Functional Domains of a Geminivirus Replication Protein*

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Tomato golden mosaic virus, a member of the geminivirus family, has a single-stranded DNA genome that is replicated and transcribed in infected plant cells through the concerted action of viral and host factors. One viral protein, AL1, contributes to both processes by binding to a directly repeated, double-stranded DNA sequence located in the overlapping (+) strand origin of replication and AL1 promoter. The AL1 protein, which occurs as a multimeric complex in solution, also catalyzes DNA cleavage during initiation of rolling circle replication. To identify the tomato golden mosaic virus AL1 domains that mediate protein oligomerization, DNA binding, and DNA cleavage, a series of truncated AL1 proteins were produced in a baculovirus expression system and assayed for each activity. These experiments localized the AL1 oligomerization domain between amino acids 121 and 181, the DNA binding domain between amino acids 1 and 181, and the DNA cleavage domain between amino acids 1 and 120. Deletion of the first 29 amino acids of AL1 abolished DNA binding and DNA cleavage, demonstrating that an intact N terminus is required for both activities. The observation that the DNA binding domain includes the oligomerization domain suggested that AL1-AL1 protein interaction may be a prerequisite for DNA binding but not for DNA cleavage. The significance of these results for AL1 function during geminivirus replication and transcription is discussed.

Geminiviruses are plant DNA viruses characterized by their single-stranded genomes and their double icosahedral particle morphology (for review, see Ref. 1). They replicate their small single-stranded genomes and their double icosahedral particle morphology (for review, see Ref. 1). They replicate their small single-stranded genomes and their double icosahedral particle morphology (for review, see Ref. 1). They replicate their small single-stranded genomes and their double icosahedral particle morphology (for review, see Ref. 1). They replicate their small single-stranded genomes and their double icosahedral particle morphology (for review, see Ref. 1). They replicate their small single-stranded genomes and their double icosahedral particle morphology (for review, see Ref. 1).

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1 The abbreviations used are: TGMV, tomato golden mosaic virus; BGMV, bean golden mosaic virus; TYLCV, tomato yellow leaf curl virus; GST, glutathione S-transferase; ACMV, African cassava mosaic virus.

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BGMV. However, the chimeric studies with TGMV and BGMV showed that interaction between the N terminus of AL1 and the cognate DNA binding motif is only part of the requirements for virus-specific origin recognition in vivo. No biochemical studies have addressed the protein domain involved in AL1-DNA binding. There is also no information regarding the various AL1 protein-protein interaction domains. In this article, we identified the domains of TGMV AL1 that mediate protein oligomerization, DNA binding, and DNA cleavage.

MATERIALS AND METHODS

Plasmid Constructs—Coding sequences corresponding to authentic AL1 and AL1 fused to the glutathione S-transferase (GST) domain were cloned into the baculovirus transfer vector pMON27025 (7) for expression in Spodoptera frugiperda Sf9 cells. The recombinant proteins were named according to their N- and C-terminal amino acids (Fig. 1). For example, AL1-120 includes AL1 amino acids 1–120. The baculovirus was named according to their N- and C-terminal amino acids (Fig. 1). For example, AL1-120 includes AL1 amino acids 1–120. The baculovirus vectors expressing vectors encoding GST (pNS313), GST-AL1-352 (pNS314), and authentic AL1-352 (pNS244) have been described previously (7, 32).

Open reading frames for C-terminal truncated AL1 proteins (Fig. 1A) were generated by inserting an Xbal linker into repaired restriction sites at TGMV A positions 2242 (Sall), 2059 (NcoI), and 1963 (EagI) to create in-frame stop codons. Truncated AL1 open reading frames were subcloned as BglII-HindIII fragments into BamHI- and HindIII-digested pMNP27025 to give pNS3388 (AL1-118), pNS3617 (AL1-119), and pNS3618 (AL1-120). Plasmid pNS3310 was digested with SalI and trimmed with T4 DNA polymerase to release a 699-base pair fragment containing the GST coding sequence. Bactuvirus expression cassettes corresponding to GST-AL1 fusion proteins (Fig. 1B) were generated by cloning this fragment into NdeI sites of pNS3388, pNS3571, and pNS3992 to give pNS3343 (GST-AL1-120), pNS3547 (GST-AL1-131), and pNS3535 (GST-AL1-133), respectively. Coding sequences for N-terminal truncated AL1 proteins (Fig. 1A) were generated by inserting an SphI linker into repaired restriction sites at TGMV A positions 2442 (Sall) and 2059 (NcoI) to create in-frame start codons. An SphI linker was also inserted into a repaired HindIII site of pMNP27025 to make pNS6448. The truncated AL1 open reading frames were subcloned as SphI-EcoRI and SphI-BamHI fragments into the same sites of pNS6448 to give pNS6516 (AL1-121-352) and pNS6469 (AL1-132-352), respectively.

Engineered restriction sites and an endogenous NcoI site at TGMV A position 2059 were used to create open reading frames for GST-AL1 fusion proteins lacking N-terminal AL1 sequences. The AL1 coding sequence was modified at TGMV A positions 2516–2517 using the in vitro mutagenesis system. The truncated AL1 open reading frames were subcloned as SphI-EcoRI and SphI-BamHI fragments into the same sites of pNS6448 to give pNS6516 (AL1-121-352) and pNS6469 (AL1-132-352), respectively.

The relative molecular masses of full-length AL1 (AL1-352) and the C-terminal truncated proteins AL1-120 and AL1-118 were determined by size exclusion chromatography of extracts from insect cells infected with the corresponding recombinant baculoviruses. Extracts were prepared by mixing cells for 30 min at 4 °C in column buffer (50 mM Tris-HCl, pH 7.5, 1 mM EDTA, and 0.15 M NaCl) supplemented with protease inhibitors (19). The extracts were clarified by centrifugation for 1 h at 100,000 × g. Approximately 0.2 mg of protein (1 mg/ml) was applied to a 50 × 1-cm column of Sepharose CL-6B in column buffer, chromatographed at 0.2 ml/min, and eluted at 0.5-ml fractions. To determine the elution positions of the various AL1 proteins, 75 μl of each fraction was analyzed by SDS-polyacrylamide gel electrophoresis and immunoblotting with anti-AL1 serum as described above. The column was calibrated with protein molecular weight markers (Sigma) individually diluted with column buffer. V, values of protein standards were determined by monitoring the column effluent at A280. The V, was determined from the elution volume of blue dextran. Relative molecular masses of AL1 proteins were estimated from linear regression analysis of V, versus the logarithm of the molecular masses of the protein standards.

In Vitro Assays for AL1 Function—DNA electrophoretic mobility shift assays and DNA cleavage assays were performed as described previously (7). For the binding assays, an 83-base pair EcoRI fragment containing the AL1-DNA binding motif (TGMV A positions 28–84) was isolated and 3’-end-labeled with [γ-32P]ATP. The radiolabeled DNA was incubated with purified GST-AL1 fusion proteins for 1 h at room temperature. DNA and protein concentrations are provided in the figure legends. The bound and free probes were resolved on 1% agarose gels, dried on Whatman DE-81 paper, and analyzed by autoradiography.

For DNA cleavage assays, a single-stranded oligonucleotide (5’-TTATTATCCGCGATGCCCAGC-3’) containing the loop and right side of the hairpin structure in the TGMV (+) strand origin was 5’-end-labeled using polynucleotide kinase and [γ-32P]ATP. Approximately 5000 cpm of labeled DNA was incubated with 100 ng of purified GST-AL1 fusion protein in 10 μl of cleavage buffer (25 mM Tris-HCl, pH 7.5, 75 mM NaCl, 5 mM MgCl2, 2.5 mM EDTA, and 2.5 mM diethiothreitol) for 30 min at 37 °C. The reactions were terminated by adding 6 μl of gel loading buffer (5% β-mercaptoethanol, 0.05% bromphenol blue) and heating to 90 °C for 2 min. The reaction products were resolved on 15% polyacrylamide denaturing gels.

ATPase assays were performed essentially as described by Desbiez et al. (18). Approximately 300 ng of GST-AL1 fusion proteins were incubated for 30 min at 57 °C in a buffer containing 25 mM Tris-HCl, pH 7.5, 20 mM NaCl, 2 mM MgCl2, 0.01% Triton X-100, 40 μM ATP, and 110 μM [γ-32P]ATP. Free phosphate was extracted and measured according to the protocol described by Seto-Young and Perlini (35) with the following modifications. The reaction was stopped with 3 volume of 5% ammonium molybdate in 2 N H2SO4, and free phosphate was extracted with an equal volume of N-butanol. Radioactivity in a 50-μl aliquot was measured by liquid scintillation.

RESULTS

The AL1 Oligomerization Domain—Recent experiments showed that TGMV AL1 interacts with itself to form a multimeric complex (19). To map the AL1 oligomerization domain, we generated a series of truncated AL1 proteins (Fig. 1A) and assayed their ability to interact with a full-length AL1 protein fused to glutathione S-transferase (GST-AL1-132-352) (Fig. 1B).

The GST-AL1-352 and truncated AL1 proteins were co-expressed in baculovirus-infected insect cells, and GST-AL1-352 was purified using glutathione-Sepharose resin. Total protein extracts from insect cells (Fig. 2, lanes 1–7) and proteins bound to glutathione resin (lanes 8–14) were resolved by SDS-polyacrylamide gel electrophoresis and visualized by immunoblotting using antibodies against AL1 and GST (Fig. 2, lanes 1, 8, and 9) and the C-terminal truncated proteins GST-AL1-132-352 and GST-AL1-120 (Fig. 2, lanes 2 and 3). GST-AL1-132-352 and GST-AL1-120 were co-expressed with GST-AL1-352 (Fig. 2, lanes 4 and 11) and GST-AL1-118 (Fig. 2, lanes 8 and 12) in insect cells infected with the same baculovirus. GST-AL1-352 was purified using glutathione-Sepharose resin. Total protein extracts from insect cells (Fig. 2, lanes 1–7) and proteins bound to glutathione resin (lanes 8–14) were resolved by SDS-polyacrylamide gel electrophoresis and visualized by immunoblotting using antibodies against AL1 and GST (Fig. 2, lanes 1, 8, and 9) and the C-terminal truncated proteins GST-AL1-132-352 and GST-AL1-120 (Fig. 2, lanes 2 and 3). However, a 1-cm deletion to amino acid 120 in AL1-120 (Fig. 2, lanes 4 and 11) abolished complex formation. Similarly, the N-terminal truncation AL1-118 (Fig. 2, lanes 5 and 12) co-precipitated with GST-AL1-352, whereas AL1-112-352 (Fig. 2, lanes 6 and 13) did not interact with GST-AL1-352. AL1-132 did not bind GST alone (Fig. 2, lanes 7 and 14).

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The TGMV AL1 Protein Domains

**Fig. 1. Diagram of the AL1 protein constructs.** A, schematic of authentic TGMV AL1 protein and truncated AL1 proteins. AL1,352 is shown as an open box, with the solid and shaded boxes marking motifs conserved among rolling circle replication initiator proteins and the ATP binding consensuses, respectively. Restriction sites used for cloning are indicated by name and position in the TGMV A genome (20) with engineered sites shown in parentheses. Solid lines correspond to truncated AL1 proteins expressed in insect cells. The proteins are designated on the left by their terminal amino acids. B, schematic of the GST-AL1 fusion proteins. AL1,352 is shown as described for A except with GST (ovals) fused to the N terminus. The truncated GST-AL1 proteins are represented by solid lines and designated on the left by their AL1 terminal amino acids.

**Fig. 2. Mapping the AL1 oligomerization domain.** A, protein extracts from baculovirus-infected cells (total, lanes 1–7) were incubated with glutathione resin and eluted in SDS sample buffer (bound, lanes 8–14). Total and bound fractions were resolved by SDS-polyacrylamide gel electrophoresis and analyzed by immunoblotting using antibodies against AL1 and GST. GST-AL1,352 was co-expressed with AL1,352 (lanes 1 and 8), AL1,181 (lanes 2 and 9), AL1,120 (lanes 3 and 10), AL1,120 (lanes 4 and 11), AL1,120 (lanes 5 and 12), and AL1,120, or AL1,120 were chromatographed through the calibrated column, and their molecular masses in parentheses. AL1,120, or AL1,120 were chromatographed through the calibrated column, and their molecular masses were thyroglobulin (669 kDa), apoferritin (443 kDa), β-amylase (200 kDa), alcohol dehydrogenase (150 kDa), albumin (66 kDa), carbonic anhydrase (29 kDa), and cytochrome c (12.4 kDa). The ratios of their Vc values and the column Ve were plotted against their reported molecular masses. The logarithmic dependence of Vc/Vt was calculated and is shown as the fitted line. Protein extracts from baculovirus-infected insect cells expressing AL1,352, AL1,181, or AL1,120 were chromatographed through the calibrated column, and their Vc/Vt values were plotted on the standard curve (B).
in vitro was mapped purified GST-AL11–352, GST-AL129–352, and GST-AL166–352 (Fig. 5B, lanes 5–8), and bound to glutathione resin (Fig. 5B, lanes 1–4). Total and bound fractions were resolved by SDS-polyacrylamide gel electrophoresis and analyzed by immunoblotting. GST-AL11–352 (lanes 1 and 5), the N-terminal truncations GST-AL129–352 (lanes 2 and 6) and GST-AL165–352 (lanes 3 and 7), and GST alone (lanes 4 and 8) were co-expressed with authentic AL11–352 oligomerization with the truncated proteins. Co-purification of AL11–352 with GST-AL11–352 (Fig. 5B, lanes 1 and 5) and GST (Fig. 5B, lanes 4 and 8) were analyzed in parallel as positive and negative controls, respectively, for specificity of interaction. Together, these results established that GST-AL129–352 (Fig. 5B, lanes 2 and 6) and GST-AL165–352 are competent for AL1 interaction but deficient for DNA binding. Thus, the functional domain for DNA binding is located between amino acids 1 and 181 and overlaps the AL1 oligomerization domain between amino acids 121 and 181.

The AL1-DNA Cleavage Domain—TGMV AL1 catalyzes site-specific DNA cleavage of the (+) strand origin of replication. The truncated GST-AL1 fusion proteins were used to map the boundaries of the DNA cleavage domain. A radiolabeled, single-stranded oligonucleotide containing the origin nick site (TGMV A positions 129–151) was incubated with full-length GST-AL11–352 and GST fusions lacking AL1 C-terminal amino acids (Fig. 5A). The mobility of a marker corresponding to the cleavage product is shown (Fig. 5A, lane 1). Cleavage products were detected for GST-AL11–352 (Fig. 5A, lane 1), GST-AL129–352 (Fig. 5A, lane 2), GST-AL165–352 (Fig. 5A, lane 3), and GST-AL11–120 (Fig. 5A, lane 4). No cleavage product was seen with GST alone (Fig. 5A, lane 5) or in the absence of protein (Fig. 5A, lane 6), indicating that the nicking activity was specific to AL1. These results showed that the DNA cleavage domain of TGMV AL1 is in the first 120 amino acids of the protein, and that GST-AL11–120 was folded correctly and functional. Although GST-AL11–213 and GST-AL11–181 formed AL1 oligomers and bound DNA, neither activity was detected for GST-AL11–120 (Figs. 2 and 4). However, because GST-AL11–120 cleaved DNA, observed with agarose gels may reflect the large size of the AL1/DNA complexes demonstrated in Fig. 2B.

The N-terminal boundary of the AL1-DNA binding domain was mapped in vitro using GST-AL129–352 and GST-AL11–352 (Fig. 1B), which lacked the first 28 or 65 amino acids of AL1, respectively. GST-AL129–352 and GST-AL11–352 were each expressed in baculovirus-infected insect cells and purified using glutathione affinity chromatography. The glutathione-eluted proteins were pure or highly enriched, as shown by Coomassie Brilliant Blue staining of SDS-polyacrylamide gels (Fig. 3, lanes 7 and 8). GST-AL11–352 (Fig. 3, lane 6) was co-electrophoresed for size comparison. The DNA binding activities of purified GST-AL11–352, GST-AL129–352, and GST-AL165–352 were compared using three concentrations of a radiolabeled TGMV A DNA fragment (positions 28–84) containing the AL1 binding site. Although shifted bands were readily detected for GST-AL11–352 (Fig. 6A, lanes 1–3), no bound DNA was observed for either GST-AL129–352 (Fig. 5A, lanes 4–6) or GST-AL165–352 (Fig. 5A, lanes 7–9) under any reaction conditions. These results demonstrated that sequences within the first 28 amino acids of AL1 are essential for protein-DNA interaction.

GST-AL11–213 and GST-AL11–120 were also analyzed for their ability to interact with authentic AL11–352 (Fig. 5B) to verify that the truncated proteins were properly folded and functional. The truncated GST-AL1 proteins were co-expressed with authentic AL1 in insect cells, extracted (Fig. 5B, lanes 1–4), and bound to glutathione resin (Fig. 5B, lanes 5–8). Immunoblot analysis showed that authentic AL11–352 co-purified with both GST-AL129–352 (Fig. 5B, lanes 2 and 6) and GST-AL165–352 (Fig. 5B, lanes 3 and 7), demonstrating AL1 oligomerization with the truncated proteins. Co-purification of AL11–352 with GST-AL11–352 (Fig. 5B, lanes 1 and 5) and GST (Fig. 5B, lanes 4 and 8) were analyzed in parallel as positive and negative controls, respectively, for specificity of interaction. Together, these results established that GST-AL129–352 (Fig. 5B, lanes 2 and 6) and GST-AL165–352 are competent for AL1 interaction but deficient for DNA binding. Thus, the functional domain for DNA binding is located between amino acids 1 and 181 and overlaps the AL1 oligomerization domain between amino acids 121 and 181.

The N-terminal boundary of the AL1-DNA binding domain. A, GST-AL1 proteins were analyzed by electrophoretic mobility shift assays for binding to a radiolabeled DNA fragment containing the TGMV AL1 binding site. Approximately 560 ng of GST-AL11–352 (lanes 1–3), GST-AL129–352 (lanes 4–6), and GST-AL165–352 (lanes 7–9) were incubated with 0.2 ng (lanes 1, 4, and 7), 0.1 ng (lanes 2, 5, and 8), or 0.05 ng (lanes 3, 6, and 9) of radiolabeled DNA. B, protein extracts from baculovirus-infected cells (total, lanes 1–4) were incubated with glutathione resin and eluted in SDS sample buffer (bound, lanes 5–8). Total and bound fractions were resolved by SDS-polyacrylamide gel electrophoresis and analyzed by immunoblotting. GST-AL11–352 (lanes 1 and 5), the N-terminal truncations GST-AL129–352 (lanes 2 and 6) and GST-AL165–352 (lanes 3 and 7), and GST alone (lanes 4 and 8) were co-expressed with authentic AL11–352.

The AL1-DNA Cleavage Domain—TGMV AL1 catalyzes site-specific DNA cleavage of the (+) strand origin of replication. The truncated GST-AL1 fusion proteins were used to map the boundaries of the DNA cleavage domain. A radiolabeled, single-stranded oligonucleotide containing the origin nick site (TGMV A positions 129–151) was incubated with full-length GST-AL11–352 and GST fusions lacking AL1 C-terminal amino acids (Fig. 5A). The mobility of a marker corresponding to the cleavage product is shown (Fig. 5A, lane 1). Cleavage products were detected for GST-AL11–352 (Fig. 5A, lane 1), GST-AL129–352 (Fig. 5A, lane 2), GST-AL165–352 (Fig. 5A, lane 3), and GST-AL11–120 (Fig. 5A, lane 4). No cleavage product was seen with GST alone (Fig. 5A, lane 5) or in the absence of protein (Fig. 5A, lane 6), indicating that the nicking activity was specific to AL1. These results showed that the DNA cleavage domain of TGMV AL1 is in the first 120 amino acids of the protein, and that GST-AL11–120 was folded correctly and functional. Although GST-AL11–213 and GST-AL11–181 formed AL1 oligomers and bound DNA, neither activity was detected for GST-AL11–120 (Figs. 2 and 4). However, because GST-AL11–120 cleaved DNA,
Purified GST alone did not cleave DNA (Fig. 6B, lane 5). lanes 2–4 were deficient for DNA cleavage activity (Fig. 6B). The amino acids of AL1, which contain motif I, are essential for DNA cleavage. These results demonstrated that sequences in the first 28 amino acids of AL1 specifically cleaved DNA containing the origin nick site (Fig. 6B). Lane 6, uncleaved substrate; lane 7, radiolabeled marker corresponding to the predicted product (M). B, cleavage results for GST-AL11–352 (lane 1) and the N-terminal truncated proteins GST-AL11–120 (lane 2), GST-AL11–120 (lanes 3), and GST AL alone (lane 5). Lane 6, uncleaved substrate; lane 7, radiolabeled marker corresponding to the predicted product (M). 3, In contrast, we showed that AL1 amino acids 1–181 located the C-terminal domain responsible for DNA binding. To verify that GST-AL11–120 and AL182–352 were active for DNA cleavage and ATP hydrolysis, respectively, indicating that the truncated proteins were properly folded and that the loss of protein interaction was due to deletion of sequences required for AL1 oligomerization. This conclusion was further supported by gel filtration data showing that the apparent and predicted monomeric molecular mass of AL11–120 are equivalent, consistent with it occurring as a single subunit in solution. In contrast, the apparent molecular masses of AL11–352 and AL11–181 complexes are approximately eight times greater than their predicted monomeric masses. However, the precise stoichiometry of the AL1 subunits could not be determined, because the complexes may have included other AL1-interacting proteins in the crude extracts. Together, these data demonstrated that native AL1 is oligomeric and that amino acids 121–181 are required and sufficient for AL1 oligomerization.

Truncated GST-AL1 proteins were used in electrophoretic mobility shift assays to map the TGMV AL1-DNA binding domain. The failure of GST-AL11–352 to bind DNA demonstrated that the first 28 amino acids of AL1 are essential for protein-DNA interactions. The loss of DNA binding activity by GST-AL11–120 but not GST-AL11–181 located the C-terminal boundary of the DNA binding domain between amino acids 121 and 181. These results showed that the functional domain for DNA binding is between AL1 amino acids 1 and 181. Hong and Stanley (39) reported that the first 57 amino acids of the C1 protein of African cassava mosaic virus (ACMV) are sufficient to repress C1 expression in tobacco protoplasts and proposed that the DNA binding domain of ACMV C1 is located in this region (39). In similar studies, we found that deletion of as little as 39 amino acids from the TGMV AL1 C terminus abrogated transcriptional regulation, 4 indicating that the DNA binding domain of TGMV AL1 is not the only requirement for repression in vivo. One potential explanation for the observed differences between the TGMV and ACMV proteins may be that the putative ACMV C1 binding site does not contain directly repeated motifs such as those found in the TGMV AL1 binding site. Thus, TGMV AL1 and ACMV C1 may contact their respective promoters differently and may repress transcription through different mechanisms.

AL1 recognition of the (+) strand origin is essential for virus-specific DNA replication (8). Chimeric virus studies showed that the N-terminal third of C1 confers virus-specific replication to closely related strains of TYLCV (31) or beet curly top virus (32). Replication studies using chimeric origins and AL1 expression cassettes established that amino acids 1–116 of TGMV and BGMV AL1 specifically recognize the repeated DNA binding motifs in their respective (+) strand origins in vivo.5 In contrast, we showed that AL1 amino acids...
1–181 are necessary for DNA binding in vitro. The additional sequences between amino acids 121 and 181 required for in vitro DNA binding may contribute essential DNA contacts that are conserved between TGMV and BGMV. Alternatively, AL1 oligomerization, which has been mapped to amino acids 121–181, may be a prerequisite for AL1-DNA binding. Chimeric studies only reveal amino acid differences involved in AL1-DNA interactions and, therefore, cannot distinguish between these possibilities.

DNA binding proteins frequently interact with DNA as dimeric or multimeric complexes (40, 41). Two observations support the idea that TGMV AL1 binds DNA as a multimer. First, the TGMV AL1 binding site contains a repeated motif, such that two AL1 subunits could interact simultaneously with the site. Protein dimer interactions with directly repeated sequences have been described for α-2 protein (42) and HAP1 (43). Second, electrophoretic mobility shift assays suggested that AL1 binds DNA as a large multimeric complex, with AL1-DNA complexes failing to enter polyacrylamide gels and only being resolved on agarose gels. Binding experiments using circularly permuted DNA fragments indicated that it is unlikely that the low electrophoretic mobility of the AL1-DNA complex is due to unusual DNA structure or bending. Several assays failed to determine the stoichiometry of the AL1-DNA complexes. Electrophoretic mobility shift assays with full-length and truncated AL1 proteins bound to DNA did not distinguish heterodimer formation (data not shown). In addition, fusion to GST, which dimerizes with itself (44), or addition of AL1 antibodies did not restore DNA binding activity to AL11–120. Based on these results, we think that it is unlikely that fusion to a heterologous protein interaction domain will restore DNA binding activity to AL11–120 and that a different strategy will be necessary to address the relationship between DNA binding and oligomerization.

AL1 initiates rolling circle replication by introducing a nick into the (+) strand origin of the viral DNA. Heyraud-Nitschke et al. (25) showed that the first 211 amino acids of the TYLCV C1 protein possess DNA cleavage activity. Our results showed that the first 120 amino acids of TGMV AL1 specifically cleaves single-stranded DNA containing the (+) strand origin in vitro and that AL1 oligomerization and DNA binding were not prerequisites for cleavage of a single-stranded DNA in vitro. However, AL1-DNA binding may be required for cleavage of the double-stranded viral genome during rolling circle replication in vivo. Three motifs in the N termini of all geminivirus AL1/CI proteins are also conserved among initiator proteins from other rolling circle systems (26, 27). Motif I (FLTY) is located between amino acids 16 and 19. Motif II (HLH) is a putative metal binding site consisting of two histidines within a region of bulky hydrophobic residues. Motif III includes a highly conserved tyrosine residue that is required for DNA cleavage and ligation by TYLCV C1 (28). Although the role of motif I is unknown, deletion of the N-terminal 28 amino acids of TGMV AL1 abolished DNA cleavage activity, suggesting that this conserved element may be essential for DNA cleavage. The loss of DNA cleavage activity by GST-AL129–352 precluded any conclusions about motifs II or III based on other N-terminal truncations of AL1.

The AL1/CI protein sequences from 17 dicot-infesting geminiviruses were compared using the EMBL Predict program (45) to determine whether the N terminus of AL1 contains any conserved structural motifs that might contribute to DNA binding, DNA cleavage, or oligomerization. This analysis revealed two sets of α-helices that are predicted with greater than 80% probability (Fig. 7A). Helices 1 and 2 are between TGMV AL1 amino acids 25–52 in the overlapping DNA binding and cleavage domains and might be involved in these activities. The sequences of both helices show a high degree of homology among different geminiviruses (Fig. 7B), especially helix 2, which is conserved at 9 of 11 positions and displays a strong amphipathic character (Fig. 7C). Most known DNA binding motifs include α-helical regions that recognize and contact DNA (46). However, the AL1 N terminus shows no obvious

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5 B. M. Orozco and L. Hanley-Bowdoin, Unpublished results.
homology to the $\alpha$-helical motifs of basic/helix-loop-helix, etc., homeodomain, zinc finger, or basic/leucine zipper proteins (for review, see Refs. 46–48). AL1 helices 1 and 2, which are separated by a 5-amino acid loop, most resemble the helix-turn-helix motif, but our sequence comparison failed to uncover a nearby third helix characteristic of most helix-turn-helix DNA binding domains (49). The second set of predicted $\alpha$-helices is located between TGMV AL1 amino acids 131 and 152 in the overlapping AL1-DNA binding and oligomerization domains (Fig. 7A). Several classes of DNA binding proteins, including members of the basic/helix-loop-helix, homeodomain, and basic/leucine zipper families, use $\alpha$-helices for dimerization as well as DNA contacts (46, 50, 51). The significance of these predicted structures in DNA binding and/or protein interactions is being investigated.

The oligomerization, DNA binding, and DNA cleavage domains are located to the N-terminal half of AL1, whereas very little is known about the C terminus of the protein. To date, the only biochemical activity that has been attributed to the AL1 C terminus is ATP and GTP hydrolysis (18). We found that deletion of only 39 amino acids from the TGMV AL1 C terminus abolished DNA replication and repression in vivo, further demonstrating the functional importance of this region. We also observed that deletion of the C-terminal 139 amino acids of AL1 enhanced DNA binding activity approximately 4-fold in vitro, 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 co-factors (43, 52, 53). The AL1 C terminus may also mediate interaction with other proteins, single-stranded DNA binding, nuclear localization, and/or attachment to the nuclear matrix. We are continuing to map the functional domains of TGMV AL1 to gain a more complete understanding of AL1 structure and function. We are also constructing a series of site-directed mutations in the N terminus of TGMV AL1 to address the functional significance of the predicted $\alpha$-helices and the relationship between DNA binding and protein oligomerization.

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