGGACGCCCATCCCTATATATAT-3' to create
pTGB-B1 (BGMV B-B1, Figure 3A). The T4 mutation was subsequently
introduced into pGAI.1B to generate the double mutant pGAI.1B-T4.
All mutations were verified by DNA sequencing. A subclone of pTG1-B1,
which contains the common region, was created by digestion with BamHI.
The resulting plasmid, pTG0.3B-B1, contained the 848-bp BamHI-ClaI fragment of TGMV B-B1. A partial tandem copy of TGMV
B-B1 was generated by inserting the 2523-bp ClaI fragment of pTG0.3B-
B1 into ClaI-digested pTG0.3B-B1. Partial tandem copies of BGMV
B-B1, BGMV B-13, and BGMV B-14 were constructed from pGAB-T4,
pGAB-T4, and pGAB-T4, respectively, as described above for wild-
type BGMV DNA B. The resulting plasmids, pGA1.3B-B1, pGA1.2B-T4,
pGA1.2B-14, and pGA1.2B-T4, contained duplicated, mutant common
regions.

Isolation and Immunoprecipitation of AL1 Protein

Bean plants (Phaseolus vulgaris cv Top Crop) were infected with BGMV-
GA by sap transmission. Systemically infected leaf tissue or the equiva-

lent from uninoculated control plants was harvested 12 days
postinfection and used to prepare whole-cell protein extracts, as de-
scribed previously by Hanley-Bowdoin et al. (1990). Whole-cell protein
extracts were also prepared from healthy beans and from recombinant
were randomly labeled using γ-32P-ATP and the Klenow fragment was included in all solutions. Virus- and component-specific probes were prepared from TGMV A, a 2.1-kb PstI fragment from TGMV B (Sambrook et al., 1989). The probes were a 1.8-kb XhoI-EcoRI fragment with 0.4 M mannitol, pH 5.5; and resuspended in 0.8% NaCl, 0.020/0.

Replication Assays

Protoplasts were prepared from the Nicotiana tabacum suspension cell line NT-1 and cultured in NT-1 medium supplemented with 0.2 mg/mL 2,4-dichlorophenoxyacetic acid (An, 1985). The protoplasts were isolated by digestion with 1% cellulase, 0.1% pectolyase Y30.4 M acid (An, 1985). The protoplasts were diluted into 8 mL of NT-1 medium supplemented with 0.2 mg/mL 2,4-dichlorophenoxyacetic acid, 0.4 M mannitol, 20 mM 2-(N-morpholino)ethanesulfonic acid, pH 5.5; washed twice with 0.4 M mannitol, pH 5.5; and resuspended in 0.8% NaCl, 0.02% KCl, 0.02% K2HPO4, 0.11% Na2HPO4. 0.4 M mannitol, pH 6.5. Replication assays were performed by electroporation (250 V, 500 μF) of 20 μg of each replicon DNA and 30 μg of sheared salmon sperm DNA into 5.0 x 10⁶ protoplasts. Protoplasts were diluted into 8 mL of NT-1 medium supplemented with 0.2 mg/mL 2,4-dichlorophenoxyacetic acid and 0.4 M mannitol. Total DNA was isolated from the protoplasts 48 hr after transfection (Junghans and Metzlaff, 1990), digested with XhoI-DpnI (TGMV A) or BglII-DpnI (TGMV B, BGMV A, and BGMV B), resolved on 1% agarose gels, and vacuum transferred and UV cross-linked to nylon membranes (MagnaGraph; MSI, Westboro, MA). The blots were analyzed by DNA gel blot hybridization according the protocol of Thomashow et al. (1980), except that 0.2% sodium pyrophosphate was included in all solutions. Virus- and component-specific probes were randomly labeled using α-32P-dATP and the Klenow fragment (Sambrook et al., 1989). The probes were a 1.6-kb XhoI-EcoRI fragment from TGMV A, a 2.1-kb PstI fragment from TGMV B, a 1.4-kb Scat-BglII fragment from BGMV A, and a 1.6-kb HindIII fragment from BGMV B.

DNA Binding Assays

DNA binding assays were prepared as described by Fontes et al. (1992). The immunocomplexes were incubated with 1 nM of radiolabeled probe DNA (≈60,000 cpm) in the absence or presence of 50 nM competitor DNA. Bound DNA was extracted from the DNA/AL1 immunocomplexes using phenol-CHCl3-isoamyl alcohol and visualized on denaturing, 6% polyacrylamide gels. Probe and competitor DNAs were prepared by restriction enzyme digestion followed by fractionation on agarose gels and purification by glass adhesion (Vogelstein and Gillespie, 1979). A 370-bp Ndel-ClaI fragment from pTG0.4B, which includes the wild-type TGMV B common region and flanking sequences, and a 489-bp BamHI-HindIII fragment from pGA0.2B, which includes the wild-type BGMV B common region and flanking sequences, were radiolabeled using α-32P-dATP and the Klenow fragment of DNA polymerase I from Escherichia coli (Sambrook et al., 1999); these fragments were used as probes in DNA binding experiments. Competitor DNAs were isolated by Ndel-ClaI digestion of pTG0.4B (wild-type TGMV B) and pTGB-B1 (mutant TGMV BT1) and by BamHI-HindIII digestion of pGA0.2B (wild-type BGMV B) and pGAB-T1 (mutant BGMV BT1).

Replication Assays

Protoplasts were prepared from the Nicotiana tabacum suspension cell line NT-1 and cultured in NT-1 medium supplemented with 0.2 mg/mL 2,4-dichlorophenoxyacetic acid and 0.4 M mannitol. Total DNA was isolated from the protoplasts 48 hr after transfection (Junghans and Metzlaff, 1990), digested with XhoI-DpnI (TGMV A) or BglII-DpnI (TGMV B, BGMV A, and BGMV B), resolved on 1% agarose gels, and vacuum transferred and UV cross-linked to nylon membranes (MagnaGraph; MSI, Westboro, MA). The blots were analyzed by DNA gel blot hybridization according the protocol of Thomashow et al. (1980), except that 0.2% sodium pyrophosphate was included in all solutions. Virus- and component-specific probes were randomly labeled using α-32P-dATP and the Klenow fragment (Sambrook et al., 1989). The probes were a 1.6-kb XhoI-EcoRI fragment from TGMV A, a 2.1-kb PstI fragment from TGMV B, a 1.4-kb Scat-BglII fragment from BGMV A, and a 1.6-kb HindIII fragment from BGMV B.

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Acknowledgments

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