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# Conserved Sequence and Structural Motifs Contribute to the DNA Binding and Cleavage Activities of a Geminivirus Replication Protein*

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**Tomato golden mosaic virus (TGMV), a member of the geminivirus family, has a single-stranded DNA genome that replicates through a rolling circle mechanism in nuclei of infected plant cells. TGMV encodes one essential replication protein, AL1, and recruits the rest of the DNA replication apparatus from its host. AL1 is a multifunctional protein that binds double-stranded DNA, catalyzes cleavage and ligation of single-stranded DNA, and forms oligomers. Earlier experiments showed that the region of TGMV AL1 necessary for DNA binding maps to the N-terminal 181 amino acids of the protein and overlaps the DNA cleavage (amino acids 1–120) and oligomerization (amino acids 134–181) domains. In this study, we generated a series of site-directed mutations in conserved sequence and structural motifs in the overlapping DNA binding and cleavage domains and analyzed their impact on AL1 function *in vivo* and *in vitro*. Only two of the fifteen mutant proteins were capable of supporting viral DNA synthesis in tobacco protoplasts. *In vitro* experiments demonstrated that a pair of predicted α-helices with highly conserved charged residues are essential for DNA binding and cleavage. Three sequence motifs conserved among geminivirus AL1 proteins and initiator proteins from other rolling circle systems are also required for both activities. We used truncated AL1 proteins fused to a heterologous dimerization domain to show that the DNA binding domain is located between amino acids 1 and 130 and that binding is dependent on protein dimerization. In contrast, AL1 monomers were sufficient for DNA cleavage and ligation. Together, these results established that the conserved motifs in the AL1 N terminus contribute to DNA binding and cleavage with both activities displaying nearly identical amino acid requirements. However, DNA binding was readily distinguished from cleavage and ligation by its dependence on AL1/AL1 interactions.**

Tomato golden mosaic virus (TGMV)[1] is a member of the geminivirus family of plant-infecting viruses characterized by twin icosahedral particles and small, single-stranded DNA genomes (reviewed in Refs. 1 and 2). The single-stranded DNA is converted to a double-stranded form in the nucleus of infected cells and then serves as a template for rolling circle replication (RCR; Refs. 3–5) and viral gene transcription (6, 7). Geminiviruses encode only a few proteins for these processes and depend on host DNA and RNA polymerases as well as their accessory factors. These characteristics make geminiviruses excellent model systems for studying plant DNA replication and transcription mechanisms.

The TGMV genome consists of two circular DNA molecules, designated as A and B (8). Both components have a conserved 5′ intergenic region (IR) that separates divergent open reading frames (9). The IR includes the plus-strand origin of replication (10) and the promoters for leftward and rightward transcription (6, 7). A directly repeated sequence in the TGMV IR is required for recognition of the plus-strand origin and negative regulation of the overlapping promoter for leftward transcription (11, 12). Related motifs are found in the genomes of most dicot-infecting geminiviruses (13), and their roles in virus-specific replication have been confirmed for bean golden mosaic virus (BGMV) and beet curly top virus (14–16). The IR also contains a hairpin with a 9-base pair loop sequence conserved among all geminiviruses that is cleaved during initiation and termination of RCR (5, 17, 18). Genetic experiments established that the hairpin structure is essential for TGMV replication (18).

TGMV encodes two proteins, AL1 and AL3, that are required for efficient viral replication. AL1 is necessary for replication, whereas AL3 enhances viral DNA accumulation by an unknown mechanism (19, 20). AL1 is a multifunctional protein that confers virus-specific recognition to its cognate plus-strand origin (15, 16, 21) and initiates RCR (17, 18, 22). It also actively represses its own transcription in a virus-specific manner (12, 16, 23, 24) and induces the expression of a host DNA synthesis protein, proliferating cell nuclear antigen, in nondividing plant cells (25). Several biochemical activities have been described for AL1 *in vitro*. TGMV AL1 binds to double-stranded DNA at the conserved repeated motif in the plus-strand origin (11, 26) and cleaves single-stranded DNA in the invariant sequence of the hairpin loop (18). Analysis of the C1 protein of tomato yellow leaf curl virus (TYLCV), a TGMV AL1 homologue, revealed that it covalently attaches to the 5′ end of the cleaved DNA by a phosphotyrosyl bond and catalyzes ligation of the cleavage products (27). ATPase activity has also been demonstrated for the AL1/C1 proteins from TYLCV and TGMV (28, 29). TGMV AL1 is involved in several protein interactions. It forms large multimeric complexes (29), binds AL3 (30), and interacts with a maize homologue of the animal cell cycle regulatory protein, retinoblastoma (31). The C1 protein from wheat dwarf virus also interacts with retinoblastoma proteins from human and maize (32–34).

In an earlier study, we used truncated proteins produced in a baculovirus expression system to map the regions of TGMV AL1 that are responsible for DNA cleavage, DNA binding and

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[1] The abbreviations used are: TGMV, tomato golden mosaic virus; IR, intergenic region; RCR, rolling circle replication; BGMV, bean golden mosaic virus; TYLCV, tomato yellow leaf curl virus; GST, glutathione *S*-transferase; nt, nucleotide(s).

TABLE I
*AL1 mutations*

| Motif | Mutation | Oligonucleotide | Baculovirus vector | Plant expression |
|---|---|---|---|---|
| Helix 1 | H1–1 | GAGAAAGTGATgCggCcgcGGACAAGGAGCAC | pNSB627 | pNSB655 |
| | H1–2 | GTAATTGAGAAAGTacTTCTTCTTTGGAC | | pNSB434 |
| Helix 2 | H2–1 | CTTCATGAAGCgCTgcaCAGATTgcTATGAATTTTTTGTTAATCGG | pNSB628 | pNSB656 |
| | H2–2 | CTCTGCAGATcTTTccGAATTTTTTGTTAATCGG | pNSB629 | pNSB657 |
| | H2–3 | CATCTTCATGtcGCTCTtcGCAGAcTTTTATGAATTTTTTGTTAATCGG | pNSB630 | pNSB658 |
| Motif 1 | M1–1 | GCACTGAGGAgcgGccgcAgcATAATTTTTGGCAT | pNSB649 | pNSB652 |
| | M1–2 | CACCTCGAGGATAcGTAAGAgcATAATTTTTGGCATT | pNSB685 | pNSB681 |
| | M1–3 | GACATGAGGAgcgGTAAGAAAATAATTTTTGGGGCATT | pNSB686 | pNSB682 |
| Motif 2 | M2–1 | GAATAAGCACGgcggccgcAGGTTGCCCATC | pNSB650 | pNSB653 |
| Motif 3 | M3–1 | GAGTATCTCCGgCTTTaTCGATGgcCGTCTTGACGTCG | pNSB651 | pNSB654 |
| | M3–2 | CTTTGTCGATcgcCGTCTTGACGTCG | pNSB687 | pNSB683 |
| | M3–3 | GAGTATCTCCGgCTTTaTCGATGTACG | pNSB688 | pNSB684 |
| | M3–4 | TACAAGAGTAgCTCCGgCTTTcgCGATGTACGTCTTGAC | pNSB741 | pNSB747 |
| | M3–5 | CTCCGTCTTTaTCGATGaACGTCTTGAC | pNSB781 | pNSB779 |
| | M3–6 | GAGTATCTCCGTCTgcaTCGATGTACG | pNSB782 | pNSB780 |

oligomerization (29). These experiments showed that the DNA cleavage domain is located in the first 120 amino acids of AL1 whereas the oligomerization domain maps to the protein center between amino acids 121 and 181. DNA binding requires a larger region that fully overlaps the DNA cleavage and oligomerization domains. Based on these experiments, we proposed that additional amino acids between position 121 and 181 are necessary for AL1/DNA binding and/or that AL1 complex formation is required for DNA binding. In this study, we identified key sequence and structural motifs in the DNA binding and cleavage domains. A series of site-directed mutations in the AL1 N terminus were analyzed for their impact on function *in vivo* and *in vitro*. These studies focused on three amino acid motifs, which are conserved among all geminivirus AL1/C1 proteins and many initiator proteins from other RCR systems (35, 36), and on a predicted helix-loop-helix motif in the N termini of AL1/C1 proteins of dicot-infecting geminiviruses (29). We also used a heterologous protein interaction domain to assess the importance of oligomerization for AL1/DNA binding and coupled DNA cleavage/ligation.

## MATERIALS AND METHODS

*Mutagenesis and Cloning of AL1 Proteins*—The plasmid pNSB148, which contains the AL1 coding sequence in a pUC118 background, was used as the template for site-directed mutagenesis (37). The oligonucleotide primers and resulting clones are listed in Table I. The sequences of fragments containing the mutations and used for subsequent cloning were verified by DNA sequence analysis. Plant expression cassettes with the mutant AL1 coding sequences were generated by subcloning *Nde*I/*Sal*I fragments (AL1 amino acids 1–120) from the mutant clones into the same sites in a wild type AL1 plant expression cassette pMON1549 (11). In pMON1549, AL1 expression is under the control of the cauliflower mosaic virus 35 S promoter with a duplicated enhancer (38) and the 3′ end from the pea E9 *rbc*S gene (39). Baculovirus expression vectors coding for AL1 proteins fused to a glutathione *S*-transferase tag (GST-AL1) were generated by digesting the mutant plant expression cassettes with *Nde*I and *Bam*HI and repairing the ends with *Escherichia coli* DNA polymerase I (Klenow). The fragments, which included complete AL1 coding regions, were inserted into the *Sma*I site of pNSB314. The pNSB314 vector contains the GST coding sequence, followed by a glycine linker, a thrombin cleavage site, and a multiple cloning site for generating in-frame fusion proteins (18). The mutant GST-AL1 expression cassettes are listed in Table I.

*Purification and in Vitro Assays for AL1 Function*—Wild type and mutant GST-AL1 fusion proteins were expressed in *Spodoptera frugiperda* (Sf9) cells using a Tn7-based baculovirus expression system (40) and purified from Sf9 cell cultures by glutathione affinity chromatography according to published protocols (18). Purified proteins were visualized by electrophoresis in 16% polyacrylamide-SDS gels and staining with Coomassie Brilliant Blue dye. Interactions between authentic AL1 and mutant GST-AL1 proteins were assayed by copurification on glutathione-Sepharose, followed by immunoblot analysis using AL1 polyclonal antisera (29, 30).

DNA gel shift assays were performed as described previously (18). An 83-base pair *Eco*RI fragment containing the AL1 DNA binding motif (TGMV A positions 28–84) was isolated from pNSB378 and 3′ end-labeled using Klenow and [$\alpha$-$^{32}$P]dATP. The radiolabeled DNA was incubated with purified GST-AL1 fusion proteins for 1 h at room temperature at the concentrations indicated in the figure legends. The bound and free probe were resolved on 1% agarose gels, dried onto DE-81 paper, and analyzed by autoradiography.

For DNA cleavage and ligation, oligonucleotides corresponding to sequences in the hairpin of the TGMV (+)-strand origin were 5′ end-labeled using polynucleotide kinase and [$\gamma$-$^{32}$P]ATP. The oligonucleotides CR13 (5′-GTTTAATATTACCGGATGGCCGC) and CR33 (5′-GCGGCCATCCGTTTAATATT) were used for assays with full-length mutant and wild type AL1 proteins. For assays with truncated AL1 proteins, only one oligonucleotide, CR13 or CR34 (5′-GCGGCCATCCGTTTAATATTACCGGATGG) was radiolabeled and the cold oligonucleotide was titrated into the reactions as described in the figure legend. Approximately 5000 cpm of each labeled DNA was incubated with ~100 ng of purified GST-AL1 fusion protein in 10 $\mu$l of cleavage buffer (25 mM Tris-HCl, pH 7.5, 75 mM NaCl, 5 mM $MgCl_2$, 2.5 mM EDTA, 2.5 mM dithiothreitol, and 250 ng of poly(dI-dC)) at 37 °C for 30 min. The reactions were terminated by adding 6 $\mu$l of gel loading buffer (95% formamide, 20 mM EDTA, 0.05% bromphenol blue) and heating to 90 °C for 2 min. The reaction products were resolved on 15% polyacrylamide denaturing gels and analyzed by autoradiography.

*Transient Replication Assays*—Protoplasts were isolated from *Nicotiana tabacum* NT-1 suspension cells, electroporated, and cultured according to published methods (11). The transfections contained 15 $\mu$g each of replicon DNA containing a partial tandem copy of TGMV B (pTG1.4B described in Ref. 14), wild type or mutant AL1 expression cassette, and an AL3 plant expression cassette (pNSB46 described in Ref. 11). Total DNA was extracted 3 days after transfection and analyzed for double-stranded viral DNA accumulation by DNA gel blot hybridization (11).

## RESULTS

*Helices 1 and 2 Are Essential for AL1 Function in Vivo and in Vitro*—Two key steps in initiation of RCR are origin recognition and generation of a free 3′-OH for priming plus-strand DNA synthesis. TGMV AL1 mediates both of these processes by binding double-stranded DNA in a sequence-specific manner and by cleaving at a unique site in the origin. Earlier studies established that the AL1 N terminus is necessary for both activities (29). This region of AL1 contains several conserved sequence and structural motifs (Fig. 1), including a highly predicted pair of $\alpha$-helices between amino acids 25 and 52 (29). The sequences of both helices are strongly conserved among dicot-infecting geminiviruses, and the second helix is amphipathic in character. This conservation suggested that the predicted $\alpha$-helices may be necessary for AL1 function. To test this hypothesis, we generated four site-directed mutants of TGMV AL1 that are modified in either helix 1 or helix 2 (Fig. 1*B*) and compared their activities to the wild type protein *in vivo* and *in vitro* (Fig. 1*B*).

The helix mutants were first tested for the ability to direct

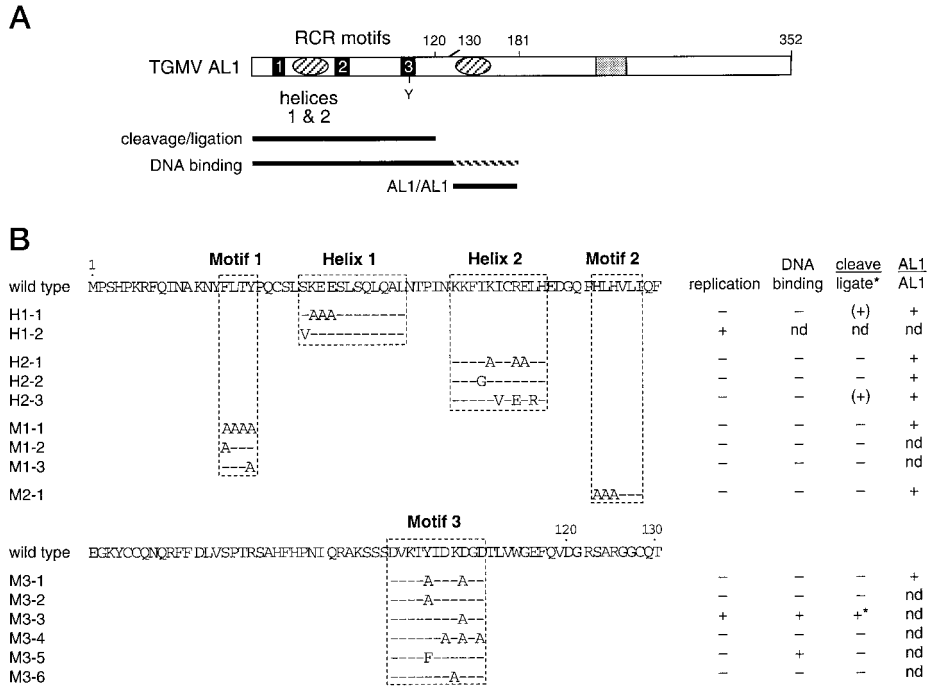


FIG. 1. **TGMV AL1 domains and mutations.** *A*, schematic of the TGMV AL1 protein. *Solid boxes* mark the location of the three motifs conserved among RCR initiator proteins, the *hatched circles* indicate predicted sets of $\alpha$-helices, and the *stippled box* shows the location of the ATP binding domain. The *Y* in motif 3 corresponds to the tyrosine residue in the active site for DNA cleavage. The *lines* below the protein mark the location of the functional domains for DNA cleavage/ligation, DNA binding, and AL1/AL1 interaction. The *numbers* indicate amino acid position numbers. *B*, the sequence of wild type TGMV AL1 from amino acids 1 to 130 is shown. Conserved sequence and structural motifs are marked by *boxes* and labeled in *bold* above the sequence. Mutations are listed on the *left*, and the amino acid changes are shown in *uppercase letters* in the *boxed* regions. The *dashes* indicate wild type amino acids. A summary of the replication, double-stranded DNA binding, DNA cleavage/ligation, and AL1/AL1 oligomerization activities displayed by each mutant AL1 protein is shown on the *right*. A (+) indicates weak activity, *nd* is not determined, and * indicates that ligation activity was tested in the cleavage assays.

replication of TGMV B DNA in tobacco protoplasts with wild type TGMV AL3 also supplied *in trans*. Helix 1-mutant 2 (H1–2), which contains a S25V change and resembles the AL1 protein of the closely related geminivirus BGMV, supported wild type levels of TGMV B replication (Fig. 2*A*, *cf. lanes 1* and *2*). In contrast, H1–1, which has alanine substitutions at three conserved charged residues in helix 1, failed to support viral DNA replication (Fig. 2*A*, *lane 3*). The mutations in helix 2 either converted three conserved charged residues to alanine (H2–1), included a I45G change (H2–2), or altered the helix to resemble BGMV AL1 (H2–3). All helix 2 mutations abolished transient replication (Fig. 2*A*, *lanes 3–5*). The mutant phenotypes established that, in general, the amino acid sequences of the predicted helices are essential for AL1 activity *in vivo*. The negative effect of the I45G replacement in H2–2, which should disrupt helix 2, suggested that the structure is also required for AL1 function.

The AL1 helix mutants defective in replication assays were expressed as GST-AL1 fusion proteins in insect cells, purified by glutathione affinity chromatography, and examined for various AL1 activities *in vitro*. H1–2 was not included in these experiments because of its wild type replication phenotype. The mutant proteins were tested for DNA binding activity in gel shift assays using a radiolabeled double-stranded DNA probe that includes the TGMV AL1 DNA binding site. None of the helix mutants bound DNA (Fig. 2*B*, *lanes 3–6*), even though wild type AL1 efficiently shifted the probe in a parallel reaction (*lane 2*). The mutant AL1 proteins were also tested for DNA cleavage activity using a radiolabeled, single-stranded oligonucleotide corresponding to the loop and right side of the hairpin in the plus-strand origin. Wild type GST-AL1 cleaved the 23-nt substrate to give a 10-nt radiolabeled product (Fig. 2*C*, *lane 2*). Mutants H1–1 (Fig. 2*C*, *lane 3*) and H2–3 (*lane 6*) displayed severely attenuated DNA cleavage activity, whereas H2–1 (*lane 4*) and H2–2 (*lane 5*) had no detectable activity. These results demonstrated that the predicted helices 1 and 2 are required both for DNA binding and cleavage by TGMV AL1.

Helices 1 and 2 are outside of the AL1 interaction domain, and their mutation should have no effect on oligomerization if the proteins are properly expressed and folded. To verify that the failure of the AL1 helix mutants to bind and cleave DNA was not due to global misfolding, the proteins were assayed for their abilities to form oligomers with authentic AL1. Wild type GST-AL1 (Fig. 3*A*, *lane 1*) and GST fusions of H1–1 (*lane 2*), H2–1 (*lane 3*), H2–2 (*lane 4*), and H2–3 (*lane 5*) were coexpressed with authentic AL1 in insect cells, as determined by immunoblotting of total protein extracts. Like wild type GST-AL1 (Fig. 3*A*, *lane 6*), all of the mutant proteins copurified with authentic AL1 on glutathione-Sepharose (*lanes 7–10*), indicating that the helix mutations did not impair AL1/AL1 interactions and, instead, specifically affected the DNA binding and cleavage activities of AL1.

*Motifs 1 and 2 Are Required for Initiation of RCR*—The N terminus of AL1 also includes three conserved sequence motifs that are found in many RCR initiator proteins (Fig. 1; Refs. 35 and 36). Laufs *et al.* (27) showed that motif 3 corresponds to the endonucleolytic active site, but the roles of motifs 1 and 2 in RCR have not been investigated. We specifically modified these motifs in TGMV AL1 and analyzed the impact of the mutations on protein function (Fig. 1*B*). In M1–1, all four motif I residues ($F^{16}LTY^{19}$) were replaced with alanine. In M1–2 and M1–3, individual aromatic residues were altered to give F16A and Y19A, respectively. The motif 2 mutant contained alanine substitutions for the core HLH sequence. Transient replication assays revealed that none of the motif 1 or 2 mutants (Fig. 4*A*, *lanes 2–5*) supported TGMV B amplification in tobacco protoplasts, establishing that both motifs are essential for AL1 activity *in vivo*.

To gain insight into the biochemical basis of the mutant replication phenotypes, we purified GST-AL1 fusion proteins corresponding to the motif 1 and 2 mutants from insect cells and tested them for DNA binding and cleavage *in vitro*. All of the mutant proteins failed to bind double-stranded DNA (Fig. 4*B*, *lanes 2–5*). Similarly, none of the mutants had detectable single-stranded DNA cleavage activity (Fig. 4*C*, *lanes 2–5*). In parallel assays, wild type GST-AL1 (*lane 1*) efficiently bound (Fig. 4*B*) and cleaved (Fig. 4*C*) the respective DNA probes. Protein interaction experiments (Fig. 3*B*) established that M1–1 (*lanes 3* and *8*) and M2–1 (*lanes 4* and *9*) can form oligomers with wild type AL1 and, thus, are not globally misfolded. Together, these results showed that motifs 1 and 2 of TGMV AL1 are required for both DNA binding and cleavage

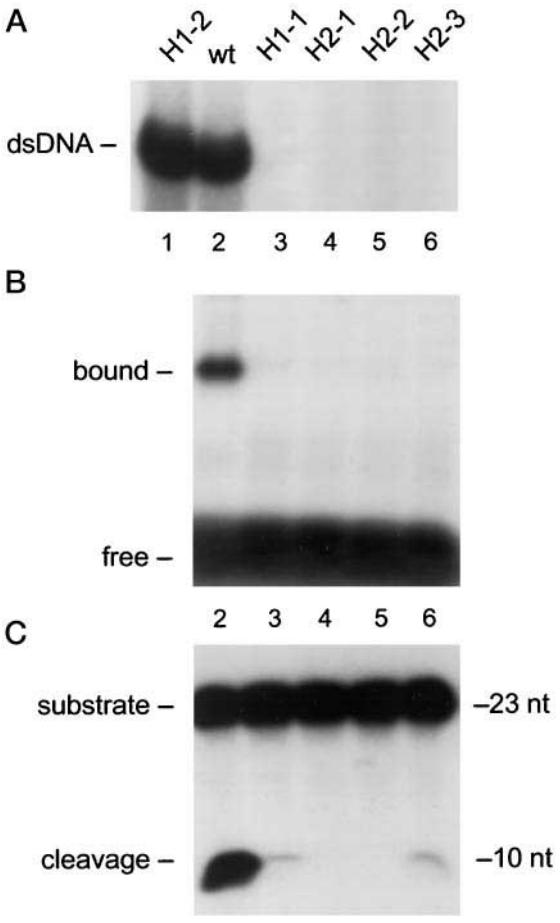


FIG. 2. **AL1 helix 1 and 2 mutations negatively affect DNA binding and cleavage.** Wild type AL1 (*lane 2*) and AL1 helix mutants H1–2 (*lane 1*), H1–1 (*lane 3*), H2–1 (*lane 4*), H2–2 (*lane 5*) and H2–3 (*lane 6*) were tested for replication (*A*), DNA binding (*B*), and DNA cleavage (*C*) activities. *A*, double-stranded DNA replication (*dsDNA*) was monitored in protoplasts cotransfected with wild type or mutant AL1 proteins, a TGMV B replicon, and a wild type AL3 expression cassette. Total DNA was isolated 3 days after transfection, digested with *Bgl*II and *Dpn*I, and analyzed by DNA gel blot hybridization using a radiolabeled TGMV B probe. *B,* GST-AL1 proteins were analyzed by gel shift assays for binding to a radiolabeled double-stranded DNA fragment containing the AL1 DNA binding site. Purified wild type or mutant GST-AL1 protein (1.5 $\mu$g) was incubated with 0.05 ng of DNA, and the complexes were resolved on agarose gels. The positions of free DNA and bound complexes are shown on the *left*. *C,* GST-AL1 proteins (0.4 $\mu$g) were analyzed for DNA cleavage of a 23-nt oligonucleotide (10 fmol) containing the TGMV loop sequence and right side of the stem. Positions and sizes of the 5′ labeled substrate and cleavage product are marked.

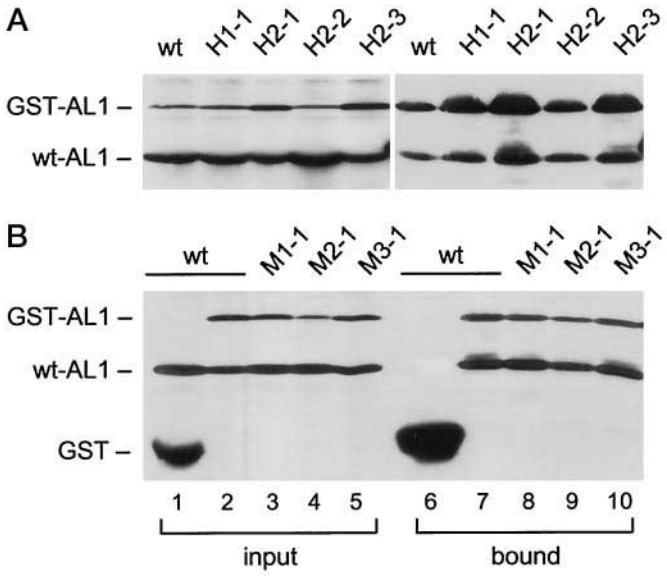


FIG. 3. **Oligomerization properties of mutant GST-AL1 proteins.** Wild type and mutant GST-AL1 proteins were coexpressed with authentic AL1 in insect cells. Total soluble protein extracts were incubated with glutathione-Sepharose, washed and eluted in SDS-sample buffer. Input (*lanes 1–5*) and bound (*lanes 6–10*) fractions were resolved by SDS-polyacrylamide gel electrophoresis and analyzed by immunoblotting. *A,* total extracts were assayed for copurification of authentic AL1 with wild type GST-AL1 (*lanes 1* and *6*), and the GST-AL1 helix mutants, H1–1 (*lanes 2* and *7*), H2–1 (*lanes 3* and *8*), H2–2 (*lanes 4* and *9*), and H2–3 (*lanes 5* and *10*). *B*, total extracts were assayed for copurification of authentic AL1 with GST (*lanes 1* and *6*), wild type GST-AL1 (*lanes 2* and *7*), and the GST-AL1 motif mutants, M1–1 (*lanes 3* and *8*), M2–1 (*lanes 4* and *9*), and M3–1 (*lanes 5* and *10*).

during initiation of RCR.

*Conserved Amino Acids in Motif 3 Contribute to DNA Binding as Well as DNA Cleavage*—Motif 3 includes several conserved amino acids that may contribute to AL1 function. We generated six TGMV AL1 mutants that modified one or more residues in motif 3 and analyzed their activities *in vivo* and *in vitro*. In M3–1, alanines were substituted for the catalytic Tyr-103 and conserved Asp-107 residues. As expected, M3–1 did not support TGMV B replication in tobacco protoplasts (Fig. 5*A*, *lane 2*) and was defective for DNA cleavage (Fig. 5*C*, *lane 2*). Gel shift assays with M3–1 revealed that double-stranded DNA binding was also attenuated over a range of protein concentrations (Fig. 5*B*, *lanes 5–7*), even though wild type GST-AL1 readily formed protein/DNA complexes at the same concentrations (*lanes 2–4*). In some experiments, low levels of DNA binding were observed with the M3–1 protein (data not shown). Protein interaction experiments showing that M3–1 can oligomerize with wild type AL1 (Fig. 3*B*, *lanes 5* and *10*) indicated that the motif 3 mutant is not globally misfolded.

The DNA binding defect of M3–1 was unexpected because of the direct involvement of motif 3 in catalysis of DNA cleavage. To better characterize the role of motif 3 and specifically Tyr-103 in DNA binding, we generated two different mutations at this position. In M3–2, Tyr-103 was changed to alanine, while M3–5 contained a phenylalanine substitution. Like M3–1, neither M3–2 or M3–5 supported viral DNA replication (Fig. 5*A*, *lanes 3* and *6*) or cleaved DNA (Fig. 5*C*, *lanes 3* and *6*), confirming that Tyr-103 is necessary for these processes. However, only M3–2 was impaired for DNA binding over a range of protein concentrations (Fig. 5*B*, *lanes 5–7*), whereas M3–5 displayed wild type DNA binding properties (*lanes 17–19*). These results demonstrated that Tyr-103 contributes to both the DNA binding and cleavage activities of TGMV AL1. These data also established that the aromatic ring of Tyr-103 is essential for DNA binding, while the hydroxyl group is required for DNA cleavage.

AL1 mutants were constructed to assess the importance of conserved charged residues in motif 3. M3–3, which contains a D107A change, supported wild type levels of TGMV B replication (Fig. 5*A*, *cf. lane 4*). Consistent with the replication data, M3–3 displayed wild type DNA binding properties (Fig. 5*B*, *lanes 11–13*) and supported efficient DNA cleavage (Fig. 5*C*, *lane 4*) and ligation (*lane 9*). Hence, the mutant phenotype of M3–1, which was altered at Tyr-103 as well as at Asp-107, was fully attributable to the Y103A substitution. In M3–4, Asp-105, Asp-107, and Asp-109 were all changed to alanine, whereas M3–6 contained a K106A substitution. Both mutants failed to support DNA replication (Fig. 5*A*, *lanes 5* and *7*), bind DNA (Fig. 5*B*, *lanes 14–16* and *20–22*), or direct cleavage (Fig. 5*C*, *lanes 5* and *7*), thereby establishing the importance of the conserved Lys-106 residue and at least one aspartic acid resi-

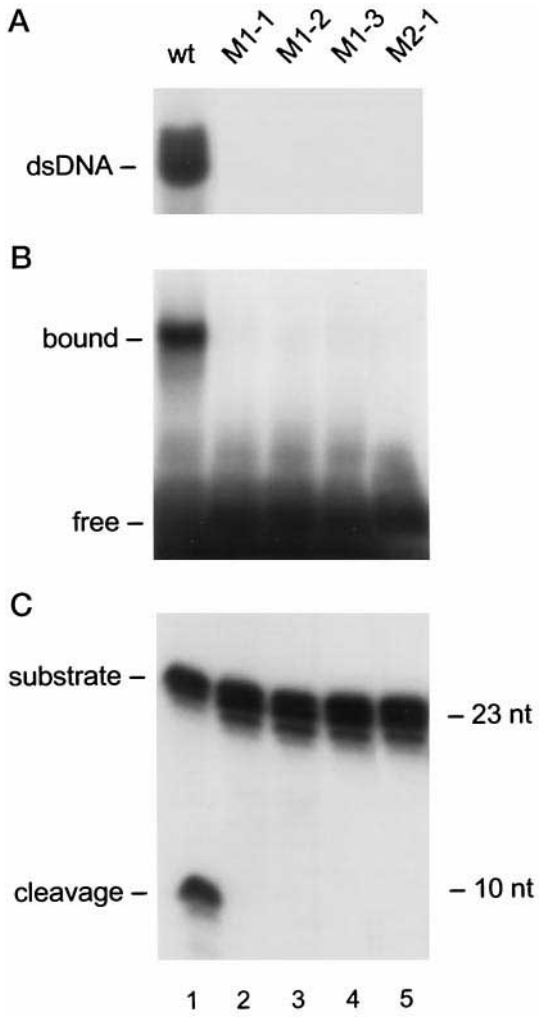


FIG. 4. **Motifs 1 and 2 are required for AL1 activity.** Wild type AL1 (*lane 1*) and AL1 motif mutants M1–1 (*lane 2*), M1–2 (*lane 3*), M1–3 (*lane 4*), and M2–1 (*lane 5*) were tested for replication (*A*), DNA binding (*B*), and DNA cleavage (*C*) activities, as described in Fig. 2. The double-stranded DNA replication product (*dsDNA*) corresponding to TGMV B is marked in *A*. The AL1/DNA complex (bound) and the probe DNA (free) are labeled in *B*. The positions and sizes of the 5′ labeled substrate and cleavage product are marked in *C*.

due in AL1 function. However, our results do not address the individual importance of Asp-105 and Asp-109 or the possibility that the three aspartic acids in motif 3 are functionally redundant.

*AL1 Oligomerization Is Required for DNA Binding*—In earlier studies (16, 29), we showed that AL1 amino acids 1–116 mediate virus-specific recognition of the AL1 binding site *in vivo*, whereas amino acids 1–181 are required for DNA binding activity *in vitro*. To reconcile these differences, we proposed that AL1 binds DNA as an oligomer and that DNA binding requires two domains: the DNA binding domain in the AL1 N terminus and the oligomerization domain between amino acids 134 and 181.[2] To better define the limits of the AL1 DNA binding domain, we expressed GST-$AL1_{1–130}$ and GST-$AL1_{1–120}$ in insect cells and purified the fusion proteins by binding to glutathione-Sepharose followed by elution with glutathione (Fig. 6*A*, *lanes 1* and *3*). The DNA binding properties of the proteins were compared in gel shift assays using a range of protein concentrations (Fig. 6B). GST-$AL1_{1–120}$ showed no DNA binding activity (Fig. 6*B*, *lanes 2–4*), whereas GST-$AL1_{1–130}$ bound DNA at all protein concentrations (*lanes 5–7*). These data established that sequences between amino acids 120 and 130 are required for AL1 DNA binding activity and that amino acids 134–181, corresponding to the AL1 oligomerization domain, do not contribute specific contacts with the DNA.

The ability of GST-$AL1_{1–130}$ to bind DNA does not rule out a requirement for oligomerization in AL1/DNA binding because GST forms dimers (41). To address the role of oligomerization in AL1/DNA binding, GST-$AL1_{1–130}$ was bound to glutathione-Sepharose and digested with thrombin to release $AL1_{1–130}$. Immunoblot analysis showed that there was no detectable GST-$AL1_{1–130}$ protein in the $AL1_{1–130}$ preparation (Fig. 6*A*, *cf. lanes 1* and *2*). Unlike GST-$AL1_{1–130}$ (Fig. 6*B*, *lanes 5–7*), $AL1_{1–130}$ displayed no detectable DNA binding activity in gel shift assays (*lanes 8–10*). The failure of $AL1_{1–130}$ to form stable protein/DNA complexes could not be attributed to GST stabilization of the truncated protein because $AL1_{1–130}$ was active in DNA cleavage assays (data not shown). Instead, these results established that AL1/DNA binding is dependent on oligomerization either through the homologous (AL1 amino acids 134–181) or a heterologous (GST) protein interaction domain. Although the size of the authentic AL1/DNA complexes is unknown, the ability of GST-$AL1_{1–130}$ to bind DNA suggests that dimerization is sufficient for AL1/DNA interactions.

*AL1 Monomers Are Sufficient for the DNA Cleavage/Ligation Reaction*—RCR requires two cleavage events and ligation to form a single-stranded, circular DNA product. To better mimic the *in vivo* events, we altered the cleavage and ligation assay to include two oligonucleotide substrates, A and B, that resulted in different length cleavage products (Fig. 7*C*). When radiolabeled substrate A was incubated with GST-$AL1_{1–120}$, two bands corresponding to the labeled 29-nt substrate and 20-nt 5′ cleavage product were observed (Fig. 7*A*, *lane 2*). A new 33-nt radiolabeled band was seen when unlabeled substrate B was titrated into the reactions (Fig. 7*A*, *lanes 3–5*), indicating that both substrates had been cleaved and that the 5′ phosphoryl donor of substrate B had been ligated to the 3′ acceptor of substrate A. In reciprocal reactions containing radiolabeled substrate B (23 nt) and unlabeled substrate A, a 10-nt cleavage product and 19-nt ligation product were observed (Fig. 7*B*, *lanes 2–5*). Interestingly, GST-$AL1_{1–120}$ ligated the 5′ phosphoryl donor of substrate B more efficiently than the shorter 5′ phosphoryl donor of substrate A. It was reported that the ligation efficiency of TYLCV C1 also increases with the length of the 5′ phosphoryl donor oligonucleotide (22).

We then asked whether AL1 oligomerization is required for the double cleavage and ligation reaction. $AL1_{1–120}$ was purified without the GST tag as described above for $AL1_{1–130}$. Immunoblot analysis verified that the thrombin-cleaved protein was free of residual GST-$AL1_{1–120}$ (Fig. 6*A*, *lanes 3* and *4*). $AL1_{1–120}$ was incubated with the two substrates and assayed for DNA cleavage and ligation. In Fig. 7 (*A* and *B*), the cleavage and ligation products were readily detectable in reactions containing $AL1_{1–120}$ (*lanes 6–9*). Thus, unlike AL1/DNA binding activity, DNA cleavage and ligation are not dependent on AL1 oligomerization.

## DISCUSSION

Geminiviruses replicate their DNA genomes by a rolling circle mechanism, analogous to some plasmids and phage in bacteria (reviewed in Refs. 42 and 43) and the parvoviruses in animal cells (44, 45). Each of these replicons encodes an initiator protein that binds double-stranded DNA and catalyzes DNA cleavage and ligation during single-stranded DNA syn-

[2] Unpublished experiments (B. M. Orozco and L. Hanley-Bowdoin) refined the N-terminal limit of the AL1 oligomerization domain to position 134.

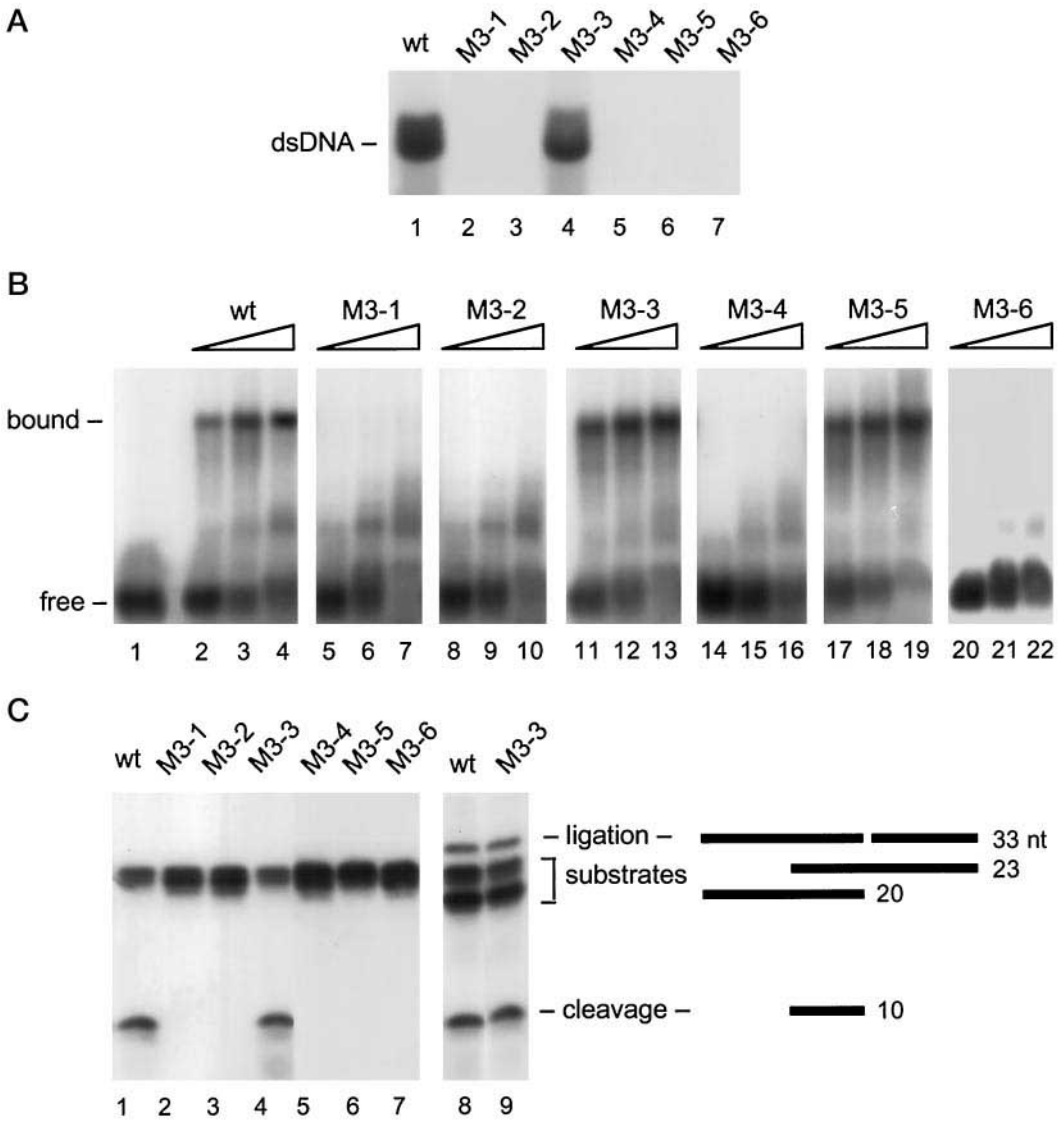


FIG. 5. **Motif 3 contributes to DNA binding as well as DNA cleavage and ligation.** *A*, wild type AL1 (*lane 1*) and the AL1 motif mutants M3–1 (*lane 2*), M3–2 (*lane 3*), M3–3 (*lane 4*), M3–4 (*lane 5*), M3–5 (*lane 6*), and M3–6 (*lane 7*) were analyzed in replication assays as described in Fig. 2*A*. The double-stranded DNA product (*dsDNA*) corresponding to TGMV B is marked. *B,* GST-AL1 proteins were analyzed by gel shift assays. Radiolabeled double-stranded DNA containing the AL1 DNA binding site (0.1 ng) was incubated alone (*lane 1*) and with wild type AL1 (*lanes 2–4*), and the AL1 motif mutants M3–1 (*lanes* 5–7), M3–2 (*lanes 8–10*), M3–3 (*lanes 11–13*), M3–4 (*lanes 14–16*), M3–5 (*lanes 17–19*), and M3–6 (*lanes 20–22*). As indicated by the *triangles*, 1, 2, and 3 $\mu$g of each GST-AL1 protein was tested in the binding reactions. The positions of free DNA and protein/DNA complexes are shown on the *left*. *C,* GST-AL1 proteins were analyzed for DNA cleavage using 10 fmol of the 23-nt substrate (*lanes 1–7*). Cleavage and ligation were detected using 10 fmol each of the 23- and 20-nt substrates (*lanes 8* and *9*). The reactions contained 0.4 $\mu$g of wild type GST-AL1 (*lanes 1* and *8*) or GST-AL1 motif mutants M3–1 (*lane 2*), M3–2 (*lane 3*), M3–3 (*lanes 4* and *9*), M3–4 (*lane 5*), M3–5 (*lane 5*), and M3–6 (*lane 6*). The positions and sizes of the 5′-labeled substrates, cleavage product, and ligation product are marked on the *right*.

thesis. The DNA binding step recruits the RCR initiator protein to the origin and mediates origin recognition. The initiator protein then cleaves the plus-strand DNA at a specific site to generate a free 3′-OH that is used to prime DNA synthesis from the minus-strand template. Upon completion of a full round of replication, a second cleavage and ligation reaction releases a circular, single-stranded copy of the genome and a new double-stranded copy. In geminivirus replication, viral AL1 or C1 proteins are the RCR initiator proteins. *In vitro* experiments established that, like other RCR initiator proteins, AL1/C1 binds double-stranded DNA and catalyzes single-stranded DNA cleavage and ligation in a sequence-specific manner (11, 17, 18). Both activities have been mapped to the N terminus of AL1/C1 (22, 29), and it was proposed that conserved structural and sequence motifs in this region are involved in DNA binding and cleavage. In this paper, we showed that these conserved motifs contribute to DNA binding and cleavage with both activities requiring nearly identical amino acids. However, DNA binding was distinguished from cleavage and ligation by its dependence on AL1/AL1 interactions.

The N terminus of TGMV AL1 contains two pairs of predicted $\alpha$-helices that are conserved among the AL1/C1 proteins of dicot-infecting geminiviruses (29). The first set of $\alpha$-helices, designated as helices 1 and 2, are located between amino acids 25 and 52. Protein/DNA interactions frequently involve contacts with amino acids in $\alpha$-helical regions. Thus, it was not unexpected that mutations in both helices abolished geminivirus replication and blocked AL1/DNA binding. However, the same mutations also impaired DNA cleavage, indicating that the predicted $\alpha$-helical regions are also required for cleavage activity. The H1–1 and H2–1 mutations, which substituted alanines for conserved charged residues in each helix, showed that the charge distribution across the two helices is essential for DNA binding and cleavage. Similarly, the negative pheno-

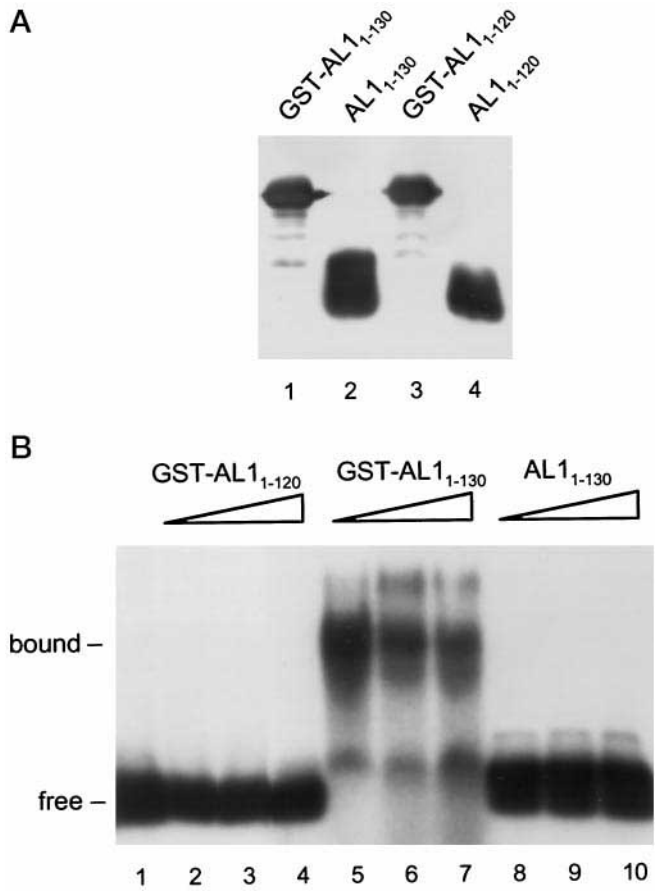


FIG. 6. **AL1/DNA binding is dependent on protein oligomerization.** *A*, truncated GST-AL1 proteins expressed in insect cells were bound to glutathione-Sepharose and eluted with free glutathione (*lanes 1* and *3*) or cleaved with thrombin (*lanes 2* and *4*) to remove the GST tag. The purified proteins GST-$AL1_{1–130}$ (*lane 1*), $AL1_{1–130}$ (*lane 2*), GST-$AL1_{1–120}$ (*lane 3*), and $AL1_{1–120}$ (*lane 4*) were fractionated by SDS-polyacrylamide gel electrophoresis and visualized by immunoblotting. *B,* the proteins were analyzed in gel shift assays for binding to a radiolabeled fragment containing the AL1 DNA binding site, as described in Fig. 2*B*. The positions of free and bound probe are shown on the *left*. The probe (0.1 ng) was incubated alone (*lane 1*), and with 3, 2, and 1 $\mu$g of GST-$AL1_{1–120}$ (*lanes 2–4*), GST-$AL1_{1–130}$ (*lanes 5–7*), or $AL1_{1–130}$ (*lanes 8–10*).

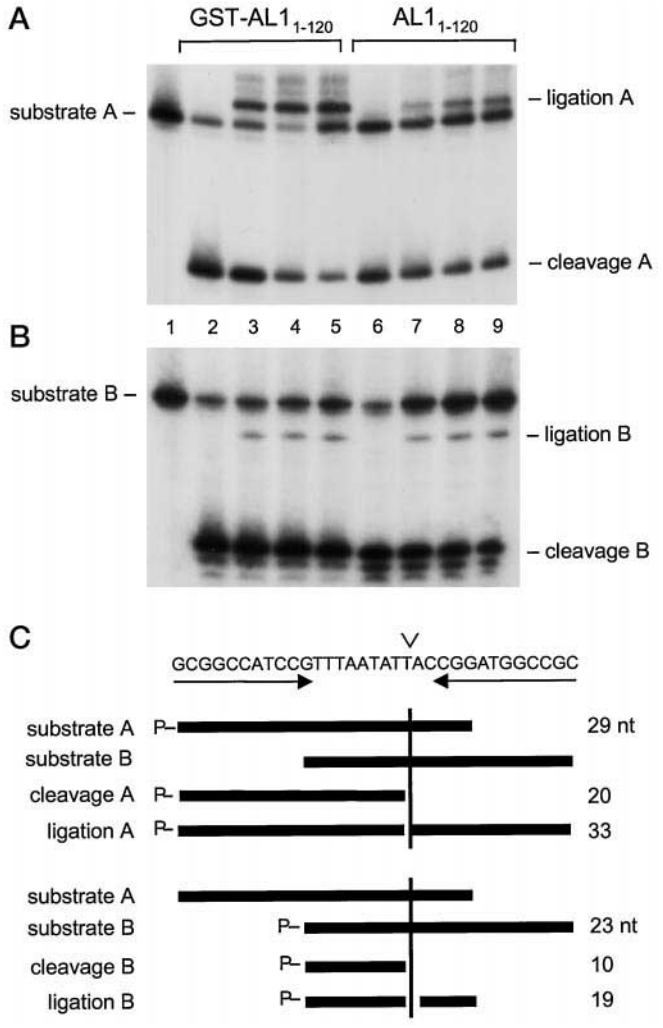


FIG. 7. **DNA cleavage and ligation do not require the AL1 oligomerization domain.** In *A* and *B*, 3 $\mu$g of purified GST-$AL1_{1–120}$ (*lanes 2–5*) and $AL1_{1–120}$ (*lanes 6–9*) were tested for DNA cleavage and ligation activities. The purified proteins are shown in Fig. 6*A*, *lanes 3* and *4*. *A,* DNA cleavage and ligation were monitored using 10 fmol of radiolabeled cleavage substrate A, as shown in *panel C*. The unlabeled cleavage substrate B was titrated into the reactions at 10 (*lanes 3* and *7*), 40 (*lanes 4* and *8*), and 120 fmol (*lanes 5* and *9*). *Lane 1* shows labeled substrate A in the absence of protein. The positions of cleavage substrates, labeled cleavage product and labeled ligation product are marked. *B,* DNA cleavage and ligation were monitored using 10 fmol of radiolabeled cleavage substrate B, as shown in *panel C*. Unlabeled substrate A was titrated into the reactions at 120 (*lanes 3* and *7*), 240 (*lanes 4* and *8*) and 600 fmol (*lanes 5* and *9*). *Lane 1* shows labeled substrate B in the absence of protein. *C*, the sequence of the TGMV plus-strand hairpin is shown. The inverted repeat that forms the stem is indicated by the *arrows below* the sequence, whereas the position of the nick site in the loop region is marked *above* the sequence. *Solid lines* are aligned under the corresponding sequence of substrate A and its labeled products. *Hatched lines* are aligned under the corresponding sequence of substrate B and its labeled products. The position of $^{32}P$ radiolabel is indicated by a *P*. Unlabeled cleavage products are not shown. The lengths of oligonucleotides are given on the *right*.

type of the H2–2 mutation, which contained an I45G change that should disrupt helix 2, suggested that the predicted helical structure is required for both activities. Substituting the BGMV AL1 helix 2 sequence into the context of the TGMV AL1 protein in mutant H2–3 also abolished DNA binding and cleavage. Although TGMV and BGMV AL1 bind related but distinct DNA sequences in their respective plus-strand origins (14), there is no evidence that DNA cleavage is a virus-specific process. The origin hairpin, which is cleaved to initiate RCR, is highly conserved among dicot-infecting geminiviruses (46) and can be exchanged between TGMV and BGMV (14). However, our data clearly showed that N-terminal domains of TGMV and BGMV AL1 are distinct in their requirements for recognition and cleavage of their cognate origins.

The N terminus of TGMV AL1 also contains three conserved amino acid motifs that are found in all geminivirus replication proteins and in many RCR initiator proteins (Fig. 1; Refs. 35 and 36). Although motif 1 (FLTY) has no known function, deletion of the first 25 amino acids of TGMV AL1 suggested that motif 1 is required for AL1/DNA binding and cleavage (29). Hence, we used site-directed mutagenesis to specifically modify motif 1 in the context of full-length TGMV AL1. Mutations that substituted alanine for all four residues (M1–1) or individually at Phe-15 (M1–2) and Tyr-18 (M1–3) abolished TGMV B replication *in vivo* and AL1 DNA binding and cleavage activities *in vitro*. These results unequivocally established the importance of motif 1 for TGMV AL1 function, but its precise role remains unclear because of the pleiotropic effect of our mutations. One possibility is that AL1 binds the hairpin before cleaving the loop and the protein/DNA contacts that mediate hairpin binding extensively overlap those necessary for origin recognition.

The motif 2 consensus sequence is HUHUUU, with U representing bulky hydrophobic amino acids (Fig. 1*B*). Based on

similarity to metalloenzymes, it was proposed that the two histidines may be involved in metal ion binding necessary for the activity of RCR initiator proteins (36). This idea is consistent with the observation that $Mg^{2+}$ or $Mn^{2+}$ is required for DNA cleavage by the TYLCV C1 protein (17). Mutation of the core histidine motif in TGMV AL1 abolished DNA replication *in vivo* and DNA cleavage and binding *in vitro*, demonstrating motif 2 is essential for AL1 activity. However, further analyses with the wild type and mutant proteins are necessary to determine whether motif 2 is a metal ion coordination site.

Motif 3, which corresponds to the catalytic site for DNA cleavage (27), contains a tyrosine residue at position 103 that is conserved in all geminivirus AL1/C1 proteins. The importance of this tyrosine for TGMV AL1 function was demonstrated by mutants containing Y103F (M3–2) or Y103A (M3–5) changes. Both mutants were defective for replication *in vivo* and DNA cleavage *in vitro*. Surprisingly, the Y103A mutation also attenuated AL1/DNA binding, whereas the Y103F mutant bound DNA comparable to wild type protein. Experiments with TYLCV C1 and BGMV AL1 proteins established that Tyr-103 is necessary for replication of these geminiviruses (27, 47). In TYLCV C1, Tyr-103 is required for DNA cleavage (27) and was shown to be involved in catalysis by experiments establishing that it is covalently attached to the 5′ end of the DNA cleavage product (27). It is not known if TYLCV C1 binds DNA or if Tyr-103 contributes to binding activity. However, our results clearly demonstrated that for TGMV AL1, the tyrosine in the cleavage active site is also essential for DNA binding and that the phenyl group is specifically required for the protein/DNA interactions.

Acidic amino acids and lysine residues are associated with the active sites of some RCR initiator proteins (48, 49) as well as some host DNA nucleases (50). TGMV AL1 contains two highly conserved charged residues, Lys-106 and Asp-107, immediately downstream of the active site tyrosine. Mutation of Lys-106 showed that it is essential for DNA replication *in vivo* and DNA binding and cleavage *in vitro*. A Lys-106 mutation in BGMV AL1 also abolished replication (47). In contrast, the D107A mutation had no detectable effect on TGMV AL1 function *in vivo* or *in vitro*. One explanation of this result is that the aspartic acids at positions 105 and 109 compensate for the D107A mutation. This idea is consistent with the inability of a TGMV AL1 mutant containing alanine substitutions for all three aspartic acids (Asp-105, Asp-107, and Asp-109) to support replication or to cleave and bind DNA. Our data do not rule out the possibility that Asp-105 and/or Asp-109 is essential for TGMV AL1 function, but we do not think that this is likely for two reasons. First, mutation of BGMV AL1 Asp-105 had no detectable impact on viral DNA replication (47), indicating that this residue is not specifically required for function. Second, geminivirus AL1/C1 proteins most commonly have an aspartic acid at position 107 and one at position 105 or 109 but not both sites, suggesting that two acidic residues in this region may be sufficient for function. Together, our results established the importance of charged residues in motif 3.

RCR involves two cleavage events and subsequent ligation of the free DNA ends. Because the TYLCV C1 active site contains a single tyrosine residue that cross-links to DNA (27), it is unclear how the second cleavage and ligation events are catalyzed during geminivirus replication. Although none of the motif 3 mutations identified amino acids that are specific for the second cleavage and ligation reaction, transient interaction between the DNA and charged residues in the AL1 active site may occur. Another possibility is that the Tyr-103 residues in separate AL1 subunits of a multimeric complex are required to catalyze both cleavage reactions and ligation. The cleavage/ligation assays were modified to resemble the *in vivo* process by requiring two cleavage reactions prior to ligation. An $AL1_{1–120}$ truncation lacking the oligomerization domain and the corresponding GST fusion, which dimerizes through the heterologous protein tag (41), both efficiently cleaved and ligated DNA in these assays. These results demonstrated that AL1 dimerization is not required for DNA cleavage and ligation and suggested that an AL1 monomer can mediate a complete RCR initiation/termination reaction. However, more extensive mutagenesis and structural analyses are necessary to determine the precise amino acid/DNA interactions involved in these processes.

Unlike DNA cleavage and ligation, AL1 DNA binding is dependent on AL1/AL1 interactions. This conclusion is supported by the observation that the region of AL1 required for DNA binding fully overlaps the oligomerization domain (29). Direct evidence for the involvement of protein interactions in AL1/DNA binding was provided by fusing a heterologous dimerization domain to a truncated AL1 lacking the homologous oligomerization domain. The GST-$AL1_{1–130}$ fusion protein, which can dimerize through the GST moiety, bound DNA, whereas the corresponding $AL1_{1–130}$ truncation, which cannot multimerize, displayed no detectable binding activity. GST-$AL1_{1–120}$ also failed to bind DNA, indicating that it did not contain the full AL1 DNA binding domain. Together, these results located the AL1 DNA binding domain between positions 1–130. Genetic experiments using chimeric TGMV and BGMV AL1 proteins mapped virus-specific origin recognition to the N-terminal 116 amino acids. The importance of amino acids 116–130 for DNA binding was not distinguished with chimeric proteins because the sequence in this region is identical for TGMV and BGMV AL1. Based on our results, it is likely that the DNA binding domains of C1 proteins from TYLCV and beet curly top virus also encompass larger regions than the domains reported for virus-specific origin recognition (15, 21).

Our mutagenesis studies established that the conserved $\alpha$-helices and cleavage motifs in the AL1 N terminus are required for both DNA binding and cleavage/ligation. However, it is striking that 11 of the 12 mutations that impaired DNA cleavage/ligation also affected DNA binding, indicating that there is considerable overlap in the amino acids contributing to these functions. One possibility is that N-terminal domain of the mutant proteins is partially unfolded, thereby affecting multiple functions. This interpretation may be correct for those mutations that destabilized $\alpha$-helical structure or prevented metal ion binding. However, three lines of evidence suggested that incorrect folding cannot account for the reduced activity of all of the AL1 mutants. First, most of the mutations were alanine substitutions and, as such, are structurally neutral changes. Second, one mutation in helix 1 and another in motif 3 resulted in fully functional proteins, demonstrating that TGMV AL1 can tolerate some changes within the conserved motifs. Third, mutations in the two helices and the three cleavage motifs did not impair AL1 oligomerization, indicating that their effects were specific for DNA binding and cleavage. Another possibility is that the strong overlap between the amino acid requirements for DNA binding and cleavage reflects an essential role for DNA binding in positioning the substrate in the active site for cleavage. Although the DNA sequences of the AL1 binding and cleavage sites are different, the amino acid contacts that mediate these DNA interactions may be related. The precise relationship between DNA binding and cleavage can best be addressed through future structural studies of wild type and mutant AL1 proteins.

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REFERENCES


1. Timmermans, M. C. P., Das, O. P., and Messing, J. (1994) *Annu. Rev. Plant Physiol.* **45,** 79–112
2. Hanley-Bowdoin, L., Settlage, S. B., Orozco, B. M., Nagar, S., and Robertson, D. (1998) *Crit. Rev. Plant Sci.*, in press
3. Saunders, K., Lucy, A., and Stanley, J. (1991) *Nucleic Acids Res.* **19,** 2325–2330
4. Stenger, D. C., Revington, G. N., Stevenson, M. C., and Bisaro, D. M. (1991) *Proc. Natl. Acad. Sci. U. S. A.* **88,** 8029–8033
5. Heyraud, F., Matzeit, V., Kammann, M., Schaefer, S., Schell, J., and Gronenborn, B. (1993) *EMBO J.* **12,** 4445–4452
6. Sunter, G., Gardiner, W. E., and Bisaro, D. M. (1989) *Virology* **170,** 243–250
7. Sunter, G., and Bisaro, D. M. (1989) *Virology* **173,** 647–655
8. Hamilton, W. D. O., Bisaro, D. M., Coutts, R. H. A., and Buck, K. W. (1983) *Nucleic Acids Res.* **11,** 7387–7396
9. Bisaro, D. M., Hamilton, W. D. O., Coutts, R. H. A., and Buck, K. W. (1982) *Nucleic Acids Res.* **10,** 4913–4922
10. Orozco, B. M., Gladfelter, H. J., Settlage, S. B., Eagle, P. A., Gentry, R., and Hanley-Bowdoin, L. (1998) *Virology* **242,** 346–356
11. Fontes, E. P. B., Eagle, P. A., Sipe, P. A., Luckow, V. A., and Hanley-Bowdoin, L. (1994) *J. Biol. Chem.* **269,** 8459–8465
12. Eagle, P. A., Orozco, B. M., and Hanley-Bowdoin, L. (1994) *Plant Cell* **6,** 1157–1170
13. Arguello-Astorga, G. R., Guevara-Gonzalez, R. G., Herrera-Estrella, L. R., and Rivera-Bustamante, R. F. (1994) *Virology* **203,** 90–100
14. Fontes, E. P. B., Gladfelter, H. J., Schaffer, R. L., Petty, I. T. D., and Hanley-Bowdoin, L. (1994) *Plant Cell* **6,** 405–416
15. Choi, I. R., and Stenger, D. C. (1995) *Virology* **206,** 904–912
16. Gladfelter, H. J., Eagle, P. A., Fontes, E. P. B., Batts, L. A., and Hanley-Bowdoin, L. (1997) *Virology* **239,** 186–197
17. Laufs, J., Traut, W., Heyraud, F., Matzeit, V., Rogers, S. G., Schell, J., and Gronenborn, B. (1995) *Proc. Natl. Acad. Sci. U. S. A.* **92,** 3879–3883
18. Orozco, B. M., and Hanley-Bowdoin, L. (1996) *J. Virol.* **270,** 148–158
19. Elmer, J. S., Brand, L., Sunter, G., Gardiner, W. E., Bisaro, D. M., and Rogers, S. G. (1988) *Nucleic Acids Res.* **16,** 7043–7060
20. Sunter, G., Hartitz, M. D., Hormuzdi, S. G., Brough, C. L., and Bisaro, D. M. (1990) *Virology* **179,** 69–77
21. Jupin, I., Hericourt, F., Benz, B., and Gronenborn, B. (1995) *FEBS Lett.* **362,** 116–120
22. Heyraud-Nitschke, F., Schumacher, S., Laufs, J., Schaefer, S., Schell, J., and Gronenborn, B. (1995) *Nucleic Acids Res.* **23,** 910–916
23. Sunter, G., Hartitz, M. D., and Bisaro, D. M. (1993) *Virology* **195,** 275–280
24. Eagle, P. A., and Hanley-Bowdoin, L. (1997) *J. Virol.* **71,** 6947–6955
25. Nagar, S., Pedersen, T. J., Carrick, K., Hanley-Bowdoin, L., and Robertson, D. (1995) *Plant Cell* **7,** 705–719
26. Fontes, E. P. B., Luckow, V. A., and Hanley-Bowdoin, L. (1992) *Plant Cell* **4,** 597–608
27. Laufs, J., Schumacher, S., Geisler, N., Jupin, I., and Gronenborn, B. (1995) *FEBS Lett.* **377,** 258–262
28. Desbiez, C., David, C., Mettouchi, A., Laufs, J., and Gronenborn, B. (1995) *Proc. Natl. Acad. Sci. U. S. A.* **92,** 5640–5644
29. Orozco, B. M., Miller, A. B., Settlage, S. B., and Hanley-Bowdoin, L. (1997) *J. Biol. Chem.* **272,** 9840–9846
30. Settlage, S. B., Miller, B., and Hanley-Bowdoin, L. (1996) *J. Virol.* **70,** 6790–6795
31. Ach, R. A., Durfee, T., Miller, A. B., Taranto, P., Hanley-Bowdoin, L., Zambriski, P. C., and Gruissem, W. (1997) *Mol. Cell. Biol.* **17,** 5077–5086
32. Xie, Q., Suarezlopez, P., and Gutierrez, C. (1995) *EMBO J.* **14,** 4073–4082
33. Collin, S., Fernandez-Lobato, M., Gooding, P. S., Mullineaux, P. M., and Fenoll, C. (1996) *Virology* **219,** 324–329
34. Xie, Q., Sanzburgos, P., Hannon, G. J., and Gutierrez, C. (1996) *EMBO J.* **15,** 4900–4908
35. Koonin, E. V., and Ilyina, T. V. (1992) *J. Gen. Virol.* **73,** 2763–2766
36. Ilyina, T. V., and Koonin, E. V. (1992) *Nucleic Acids Res.* **20,** 3279–3285
37. Kunkel, T. A. (1985) *Proc. Natl. Acad. Sci. U. S. A.* **82,** 482–492
38. Kay, R., Chan, A., Daly, M., and McPherson, J. (1987) *Science* **236,** 1299–1302
39. Coruzzi, G., Broglie, R., Edwards, C., and Chua, N.-H. (1984) *EMBO J.* **3,** 1671–1679
40. Luckow, V. A., Lee, S. C., Barry, G. F., and Olins, P. O. (1993) *J. Virol.* **67,** 4566–4579
41. Mannervik, B., and Danielson, U. H. (1988) *CRC Crit. Rev. Biochem.* **23,** 283–337
42. Kornberg, A., and Baker, T. A. (1992) *DNA Replication,* 2nd Ed., W. H. Freeman and Co., New York
43. Del Solar, G., Moscoso, M., and Espinosa, M. (1993) *Mol. Microbiol.* **8,** 789–796
44. Snyder, R. O., Samulski, R. J., and Muzyczka, N. (1990) *Cell* **60,** 105–113
45. Ni, T. H., Zhou, X. H., Mccarty, D. M., Zolotukhin, I., and Muzyczka, N. (1994) *J. Virol.* **68,** 1128–1138
46. Stanley, J. (1995) *Virology* **206,** 707–712
47. Hoogstraten, R. A., Hanson, S. F., and Maxwell, D. P. (1996) *Mol. Plant Microbe Interaction* **9,** 594–599
48. Noirot-Gros, M. F., Bidnenko, V., and Ehrlich, S. D. (1994) *EMBO J.* **13,** 4412–4420
49. Nuesch, J. P. F., Cotmore, S. F., and Tattersal., P. (1995) *Virology* **209,** 122–135
50. Soengas, M. S., Esteban, J. A., Lazaro, J. M., Bernad, A., Blasco, M. A., Salas, M., and Blanco, L. (1992) *EMBO J.* **11,** 4227–4237