The Multifunctional Character of a Geminivirus Replication Protein Is Reflected by Its Complex Oligomerization Properties*

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Beverly M. Orozco‡, Ling-Jie Kong, Lou Ann Batts, Sharon Elledge, and Linda Hanley-Bowdoin

From the Department of Biochemistry, North Carolina State University, Raleigh, North Carolina 27695-7622

Tomato golden mosaic virus (TGMV), a member of the geminivirus family, encodes one essential replication protein, AL1, and recruits the rest of the DNA replication apparatus from its plant host. TGMV AL1 is an oligomeric protein that binds double-stranded DNA and catalyzes cleavage and ligation of single-stranded DNA. The oligomerization domain, which is required for DNA binding, maps to a region that displays strong sequence and structural homology to other geminivirus Rep proteins. To assess the importance of conserved residues, we generated a series of site-directed mutations and analyzed their impact on AL1 function in vitro and in vivo. Two-hybrid experiments revealed that mutation of amino acids 157–159 inhibited AL1–AL1 interactions, whereas mutations at nearby residues reduced complex stability. Changes at positions 157–159 also disrupted interaction between the full-length mutant protein and a glutathione S-transferase-AL1 oligomerization domain fusion in insect cells. The mutations had no detectable effect on oligomerization when both proteins contained full-length AL1 sequences, indicating that AL1 complexes can be stabilized by amino acids outside of the oligomerization domain. Nearly all of the oligomerization domain mutants were inhibited or severely attenuated in their ability to support AL1-directed viral DNA replication. In contrast, the same mutants were enhanced for AL1-mediated transcriptional repression. The replication-defective AL1 mutants also interfered with replication of a TGMV A DNA encoding wild type AL1. Full-length mutant AL1 was more effective in the interference assays than truncated proteins containing the oligomerization domain. Together, these results suggested that different AL1 complexes mediate viral replication and transcriptional regulation and that replication interference involves multiple domains of the AL1 protein.

Geminiviruses are a large family of plant viruses with circular, single-stranded DNA genomes that replicate in the nuclei of infected cells (reviewed in Ref. 1). The single-stranded genome is converted to a double-stranded DNA that serves as the template for rolling circle replication (2–4) and transcription (5, 6). Geminiviruses do not encode their own polymerases and, instead, rely on host enzymes for viral DNA and RNA synthesis. These characteristics make geminiviruses excellent model systems for studying plant DNA replication and transcription mechanisms.

The geminivirus, tomato golden mosaic virus (TGMV), has a bipartite genome that encodes seven open reading frames that are divergently transcribed. The 5’-intergenic region separating the transcription units is nearly identical between the two DNA components and includes the plus strand origin of replication (7, 8). The promoter for complementary sense transcription overlaps the replication origin (5, 9) and shares some of the cis-elements involved in origin function (10). A directly repeated sequence, GTTAG, is required for origin recognition (11) and transcriptional repression of the complementary sense (AL1) promoter (10). Similarly, the TATA-box and G-box transcription factor binding sites in the AL1 promoter act as replication enhancer elements (12). In contrast, three elements in the TGMV intergenic region are necessary for origin function but have little or no effect on AL1 promoter activity. A hairpin structure with a 9-base pair loop sequence that is conserved among all geminiviruses is essential for replication and contains the cleavage site for initiating plus strand DNA synthesis (4, 13, 14). A conserved sequence between the AL1 binding site and the hairpin, the AG-motif, is also required for replication (8). The third element, the CA motif, is located outside of the minimal origin but its deletion reduced replication 20-fold (8). The role of the AG- and CA-motifs in TGMV origins is not known, but one possibility is that they bind host factors that facilitate initiation of plus strand DNA replication.

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 (15, 16) (AL1 homologues are also designated C1 or Rep.) AL1 is a multifunctional protein that mediates both virus-specific recognition of its cognate origin (17) and transcriptional repression by binding to the directly repeated sequence in the intergenic region (10, 12). AL1 initiates and terminates plus strand replication (13, 14, 18) and induces the accumulation of a host replication factor, proliferating cell nuclear antigen, in infected cells (19). Recombinant AL1 specifically binds double-stranded DNA (11, 20), cleaves and ligates single-stranded DNA in the invariant sequence of the hairpin loop (14, 21), and hydrolyzes ATP (22, 23). TGMV AL1 also interacts with itself (23), the viral replication enhancer protein AL3 (24), and a maize homologue of the cell cycle regulatory protein, retinoblastoma (25).

We previously mapped the TGMV AL1 domains for double-stranded DNA binding, single-stranded DNA cleavage and ligation, and AL1 oligomerization (23, 26). The DNA cleavage/ligation domain was located to the first 120 amino acids, and the oligomerization domain was mapped between amino acids 120 and 181. DNA binding activity required amino acids 1–130

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‡ To whom correspondence should be addressed. Tel.: 919-515-5736; Fax: 919-515-2047.

1 The abbreviations use are: TGMV, tomato golden mosaic virus; AD, activation domain; GST, glutathione S-transferase; MSV, maize streak virus.
TABLE I

<table>
<thead>
<tr>
<th>Mutation</th>
<th>Oligonucleotide</th>
<th>Baculovirus vector</th>
<th>Yeast GAL4-AD</th>
<th>Plant expression</th>
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For transcriptional repression assays, N. benthamiana protoplasts were transfected with 15 μg of luc reporter construct (pNSB114), 15 μg of AL1 expression cassette, and 36 μg of sheared salmon sperm DNA (10). Luciferase activity in total soluble protein extracts was measured 36 h post-transfection and standardized for protein concentration. Repression was determined as the ratio of luc activity in the absence versus presence of AL1. Each repression experiment was assayed in triplicate in at least three independent experiments.

**MATERIALS AND METHODS**

**Mutagenesis and Cloning of AL1 Proteins—** Constructs are listed in Table I. The plasmid pNSB148, which contains the AL1 coding sequence in a pUC118-background, was used as the template for site-directed mutagenesis (27). The oligonucleotide primers and resulting clones are also listed in Table I. DNA fragments containing the mutations were verified by DNA sequence analysis. Plant expression cassettes for mutant AL1 proteins were generated by subcloning SalI/XcoI fragments (TGMV A position 2442 and 2059) from the mutant clones into the same sites in the wild type AL1 plant expression cassette pMON1549 (11). In pMON1549, AL1 expression is under the control of the cauliflower mosaic virus 35S promoter with a duplicated enhancer region and the pea E9 rbcS 3'-end.

Baculovirus vectors were generated for expression of mutant and truncated AL1 proteins in insect cells. Expression vectors coding for mutant AL1 proteins were made by subcloning BglII/BamHI inserts from the mutant plant expression cassettes into the BamHI site of pMON27025 (26). Expression vectors for the truncated proteins, AL1119–352 (pNSB516), AL11–120 (pNSB388), and AL11–180 (pNSB517), have been described previously (23). The N-terminal truncations, AL11–120 and AL11–135, were generated by a double-stranded oligonucleotide containing an SphI site in the NotI site of plasmid pNSB593 and pNSB595 to create in-frame stop codons. AL11–352 was created by inserting an SphI linker with a start codon (New England Biolabs, Beverly, MA) into the SphI site of pMON1539 (29). SphI/BamHI fragments from the resulting clones were inserted into the same sites in the baculovirus vector pNSB488 (23), to give pNSB606 (AL11–352), pNSB606 (AL11–352), and pNSB633 (AL11–352). The C-terminal truncation, AL11–156 (pNSB646), was generated by digesting pMON1539 with NdeI and SphI, repairing with Klenow, and subcloning into the filled BamHI site of pMON27025 to create an in-frame stop codon. The AL11–140 truncation, pNSB708, was created by inserting an XhoI linker into the repaired BssHI of pNSB609, generating an in-frame stop codon.

Yeast expression cassettes were generated using the pSA1-1 and pACT2 vectors from CLONTECH (Palo Alto, CA). The BamHI/NdeI fragment of pMON1539 was cloned into the same sites as pSA2-1 to give pNSB736, which contained the GAL4 DNA binding domain fused to wild type AL1 sequences. The ends of the same BamHI/NdeI fragment were repaired with Klenow and cloned into the SmaI site of pACT2 to give pNSB735. The AatII/BamHI fragment of pNSB735 was then replaced with the AatII/BamHI fragment from pMON1549. The resulting clone, pNSB809, contained the GAL4 activation domain (AD) fused to wild type AL1 sequences. Mutant AL1 yeast expression cassettes were created by replacing the wild type AatII/BamHI fragment of pNSB735 with mutant AatII/BamHI fragments from the corresponding plant expression cassettes.

**Transient Replication and Repression Assays—** Protoplasts were isolated from Nicotiana tabacum (BY-2) or Nicotiana benthamiana suspension cells, electroporated, and cultured according to published methods (11). For replication assays, N. tabacum transfections included 15 μg each of replicon DNA containing a partial tandem copy of TGMV B (pTG1.4B, Ref. 17), wild type or mutant AL1 plant expression cassette, and an AL3 plant expression cassette (pNSB46, Ref. 11). For the interference assays, 2 μg of replicon DNA containing a partial tandem copy of TGMV A (pMON1565, Ref. 14) was cotransfected with 40 μg of mutant AL1 expression cassette or the empty expression vector (pMON921, Ref. 11). Total DNA was extracted 3 days post-transfection and analyzed for double- and single-stranded viral DNA accumulation by DNA gel blot hybridization (11). The viral DNA was quantified by phosphorimager analysis in a minimum of three independent experiments.

**AL1 Interactions—** Recombinant proteins were produced in Spodoptera frugiperda SF9 cells using a baculovirus expression system according to published protocols (14, 24). Protein extracts from cells co-expressing authentic and GST-AL1 fusion proteins were assayed for AL1 oligomerization by co-purification on glutathione-Sepharose (24). Purification was monitored by SDS-polyacrylamide electrophoresis followed by transfer to nitrocellulose membrane (Schleicher and Schuell) and immunoblotting using the ECL detection system (Amersham Pharmacia Biotech). Primary antibodies were rabbit polyclonal anti-GST (Upstate Biotechnology Inc.) and anti-AL1 antisera (24).

The Saccharomyces cerevisiae strain Y187 (MATa, ura3–52, his3–200, ade2–1, trp1–901, leu2–3, ura3, galΔA, met+, gal80Δ, URA3, GAL1) was transformed using lithium acetate/polyethylene glycol 3000. The DNAs were pNSB736, which expresses the GAL4 binding domain wild-type AL1 fusion, and either pNSB809, which produces the GAL4 AD-wild type AL1 protein, or the equivalent cassettes corresponding to the GAL4 AD-AL1 mutants. For β-galactosidase assays, yeast transformants were grown to an A600 of 0.5 in 3 ml of synthetic dropout medium lacking tryptophan and leucine (31). Yeasts were pelleted at 10,000 × g for 5 min, rinsed with Z buffer (0.1 M NaPO4, pH 7.0, 10 mM KCl, 1 mM MgSO4, 40 mM β-mercaptoethanol) and resuspended in 300 μl of Z buffer. The cells were subjected to three freeze/thaw cycles in liquid nitrogen and centrifuged at 5000 × g for 2 min. The supernatant (150 μl) was assayed for β-galactosidase activity in a total reaction volume of 250 μl using the substrate o-nitrophenyl β-D-galactopyranoside, as described by CLONTECH. Accumulation of the o-nitrophenol product was measured at A420 using a BioKinetics microplate reader (Bio-Tek Instrument Inc.) at 37 °C. Protein concentrations were measured by Bradford assays (Bio-Rad). The enzyme-specific activity (1 unit = 1.0 μm product/min at pH 7.3 at 37 °C) resulting from interaction between two-hybrid cassettes carrying wild type AL1 sequences was determined using purified β-galactosidase (Sigma) as the standard. The relative activities of the mutants were normalized against the wild type AL1 interaction level, which was set to 100%. The β-galactosidase specific activity for wild type AL1 was adjusted for background from pNSB736 alone. The different constructs were tested in a minimum of two experiments, each of which assayed four independent transformants for each construct.

For immunoblot analysis, individual yeast transformants were grown in 5 ml of medium containing 1% yeast extract, 2% bacto-peptone, 2% glucose, pH 5.8 (31) to an A600 of 1. An equal volume of crushed ice was added and the culture was centrifuged at 1000 × g for...
5 min. The resulting pellet was washed once with ice cold water and resuspended in 80 μl of modified radioimmune precipitation buffer (150 mM NaCl, 1% (v/v) Nonidet P-40, 0.5% (w/v) sodium deoxycholate, 50 mM Tris-HCl, pH 7.5 (32), containing 1% (w/v) SDS) and protease inhibitors (6 μg/ml peptatin A, 10 μg/ml leupeptin, 20 μg/ml aprotinin, 8 mM benzamidine, 1 mM phenylmethylsulfonyl fluoride). Glass beads (40 μl, 425–600 μm, Sigma) were added and the sample was vortexed at maximum speed for four 30-s intervals separated by 2-min intervals on ice. The sample was then centrifuged at 5000 × g for 2 min at 4 °C. The supernatant was recovered, and the protein concentration was determined using Bradford assays. Total protein (100 μg) was resolved on 12% polyacrylamide/SDS gels and analyzed by immunoblotting using a GAL4 AD monoclonal antibody at 0.4 μg/ml (CLONTECH).

RESULTS

The Limits of the AL1 Oligomerization Domain—The domains for TGMV AL1 DNA binding and DNA cleavage/ligation activities are well defined, and key structural and sequence motifs have been identified for these functions (23, 26), whereas the AL1 oligomerization domain has only been broadly located to the center of the protein (23). In this paper, closely spaced N- and C-terminal truncations were generated to define the limits of the AL1 oligomerization domain (Fig. 1A). A GST fusion corresponding to full-length AL1 (GST-AL1) was co-expressed with the truncated AL1 proteins in baculovirus-infected insect cells, and protein complexes were purified on glutathione-Sepharose resin. Total extracts and purified proteins were resolved by SDS-polyacrylamide gel electrophoresis, and proteins were visualized by immunoblotting with AL1 and GST polyclonal antisera. As reported previously (23), the C-terminal truncation AL11–180 (Fig. 1B, lanes 3 and 6) copurified with full-length GST-AL1. Further deletion of the C terminus to amino acid 168 (lanes 2 and 5) or 158 (lanes 1 and 4) abolished interactions with GST-AL1, demonstrating that the C-terminal limit of the oligomerization domain is between position 168 and 180. The N-terminal truncations to amino acids 134 (Fig. 1C, lanes 3 and 6), 147 (lanes 2 and 5), and 160 (lanes 1 and 4) showed a gradual disappearance of interaction with GST-AL1. AL1134–352 consistently displayed interactions with GST-AL1, whereas AL1147–352 and AL1160–352 interactions varied between weak to background levels, making it more difficult to define the N-terminal limit.

Contacts outside the oligomerization domain may contribute to complex stability or multimerization and account for the low level interactions observed with AL1147–352 and AL1160–352. To address this possibility, we asked whether full-length AL1 copurified when only the oligomerization domain was fused to GST (GST-AL1139–180). Full-length AL1 interacted with GST-AL1139–180 but not with GST alone (Fig. 2B, lanes 1 and 2), demonstrating that amino acids 119–180 are sufficient for oligomerization. We then examined the abilities of the N-terminal AL1 truncations to bind GST-AL119–180. In this assay, deletion to positions 119 (Fig. 1D, lanes 1 and 5) and 134 (lanes 2 and 6) did not affect oligomerization, whereas further deletion to positions 147 (lanes 3 and 7) and 160 (lanes 4 and 8) abolished interactions with GST-AL119–180. Together, these results showed that AL1 amino acids 134–180 contain the oligomerization domain and that sequences outside the domain contribute stabilizing contacts.

Mutations in the Oligomerization Domain Affect AL1 Complex Stability—Alanine substitutions were generated in conserved charged or hydrophobic residues within the oligomerization domain to identify key amino acids that contribute to AL1 interactions (Fig. 3, the mutations are designated by the corresponding wild type sequence and the position of the last amino acid that was altered; dashes indicate amino acids that were not changed). Alanine was selected because it is structurally neutral and should not interfere with normal protein folding. The mutations are within a region that includes a pair of predicted α-helices (26) and downstream sequences required for oligomerization. All of the mutant AL1 proteins co-purified with GST-AL1 on glutathione resin when co-expressed in insect cells (Fig. 2A), showing that they formed stable complexes with the wild type protein. Similar results were observed when both the test and GST fusion proteins carried the mutations (data not shown). In contrast, when interactions between the mutant AL1 proteins and GST-AL119–180 were examined mutant EKY159 (Fig. 2B, lane 6) was defective for AL1 interactions. Thus, sequences outside of the oligomerization domain in the full-length AL1 masked the effect of the EKY159 mutation in Fig. 2A, which is consistent with our previous conclusion that sequences outside the oligomerization domain stabilize...
Fig. 2. Oligomerization of the AL1 mutants in insect cells. Protein interactions were assessed as described in Fig. 1B. A, a mutant AL1 proteins coexpressed with full-length GST-AL1 were extracted (top) and bound to glutathione-Sepharose (bottom). Lanes correspond to wild type AL1 (lane 1), E-N140 (lane 3), KEE146 (lane 4), REK154 (lane 5), EKY159 (lane 6), Q-HN165 (lane 7), N-DR172 (lane 8), and K-E179 (lane 9). Wild type AL1 protein was also coexpressed with GST alone (lane 2). B, mutant AL1 proteins were coexpressed with GST-AL1119–180. The lanes are as described in A. C, the mutant EKY159 was coexpressed with GST fusion proteins corresponding to full-length and truncated AL1. AL1 protein in total extracts (lanes 1–4) and bound to glutathione-Sepharose (lanes 5–8) are shown. Lanes correspond to assays with full-length GST-AL1 (lanes 1 and 5), GST-AL1119–180 (lanes 2 and 6), GST-AL1119–352 (lanes 3 and 7), and GST-AL1119–180 (lanes 4 and 8). wt, wild type.

Fig. 3. Site-directed mutations in AL1. The AL1 sequence between amino acids 115 and 180 is shown, with the locations of the oligomerization domain, predicted α-helices, and a conserved sequence indicated. The boxed region indicates the positions of the alanine substitutions. Mutations (shown on the left) are designated by the corresponding wild type sequence and the position of the last amino acid that was altered. Dashes indicate amino acids that were not changed.

All interactions. The oligomerization mutant, EKY159, was assayed with truncated GST-AL1 proteins to locate the stabilizing region. As shown above, EKY159 bound full-length GST-AL1 (Fig. 2C, lanes 1 and 5) but not GST-AL1119–180 (lanes 4 and 8). EKY159 also bound an N-terminal truncation, GST-AL1119–352 (lanes 3 and 7), whereas no interaction was detected with a C-terminal truncation, GST-AL1119–180 (lanes 2 and 6), demonstrating that the AL1 C terminus contributes stabilizing contacts.

The quantitative impact of each mutation on AL1 oligomerization was measured using a yeast two-hybrid system. Expression cassettes for wild type or mutant AL1 fused to the GAL4 DNA binding domain were cotransformed into yeast with cassettes corresponding to either wild type or mutant AL1 fused to the GAL4 activation domain (on the left). Interactions between the AL1 proteins were assayed by measuring β-galactosidase activity in total protein extracts. The error bars correspond to two standard errors. B, total proteins were extracted from yeast and fractionated by polyacrylamide gel electrophoresis. The activation domain-AL1 fusion proteins (AD-AL1) were visualized by immunoblotting with a monoclonal antibody to the GAL4 activation domain. The lanes correspond to fusions with wild type AL1 (lane 2), Q-HN165 (lane 3), D120 (lane 4), RS-R125 (lane 5), QT130 (lane 6), ND133 (lane 7), E-N140 (lane 8), KEE146 (lane 9), REK154 (lane 10), EKY159 (lane 11), Q-HN165 (lane 12), N-DR172 (lane 13), and K-E179 (lane 14). In lane 1, which contained extract from cells transfected with the vector pACT-2, the peptide corresponding to GAL4 activation domain alone migrated further in the gel and is not shown in the blot.

Fig. 4. Interaction of the AL1 mutants in yeast two-hybrid assays. A, an expression cassette for wild type AL1 fused to the GAL4 DNA binding domain was cotransformed into yeast with cassettes corresponding to either wild type or mutant AL1 fused to the GAL4 activation domain (on the left). Interactions between the AL1 proteins were assayed by measuring β-galactosidase activity in total protein extracts. The error bars correspond to two standard errors. B, total proteins were extracted from yeast and fractionated by polyacrylamide gel electrophoresis. The activation domain-AL1 fusion proteins (AD-AL1) were visualized by immunoblotting with a monoclonal antibody to the GAL4 activation domain. The lanes correspond to fusions with wild type AL1 (lane 2), Q-HN165 (lane 3), D120 (lane 4), RS-R125 (lane 5), QT130 (lane 6), ND133 (lane 7), E-N140 (lane 8), KEE146 (lane 9), REK154 (lane 10), EKY159 (lane 11), Q-HN165 (lane 12), N-DR172 (lane 13), and K-E179 (lane 14). In lane 1, which contained extract from cells transfected with the vector pACT-2, the peptide corresponding to GAL4 activation domain alone migrated further in the gel and is not shown in the blot.
mants and examined the levels of the AD-AL1 proteins on immunoblots probed with a monoclonal antibody against the AD. Two bands were detected on the immunoblot (Fig. 4B), both of which were precipitated by AL1 antibodies (data not shown). A doublet was also observed for the GAL4 AD alone in our system (data not shown). Although there was some variation in protein amounts, all of the mutants (Fig. 4B, lanes 3–14) accumulated to similar or greater levels than the fusion protein carrying wild type AL1 sequences (lane 2). Thus, the lack of interaction displayed by mutant EKY159 (lane 11) and the reduced interactions seen with mutants E-N140 (lane 8), KEE146 (lane 9), REK154 (lane 10), and N-DR172 (13) were not attributable to reduced protein levels. Together, these results confirmed the strong negative effect of the EKY159 mutation on AL1 oligomerization and revealed additional contacts that were not detected by the GST copurification assays.

The AL1 homologues of most dicot-infecting geminiviruses contain a 14-amino acid stretch of near sequence identity immediately upstream of the oligomerization domain (Fig. 3). We asked if alanine substitutions in this conserved sequence quantitatively impacted AL1 interactions in yeast two-hybrid assays (Fig. 4A). Mutation FQ118 had no effect on AL1 interactions, and mutations D120 (67%), QT130 (77%) and ND133 (79%) only moderately affected interactions, consistent with their location outside of the domain. Mutation RS-R125 was more severely impaired, reducing AL1 oligomerization to 27% of wild type. However, this mutation also interfered with AL1 DNA binding and cleavage activities in vitro (data not shown), indicating that it is pleiotropic in character.

**Mutations in the AL1 Oligomerization Domain Impair Viral DNA Replication**—Earlier experiments demonstrated that AL1-DNA interaction is a necessary step in geminivirus replication (11). Because AL1 complex formation is required for DNA binding (26), we assessed the effects of the oligomerization mutations on TGMV replication in transient assays. Plant expression cassettes for wild type and mutant AL1 proteins were transfected into tobacco protoplasts with TGMV B DNA and an expression cassette for AL3. AL1 expression was regulated by the CaMV 35S promoter to separate replication from transcription effects of the mutant AL1 proteins. Accumulation of double-stranded TGMV B DNA was examined 3 days later on DNA gel blots. Eleven of the twelve AL1 mutants were impaired in their ability to direct viral DNA replication (Fig. 5A, lanes 2–12) when compared with wild type AL1 (lane 1). Only mutant K-E179 supported near wild type replication levels (lane 13). Mutations REK154 (lane 9), EKY159 (lane 10), Q-HN165 (lane 11), and N-DR172 (lane 12) within the oligomerization domain (lanes 9–12) as well as mutations FQ118 (lane 2), D120 (lane 3), and RS-R125 (lane 4) in the upstream conserved sequence abolished replication. Low levels of TGMV B DNA synthesis were observed for mutants QT130 (lane 5), ND133 (lane 6), E-N140 (lane 7), and KEE146 (lane 8), all of which were modified in the predicted α-helices. The results in Fig. 5A are not due to problems in stable protein production from the mutant AL1 expression cassettes because the same constructs were active in transcriptional repression (Fig. 5B) and replication interference assays (Fig. 6A). Thus, these results demonstrated that the ability of AL1 to support viral DNA replication is very sensitive to mutations in its oligomerization domain and the conserved N-terminal sequences. The high degree of sensitivity may reflect changes in AL1 complexes that are detrimental for replication but cannot be detected in yeast two-hybrid assays, which only measure interaction strength.

**Mutations in the AL1 Oligomerization Domain Enhance Repression of the AL1 Promoter**—We also examined the abilities of the oligomerization mutants to repress complementary sense transcription, which is dependent on the ability of AL1 to bind viral DNA (10). The AL1 promoter fused to the luciferase reporter gene (luc) was transfected into N. benthamiana protoplasts in the presence of plant expression cassettes for wild type and mutant AL1 proteins or the corresponding empty expression cassette. In these experiments, wild type AL1 repressed transcription from the AL1 promoter ~20-fold. In Fig. 5B, repression activities of the mutant AL1 proteins were standardized to wild type (100%). All but one of the mutants that showed reduced viral DNA replication repressed transcription 40–80-fold, i.e. 2–4-fold greater than wild type AL1 repression. RS-R125 was reduced for repression, consistent with its lack of detectable DNA binding activity in vitro. K-E179, which supported normal replication levels, repressed transcription similar to wild type AL1. These results suggested that enhanced repression may be a consequence of a decreased capacity to support viral DNA replication. However, mutations outside of the oligomerization domain in the DNA cleavage (Y104F) and ATP binding (GK229ALE) sites did not display enhanced repression activity (Fig. 5B) even though they are impaired for replication (22, 26, 33). Consequently, increased repression activity correlated specifically with changes in the AL1 oligomerization domain and upstream conserved sequence.

**AL1 Oligomerization Mutants Interfere with Viral DNA Replication**—Because AL1 forms a large protein complex (23), the incorporation of a mutant subunit that alters complex stability
and/or conformation may interfere with the function of wild type AL1 during viral DNA replication. To test this idea, a TGMV A replicon carrying wild type AL1 sequences was transfected into tobacco protoplasts with a 20-fold excess of an empty or mutant AL1 expression cassette. Coexpression of the oligomerization domain mutants, REK154 (Fig. 6A, lane 5), EKY159 (lane 6), Q-HN165 (lane 7), and N-DR172 (lane 8) resulted in single-stranded DNA accumulation between 5 and 25% and double-stranded DNA accumulation between 8 and 29% relative to wild type replication levels (lane 1). Two mutants altered in the conserved sequence upstream of the oligomerization domain, FQ118 (lane 2) and D120 (lane 3), also interfered with replication, decreasing single- and double-stranded DNA accumulation ~20-fold. As a reference point, the previously described Y104F trans-dominant-negative mutant (34), which is defective for DNA cleavage activity (26), attenuated replication to similar levels in our assay system (lane 9). The mutation RS-R125 (lane 5) was the least detrimental, reducing single- and double-stranded DNA by about 50%. RS-R125 is also impaired for AL1 DNA binding (data not shown), which may have reduced the effectiveness of the dominant-negative phenotype.

To better understand the contributions of different AL1 domains to replication interference, we tested the abilities of various AL1 truncations to block viral DNA replication in transient assays. A GST fusion containing only the AL1 oligomerization domain reduced viral replication ~2-fold (Fig. 6B, lane 2). The C-terminal truncation AL1291–180 (lane 3), which lacks the ATPase domain, also reduced replication ~50%. Similarly, an N-terminal truncation lacking the DNA binding and cleavage domains and carrying the N-DR172 mutation lowered replication ~3-fold (lane 4). None of the truncated proteins were as detrimental to viral replication as full-length N-DR172, which reduced replication ~10-fold in parallel assays (lane 5). Together, these results indicated that the mechanism of interference is complex, involving multiple domains of the AL1 protein.

**DISCUSSION**

Small DNA viruses typically encode a protein that mediates initiation of viral replication and also acts as a transcriptional regulator. Protein interactions play key roles in both processes and the ability to form homomultimers is often a requirement. Replication complexes are generally large oligomeric complexes. Large T-antigen forms a double hexamer around the replication origin of simian virus 40. Papillomavirus E1 also assembles as a hexameric complex at its replication origin (35). In contrast, transcription factors frequently bind DNA as dimers or tetramers (36–38). TGMV AL1 is also a multifunctional protein that initiates rolling circle replication and negatively regulates its own transcription. Earlier experiments showed that it forms large multimeric complexes in solution and is dependent on oligomerization for DNA binding. In this study, we characterized the TGMV AL1 oligomerization domain and showed that mutations in the domain differentially affected AL1 replication and transcription activities. Based on our results, we propose that different AL1 complexes are involved in viral replication and transcriptional regulation.

Copurification experiments using truncated proteins and GST fusions established that the N-terminal boundary of the TGMV AL1 oligomerization domain is between amino acids 134 and 147 and the C-terminal boundary is between amino acids 168 and 180. These limits are consistent with data showing that an AL1 fragment containing amino acids 119–180 is sufficient for oligomerization. There is limited information suggesting that the oligomerization domains of other geminivirus replication proteins also map to a similar region. The master-virus, maize streak virus (MSV), encodes two proteins, rep and
repA, that are analogous to full-length and a truncated version of AL1, respectively. The shared multimerization domain of MSV rep and repA has been broadly mapped between amino acids 73 and 213 (39), with the C-terminal border overlapping the C terminus of the TGMV AL1 oligomerization domain.

The TGMV AL1 oligomerization domain has no sequence homology to known protein interaction domains. However, two α-helices between amino acids 132 and 154 are predicted at greater than 80% probability (Fig. 3 and Ref. 23). Several classes of DNA-binding proteins, including members of the basic/helix-loop-helix, homeodomains and basic/leucine zipper families, use α-helices for dimerization contacts (40–42). Deletion of the predicted α-helices greatly reduced AL1 interactions, indicating that this region contributes to oligomerization. The importance of the predicted helical structures is further supported by their absolute conservation across replication proteins from all three geminivirus subgroups (Fig. 7). This structural conservation is underscored by the observation that position, length, and spacing of the helices are maintained even in the absence of sequence conservation (Fig. 7, cf. helix 3 sequences for TGMV and MSV). However, the ability of AL1134–352, which lacks the first two amino acids of helix 3, to form oligomers indicated that there is some flexibility in the length of the helical region. There is also evidence that the primary amino acid sequence of the α-helical region contributes to oligomerization. Alanine substitutions in both helices 3 and 4, which are structure neutral replacements, attenuated AL1 interactions in yeast by 20–50%. The helix 3 mutations (ND133) targeted amino acids conserved among closely related geminivirus replication proteins, whereas the helix 4 mutations (KEE146 and REK154) included conserved or similar amino acids among diverse groups of geminiviruses (Fig. 7).

The most detrimental oligomerization mutations, EKY159 and N-DR172, were located outside of the helical region (Fig. 7). The importance of these sequences was further confirmed by the inability of the C-terminal truncations, AL11–168 and AL11–168, to form oligomers. The conserved aromatic residue mutated in EKY159 may provide a critical contact for AL1 interactions because tyrosine and phenylalanine residues are commonly found at protein interfaces (43). N169 altered in N-DR172 is also absolutely conserved and, thus, may be part of the interaction interface. The oligomerization sequence defined by the EKY159 and N-DR172 mutations is flanked by an invariant proline at TGMV AL1 position 156 and up to six prolines at the C-terminal border. The position of these proline residues is of particular interest because unstructured or looped regions frequently delimit functional domains (44–46). One possibility is that the AL1 oligomerization domain consists of two regions, a major region located between the conserved prolines and an N-terminal α-helical region. According to this model, the major region contributes the primary contacts necessary for oligomerization, whereas the α-helices provide stabilizing interactions. Because of the strong sequence and structural conservation throughout these regions, it is likely that the replication proteins from diverse geminiviruses interact through very similar mechanisms. The ability of replication proteins from different begomaviruses to form heteromultimers supports this idea (24).

Several lines of evidence suggested that contacts outside the oligomerization domain influence AL1 interactions. The N-terminal truncation, AL1147–352, bound full-length GST-AL1 but did not interact with a GST fusion containing only the oligomerization domain. Similarly, EKY159 interacted with full-length AL1 but not with truncated proteins and was active in repression and interference assays, both of which are dependent on oligomerization. Analysis of N- and C-terminal truncations revealed that the pertinent sequences are located between AL1 amino acids 181 and 352. However, the detrimental effects of the D120 and QT130 mutations on AL1 interactions in yeast indicated that N-terminal sequences can also influence oligomerization. Although the AL1 oligomerization domain is essential for multimerization, sequences outside of the domain enhance or facilitate interactions, possibly by contributing to a more favorable conformation for protein interactions or by providing stabilizing amino acid contacts. Recent studies of p53 heteromultimers provide precedence for sequences outside an oligomerization domain impacting protein complex formation (47).

Mutations in the oligomerization domain and the adjacent conserved sequence impacted AL1 function in vivo. Mutations in the major region reduced replication below detectable levels, whereas mutations in the α-helices attenuated replication 8–10-fold compared with wild type levels. These results are

**Fig. 7. Sequence and structural conservation of the AL1 oligomerization domain.** The oligomerization domain of TGMV AL1 is aligned with the equivalent regions of other geminivirus replication proteins. Conserved residues are shown in white type. The consensus at the bottom shows conserved residues or functional groups (Φ, aliphatic; +, basic; −, acidic; ε, charged; ϕ, aromatic). The filled triangles indicate the outer limits of the TGMV AL1 oligomerization domain, whereas the open triangles mark the end points of the nearest truncation within the domain. The predicted α-helices are boxed, whereas the major region is indicated by the line. The circles indicate mutations that were detrimental to AL1 oligomerization, with the filled circles marking the mutations with the strongest effects. Begomoviruses: TGMV (K02029), bean golden mosaic virus-Guatemalan isolate (BGMV-GA, M91994), African cassava mosaic virus-Nigerian isolate (ACMV-N, X17095), tobacco yellow leaf curl virus-Israeli isolate (TYLCV-IS, X15566), and squash leaf curl virus (SqLCV, M38183). Curtovirus: beet curly top virus-CFH isolate (BCTV-CFH, U02311). Mastreviruses: tobacco yellow dwarf virus (TYDV, M81103), maize streak virus-Nigerian isolate (MSV-NI, K02026). The protein sequences are TGMV AL1 amino acids 132–180, BGMV-GA AL1 amino acids 131–179, ACMV-N C1 amino acids 130–178, TYLCV-IS C1 amino acids 129–177, SqLCV AL1 amino acids 129–177, BCTV-CFH C1 amino acids 127–175, TYDV Rep amino acids 126–174, and MSV-NI Rep amino acids 139–187.

consistent with yeast two-hybrid data demonstrating that AL1 complexes are more sensitive to changes in the major region than in the α-helices. However, we did not observe a tight correlation between replication activity and oligomerization efficiency. For example, QH-N165, which is mutated in the major sequence, displayed wild type oligomerization activity in yeast assays but failed to support TGMV replication in vivo. The simplest explanation for this lack of correlation is that the mutant proteins were not properly expressed or folded in vivo. We were unable to rule out this possibility by directly measuring AL1 protein, which is expressed below the limits of detection in tobacco protoplasts. However, the abilities of the mutants to efficiently repress the AL1 promoter and interfere with viral replication in protoplasts established that partially functional proteins were produced in vivo. Another possibility is that some of the mutations were pleiotrophic in character. This is true for RS-R125, which is impaired for DNA binding and cleavage as well as for oligomerization. These activities, all of which are required for viral replication in vivo, are mediated by overlapping domains in the AL1 protein. The lack of correlation could also reflect differences in sensitivity to changes in complex structure and size in vivo versus in vitro. Earlier experiments showed that AL1 forms a complex of ~8 subunits in insect cells, and it was proposed that AL1 may act as part of large complex in plant cells (24). In contrast, yeast dihybrid and GST-AL1 co-purification assays only require dimer formation to give a positive signal and, thus, provide minimal information about the character and alterations in a large protein complex.

In contrast to the replication results, nearly all the mutations enhanced AL1-mediated transcriptional repression. The enhanced repression activity of EKY159 was surprising given its failure to interact in two-hybrid assays and the dependence of AL1/DNA binding on oligomerization (26). However, immunohistochemical studies (19) have indicated that the local concentration of AL1 is high in infected cells and may be sufficient to drive interactions of the full-length EKY159 protein in vivo, analogous to our results in insect cells. The only mutants that did not display enhanced repression activity were K-E179, which had no detectable effect on AL1 function, and RS-R125, which showed reduced repression, most likely because it was impaired for DNA binding. There are examples of point mutations in papillomavirus E1 and E2 proteins (48, 49) that differentially affect replication and transcription. AL1 is unusual in that all of the mutations resulted in increased transcriptional repression concomitant with decreased viral replication. This suggests that the mutations affected the overall structure of the AL1 complex rather than specific interactions with host factors. Thus, different AL1 complexes may be required for the two activities. Replication initiation factors generally function as large protein complexes, whereas transcription factors frequently act as dimers or tetramers. Alternatively, AL1 function may be modulated by two conformational states, similar to p53 (50), with the formation of repression complexes favored or less sensitive to changes in the oligomerization domain than replication complexes. A conformational change might make a region that contacts the transcription apparatus more accessible and facilitate active repression (12). Changes in complex size or conformation are both consistent with the observed lack of correlation between the strength of AL1 interactions in yeast and the level of transcriptional repression in protoplasts.

AL1 oligomerization domain mutants also interfered with viral replication, possibly through interactions with the wild type AL1 protein. This idea is supported by the observation that EKY159 was least effective at inhibiting replication and most severely impaired for AL1 interactions in yeast and insect cells. Dominant negative replication mutants with altered protein oligomerization domains have also been reported for mammalian viruses. Mutations that affect double hexamer assembly of SV40 large T-antigen block wild type DNA replication (51). Expression of the NS1 oligomerization domain was sufficient to impair parvovirus replication (52). We were unable to duplicate this effect with AL1 because the oligomerization domain alone could not be stably expressed and a GST fusion with the oligomerization domain only reduced replication 2-fold. Two mutants, FQ118 and D120, with changes outside of the oligomerization domain also greatly reduced replication. Although both showed enhanced repression, like the oligomerization domain mutants, these mutations are located in the conserved sequence that includes overlapping domains for several AL1 activities that could contribute to interference. Similarly, a mutant with a substitution in the DNA cleavage active site (Y104F) strongly attenuated replication. Interestingly, AL1 truncations displayed weaker interfering activity than mutant full-length proteins. For example, the mutant N-D172 reduced replication better in the context of the full-length protein than in an N-terminally truncated protein lacking the DNA binding and cleavage domains. One explanation for these results is that DNA binding augments the dominant-negative phenotype by repressing expression of the wild type protein or blocking functional complexes from the origin. This idea is supported by the observations that the RS-R125 mutant, which is impaired for DNA binding and repression, is a weak trans-dominant-negative mutant. Deletions in the N and C termini may have also disrupted interactions with host factors that contribute to the efficiency of the dominant-negative phenotype (53). Although further studies will be necessary to discern the precise mechanisms, it is clear that geminivirus replication interference is a complex process involving multiple interactions that are most effectively mediated by full-length AL1 protein.

There is considerable interest in using AL1 trans-dominant-negative mutants to confer geminivirus resistance to transgenic crop plants. The efficacy of this approach was demonstrated by transgenic plants expressing mutant versions of the TYLCV and ACMV AL1 homologues (54–56). Most approaches have focused on using mutant proteins modified in the catalytic domains for DNA cleavage or ATP hydrolysis. However, the oligomerization mutants have several features that suggest that they may be better candidates for engineering geminivirus-resistant plants. They blocked wild type replication activity as effectively as a DNA cleavage site mutant in protoplast assays. They also were enhanced repressors of the AL1 promoter, thereby providing an additional level of control over the wild type virus. In addition, some of the mutations significantly reduced the ability of AL1 to bind the maize Rb homologue. This characteristic may facilitate the generation of transgenic plants that express high levels of AL1 through multiple generations. To date, this has not been achieved, presumably because of the ability of AL1 to modify plant gene expression and cell cycle controls (19, 25). Future studies will ask if the AL1 oligomerization mutants can be stably expressed in transgenic plants at levels sufficient to confer geminivirus resistance.

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Beverly M. Orozco, Ling-Jie Kong, Lou Ann Batts, Sharon Elledge and Linda Hanley-Bowdoin

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