A Geminivirus Replication Protein Is a Sequence-Specific DNA Binding Protein

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The genome of the geminivirus tomato golden mosaic virus (TGMV) consists of two circular DNA molecules designated as components A and B. The A component encodes the only viral protein, AL1, that is required for viral replication. We showed that AL1 interacts specifically with TGMV A and B DNA by using an immunoprecipitation assay for AL1:DNA complex formation. In this assay, a monoclonal antibody against AL1 precipitated AL1:TGMV DNA complexes, whereas an unrelated antibody failed to precipitate the complexes. Competition assays with homologous and heterologous DNAs established the specificity of AL1:DNA binding. AL1 produced by transgenic tobacco plants and by baculovirus-infected insect cells exhibited similar DNA binding activity. The AL1 binding site maps to 52 bp on the left side of the common region, a 235-bp region that is highly conserved between the two TGMV genome components. The AL1:DNA binding site does not include the putative hairpin structure that is conserved in the common regions or the equivalent 5' intergenic regions of all geminiviruses. These studies demonstrate that a geminivirus replication protein is a sequence-specific DNA binding protein, and the studies have important implications for the role of this protein in virus replication.

INTRODUCTION

Geminiviruses are a family of plant viruses characterized by their circular, single-stranded DNA genomes, which replicate via double-stranded intermediates in the nuclei of infected cells (Rushing et al., 1987; Davies and Stanley, 1989; Lazarowitz et al., 1989; Horns and Jeske, 1991). The double-stranded form of viral DNA is assembled into nucleosomes and transcribed in infected plant cells (Abouzid et al., 1988; Hanley-Bowdoin et al., 1988; Sunter and Bisaro, 1989). Geminiviruses encode a single protein required for their replication and must recruit the remainder of their DNA replication machinery from the host plant (Elmer et al., 1988a; Etessami et al., 1991). These attributes suggest that geminiviruses are excellent models for studying nuclear DNA replication in plant cells. Geminiviruses are unique among plant viruses in their capacity to serve as models for plant DNA replication because all other known plant viruses, including other plant DNA viruses, replicate via RNA intermediates.

The geminivirus tomato golden mosaic virus (TGMV) has a bipartite genome consisting of two 2.5-kb circles designated A and B. Figure 1 depicts the two TGMV genome components, both of which have been cloned, sequenced, and shown to be required for productive infection of plants (Bisaro et al., 1982; Hamilton et al., 1983, 1984). Agrobacterium-mediated transformation has been used to create plants with stably integrated

copies of TGMV A or B component DNAs in their genomes. The presence of extrachromosomally replicating viral DNA and virus particles in transgenic plants containing linear tandem copies of TGMV A demonstrated that the A component encodes all of the information necessary for viral replication and encapsidation (Rogers et al., 1986; Sunter et al., 1987). The B component cannot replicate in the absence of the A component and, instead, contributes functions essential for movement (Rogers et al., 1986; Hayes and Buck, 1989). The six open reading frames (ORFs) of TGMV have been characterized functionally in infectivity and replication assays by adapting Agrobacterium-mediated transformation to inoculate cloned, mutant viral DNAs at high efficiency (Brough et al., 1988; Elmer et al., 1988b; Gardiner et al., 1988). These experiments demonstrated that the AL1 gene encodes the only viral protein required for replication (Elmer et al., 1988a). Transgenic tobacco plants, which express AL1 in the absence of all other TGMV proteins, can complement TGMV mutants with defective AL1 ORFs and support replication of TGMV B DNA (Hayes and Buck, 1989; Hanley-Bowdoin et al., 1990). Both single- and double-stranded forms of TGMV B were detected in leaf disc assays using transgenic plant material expressing AL1 (Hanley-Bowdoin et al., 1990), thereby establishing that AL1 is not only required, but is sufficient, for viral single- and double-stranded DNA synthesis in the presence of host proteins.

The AL1 ORF of TGMV and the equivalent ORFs of other bipartite geminivirus genomes are highly conserved at the

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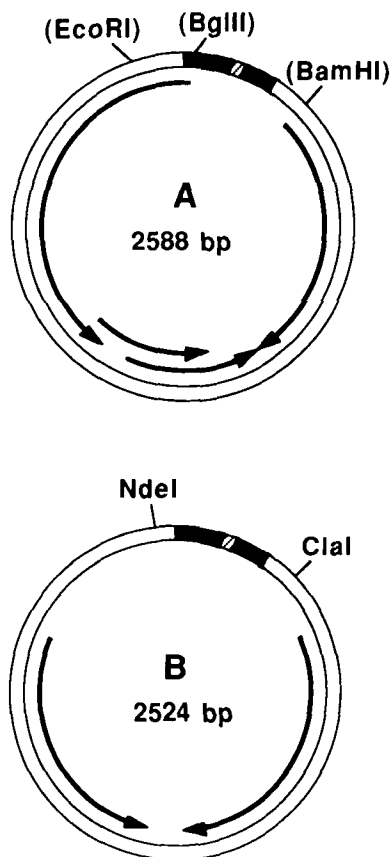[1] To whom correspondence should be addressed.

Figure 1. The TGMV Genome.

The A and B components of the TGMV genome are depicted. TGMV encodes six proteins, AR1, AL1, AL2, AL3, BR1, and BL1, whose ORFs and directions of transcription are designated by arrows. The filled boxes represent the highly conserved common regions of A and B; the hatched areas indicate the putative stem-loop structures of the common regions. Restriction sites that were used in this study are marked. Their positions are indicated relative to the left border of the common region (position 0). The BglII and BamHI sites are engineered sites at positions 28 and 326, respectively. The EcoRI site was acquired from vector polylinker sequences and is adjacent to the PstI site at TGMV A position 2460.

amino acid sequence level (Mullineaux et al., 1985). The homologous gene product of African cassava mosaic virus (ACMV) is also required for viral DNA replication (Davies and Stanley, 1989; Etessami et al., 1991). The amino acid conservation extends to include monopartite geminiviruses, which encode a split ORF that is homologous to AL1. A spliced mRNA specifying the homologous protein has been detected in plants infected by wheat dwarf virus (Schalk et al., 1989) or Digitaria streak virus (Accotto et al., 1989). In addition, the replication functions for maize streak virus are located on the left side of its genome (Lazarowitz et al., 1989). These results strongly suggest that the AL1 homologs of other geminiviruses are also

involved in viral DNA replication. The precise role of AL1 and its homologs in geminivirus DNA replication has not been determined. One possibility is that these proteins mediate initiation of viral replication by introducing a site-specific nick to prime rolling circle replication, similar to φX174 gene A protein (Eisenberg and Kornberg, 1979; Meyer and Geider, 1979). Recent studies have provided evidence for a rolling circle mechanism for geminivirus replication (Saunders et al., 1991; Stenger et al., 1991). Alternatively, geminivirus replication proteins may be involved in unwinding origin DNA to facilitate loading of DNA polymerase complexes, in a manner analogous to SV40 T antigen (Goetz et al., 1988). There is one report of amino acid sequence homology between geminivirus replication proteins and a family of helicases (Gorbalenya and Koonin, 1989). In either case, an early step in the initiation of DNA replication would involve binding of the geminivirus replication protein to the viral origin of replication. We have developed an immunoprecipitation assay for AL1:DNA binding and used it to examine DNA binding by TGMV AL1 produced in transgenic plants and baculovirus-infected insect cells. We demonstrate here that TGMV AL1 is a sequence-specific DNA binding protein and that its binding site is located on the left side of the viral common region. The function of AL1 in geminivirus replication is discussed with respect to these results and other published studies.

RESULTS

Synthesis of Recombinant TGMV AL1 Protein in Insect Cells

The involvement of AL1 in TGMV replication was first demonstrated by Elmer et al. (1988a). Since these early experiments, no progress has been made in defining the function of AL1 during geminivirus replication. The primary reason for the lack of progress is the difficulty in addressing AL1 function using TGMV-infected plants because of their diseased state and the low abundance of the protein. Immunoblot analysis showed that AL1 constitutes <0.01% of soluble protein extracts from TGMV-infected tissue (Hanley-Bowdoin et al., 1990). To facilitate functional studies, AL1 was expressed in a variety of systems, including Escherichia coli, transgenic plants, and insect cells. The initial studies on AL1 protein produced in E. coli and transgenic plants have been reported previously (Hanley-Bowdoin et al., 1990). These experiments indicated that it is not practicable to use the bacterial produced AL1 protein for functional analysis because it partitions to refractile bodies and is intractable to solubilization. As a consequence, there is no evidence that E. coli-produced AL1 is functional. In contrast, complementation studies of transgenic Nicotiana benthamiana plants expressing the AL1 gene under the control of the cauliflower mosaic virus 35S promoter established that the recombinant plant protein is functional for TGMV replication. However, the amount of AL1 protein produced by

transgenic plants is low, accumulating to levels similar to those observed in infected tissue.

To overcome this problem, we constructed a recombinant baculovirus containing AL1 under the control of the polyhedrin promoter (Luckow, 1991) and examined AL1 expression in insect cells by protein gel blot hybridization. Figure 2 shows that soluble protein extracts from Sf9 cells infected with the recombinant AL1 baculovirus contain a 40-kD protein that cross-reacts with a rabbit anti-AL1 polyclonal antiserum (lane 2). The insect-produced protein coelectrophoresed with AL1 from E. coli and transgenic plants (cf. lanes 1, 2, and 3). No cross-reacting material was detected in insect cells infected with wild-type baculovirus (results not shown). The identity of the recombinant protein was established further by immunoprecipitation with a mouse monoclonal antibody produced against AL1 (AL1 Ab), followed by detection of AL1 on immunoblots using anti-AL1 polyclonal serum. AL1 Ab precipitated AL1 from transgenic N. benthamiana plants (lane 3) and Sf9 cells infected with the recombinant AL1 baculovirus (lane 5) but not from wild-type plants (lane 4). Comparison of the amounts of AL1 immunoprecipitated in lanes 3 and 5 reveals that insect cells produce at least 70-fold more AL1 than transgenic plants per mass of total soluble protein. The higher levels of AL1 in baculovirus-infected insect cells suggest that they may be a better source of AL1 protein for functional studies.
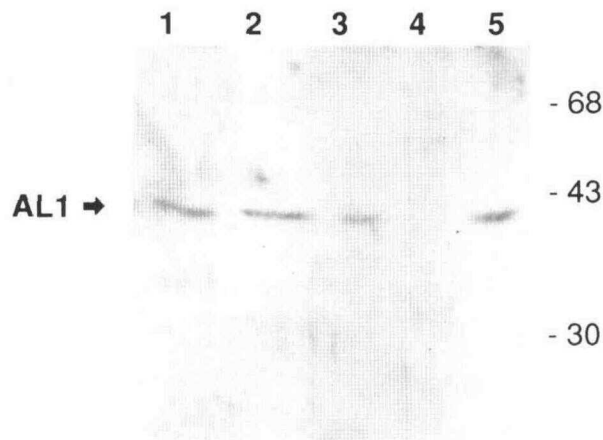

Figure 2. Synthesis of Recombinant AL1 Protein by Insect Cells, Transgenic Plants, and Bacteria.

AL1 was expressed in E. coli, baculovirus-infected Sf9 cells, and transgenic N. benthamiana plants. AL1 (10 ng) was purified from E. coli refractile bodies and resolved by SDS-PAGE (lane 1). Proteins from whole cell extracts of insect cells (50 µg; lanes 2 and 5), transgenic N. benthamiana plants (3.5 mg; lane 3), and wild-type plants (3.5 mg, lane 4) were resolved. In lanes 1 and 2, the extracts were loaded directly onto polyacrylamide gels followed by protein gel blot analysis. In lanes 3 to 5, AL1 was immunoprecipitated using AL1 Ab and rabbit anti–mouse IgG coupled to Sepharose beads prior to immunoblot analysis. AL1 protein is indicated on the left. The sizes and positions of protein molecular mass markers are shown on the right in kD.

AL1 Synthesized in Transgenic Plants Binds to TGMV DNA

The replication proteins of many DNA viruses bind to their respective viral origins of replication and perform catalytic functions that are involved in the initiation of DNA synthesis. By analogy, if AL1 is involved in the early events of replication, it may also bind to the TGMV origin of replication. Furthermore, if AL1 binding is a prerequisite for initiation of DNA replication, the A and B components of the TGMV genome are likely to have closely related origins with a conserved AL1 binding site. A region of strong sequence homology between TGMV A and B is the 235-bp common region (Figure 1—CR; Hamilton et al., 1984), which has been proposed to contain the viral replication origin (Rogers et al., 1989). Consequently, we investigated the potential binding of AL1 to the TGMV common region and adjacent DNA sequences.

Initial studies using a gel mobility shift assay for DNA binding failed to demonstrate interaction between AL1 and the common region. Nuclear extracts from wild-type and transgenic AL1 plants gave complicated gel retardation patterns with a TGMV A DNA fragment that included the common region. The patterns for wild-type and transgenic AL1 extracts were indistinguishable, reflecting a high level of host protein binding to the common region DNA fragment (E. Fontes and L. Hanley-Bowdoin, unpublished results). The common region overlaps the divergent promoters for AR1 and AL1 transcription (Hanley-Bowdin et al., 1988) and is expected to include binding sites for host transcription factors. In addition, host proteins involved in DNA replication may bind to TGMV common region sequences. These experiments demonstrated that a more sensitive and specific assay for AL1:DNA binding was necessary. We approached this problem by using AL1 Ab to enrich for AL1 protein prior to DNA binding.

Immunoprecipitation of AL1 concentrates the protein and may facilitate its binding to DNA. In addition, the immunoprecipitation assay strongly favors specific interactions because the protein/DNA complexes are formed and washed extensively in the presence of nonspecific competitor DNA. Figure 3 demonstrates that the immunoprecipitation assay detected complex formation between AL1 and TGMV fragments that included common region sequences. A 471-bp, (Eco-RI)-(BamHI) fragment from TGMV A (prA1; Figure 1) and a 382-bp, NdeI-ClaI fragment from TGMV B (prB; Figure 1) were used as probes in Figure 3. Both DNA probes included the common region and adjacent sequences. In Figure 3A, radiolabeled prA1 DNA (lane 1) bound to AL1:AL1 Ab immunocomplexes isolated from whole cell extract that was prepared from transgenic plants expressing AL1 (lane 3). Probe prA1 did not bind to wild-type extracts immunoprecipitated with AL1 Ab (lane 2) or to transgenic extracts immunoprecipitated with a monoclonal antibody (NS Ab) against a 70-kD ribonucleoprotein (lane 4; Billings et al., 1982). In Figure 3B, equivalent results were obtained with radiolabeled prB DNA (lane 1), which bound in the reaction containing immunoprecipitates formed with transgenic plant extract and AL1 Ab (lane 3) and not in reactions containing
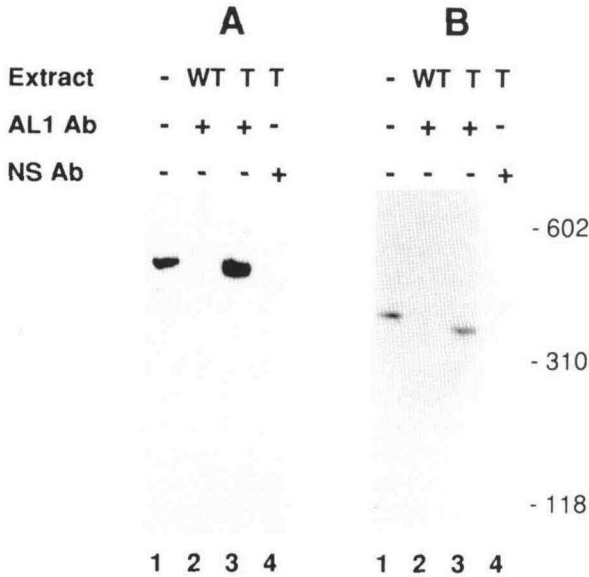
A   **B**

Extract	-	WT	T	T	-	WT	T	T
AL1 Ab	-	+	+	-	-	+	+	-
NS Ab	-	-	-	+	-	-	-	+

- 602

- 310

- 118

    1 2 3 4   1 2 3 4

Figure 3. AL1 Synthesized by Transgenic Plants Binds to TGMV DNA.

AL1 binding assays were performed using ^{32}P-labeled TGMV DNA probes and whole cell extracts from wild-type *N. benthamiana* plants (WT, lane 2) or transgenic *N. benthamiana* plants expressing functional AL1 (T, lanes 3 and 4). The input probes were electrophoresed in lane 1 of each panel. The immunoprecipitation reactions contained the components indicated at the top of each lane. AL1 Ab (lanes 2 and 3) is a monoclonal antibody specific for AL1 protein, whereas NS Ab (lane 4) is a monoclonal antibody against a 70-kD human ribonucleoprotein. The numbers on the right correspond to the sizes and positions of ^{32}P-φX174 HaeIII-digested DNA markers.
(A) The binding activities of the protein extracts with probe DNA, prA1, from TGMV A were assayed. Probe prA1 is a 471-bp, EcoRI-BamHI fragment (Figure 1, TGMV A) that was isolated from pNSB2.
(B) The binding activities of the protein extracts with probe DNA, prB, from TGMV B were assayed. Probe prB is a 382-bp, NdeI-ClaI fragment (Figure 1, TGMV B) that was isolated from pMON393.

wild-type extracts and AL1 Ab (lane 2) or transgenic extracts and NS Ab (lane 4). The difference in the mobilities of prB in lanes 1 and 3 is due to variable salt concentration between the two samples. These results establish that AL1 binds to both TGMV A and B DNAs. The AL1 binding site is most likely in the common region because this is the only conserved sequence that is shared by the TGMV A and B fragments used as probes in this experiment.

The Specificity and Selectivity of AL1:DNA Interaction

The sequence specificity of AL1 binding to TGMV A was investigated in competition studies between radiolabeled probes and excess unlabeled DNA. These experiments were performed using whole cell extracts isolated from baculovirus-infected insect cells synthesizing AL1. Figure 4A shows that

AL1 immunoprecipitated from insect cells by AL1 Ab bound to the radiolabeled TGMV A probe, prA1, in the absence of competitor DNA (lane 3). No AL1:prA1 complexes were detected in an equivalent reaction containing NS Ab (lane 2), thereby demonstrating that AL1 produced in insect cells also binds to TGMV DNA. In contrast to the result in lane 3, a 50-fold molar excess of unlabeled prA1 completely abolished binding of ^{32}P-prA1 by AL1 (lane 6). A 25-fold excess of prA1 was also sufficient to abolish binding (results not shown), demonstrating the sensitivity of ^{32}P-prA1:AL1 binding to the presence of the homologous unlabeled DNA. No competition with prA1:AL1 binding (lanes 4 and 5) was observed with a 50-fold excess of poly(dI-dC). The different competition efficiencies of the competitor DNAs establish that AL1 binds specifically and with a significantly higher affinity to prA1 than to poly(dI-dC). DNA fragments that included the entire A and B component DNAs except for the prA1 and prB sequences also did not compete in immunoprecipitation assays for AL1:prA1 binding (results not shown), indicating that there are no equivalent AL1 binding sites located elsewhere on the TGMV genome. In addition, these experiments suggested that AL1 and viral DNA are not covalently linked because proteinase K (250 µg/mL) digestion of the protein/DNA complex prior to phenol/CHCl$_3$/isoamyl alcohol extraction did not significantly increase the yield of bound DNA (Figure 4A, lane 5).

Figure 4B also demonstrates the specificity of AL1:DNA binding and delimits the AL1 binding site further. We took advantage of unique restriction sites in prA1 to examine the capacities of different prA1 subfragments (Figure 4C) to bind AL1 immunocomplexes prepared from baculovirus-infected insect cells. Probe prA1 radiolabeled at its 3' ends was digested with PvuI, SspI, AluI, or DdeI and the digested DNA was added to AL1 binding reactions. After incubation, bound DNA was separated from free DNA and analyzed by denaturing PAGE. PvuI digestion of prA1 generated two fragments (Figure 4B, lane 4). In lane 5, the AL1 immunocomplexes selectively bound to the 291-bp PvuI digestion product that includes TGMV A sequences from positions 2460 to 156. SspI digestion of prA1 yielded two fragments (lane 6) but only one of the fragments, a 272-bp DNA that extends from TGMV A positions 2460 to 135, bound to AL1 immunocomplexes (lane 7). The 181- and 200-bp fragments, which result from PvuI and SspI digestion and are absent in lanes 5 and 7, were isolated in reactions in which prA1 was digested after binding to AL1 immunocomplexes (data not shown). This result verified that the smaller DNAs would have been recovered by our protocol if they had bound to AL1 immunocomplexes. The result also demonstrates that AL1 binding to intact prA1, the 291-bp PvuI fragment, and the 272-bp SspI fragment is specific. In contrast, no specific interaction between AL1 and viral DNA was detected in binding reactions that contained AluI-digested prA1 (lane 9), suggesting that AluI digestion destroyed the AL1 binding site of prA1. DdeI was also used to digest ^{32}P end-labeled prA1 to give five fragments such that only the two terminal fragments were radiolabeled (lane 10). No binding of the terminal DdeI fragments to AL1 immunocomplexes was detected (lane 11).
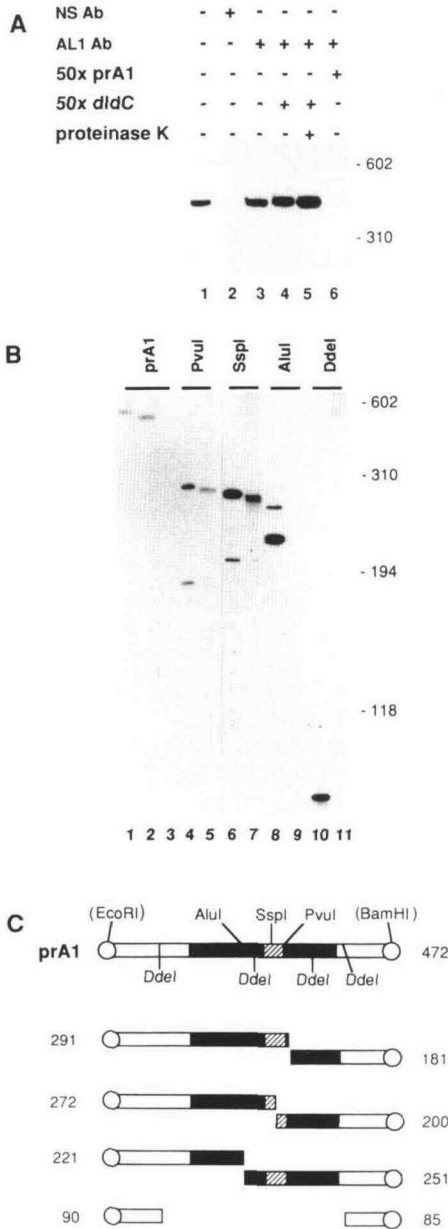
A

NS Ab   - + - - - -
AL1 Ab   - - + + + +
50x prA1   - - - - - +
50x dIdC   - - - + + -
proteinase K - - - - + -

- 602

- 310

1 2 3 4 5 6

B

prA1   PvuI   SspI   AluI   DdeI

- 602

- 310

- 194

- 118

1 2 3 4 5 6 7 8 9 10 11

C

(EcoRI)   AluI   SspI   PvuI   (BamHI)
prA1   O━━━━━━━━━━━━━━━━━━━━━━━O 472
             DdeI   DdeI   DdeI DdeI

291 O━━━━━━━━━━━━━━
                    ━━━━━━O 181

272 O━━━━━━━━━━━━
                    ━━━━━━O 200

221 O━━━━━━━━━━
                    ━━━━━O 251

90   O━━   ━━O 85

Figure 4. Specificity and Selectivity of DNA Binding by AL1.

AL1 binding assays were performed using the ³²P-labeled TGMV DNA, prA1, or its digestion products **(C)** and whole cell extract from Sf9 cells infected with a recombinant baculovirus expressing AL1.
(A) The DNA binding specificity of AL1 is shown by using homologous and heterologous unlabeled DNAs as competitors. As indicated at the top of each lane, the DNA binding reactions contained ³²P-prA1 and insect cell extract plus NS Ab (lane 2), AL1 Ab (lane 3), AL1 Ab and 50-fold molar excess of poly(dI-dC) (lanes 4 and 5), or AL1 Ab and a 50-fold molar excess of unlabeled prA1 (lane 6). The reaction in lane 5 was digested with 250 μg/mL proteinase K prior to extraction. The probe prA1 was electrophoresed in lane 1.
(B) The selectivity of AL1-DNA binding is shown by restriction digestion of probe prA1. The probe prA1 was electrophoresed in lane 1. The

From the results in Figure 4B, we can conclude that the AL1 binding site is located on the left side of prA1 between TGMV A positions 2460 and 135 (EcoR1 and SspI sites) and overlaps or is in close proximity to position 84 (AluI site). These results also indicate that binding of AL1 to viral DNA is not mediated by the putative hairpin structure in the TGMV common region because prA1 DNA retained its capacity to bind AL1 after digestion at the SspI site in the loop of the structure. In experiments not shown, binding by transgenic plant-produced AL1 to ³²P-prA1 was competed by a 50-fold excess of unlabeled prA1 but not by a 50-fold excess of poly(dI-dC). Furthermore, AL1 from transgenic plants showed the same binding selectivity for the 291-bp PvuI and 272-bp SspI digestion products as the insect cell-produced protein. AL1 from transgenic plants also did not interact with prA1 digested with AluI. These results establish that AL1 synthesized by transgenic plants and insect cells have the same DNA binding specificity.

A 52-bp Sequence to the Left of the Hairpin Structure of TGMV A Includes the AL1 Binding Site

The strongest region of conservation between the common regions of TGMV A and B extends from positions 55 to 160. It is likely that the AL1 binding site, which was positioned between TGMV A 2460 and 135 by the experiment shown in Figure 4B, is located in this conserved region. To test this possibility, we examined AL1 binding to probes isolated from subclones of the TGMV A common region. Figure 5 shows that AL1 immunocomplexes from transgenic plants (Figure 5A, lane 2) and insect cells (Figure 5B, lane 2) bind to a TGMV A fragment, prA3, that includes TGMV A sequences from positions 28 to 135 (Figure 5C). Binding to prA3 is mediated by AL1 because no binding activity was detected in AL1 Ab immunoprecipitates of extracts from wild-type plants (Figure 5A, lane 3) or from Sf9 cells infected with a recombinant baculovi-

binding reactions in lanes 2 and 3 contained insect cell extract, intact prA1, and AL1 Ab (lane 2) or NS Ab (lane 3). In lanes 4 to 11, probe prA1 was digested with either PvuI (lanes 4 and 5), SspI (lanes 6 and 7), AluI (lanes 8 and 9), or DdeI (lanes 10 and 11), as indicated. Lanes 1, 4, 6, 8, and 10 show the digestion products of prA1. Lanes 2, 5, 7, 9, and 11 show binding reactions that contained insect cell extract and AL1 Ab. The numbers to the right of each panel correspond to the sizes and positions of ³²P-φX174 HaeIII-digested DNA markers.
(C) The DNA probe prA1 and its digestion products are shown. Common region sequences are indicated by the filled boxes with the putative stem-loop structure designated by the hatched area. The BamHI and EcoRI restriction sites delimiting the probes correspond to those shown in Figure 1. The positions of the PvuI (TGMV A position 156), SspI (position 135), AluI (position 84), and DdeI (positions 2539, 105, 198, 247) restriction sites in prA1 (top) are also given. The sites of the ³²P radiolabel are marked by the circles. The sizes in base pairs of each radiolabeled fragment are given.
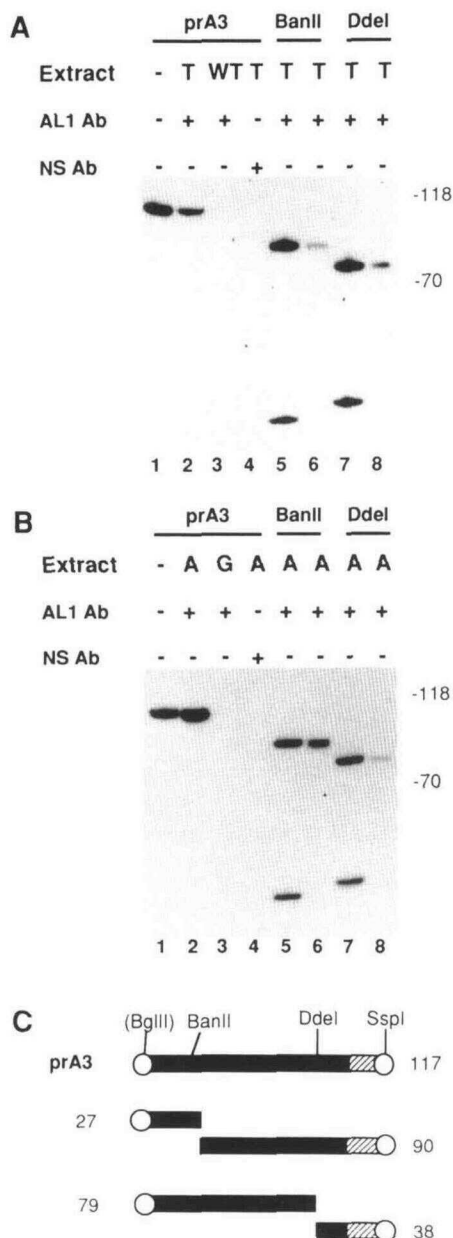
A

Extract	-	T	WT	T	T	T	T	T
AL1 Ab	-	+	+	-	+	+	+	+
NS Ab	-	-	-	+	-	-	-	-

(prA3 | BanII | DdeI)

-118

-70

1 2 3 4 5 6 7 8

B

Extract	-	A	G	A	A	A	A	A
AL1 Ab	-	+	+	-	+	+	+	+
NS Ab	-	-	-	+	-	-	-	-

(prA3 | BanII | DdeI)

-118

-70

1 2 3 4 5 6 7 8

C

prA3 (BglII) BanII   DdeI   SspI   117

27   90

79   38

Figure 5. The AL1 Binding Site Is Located in a 52-bp Region of the TGMV Common Region.

AL1 binding assays were performed using ^{32}P-labeled TGMV DNA, prA3, or its digestion products **(C)** and whole cell extract from *N. benthamiana* plants or baculovirus-infected Sf9 cells.

(A) Binding assays are shown containing extracts from transgenic plants expressing AL1 (T) or wild-type plants (WT), as indicated at the top of each lane.

(B) Binding assays are shown containing extracts from Sf9 cells expressing AL1 (A) or β-galactosidase (G) from recombinant baculoviruses, as indicated at the top of each lane.

The intact probe prA3 was electrophoresed in lane 1 of **(A)** and **(B)**. The binding reactions in lanes 2 to 4 contained prA3 and AL1 Ab (lanes 2 and 3) or NS (lane 4). Probe prA3 was digested with either BanII

rus expressing a β-galactosidase gene (Figure 5B, lane 3). In addition, no binding was detected when NS Ab was used to precipitate extracts from transgenic plants (Figure 5A, lane 4) or from insect cells expressing AL1 (Figure 5B, lane 4). In contrast to the results with prA3, no AL1 binding was detected to a fragment that included TGMV A sequences from 136 to 325 (results not shown). These results show that the AL1 binding site is contained entirely within probe prA3. They also establish unequivocally that AL1 binding is not dependent on the putative hairpin structure in the common region because the use of separately cloned and purified fragments as probes precluded the formation of nicked stem-loop structures during the binding incubation period.

The DNA sequences in prA3 that are responsible for AL1 binding were defined further by digestion with BanII or DdeI (Figure 5C) followed by immunoprecipitation in DNA binding assays containing extracts from transgenic plants (Figure 5A) and insect cells expressing AL1 (Figure 5B). BanII digestion of prA3 resulted in two fragments (lanes 5), but only the larger fragment (TGMV A positions 53 to 135) was selectively bound by AL1 immunocomplexes (lanes 6). DdeI digestion also gave two fragments (lanes 7) with only the larger fragment (positions 28 to 105) binding AL1 immunocomplexes (lanes 8). These results locate the AL1 binding site to a 52-bp sequence in the common region of TGMV A between positions 53 and 105. Controls equivalent to those described above for Figure 4 established that we would have detected the smaller BanII and DdeI digestion products if AL1 had bound these fragments. However, it is possible that sequences flanking the 52-bp sequence are involved in stabilizing AL1:DNA interactions because intact prA3 was immunoprecipitated more efficiently than prA3 digested with BanII or DdeI (cf. lanes 2 with lanes 6 and 8).

DISCUSSION

DNA replication plays a central role in plant growth and development. Knowledge of the biochemical mechanisms that

(lanes 5 and 6) or DdeI (lanes 7 and 8). Lanes 5 and 7 show the digestion products of prA3. Lanes 6 and 8 show binding reactions that contained transgenic plant or insect cell extract and AL1 Ab. The numbers to the right in **(A)** and **(B)** correspond to the sizes and positions of ^{32}P-φX174 HaeIII-digested DNA markers.

(C) The DNA probe prA3 and its digestion products are shown. Probe prA3 was isolated from pNSB73. Common region sequences are indicated by the filled boxes with the putative stem-loop structure designated by the hatched area. The BglII site was derived from vector polylinker sequences and is immediately adjacent to TGMV A position 28. The positions of the SspI site (TGMV A position 135), BanII (position 53), and DdeI (position 105) restriction sites in prA3 (top) are also given. The sites of the ^{32}P radiolabel are marked by the circles. The sizes in base pairs of each radiolabeled fragment are given.

mediate and control plant DNA replication is critical to our understanding of these key processes in plants. Historically, DNA viruses have served as important model systems for studying DNA replication. Studies of φX174 (Kornberg and Baker, 1992) and SV40 (Kelly, 1988) replication have been instrumental in identifying the enzymes and biochemical mechanisms involved in DNA replication in bacterial and animal cells, respectively. A similar approach has been difficult to implement for plants because nearly all plant viruses, including most plant DNA viruses, replicate via RNA intermediates. Geminiviruses are the only known exceptions and have many advantages for studying DNA replication in higher plants. Geminivirus genomes are small, replicate to high copy number, assemble into nucleosomes, and are transcribed in the nuclei of infected plant cells (Davies and Stanley, 1989; Lazarowitz et al., 1989). A single viral protein with no homology to any known DNA polymerase is required for their replication (Elmer et al., 1988a; Etessami et al., 1991). These characteristics suggest that geminiviruses use host replication machinery and are excellent model systems for chromosomal replication in plant nuclei. In this paper, we identify a molecular event that is associated with geminivirus replication, i.e., sequence-specific interaction between a geminivirus replication protein and its genome. We show that TGMV AL1 binds specifically to TGMV DNA and that its binding site is located in a 52-bp sequence on the left side of the common region, a 235-bp region that is strongly conserved between the A and B components of the viral genome.

Many DNA viruses encode proteins that bind specifically to their origins of replication and help mediate the initiation of DNA replication. The origin of TGMV replication has not been characterized, but it is most likely located in the common region of the TGMV genome (Rogers et al., 1989). Deletion studies showed that 2106 bp of TGMV A are dispensable for replication (Elmer et al., 1988a; Gardiner et al., 1988; T. Watson and L. Hanley-Bowdoin, unpublished results). The remaining 482 bp of TGMV A DNA include the 235-bp common region, the bidirectional polyadenylation site, and 70 bp of AL2 coding sequence. A 413-bp deletion that includes the TGMV B common region abolishes replication, and site-directed mutations in the TGMV B common region impact replication (Revington et al., 1989). A TGMV A fragment derived from the common region can support episomal replication in the absence of other viral DNA sequences when AL1 is provided in *trans* (H. Gladfelter and L. Hanley-Bowdoin, unpublished results). Collectively, these results argue strongly that the common region contains the TGMV origin of replication. A corollary of this hypothesis is that if AL1 binds to the TGMV origin of replication, its binding site is in the common region.

We have provided three lines of evidence that AL1 binds to the TGMV common region and that this binding is specific. First, AL1 binding activity was only observed when protein extracts containing AL1 and a monoclonal antibody against AL1 were used in an AL1:DNA immunoprecipitation assay. No binding was detected with equivalent protein extracts lacking AL1, or when an unrelated monoclonal antibody was used. Second, AL1 binding was only competed by DNA that was

homologous to probe DNA containing the AL1 binding site. Poly(dI-dC) and unrelated TGMV A and B DNA fragments did not compete for AL1 binding. Finally, AL1 selectively bound to overlapping fragments of the common region in the presence of other common region fragments. All of the common region DNAs that bound AL1 shared a 52-bp BanII-DdeI fragment. No binding was detected when the 52-bp sequence was digested internally with the restriction endonuclease AluI. These results establish that AL1 binds specifically to the common region and that its binding site is contained within a 52-bp BanII-DdeI fragment of the common region, most likely overlapping or near the AluI site at TGMV A position 84. The AL1 binding domain has not been defined further by DNA footprinting techniques because host DNA binding activities that mask AL1 binding in crude extracts interfere with footprinting. It is not technically feasible to detect a footprint of the immunoprecipitated AL1:DNA complex because of the relatively low sensitivity of the footprinting assay. The AL1 binding site will be characterized further in future studies using purified AL1 protein for footprinting and by mutational analysis of the 52-bp region. Purified AL1 protein will also be used to address the potential role of cooperativity in AL1:DNA binding and AL1 binding to supercoiled DNA, both of which cannot readily be assessed using our immunoprecipitation assay.

We have demonstrated sequence-specific AL1:DNA binding for both transgenic plant and baculovirus-infected insect cell extracts. The AL1 proteins produced by transgenic plants and insect cells are indistinguishable with respect to their DNA binding properties. These results suggest that plant proteins do not affect the specificity of AL1 binding, either by interacting with AL1 or by modifying the protein in a plant-specific manner. However, it is possible that a protein evolutionarily conserved between plants and insects may be involved in the AL1:DNA interaction. Several cDNAs encoding proteins that are involved in DNA replication and the cell cycle and which show evolutionary and functional conservation with their animal and yeast homologs have been isolated from plants (Suzuka et al., 1989; Feiler and Jacobs, 1990; Colasanti et al., 1991; Ferreira et al., 1991; Hata et al., 1991). In our immunoprecipitation assay, we did not detect any association between AL1 and a plant or an insect protein, as analyzed by silver-stained protein gels (results not shown). However, the sensitivity of silver staining may not be sufficient to detect AL1-associated proteins in crude extracts.

Figure 6 shows that the 52-bp fragment containing the AL1 binding site has several important features. (1) It is conserved at 51 nucleotide positions between TGMV A and B DNA, indicating that the specific contacts that mediate AL1:DNA binding are the same for both genome components. It is likely that the single nucleotide difference, which is at the extreme left edge of the 52-bp region, is not involved in specific contact formation. (2) The 52-bp region is to the left of the putative hairpin structure and the conserved nonanucleotide loop sequence that have been found in the common regions or 5' intergenic regions of all geminiviruses (Lazarowitz, 1987). Our results establish that the hairpin structure of TGMV is not required for

```
GATGCGATGGCATTTTTGTAATTAAGAGGCTTACTACCAATTGAGGAGGGGCT
--G-T----------G-------G----------------GTTT------
```

```
BanII                              Alu I                          DdeI
CCAAAAGTTATATGAATTGGTAGTAAGGTAGCTCTTATATATTAGAAGTTCCT
A-------•----------------------------------------------
```

```
                            SspI
AAGGGGCACGTGGCGGCCATCCG*TTTAATATTACCGGATGGCCGCGCGATCG
--------------------T-----------------------
```

```
TCA*CCCGACCCGCTTCCGC*AATTACGCCGCATTGTCGTCTAAGTGGTCCCG
--CT---------TG----TG----G------------G-C-CT----GTG-
```

```
CA**TATGTGAAGGGCCAATCATATTT
TCCCCT----*TTAA----------
```

Figure 6. AL1 Binding Site in the Common Region of TGMV.

The DNA sequences of the common regions of TGMV A (top line) and B (bottom line) are given. In the DNA sequences for TGMV B, — indicates identity with the TGMV A DNA sequence. Deletions in the two sequences relative to each other are indicated (*). The inverted repeats that form the stem of the putative hairpin are designated by the lines over the sequence. The AL1 transcription start site (●) and its upstream TATA box (underlined) are marked. The current limits of the AL1 binding site on TGMV A are enclosed by the box and the BanII and DdeI sites. The AluI site, which when digested abolishes all AL1 binding, and the SspI site in the hairpin are also marked.

AL1 binding. (3) The 52-bp region overlaps the AL1 gene transcription start site and promoter. Perhaps AL1 also has a dual role in replication and transcription of TGMV. (4) The 52-bp region of TGMV shows weak homology to DNA sequences on left sides of the common regions of other bipartite geminivirus DNAs. This observation implies that if the binding sites for other geminivirus replication proteins are located in these homologous regions their DNA binding motifs may be related, but not identical, to each other.

The replication proteins of the closely related geminiviruses TGMV, squash leaf curl virus (SqLCV), and ACMV are not interchangeable even though there is strong sequence and, presumably, functional homology between these proteins. The TGMV A and SqLCV A components both replicate in N. benthamiana leaf discs but cannot support replication of the heterologous B component in the same assay (Lazarowitz et al., 1991). The B components only replicate in leaf discs in the presence of their homologous A component. Similarly, the release and replication of stably integrated ACMV B are only supported by its homologous A component and not by TGMV A (Stanley et al., 1990). These results demonstrate that there is specificity in the interactions between a geminivirus genome and its replication protein. This specificity is not due to differences in host protein/DNA binding because N. benthamiana supports replication of all three viruses and, instead, probably reflects specific interactions between a given geminivirus replication protein and its genome. This specificity may be due

to sequence-specific DNA binding of the replication protein to its cognate genome. However, it is also possible that sequences involved in other aspects of replication, e.g., catalysis, confer specificity between a given geminivirus and its replication protein. Gene A protein recognizes two sites in the plus-strand origin of φX174, one that is required for DNA binding and a second that includes the cleavage site that primes rolling circle replication (Kornberg and Baker, 1992). Future studies that compare the replication protein/DNA binding motifs of different geminiviruses and that determine if there are other sequence-specific motifs required for replication protein function will be necessary to resolve these possibilities.

The demonstration that AL1 binds to the common region of TGMV in a sequence-specific manner suggests that AL1 is likely to be involved in the initiation of replication and has important implications for its function. First, AL1 binding may be one of the first events in replication and a prerequisite for the assembly of the replication complex at the viral origin of replication. Subsequent to binding, AL1 could function in initiation as a sequence-specific endonuclease to initiate rolling circle replication, as a helicase to unwind origin DNA, or by some unknown mechanism. Second, the putative hairpin structure in the common region is involved in a replication step other than AL1 binding. Experiments by Revington et al. (1989), which showed that the insertion of 8 bp into the loop of the TGMV B hairpin eliminates replication, indicate that the hairpin is required for replication. The hairpin structure may have a role in the catalytic function of AL1, possibly serving as a signal for or facilitating the introduction of a single-stranded nick at the origin. Recent experiments suggest that rolling circle replication of beet curly top virus initiates in the hairpin, possibly via a nick (Stenger et al., 1991). Alternatively, the hairpin may not exist in duplex DNA and, instead, may be important in the initiation of minus-strand DNA synthesis on the single-stranded, plus-sense DNA template or in protecting the 5' end of the nicked, plus-sense DNA from degradation during rolling circle replication. Hairpin structures are functional elements in the minus-strand origins of several single-stranded DNA bacteriophages (Kornberg and Baker, 1992). Last, the location of the AL1 binding site relative to the AL1 promoter suggests that AL1 may also be involved in its own transcription, possibly as a regulatory factor. Many eukaryotic origins of replication overlap transcriptional elements, and the proteins that bind these overlapping sequences frequently function both in replication and transcription (DePamphilis, 1988).

We have demonstrated that AL1 protein produced by transgenic plants binds to TGMV DNA in a sequence-specific manner. Earlier studies established that the same protein is functional for replication (Hanley-Bowdoin et al., 1990). We have also shown that the DNA binding properties of AL1 synthesized by baculovirus-infected insect cells are indistinguishable from those of the functional plant protein. The baculovirus expression system has been used previously to produce functional plant proteins, including maize Ac transposase (Kunze and Starlinger, 1989), potato patatin (Andrews et al., 1988), and cowpea mosaic virus 24K protease (Van Bokhoven et al., 1991).

It is probable that TGMV AL1 synthesized by insect cells is also enzymatically active. The baculovirus expression system produces at least 70-fold more AL1 protein than transgenic plants and, as a consequence, will be an excellent source of AL1 for future studies on the catalytic role of the protein in TGMV replication.

METHODS

Enzymes and Materials

Calf intestine alkaline phosphatase, *Escherichia coli* DNA polymerase (Klenow fragment), and proteinase K were from Boehringer Mannheim. T4 polynucleotide kinase was from Stratagene. All other enzymes were from New England Biolabs, Inc. (Beverly, MA). Poly(dl-dC) was from Sigma or Boehringer Mannheim. The Geneclean II and Mermaid DNA purification kits were from BIO 101, Inc. (Vista, CA). Immunobeads and AMPPD were purchased from Bio-Rad and TROPIX, Inc. (Bedford, MA), respectively.

Plasmids and Probes

The position numbers used to describe the following clones refer to the nucleotide coordinates of the TGMV A sequence determined by Hamilton et al. (1984). In this numbering scheme, TGMV A is numbered from 1 to 2588 with the common region delimited by positions 1 to 235.

The A component of TGMV was cloned as an EcoRI fragment into pUC119 to give pMON424 (Elmer et al., 1988a). Plasmid pMON424 was modified by site-directed mutagenesis (Kunkel, 1985) to insert the sequence GATCTGGATCCAT at position 326. The resulting plasmid, pMON437, contains new BglII, BamHI, and NdeI sites immediately upstream of the AR1 gene. A 467-bp PstI-BamHI fragment from pMON437 was subcloned into the Stratagene vector pBSII KS−, digested with same enzymes, to give pNSB2. The TGMV A fragment in pNSB2 includes the entire 235-bp common region flanked by 131 bp of AL1 coding sequence and 91 bp of AR1 upstream sequence. Plasmid pMON477 was derived from pMON437 by a second round of site-directed mutagenesis to introduce the trinucleotides GTA and TCT at positions 16 and 28 to create NdeI and BglII sites, respectively (Elmer et al., 1988a). A BglII-SspI fragment from pMON477 was subcloned into a pBSII SK− derivative digested with NdeI, repaired with Klenow, and then digested with BglII. (The pBSII SK− derivative had been modified by insertion of a BglII linker into a SmaI site and a NdeI linker into a Klenow-repaired EcoRI site. The EcoRI site was restored in the derivative.) The resulting clone, pNSB73, contains a 108-bp fragment from TGMV A positions 28 to 135 on the left side of the common region. Plasmid pMON393 contains TGMV B DNA cloned as a tandem one and one half copy into the binary plant transformation vector pMON505 (Elmer et al., 1988b; Hanley-Bowdoin et al., 1989).

A baculovirus transfer vector for AL1 expression was constructed by insertion of a 1.2-kb BglII-BamHI TGMV fragment, which includes the AL1 ORF, downstream of the baculovirus polyhedrin promoter of pVL1392 to give pMON1680. The TGMV fragment in pMON1680 is identical to the insert in pMON455, the binary plant transformation vector used in the construction of transgenic plants that express AL1 (Hanley-Bowdoin et al., 1990). Plasmid pVL1392 is a derivative of pVL941

(Luckow and Summers, 1989) with a polylinker containing the restriction sites BglII, PstI, EagI, NotI, XbaI, KpnI, SmaI, and BamHI inserted downstream of the polyhedrin promoter.

DNA probes were prepared from pMON393, pNSB2, and pNSB73 by restriction enzyme digestion as indicated in Figures 3 to 5. The DNA fragments were fractionated on agarose gels followed by purification via glass adhesion (Vogelstein and Gillespie, 1979) using a Geneclean II or Mermaid Kit. The purified fragments were radiolabeled using γ-^{32}P-ATP and T4 polynucleotide kinase or α-^{32}P-ATP and *E. coli* DNA polymerase (Klenow fragment; Sambrook et al., 1989).

Recombinant Protein Production and Isolation

Recombinant baculovirus was isolated by cotransfecting 2 μg of pMON1680 DNA with 1 μg of genomic DNA from the E2 strain of the *Autographa californica* nuclear polyhedrosis virus (Smith and Summers, 1979) into *Spodoptera frugiperda* Sf9 cells (Summers and Smith, 1987). Putative occlusion minus recombinant viruses were confirmed by DNA dot blot hybridization using an AL1-specific probe (Luckow and Summers, 1988). Recombinant viruses were purified by three rounds of plaque purification and were further characterized by radiolabeling of virus-infected Sf9 cells with ^{35}S-methionine (Luckow and Summers, 1988). One virus isolate, designated vMON1680.A10, expressed AL1 and was used for all subsequent experiments. A 10-L bioreactor (Applikon Dependable Instruments, Foster City, CA) was used for the large-scale propagation of Sf9 insect cells infected with vMON1680.A10. Production of recombinant AL1 protein in *E. coli* and transgenic *Nicotiana benthamiana* plants (line 9985) has been described previously (Hanley-Bowdoin et al., 1990).

Whole cell protein extracts were prepared from *N. benthamiana* plants, as described previously (Green et al., 1988; Hanley-Bowdoin et al., 1990). Whole cell extracts from insect cells were prepared from Sf9 cultures harvested 48 hr after vMON1680.A10 infection, washed with Grace's medium (Grace, 1962), resuspended in extraction buffer, and lysed by adjusting to 0.5 M NaCl. The remaining steps were identical to those in the plant extraction protocol (Green et al., 1988). The protease inhibitor mixture for the insect extracts contained 0.8 mM phenylmethysulfonyl fluoride, 1 μg/mL aprotinin, 0.5 μg/mL leupeptin, and 0.7 μg/mL pepstatin.

Antibodies and Protein Analysis

The mouse monoclonal antibody AL1 Ab was raised using standard techniques (Goding, 1986) against a combination of AL1 protein produced by *E. coli* and by vMON1680.A10-infected Sf9 cells. Ascites fluid containing AL1 Ab was produced in BALB/c mice injected intraperitoneally with 3 × 10^7 hybridoma cells. The rabbit polyclonal antiserum, which was raised against AL1 protein produced in *E. coli*, has been described previously (Hanley-Bowdoin et al., 1990).

AL1 was immunoprecipitated from 3.5 mg of plant cell extract or 50 μg of insect cell extract using 15 μg of AL1 Ab and rabbit anti-mouse IgG coupled to Sepharose beads (Harlow and Lane, 1988). The immunocomplexes were analyzed by SDS-PAGE (Laemmli, 1970) and immunoblot analysis using a rabbit polyclonal antibody against AL1 (Hanley-Bowdoin et al., 1990) and a goat anti-rabbit IgG alkaline phosphatase conjugate. Alkaline phosphatase activity was detected with the chemiluminescent substrate AMPPD (Bronstein et al., 1989). In control experiments, protein extracts were immunoprecipitated with NS Ab, a monoclonal antibody (designated 2.73) against a 70-kD ribonucleoprotein (Billings et al., 1982).

DNA Binding Assay

The DNA binding assay was adapted from the protocol of McKay (1981). AL1 was immunoprecipitated from protein extracts as described above, and the immunocomplexes were resuspended in 50 μL of binding buffer (25 mM Tris-HCl, pH 7.5, 40 mM KCl, 10 mM MgCl$_2$, 0.1 mM EDTA, 1 mM DTT, 5% [v/v] glycerol, 2.5 mM ATP). A standard binding reaction (60 μL) contained 50 μL of resuspended immunobead complexes, 100 ng of poly(dI-dC), and 5 fmoles (100,000 cpm) of radiolabeled intact or restricted probe. The binding reactions were incubated at room temperature for 30 min, and the protein/DNA complexes were washed twice with 500 μL of binding buffer containing 0.5% (w/v) Nonidet P-40 and 1% (w/v) BSA and once with 500 μL of binding buffer supplemented with 50 μg/mL sheared salmon sperm DNA. The bound DNA was extracted from the complexes using phenol/CHCl$_3$/isoamyl alcohol (25:24:1) and precipitated with ethanol or recovered by glass adhesion (Vogelstein and Gillespie, 1979) using the Mermaid kit. The DNA was analyzed on denaturing 6% polyacrylamide gels followed by autoradiography. In competition assays, 25- or 50-fold molar excess of unlabeled probe or unrelated DNA was included.

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