JOURNAL OF VIROLOGY, Oct. 1996, p. 6790–6795 Vol. 70, No. 10
0022-538X/96/$04.00+0

Interactions between Geminivirus Replication Proteins

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Received 14 March 1996/Accepted 20 June 1996

Geminiviruses are small DNA viruses that replicate in the nuclei of infected plant cells. The closely related geminiviruses tomato golden mosaic virus and bean golden mosaic virus each encode a protein, AL1, that catalyzes the initiation of rolling-circle replication. Both viruses also specify a second replication protein, AL3, that greatly enhances the level of viral DNA accumulation. Using recombinant proteins produced in a baculovirus expression system, we showed that AL1 copurifies with a protein fusion of glutathione *S*-transferase (GST) and AL1, independent of the GST domain. Similarly, authentic AL3 cofractionates with a GST-AL3 fusion protein. These results demonstrated that both AL1 and AL3 form oligomers. Immunoprecipitation of protein extracts from insect cells expressing both AL1 and AL3 showed that the two proteins also complex with each other. None of the protein interactions displayed virus specificity; the tomato and bean golden mosaic virus proteins complexed with each other. The addition of heterologous replication proteins had no effect on the efficiency of geminivirus replication in transient-replication assays, suggesting that heteroprotein complexes might be functional. The significance of these protein interactions is discussed with respect to geminivirus replication in plant cells.

Geminiviruses are one of only two families of plant viruses with true DNA replication cycles (35). They replicate their small, circular DNA genomes through double-stranded DNA intermediates in plant nuclei by a rolling-circle mechanism (29, 30). Geminiviruses encode only a few proteins for their replication and recruit most of their replication enzymes from their plant hosts (7, 16, 25). Members of the adenovirus, polyomavirus, and papillomavirus families also rely heavily on host replication factors and have contributed significantly to our understanding of DNA replication in mammalian cells (17). Geminiviruses offer the same potential for plants.

Geminiviruses are divided into three subgroups on the basis of their insect vectors, host ranges, and genome structures (28). Tomato golden mosaic virus (TGMV) and bean golden mosaic virus (BGMV), two subgroup III geminiviruses, are transmitted by whiteflies, infect dicots, and have bipartite genomes consisting of two 2.6-kb circular components designated A and B (10, 15). The viral proteins involved in replication, AL1 and AL3, are encoded by the A component, which can replicate independently of B DNA. AL1, the only essential viral replication protein (7, 16), is multifunctional. It is required for positive-strand DNA synthesis and serves as the origin recognition protein (12, 13, 22) and a site-specific endonuclease to initiate rolling-circle replication (21, 26). AL1 also acts as a negative transcriptional regulator of its own expression (6, 32) and induces the accumulation of host proteins necessary for DNA replication in infected plant cells (25). The AL3 protein, which is not required for replication, is an accessory factor that increases the DNA accumulation of subgroup II and III geminiviruses ca. 50-fold in protoplast replication assays (7, 32), suggesting that it contributes an activity important for efficient viral replication.

Protein interactions are crucial for the initiation of chromosomal (2, 3) and viral (5, 14, 24) replication in both prokaryotes and eukaryotes. Protein interactions are frequently necessary for efficient origin binding or for the induction of DNA structural changes necessary for the initiation of replication. In geminivirus replication, different protein complexes or distinct subunits within a complex may be responsible for the various activities associated with AL1. The enhancing effect of AL3 on viral DNA accumulation may be due to direct protein interactions with AL1. We have begun to address these possibilities by asking if AL1 and AL3 interact with themselves and each other.

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MATERIALS AND METHODS

Plasmid constructs and recombinant baculoviruses. The position numbers describing the following constructs refer to the nucleotide coordinates of the TGMV sequence determined by Hamilton et al. (15). The coordinates for BGMV refer to the sequence of the Guatemalan isolate (10).

Plant expression cassettes containing TGMV AL1 (TAL1) and TGMV AL3 (TAL3) under the control of the cauliflower mosaic virus 35S promoter have been described previously (12). For plant expression of BGMV AL1 (BAL1), a 1,059-bp PCR product (BGMV A DNA positions 1595 through 2647 to 7) was cloned into pUC118 to give pNSB149, sequenced, and isolated as a *Bam*HI fragment. The fragment was cloned into the *Bgl*II site of pMON921 (11) to give pNSB152. For BGMV AL3 (BAL3), a 397-bp PCR product (BGMV A DNA positions 1124 to 1521) was cloned, sequenced, and isolated as an *Nco*I-*Bam*HI fragment. The fragment ends were filled in with *Escherichia coli* DNA polymerase I (Klenow fragment) and inserted into a filled *Bam*HI site of pMON921 to give pNSB501. Viral replication was assayed with the pUC-based plasmids pMON1565 (7) and pGA1.2A (11), which contain partial tandem copies of TGMV and BGMV A DNA, respectively.

Baculovirus transfer vectors containing the polyhedrin promoter and the simian virus 40 poly(A) site were constructed for the authentic viral proteins TAL3, BAL1, and BAL3 and for the glutathione *S*-transferase (GST) fusion proteins GST-TAL3, GST-BAL3, and GST-BAL1. The TAL3 vector, pNSB298, was constructed by inserting a filled 429-bp *Nco*I-*Bst*BI fragment (TGMV A DNA positions 1036 to 1465) (11) into pMON27025 (23) that had previously been digested with *Hin*dIII and filled in with Klenow fragment. The GST-TAL3 vector, pNSB363, was constructed by insertion of the same 429-bp *Nco*I-*Bst*BI TGMV A fragment into the *Sma*I site of pNSB314, a GST baculovirus transfer vector (26). For the BAL1 vector, a filled 1,059-bp *Bam*HI fragment from pNSB149 (described above) was cloned into pMON27025 that had previously been digested with *Bam*HI and filled in with Klenow fragment to give pNSB377. For insect cell expression of GST-BAL1, pNSB149 was modified by site-directed mutagenesis to create an *Nde*I site at the translation start site of BAL1. The BAL1 coding sequence was isolated as an *Nde*I-*Hin*dIII fragment, reacted with Klenow fragment, and inserted into a filled *Bam*HI site of pNSB310, a GST vector in pUC119 (26). The resulting plasmid, pNSB471, contains an in-frame fusion of the GST and BAL1 coding sequences. A 2.0-kb *Sac*I fragment including the GST-BAL1 fusion was isolated from pNSB471, trimmed with T4 DNA polymerase, and inserted into a filled *Hin*dIII site of pMON27025 to give the

insect cell expression plasmid pNSB473. For insect cell expression of BAL3, the filled 397-bp *Nco*I-*Bam*HI fragment described for pNSB501 was cloned into a filled *Hin*dIII site of pMON27025 to give pNSB431. The same 397-bp fragment was cloned into the *Sma*I site of pNSB314 for expression of GST-BAL3. Baculovirus DNA corresponding to each of the transfer vectors was generated by Tn7-mediated transposition with the bacmid plasmid bMON14242, transfected into *Spodoptera frugiperda* Sf9 cells, and screened for recombinant protein expression as described previously (23, 26). Recombinant baculoviruses corresponding to authentic TAL1, the GST-TAL1 fusion protein, and GST have been described elsewhere (13, 26). The recombinant baculovirus containing the β-glucuronidase (GUS) coding sequence was provided by V. Luckow (Monsanto Company).

Protein expression, extraction, and analysis. Sf9 cells (10^6/ml) were infected with recombinant baculoviruses in either a 15-ml shaker culture or a 25-mm^2 T flask at a multiplicity of infection of 5. Cells were harvested 48 h postinfection and lysed in extraction buffer (EB) (25 mM Tris-HCl [pH 7.5], 1 mM EDTA, 1 mM dithiothreitol, 1% Nonidet P-40, 10 μg of pepstatin per ml, 50 μg of leupeptin per ml, 1 mM phenylmethylsulfonyl fluoride). Lysates were subjected to ultracentrifugation at 200,000 × *g* for 1 h immediately prior to all experiments to remove large protein aggregates. GST fusion proteins were purified by incubation with glutathione-Sepharose 4B (Pharmacia Biotech Inc., Piscataway, N.J.) which had been treated with 3% bovine serum albumin and equilibrated in EB. Proteins were eluted with a mixture containing 10 mM glutathione and 50 mM Tris (pH 8.0) and heated in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer (50 mM Tris [pH 6.8], 100 mM dithiothreitol, 2% SDS, 0.1% bromophenol blue, 10% glycerol) for 5 min at 100°C prior to fractionation by SDS-PAGE.

Immunoprecipitations were performed by incubating protein extract with either anti-AL1 or anti-AL3 polyclonal serum for 2 h or overnight on ice. Protein-antibody complexes were mixed with protein A-Sepharose (Pharmacia Biotech Inc.) in EB at 4°C for 2 h and then washed with buffer containing 50 mM Tris-HCl (pH 7.5), 50 mM EDTA, and 0.1% Triton X-100. Bound proteins were eluted with SDS-PAGE sample buffer at 100°C. Proteins resolved on SDS-polyacrylamide gels were transferred to nitrocellulose membranes (Schleicher and Schuell, Keene, N.H.) and analyzed by immunoblotting with the enhanced chemiluminescence detection system (Amersham Life Science, Arlington Heights, Ill.). Primary antibodies were rabbit polyclonal anti-GST (Upstate Biotechnology Inc.), anti-β-glucuronidase (Clontech, Palo Alto, Calif.), anti-TAL3 (27) or anti-TAL1 (16) antiserum.

Replication assays. Transient-replication assays were performed with protoplasts prepared from *Nicotiana tabacum* suspension cells as described elsewhere (12). Protoplasts were transfected with 10 μg of either the TGMV A or BGMV A replicon and 10 μg of expression cassettes for TAL1, TAL3, BAL1, or BAL3. Total DNA was isolated 48 h after transfection and digested with *Xho*I and *Dpn*I for TGMV A or *Bgl*II and *Dpn*I for BGMV A. Digested DNAs were resolved on 1% agarose gels and transferred to nylon membranes. A TGMV A-specific probe was prepared from a 1.8-kb *Eco*RI-*Xho*I fragment of pMON1565. A 1.3-kb *Nco*I-*Sca*I fragment of pGA1.2A was used to generate a BGMV A-specific probe. Assays were repeated three separate times.

RESULTS

TAL1 and TAL3 both form oligomers. Many replication proteins and transcriptional regulators function in oligomeric complexes. We asked whether AL1 oligomerizes by coexpressing authentic TAL1 (13) with a fusion protein of TAL1 and GST (GST-TAL1), and we assayed for copurification of the two proteins. Both proteins were expressed efficiently in insect cells coinfected with recombinant baculoviruses encoding either TAL1 or GST-TAL1 (Fig. 1A, lane 3). When the protein extract was incubated with glutathione resin, both GST-TAL1 and TAL1 were detected in the bound fraction (Fig. 1A, lane 6), indicating that the two proteins formed a complex.

Two control experiments were performed to verify that the TAL1–GST-TAL1 interactions were specific. First, extracts from insect cells coinfected with baculoviruses corresponding to GST alone and TAL1 were incubated with glutathione resin. Both proteins were apparent in the input fraction (Fig. 1A, lane 1), but only GST was detected in the bound fraction (lane 4), establishing that TAL1 does not bind to GST alone. The second control tested whether GST-TAL1 showed nonspecific aggregation with any coexpressed protein. Insect cells coinfected with recombinant baculoviruses containing GST-TAL1 and GUS expression cassettes expressed both proteins (Fig. 1A, lane 2), but only GST-TAL1 bound the glutathione resin (lane 5). These experiments established the specificity of the TAL1–GST-TAL1 interactions and demonstrated that TAL1 possesses the ability to form dimers or higher-order complexes.

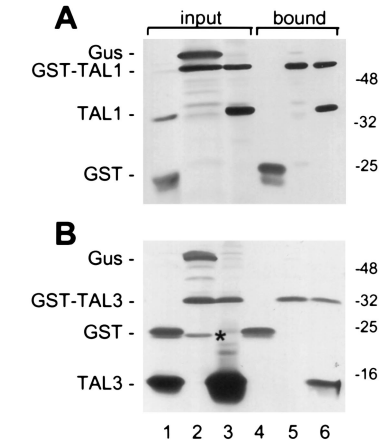

FIG. 1. TAL1 and TAL3 both oligomerize. Protein extracts from baculovirus-infected cells (input, lanes 1 to 3) were incubated with glutathione resin and eluted with glutathione (bound, lanes 4 to 6). Equivalent amounts of input and bound fractions were resolved by SDS-PAGE and analyzed by immunoblotting. (A) Insect cells were coinfected with baculoviruses for TAL1 and GST (lanes 1 and 4), GUS and GST-TAL1 (lanes 2 and 5), or TAL1 and GST-TAL1 (lanes 3 and 6). The blot was incubated with antisera to AL1, GST, and GUS. (B) Insect cells were coinfected with baculoviruses for TAL3 and GST (lanes 1 and 4), GUS and GST-TAL3 (lanes 2 and 5), or TAL3 and GST-TAL3 (lanes 3 and 6). The blot was incubated with antisera to AL3, GST, and GUS. The identities of immunoreactive proteins are given on the left. A proteolytic breakdown product corresponding to GUS is marked by an asterisk in lane 2. The molecular masses (in kilodaltons) of protein markers are shown on the right.

We also asked if AL3 has the capacity to multimerize. TAL3 was coexpressed with GST-TAL3 in insect cells and analyzed for protein complex formation by glutathione affinity chromatography. Authentic TAL3 was more apparent than GST-TAL3 in the input fraction, even though GST-TAL3 was recognized by both the anti-AL3 and the anti-GST sera used to probe the blot (Fig. 1B, lane 3). TAL3 copurified with GST-TAL3, but the ratio of TAL3 to GST-TAL3 decreased relative to the input fraction (Fig. 1B, compare lanes 3 and 6), suggesting that only a portion of TAL3 complexed with GST-TAL3. When TAL3 was coexpressed with GST alone (Fig. 1B, lane 1), it was not detected in the glutathione-bound fraction (lane 4), indicating that TAL3 does not interact with GST. Similarly, when GUS was coexpressed with GST-TAL3 (Fig. 1B, lane 2), it was not seen in the bound fraction (lane 5), further establishing the specificity of TAL3–GST-TAL3 interactions. These results demonstrated that TAL3 can also form dimers or higher-order complexes.

AL1 and AL3 oligomerization are both evolutionarily conserved. The AL1 proteins from different geminiviruses display functional conservation in their DNA binding (11) and cleavage (21, 26) activities. We asked if AL1 protein complex formation is also conserved by determining if BAL1 oligomerizes. BAL1 and GST-BAL1 were coexpressed and examined for interaction by glutathione affinity chromatography. As a positive control, TAL1–GST-TAL1 complexes were analyzed in parallel (Fig. 2A, lanes 1 and 7). The BGMV proteins were more difficult to detect with the anti-TAL1 antiserum, presumably because of reduced affinity for the heterologous protein. However, both proteins were readily apparent in input (Fig. 2A, lane 4) and bound (lane 10) fractions, indicating that BAL1 complexes with GST-BAL1. Like TAL1 (Fig. 2A, lanes 3 and 9), BAL1 did not interact with GST alone (lanes 6 and 12).

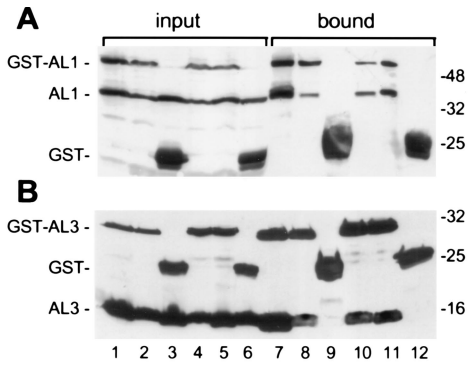

FIG. 2. BAL1 and BAL3 both oligomerize and interact with their TGMV homologs. Protein extracts from baculovirus-infected cells (input, lanes 1 through 6) were incubated with glutathione resin and eluted with glutathione (bound, lanes 7 through 12). Input and bound fractions were resolved by SDS-PAGE and analyzed by immunoblotting. (A) Insect cells were coinfected with baculoviruses for GST-TAL1 and TAL1 (lanes 1 and 7), GST-TAL1 and BAL1 (lanes 2 and 8), GST and TAL1 (lanes 3 and 9), GST-BAL1 and BAL1 (lanes 4 and 10), GST-BAL1 and TAL1 (lanes 5 and 11), or GST and BAL1 (lanes 6 and 12). The blot was incubated with anti-AL1 and anti-GST sera. (B) Insect cells were coinfected with baculoviruses for GST-TAL3 and TAL3 (lanes 1 and 7), GST-TAL3 and BAL3 (lanes 2 and 8), GST and TAL3 (lanes 3 and 9), GST-BAL3 and BAL3 (lanes 4 and 10), GST-BAL3 and TAL3 (lanes 5 and 11), or GST and BAL3 (lanes 6 and 12). The immunoblot was incubated with anti-AL3 and anti-GST sera. Identities of immunoreactive proteins are given on the left. The molecular masses (in kilodaltons) of protein markers are shown on the right.

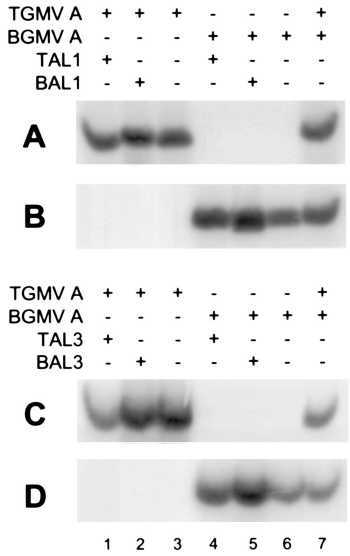

FIG. 3. Expression of heterologous AL1 or AL3 does not affect viral replication. Plasmids containing partial tandem copies of TGMV A or BGMV A and 35S expression cassettes for TAL1, BAL1, TAL3, or BAL3 were electroporated into tobacco protoplasts in the combinations indicated above panels A and C. Total DNA was isolated 2 days posttransfection and digested with *Dpn*I and *Xho*I for TGMV A or *Dpn*I and *Bgl*II for BGMV A. Digested DNA was resolved on agarose gels and analyzed by DNA hybridization with ^{32}P-labeled probes specific for TGMV A (A and C) or for BGMV A (B and D). Accumulation of double-stranded viral DNA is shown.

We also examined the conservation of AL3 oligomerization by investigating the protein interactions of BAL3. We coexpressed BAL3 with its corresponding GST fusion protein, GST-BAL3 (Fig. 2B, lane 4). When GST-BAL3 was purified from these extracts, BAL3 was observed in the bound fraction (lane 10). BAL3 did not show any affinity for GST alone (Fig. 2B, lanes 6 and 12). Together, these experiments indicated that AL1 and AL3 oligomerizations are evolutionarily conserved functions.

Neither AL1 nor AL3 oligomerization displays virus specificity. Although TAL1 and BAL1 show 82% similarity in their amino acid sequences, they possess different DNA recognition and replication specificities (12). We asked if AL1 protein complex formation is also virus specific by determining if BAL1 can interact with GST-TAL1 and if TAL1 can interact with GST-BAL1. When BAL1 was expressed with GST-TAL1 (Fig. 2A, lane 2), it copurified with the heterologous GST fusion protein (lane 8). Similarly, TAL1 cofractionated with GST-BAL1 (Fig. 2A, lanes 5 and 11). The efficiencies of AL1 interactions for the homologous and heterologous proteins were similar (Fig. 2A, compare lane 7 with lane 8 and lane 10 with lane 11). These experiments demonstrated that AL1 oligomerization is not a virus-specific process.

TAL3 and BAL3 are 86% similar in their amino acid sequences and functionally interchangeable in transient-replication assays (18, 34). To examine the virus specificity of AL3 oligomerization, we analyzed the interactions of BAL3 with GST-TAL3 and of TAL3 with GST-BAL3. When BAL3 and GST-TAL3 were coexpressed in insect cells (Fig. 2B, lane 2), the two proteins cofractionated on glutathione resin (lane 8). TAL3 also copurified with GST-BAL3 (Fig. 2B, lanes 5 and 11). As with AL1, the efficiencies of AL3 oligomerization for homologous and heterologous proteins were similar (Fig. 2B, compare lane 7 with lane 8, and lane 10 with lane 11). These results established that AL3 oligomerization is not virus specific.

Heterologous interactions between TGMV and BGMV replication proteins might impair their activities in vivo, an effect analogous to the ability of mutant simian virus 40 large T antigen to inactivate its wild-type counterpart (9). We addressed this possibility by asking if BAL1 or BAL3 affects TGMV A replication and if TAL1 or TAL3 affects BGMV A replication (Fig. 3). Replication was assayed by release and amplification of unit-length A component DNA from partial tandem copies of TGMV or BGMV A DNA in tobacco protoplasts (8). Viral replication proteins were produced from cauliflower mosaic virus 35S promoter expression cassettes (11) and/or A component DNA (7). When TGMV A replicon DNA was introduced into protoplasts either alone (Fig. 3A, lane 3) or with 35S-TAL1 (lane 1), 35S-BAL1 (lane 2), or BGMV A (lane 7), similar levels of double-stranded DNA accumulation were detected in all four samples by DNA gel blot analysis. Transfection of protoplasts with BGMV A replicon DNA either alone (Fig. 3B, lane 6) or in the presence of 35S-TAL1 (lane 4), 35S-BAL1 (lane 5), or TGMV A (lane 7) also resulted in equivalent levels of BGMV A accumulation. Analogous results were obtained for AL3, with the levels of TGMV A (Fig. 3C) or BGMV A (Fig. 3D) double-stranded-DNA accumulation being similar under all assay conditions. Thus, the presence of heterologous AL1 or AL3 protein had no detectable effect on double-stranded-DNA accumulation in vivo.

TAL1 and TAL3 interact. Previous experiments found that TAL3 can alleviate the effects of a mutation within an AL1 binding site in the origin, possibly by interacting with or modifying AL1 function (11). To test whether the two TGMV replication proteins interact, we immunoprecipitated protein extracts from cells coexpressing TAL1 and TAL3 with increasing amounts of anti-AL1 or anti-AL3 serum. Polyclonal antibodies against AL1 quantitatively immunoprecipitated TAL1 at the highest antibody concentration (Fig. 4A, lane 6). It was apparent from a lighter exposure of the immunoblot that less

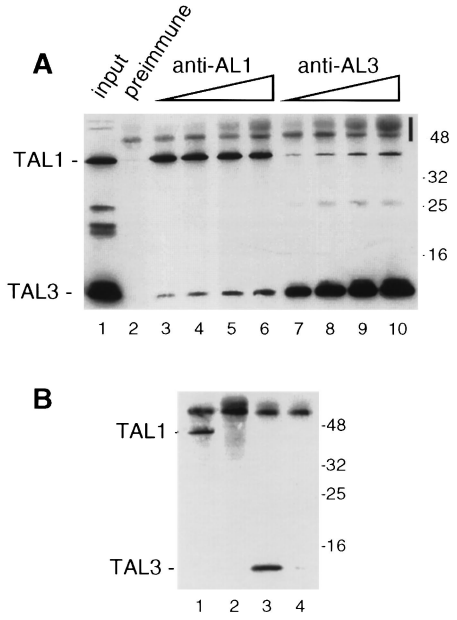

FIG. 4. TAL1 and TAL3 interact. Protein extract from baculovirus-infected cells was immunoprecipitated with anti-AL1 or anti-AL3 polyclonal serum. The precipitated proteins were resolved by SDS-PAGE and analyzed by immunoblotting with anti-AL1 and anti-AL3 sera. The identities of immunoreactive proteins are given on the left. (A) Extract (10 μl) from cells expressing TAL1 and TAL3 was incubated with 1 μl of preimmune serum (lane 2), a titration of anti-AL1 serum (lane 3, 0.1 μl; lane 4, 0.2 μl; lane 5, 0.5 μl; lane 6, 1 μl), or a titration of anti-AL3 serum (lane 7, 0.1 μl; lane 8, 0.2 μl; lane 9, 0.5 μl; lane 10, 1 μl). Lane 1 was loaded with an equivalent amount of total protein extract. The large subunit of rabbit immunoglobulin G is marked by the vertical bar on the right. (B) Extracts (10 μl) from cells expressing TAL1 (lanes 1 and 2) or TAL3 (lanes 3 and 4) were incubated with 1 μl of anti-AL1 serum (lanes 1 and 4) or 1 μl of anti-AL3 serum (lanes 2 and 3). The molecular masses (in kilodaltons) of protein markers are shown on the right.

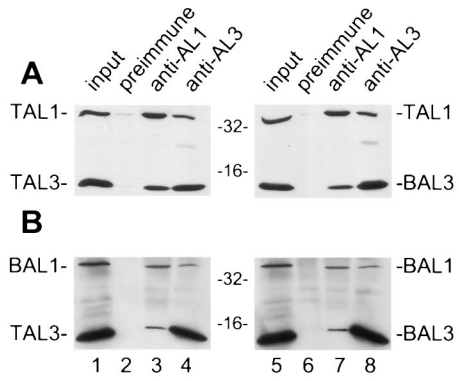

FIG. 5. BAL1 and BAL3 interact with each other and with TAL1 and TAL3. Protein extracts (50 μl) from baculovirus-infected cells were immunoprecipitated with 1 μl of preimmune (lanes 2 and 6), anti-AL1 polyclonal (lanes 3 and 7), or anti-AL3 polyclonal (lanes 4 and 8) serum. The precipitated proteins were resolved by SDS-PAGE and analyzed by immunoblotting with anti-AL1 and anti-AL3 sera. (A) Cells were coinfected with baculoviruses for TAL1 and TAL3 (lanes 1 to 4) or TAL1 and BAL3 (lanes 5 to 8). (B) Cells were coinfected with baculoviruses for BAL1 and TAL3 (lanes 1 to 4) or BAL1 and BAL3 (lanes 5 to 8). The identities of immunoreactive proteins are given on the left and right. The molecular masses (in kilodaltons) of protein markers are shown between lanes 4 and 5.

TAL1 was precipitated at the lower antibody concentrations (data not shown). The AL1 antibodies also fractionated TAL3 with the immunoprecipitated TAL1 (Fig. 4A, lanes 3 to 6). More TAL3 was detected in the precipitated fractions at higher antibody concentrations, indicating that there was a correlation between the amount of TAL3 in the precipitated fraction and the efficiency of TAL1 immunoprecipitation. In contrast, preimmune serum corresponding to the AL1 polyclonal antibody immunoprecipitated trace amounts of both proteins (Fig. 4A, lane 2), most likely reflecting a low level of nonspecific binding to the immunobeads. Coimmunoprecipitation of TAL3 with TAL1 also occurred with a second anti-AL1 polyclonal serum and an anti-AL1 monoclonal antibody (data not shown), indicating that the phenomenon was not dependent on a particular antiserum. No significant cross-reactivity was observed in immunoprecipitations of TAL3-containing extracts with anti-AL1 serum (Fig. 4B, lanes 3 and 4).

Extracts from cells coexpressing TAL1 and TAL3 were also incubated with anti-AL3 antiserum. The amount of immunoprecipitated TAL3 was directly proportional to antibody concentration (Fig. 4A, lanes 7 to 10), with quantitative TAL3 precipitation at the highest concentration (lane 10). TAL1 coimmunoprecipitated with TAL3 at all antibody concentrations (Fig. 4A, lanes 7 to 10). As was observed for TAL3 coimmunoprecipitation with TAL1, the amount of precipitated TAL1 was greatest at the highest AL3 antibody concentration (lane 10). The anti-AL3 serum showed no cross-reactivity in immunoprecipitations with AL1-containing extracts (Fig. 4B, lanes 1 and 2). In addition, preimmune serum corresponding to the anti-AL3 antibody precipitated neither protein (see Fig. 5A, lane 2). Together, these data showed that coimmunoprecipitation of TAL1 and TAL3 is due to interaction between the TGMV replication proteins.

AL1-AL3 interactions are conserved and do not display virus specificity. We asked if the TAL1-TAL3 interaction could be extended to the BGMV replication proteins. BAL1 and BAL3 were coexpressed and analyzed for interaction by the coimmunoprecipitation assay. As a positive control, TAL1 and TAL3 interactions were retested in parallel (Fig. 5A, lanes 1 to 4). The levels of BAL3 in the extracts were comparable to those seen for TAL3 (Fig. 5B, lanes 1 and 5), but less BAL1 was detected than TAL1 (Fig. 5A, lane 1, and 5B, lane 1). Nevertheless, both anti-AL1 (Fig. 5B, lane 7) and anti-AL3 (lane 8) sera coimmunoprecipitated BAL1 and BAL3, whereas preimmune sera showed no effect (lanes 2 and 6). These results established that BGMV replication proteins also interact, indicating that AL1-AL3 interaction may be a common property of subgroup III geminiviruses.

Because of the interchangeability of AL3 proteins in replication assays, we predicted that AL1-AL3 interactions would not be virus specific. This hypothesis was tested in coimmunoprecipitation experiments with extracts from cells coexpressing either TAL1 and BAL3 (Fig. 5A, lane 5) or BAL1 and TAL3 (Fig. 5B, lane 1). When TAL1 was expressed with BAL3 and immunoprecipitated with anti-AL1 serum, TAL1 and BAL3 were apparent in the immunoprecipitate (Fig. 5A, lane 7). Similarly, anti-AL3 serum immunoprecipitated TAL1 with BAL3 (Fig. 5A, lane 8). No BAL3 was detected with the preimmune serum (Fig. 5A, lane 6). The converse experiment with BAL1 and TAL3 also showed coimmunoprecipitation of the two proteins (Fig. 5B, lanes 1 to 4). These results supported our hypothesis that AL1 and AL3 proteins from different geminiviruses can interact.

DISCUSSION

Protein complex formation is an important feature of origin recognition and initiation of DNA replication in bacteria,

fungi, and mammals (31). There is less information regarding the protein complexes involved in plant DNA replication. Recent studies have begun to define the mechanisms that underlie origin recognition and initiation of geminivirus replication in plants (35). All geminiviruses encode AL1 or C1, a protein that catalyzes the initiation of rolling-circle replication (21, 26). Subgroup II and III geminiviruses specify a second protein, AL3 or C3, that greatly enhances the level of viral DNA accumulation (7, 33). We demonstrated that three types of interactions can occur between geminivirus replication proteins: AL1-AL1, AL3-AL3, and AL1-AL3 interactions. Thus, AL1 can be included in the class of replication initiator proteins that form oligomers and interact with replication enhancer proteins. The mechanism of action of AL3 remains unknown but may involve interaction with AL1 and oligomerization with itself.

We used baculovirus-mediated expression of recombinant proteins to demonstrate that both TAL1 and TAL3 multimerize. In these experiments, TAL1 interacted with GST-TAL1 and TAL3 complexed with GST-TAL3. Both interactions were specific; neither TAL1 nor TAL3 interacted with GST alone, and neither GST fusion protein complexed with an unrelated overexpressed protein. In addition, GST-AL1 did not complex with AL3 and GST-AL3 did not interact with AL1 (data not shown), demonstrating that nonspecific aggregation was not typical of the recombinant proteins and could not explain the AL1 or AL3 multimerization results. Silver-stained profiles of glutathione affinity-purified GST-TAL1 (26) or GST-TAL3 (data not shown) showed no protein contaminants that were not also present in GST preparations. Thus, if an insect protein was necessary for TAL1 or TAL3 complex formation, it was not stoichiometrically represented in the complex. Analysis of the BGMV replication proteins showed that both AL1 and AL3 interactions are evolutionarily conserved and do not display virus specificity. These data strongly suggested that both AL1 and AL3 oligomerization are authentic processes that occur through direct protein interactions.

We detected AL1-AL3 complexes by coimmunoprecipitation from extracts of insect cells expressing AL1 and AL3. TAL1 interacted with BAL3 as well as with TAL3, and BAL1 also complexed with both AL3 proteins. Like AL1 and AL3 oligomerization, AL1-AL3 interactions are not virus specific. It was difficult to rule out the involvement of an insect protein in AL1-AL3 interactions because the purity of the complexes could not be assessed because of the presence of immunoglobulin proteins. We were not able to utilize glutathione affinity chromatography to demonstrate interactions between GST-AL1 and AL3 or GST-AL3 and AL1 because the GST moiety interfered with AL1-AL3 complex formation (data not shown). However, the involvement of an insect protein would require that it interact specifically with both AL1 and AL3, which is unlikely. Thus, it is probable that AL1 and AL3 interact directly.

There are several mechanisms whereby oligomerization could contribute to AL1 replication and transcription activities. A single tyrosine residue has been identified as essential for AL1-catalyzed DNA cleavage and ligation (20). Therefore, AL1 dimerization would provide the two active-site tyrosines presumably required for these processes during rolling-circle replication. There are instances of dimerization of replication initiator proteins in other rolling-circle systems (36). In addition, AL1 may bind DNA as a dimer, with each subunit contacting one of the repeated motifs in its DNA binding site (11). Oligomerization might also be involved in AL1 binding to a second site in the common region that matches its first binding site at 14 of 17 positions (1), possibly allowing a single protein complex to contact both sites simultaneously. The *E. coli* DnaA (3) and Epstein-Barr virus EBNA I (14) proteins both form large oligomers that contact multiple sites in their origins.

Neither AL1 nor AL3 displayed virus-specific oligomerization, suggesting that the protein domains required for oligomerization are conserved between TGMV and BGMV. Geminiviruses frequently occur as mixed infections in the field (4). Thus, it is likely that both AL1 and AL3 heteroform multimers in vivo and are functional for viral replication. This idea is supported by our experiment showing that both TGMV A and BGMV A replicated to wild-type levels in the presence of heterologous A components or replication proteins provided from plant expression cassettes. It is unlikely that the lack of interference is due to a problem inherent in our protoplast experiment. An earlier study (26) showed that replication of a TGMV origin mutant was severely impaired when the mutant was coelectroporated with wild-type TGMV DNA, indicating that most transfected cells received both viral replicons. We used similar assay conditions to examine the effects of heterologous AL1 and AL3 proteins on replication and should have detected interference if it had occurred. Previous studies also showed that plant expression cassettes for AL1 and AL3 support efficient replication of TGMV (26) and BGMV (14a) in *trans*. Thus, the lack of interference cannot be attributed to a failure to produce the heterologous viral proteins. In addition, low levels of protein production cannot explain our results because wild-type levels of TGMV A and BGMV A replication, which are absolutely dependent on both TAL1 and BAL1, were observed in the same protoplast assay (Fig. 3, lanes 7). Hence, the best interpretation of our results is that the presence of a heterologous AL1 or AL3 protein does not interfere with viral replication. One explanation of our data is that the AL1 protein may be produced in excess during infection, such that the fraction that forms homomultimers is sufficient to support wild-type replication levels. This explanation is inconsistent with the presence of low steady-state levels of AL1 in infected plants (16) and the tight control of its expression (6, 32). Alternatively, the inclusion of an AL1 subunit that is unable to bind DNA may not result in a nonfunctional complex. Independent of the mechanism, these results imply that AL1 proteins deficient for DNA binding do not act as transdominant negative mutants of viral replication.

The mechanism by which AL3 enhances viral DNA accumulation may involve its ability to interact with AL1. The AL3 protein sequence shows no homology to any known enzymatic motifs. Therefore, it is more likely that the structure of the AL1-AL3 complex, rather than a catalytic activity of AL3 that affects AL1, is important for replication. AL3 might increase the affinity of AL1 for the origin, an effect analogous to that of E2 on E1 during papillomavirus replication (24). This idea is supported by an earlier study showing that AL1 overcame a detrimental binding-site mutation within the TGMV origin only when AL3 was present (11). Another possibility is that AL3 directs AL1 to its cleavage site in the origin during the replication of subgroup II and III geminiviruses. In these viruses, the AL1 DNA binding site is located upstream and distal from the hairpin where AL1 catalyzes DNA cleavage. This separation is likely to have an adverse effect on replication unless a mechanism exists to direct bound AL1 to its cleavage site. AL3 may enhance replication by performing this function. This model accounts for the lack of an AL3 homolog in subgroup I geminiviruses, whose C1 binding sites are thought to be located in the stems of their hairpins (1) and which thus would not require an AL3-like protein to direct C1 to its nick site. AL1-AL3 complex formation might also influence the DNA cleavage and ligation (21, 26) or putative helicase (19) activi-

ties of AL1. Because GST-TAL1 and TAL3 do not interact in insect cells, we have not been able to determine the effects of AL3 on the previously demonstrated DNA binding or cleavage activity of GST-AL1 (26).

The detection of geminivirus replication protein complexes in insect cell extracts suggests, but does not prove, the presence of equivalent complexes in infected plants. It was not technically feasible to directly address the existence of these complexes in geminivirus-infected plants because the organization of the viral genome precluded the expression of GST fusion proteins in infected plants and the authentic proteins are not extractable at high concentrations from infected or transgenic plants. However, several lines of evidence support the existence of geminivirus replication protein complexes in plants. First, GST-TAL1 synthesized in insect cells is active for DNA binding and cleavage (26), suggesting that protein complex formation also reflects normal protein function. Second, the ability of heterologous AL1 and AL3 to interact in insect cells is consistent with the capacity of AL3 to enhance the replication of unrelated geminiviruses in plant cells (18, 34). Third, the amounts of AL1 and AL3 in infected plant tissue are comparable (27), indicating that a stoichiometric complex between the two proteins could be formed in plants. Last, AL1 and AL3 are both present in the nuclei of infected plant tissue (25) and thus have an opportunity for interaction in plant cells. The importance of protein interactions during geminivirus replication and infection in plants can best be addressed in future experiments when AL1 and AL3 mutants defective for protein interactions become available.

ACKNOWLEDGMENTS

We thank Patricia Eagle, Kent Burkey, Ralph Dewey, and Beverly Orozco for critically reading the manuscript. We are grateful to Verne Luckow (Monsanto Company) for the recombinant baculovirus for β-glucuronidase.

This research was supported by Public Health Service—National Institutes of Health grant GM-16681-02 (to S.B.S.) and U.S. Department of Agriculture grant 95-37301-8786 (to L.H.-B.).

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