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A DNA Sequence Required for Geminivirus Replication Also Mediates Transcriptional Regulation

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Tomato golden mosaic virus (TGMV), a member of the geminivirus family, requires a single virus-encoded protein for DNA replication. We show that the TGMV replication protein, AL1, also acts during transcription to specifically repress the activity of its promoter. An earlier study established that AL1 binds to a 13-bp sequence (5′-GGTAGTAAGGTAG) that is essential for activity of the TGMV replication origin. Analysis of AL1 binding site mutants in transient expression assays demonstrated that the same site, which is located between the transcription start site and TATA box in the AL1 promoter, also mediates transcriptional repression. These experiments revealed that the repeated motifs in the AL1 binding site contribute differentially to repression, as has been observed previously for AL1–DNA binding and viral replication. Introduction of the AL1 binding site into the 35S promoter of the cauliflower mosaic virus was sufficient to confer AL1-mediated repression to the heterologous promoter. Analysis of a truncated AL1 promoter and of mutant AL1 proteins showed that repression does not require a replication-competent template or a replication-competent AL1 protein. Transient expression studies using two different *Nicotiana* cell lines revealed that, although the two lines replicate plasmids containing the TGMV origin similarly, they support very different levels of AL1-mediated repression. These results suggest that geminivirus transcriptional repression and replication may be independent processes.

INTRODUCTION

Geminiviruses are a family of plant viruses characterized by twin icosahedral capsids and circular, single-stranded DNA genomes (reviewed by Stanley, 1991; Lazarowitz, 1992). Their genomes replicate through double-stranded intermediates via a rolling-circle mechanism in infected plant cells (Saunders et al., 1991; Stenger et al., 1991; Heyraud et al., 1993). The double-stranded genomic DNA is assembled into nucleosomes and transcribed in infected plant nuclei (Rushing et al., 1987; Hanley-Bowdoin et al., 1989; Sunter et al., 1989; Pilartz and Jeske, 1992). Geminivirus replication and transcription rely on only a few viral proteins (Elmer et al., 1988; Sunter et al., 1990; Etessami et al., 1991; Lazarowitz et al., 1992) that must act with plant enzymes to catalyze these processes. Thus, the study of geminiviruses is likely to lead to the identification and characterization of host factors whose primary functions are in replication and transcription of the plant nuclear genome. The geminivirus family represents a unique viral model system to study the relationship between plant DNA replication and transcription because no other known plant viruses replicate through DNA intermediates and are transcribed in plant nuclei.

Geminiviruses can be separated into three subgroups based on their insect vector, host range, and genome structure (Lazarowitz, 1992). Tomato golden mosaic virus (TGMV) is a member of the geminivirus subgroup that is transmitted by whiteflies, infects dicotyledonous plants, and has bipartite genomes with the DNA components designated A and B (Bisaro et al., 1982). TGMV A and B DNAs are arranged similarly with divergent transcription units separated by a 5′ intergenic region that includes the ~200-bp common region (Figure 1A). The common region, which is highly conserved between the two genome components, contains the viral origin of replication (Lazarowitz et al., 1992) and transcriptional elements (Hanley-Bowdoin et al., 1989; Sunter and Bisaro, 1989; Sunter et al., 1989). The common region also includes a 30-bp sequence that has the potential to form a stem-loop structure and is essential for replication (Revington et al., 1989). TGMV A specifies all of the viral functions necessary for replication and encapsidation (Rogers et al., 1986; Sunter et al., 1987), whereas TGMV B contributes activities essential for movement (Brough et al., 1988). TGMV A encodes the three viral proteins known to be involved in replication and transcription. The AL1 gene encodes the only TGMV protein that is essential for replication (Elmer et al., 1988; Hayes and Buck, 1989; Hanley-Bowdoin et al., 1990). The AL3 gene specifies a protein that greatly enhances the levels of viral DNA accumulation (Elmer et al., 1988; Sunter et al., 1990). The AL2 gene encodes a factor that activates rightward transcription of the A and B genome components (Sunter et al., 1990; Sunter and Bisaro, 1992).

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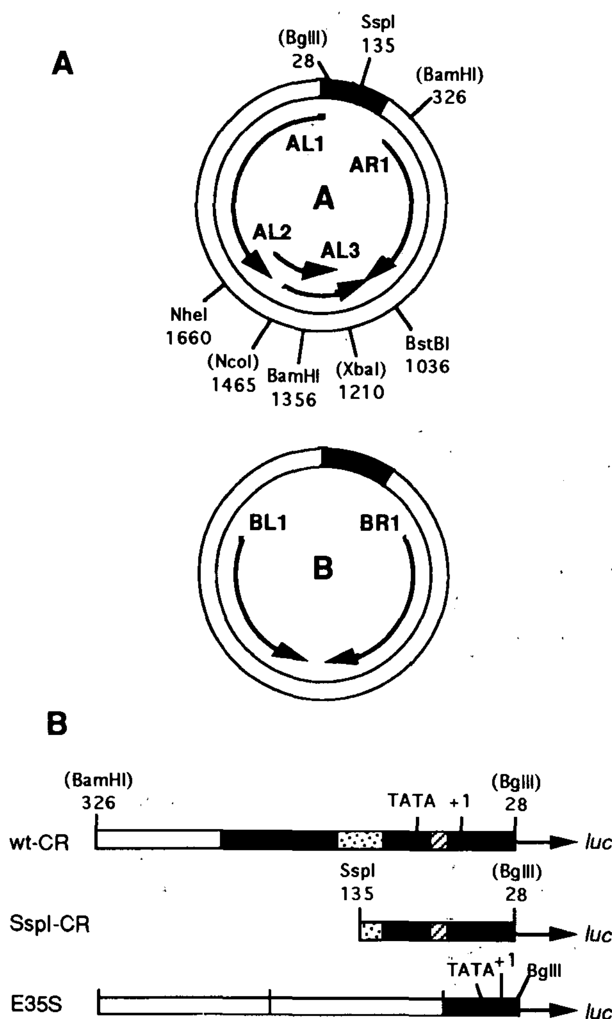

Figure 1. Diagram of TGMV and Reporter Constructs.

(A) The A and B components of the TGMV genome are represented. The AL1, AL2, AL3, AR1, BL1, and BR1 open reading frames are indicated by arrows. The black boxes represent the common region. Restriction sites that were used in the construction of *luc* reporter plasmids and the plant expression cassettes are marked. Engineered sites are indicated by parentheses. The site positions are labeled relative to the sequence of TGMV A determined by Hamilton et al. (1984).
(B) *luc* reporter constructs. Wt-CR (pNSB114) contains TGMV A sequences (28 to 326) between the engineered BglII and BamHI sites in **(A)**. SspI-CR (pNSB115) is a truncated form of wt-CR that terminates at the SspI site in the stem-loop motif. The orientations of wt-CR and SspI-CR are reversed relative to the TGMV A genome, such that transcription is shown 5' to 3'. The dotted boxes represent the stem-loop sequence motif located in the common region (black boxes). The AL1 binding site is shown by hatched boxes. E35S (pMON8796) contains the duplicated enhancer region (−343 to −90; open boxes) and the promoter region (+1 to −90; black box) from the CaMV 35S promoter (Kay et al., 1987). The transcription start sites (+1) and the TATA motifs in the AL61 promoter of wt-CR and SspI-CR and of the E35S promoter are marked. The *luc* coding region and direction of transcription are designated by the arrows.

RNAs transcribed from TGMV A and B have been identified in infected plants (Hanley-Bowdoin et al., 1989; Sunter and Bisaro, 1989; Sunter et al., 1989). The 3' ends of the viral transcripts are polyadenylated, and their 5' ends frequently are positioned downstream of TATA consensus sequences, suggesting that they are transcribed by host RNA polymerase II. A single, major RNA species (AR319) results from rightward transcription of TGMV A. (TGMV RNAs are designated by the genomic positions of their 5' ends [Hanley-Bowdoin et al., 1989].) The transcription start site and upstream TATA box corresponding to the promoter for the AR319 RNA are located outside of the common region. Transcription of the leftward open reading frames of TGMV A is complex, resulting in at least five overlapping RNAs. Only one RNA, AL61, encodes a full-length AL1 protein; the other AL RNAs begin 3' of the AL1 AUG initiator codon. The 5' end of AL61 RNA is positioned downstream of a TATA consensus sequence that overlaps the left side of the TGMV minimal origin of replication (H.J. Gladfelter and L. Hanley-Bowdoin, manuscript in preparation). Transcription of TGMV B is similar to the A component, with a single RNA derived from the right side and multiple, overlapping RNAs arising from the left side (Sunter et al., 1989). RNA mapping experiments with other geminiviruses suggest that complex transcription patterns may be a common feature of geminivirus expression (Morris-Krisinich et al., 1985; Townsend et al., 1985; Accotto et al., 1989; Dekker et al., 1991; Frischmuth et al., 1991; Mullineaux et al., 1993).

TGMV encodes two proteins known to regulate gene expression. The AL2 protein is necessary to activate rightward expression of both genome components (Sunter et al., 1990). Recent experiments show that activation of TGMV rightward expression occurs at the level of transcription (Sunter and Bisaro, 1992). Regulation of rightward gene expression has also been observed for a second bipartite geminivirus, African cassava mosaic virus (Haley et al., 1992), and for geminiviruses with single genome components (Hofer et al., 1992; Zhan et al., 1993). In contrast, recent studies show that leftward expression of the AL1 open reading frame in the bipartite geminiviruses TGMV (Sunter et al., 1993) and African cassava mosaic virus (Haley et al., 1992) is autoregulated. These studies did not address the level or mechanism of AL1-mediated regulation of leftward expression. We showed previously that AL1 binds specifically to the TGMV common region (Fontes et al., 1992) and that the AL1 recognition sequence is essential for replication origin function (Fontes et al., 1994a). The location of the AL1 recognition sequence, which is positioned between the transcriptional start site and TATA box of the AL61 promoter region, suggests that the same site may mediate regulation of leftward gene expression. We examined this possibility in transient assays that determined the effect of AL1 on the regulation of its promoter. We showed that AL1 specifically represses TGMV leftward expression. These studies demonstrate that repression occurs, at least partially, at the level of transcription through the interaction of AL1 with its binding site in the AL61 promoter. We also examined the relationship between AL1 function in repression and replication.

RESULTS

AL1 Specifically Inhibits Its Own Expression

AL1 is the only virus-encoded protein that is required for TGMV replication (Elmer et al., 1988; Hanley-Bowdoin et al., 1990). Recent experiments, which showed that TGMV AL1 binds with high affinity to a site in the promoter of its gene (Fontes et al., 1994a), suggest that AL1 may also function in transcription. We examined this possibility by using a wild-type (wt) common region (CR) construct, wt-CR (Figure 1B), which includes the luciferase (*luc*) reporter gene under the control of the promoter elements for the AL61 RNA, the only TGMV mRNA that encodes the full-length AL1 protein (Hanley-Bowdoin et al., 1989; Sunter et al., 1989). The wt-CR construct contains 32 nucleotides of AL1 5′ leader sequence and 266 nucleotides of 5′ nontranscribed DNA (TGMV A positions 28 to 326). The wt-CR construct was transfected into *Nicotiana benthamiana* (NB) protoplasts either alone or in the presence of plant expression cassettes corresponding to each TGMV A leftward open reading frame under the control of the cauliflower mosaic virus (CaMV) E35S promoter (Kay et al., 1987) and the 3′ end of the ribulose bisphosphate carboxylase small subunit (*rbcS*) E9 gene (Coruzzi et al., 1984). When transfected alone, wt-CR supported high levels of Luc activity (Figure 2). In contrast, expression from wt-CR was repressed 20-fold in protoplasts cotransfected with an AL1 expression cassette (pMON1549). Expression from wt-CR was not altered, as was determined by using a two-tailed Student's *t* test ($P > 0.1$), when cotransfected with either an AL2 (pNSB28) or an AL3 (pNSB46) expression cassette. In these transfections, the lack of effect was not a result of the failure to synthesize AL2 or AL3 protein because the same expression cassettes complemented TGMV AL2 or AL3 mutants, respectively, in protoplasts (data not shown). These results demonstrated that AL1, but not AL2 or AL3, represses expression from the AL61 promoter region. Similar results have been published for TGMV by Sunter et al. (1993).

We compared the activity and regulation of the AL61 promoter with the CaMV 35S promoter containing a duplicated enhancer (E35S), which is known to be a strong promoter in plants (Kay et al., 1987). The wt-CR construct resulted in only threefold lower Luc activity than the E35S *luc* construct (Figure 2). This result established that the AL61 promoter is also a strong plant viral promoter. The AL1 expression cassette had no impact on the activity of the E35S promoter ($P > 0.1$), indicating that the regulation of the two promoters is different. Thus, AL1-mediated regulation is specific for the AL1 promoter.

To further delineate the region of TGMV A that is responsive to repression by AL1, we constructed a *luc* reporter plasmid, SspI-CR (Figure 1B), that contains only 107 bp of TGMV A DNA flanking the AL61 transcription start site (TGMV A positions 28 to 135). Cotransfection of SspI-CR with the AL1 expression cassette resulted in a 14-fold repression of Luc activity (Figure 2). This level of repression was not statistically

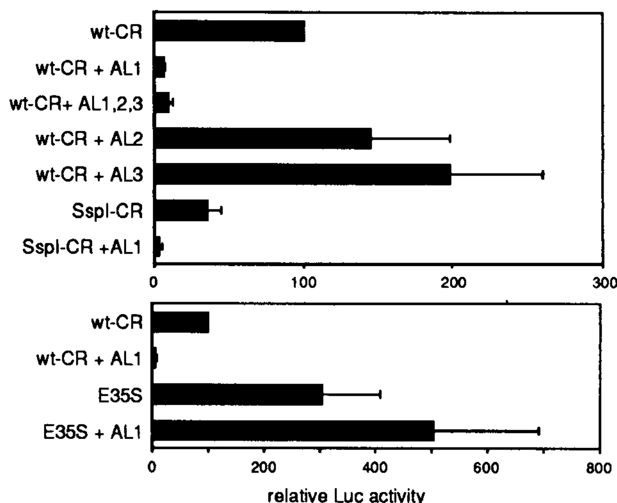

Figure 2. TGMV AL1 Specifically Represses Expression from the AL61 Promoter Region.

NB protoplasts were electroporated with 10 μg of each construct indicated on the left. The *luc* reporter constructs were wt-CR (pNSB114), SspI-CR (pNSB115), and E35S (pMON8796) depicted in Figure 1B. The plant expression cassettes contained the open reading frames corresponding to AL1 (pMON1549), AL1,2,3 (pMON1548), AL2 (pNSB28), or AL3 (pNSB46). The Luc-specific activity resulting from transfection of wt-CR alone was arbitrarily set at 100, and all other results were standardized against wt-CR to give relative Luc activity. The lines indicate two standard errors. The relative Luc activities were compared using a two-tailed Student's *t* test. The relative Luc activities of wt-CR, wt-CR + AL2, and wt-CR + AL3 transfection or of E35S and E35S + AL1 transfections were not statistically different ($P > 0.1$).

different ($P > 0.1$) from that detected for wt-CR, thereby indicating that the AL1-responsive element(s) is located in the 107-bp TGMV fragment of SspI-CR. Interestingly, comparison of Luc activity from the wt-CR and SspI-CR constructs in the absence of AL1 indicated that there is a transcriptional activation element between TGMV A positions 136 and 326 that is absent in SspI-CR. The threefold difference in Luc activity between wt-CR and SspI-CR, which was seen in five independent experiments and was significant at $P < 0.01$ in a two-tailed Student's *t* test, could not be attributed to differences in the abilities of the two templates to replicate in NB protoplasts because AL1 was not present in these assays.

Repression Is Mediated by the AL1 DNA Binding Site

The AL1 protein binds specifically to the left side of the TGMV common region to a 13-bp sequence in the viral origin of replication (Fontes et al., 1994a). The position of the AL1 binding site between the AL61 transcription start site and its TATA box (Figure 3A) suggested that this site may also function in transcription, possibly mediating AL1 repression. To test this hypothesis, we introduced mutations into the AL1 binding site

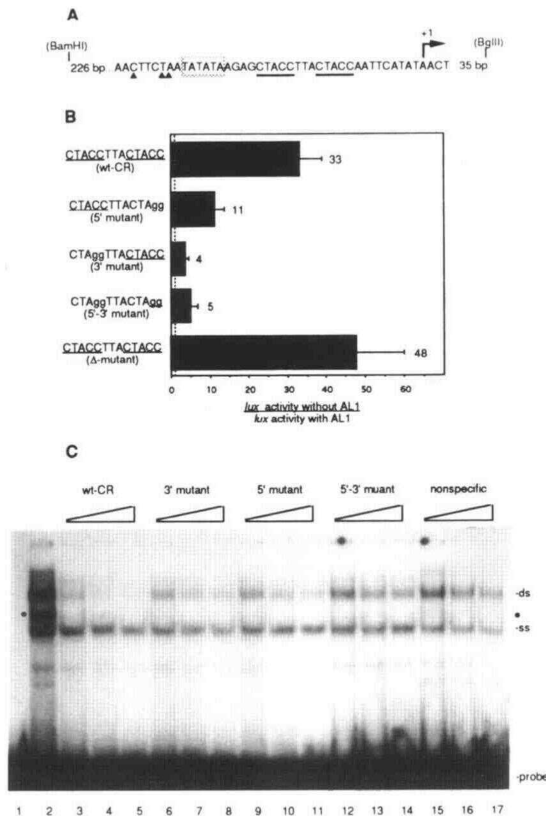

Figure 3. Mutations in the AL1 Binding Site Impair Repression.

(A) The DNA sequence surrounding the transcription start site (+1) and the TATA box (box) of the AL61 promoter is shown. The directly repeated motifs of the AL1 binding site are underlined. The triangles indicate nucleotides that are deleted in the Δ-mutant (pNSB189). The restriction sites indicate the limits of the TGMV A DNA insert of wt-CR (Figure 1B). The sequence of the AL61 promoter region is oriented opposite of the TGMV A genome such that transcription is shown 5′ to 3′.

(B) NB protoplasts were electroporated with 10 μg of a *luc* reporter construct and 10 μg of the AL1 plant expression cassette (pMON1549). The *luc* reporter constructs were wt-CR (pNSB114), 5′ mutant (pNSB218), 3′ mutant (pNSB256), 5′-3′ mutant (pNSB255), and Δ-mutant (pNSB189). The AL1 binding site sequence of each construct is indicated at left, with mutations shown by lowercase letters and intact 5-bp repeat motifs marked by underlining. Repression was measured as the ratio of the AL61 promoter activity from each reporter construct in the absence versus the presence of the AL1 expression cassette. A ratio >1 is repression, <1 is activation, and of 1 is no change (dotted line). The lines indicate two standard errors. The relative Luc activities were compared using a two-tailed Student's *t* test. The repression levels of wt-CR and Δ-mutant and of 3′ mutant and 5′-3′ mutant were not statistically different ($P > 0.1$).

(C) The synthetic oligonucleotides 5′-AGCTTCCCAATTGGATGTAAGGATGGGGG and 5′-GATCCCCCCATCCTTACATCCAATTGGGA, containing the AL1 binding site (underlining marks the repeated motifs), were annealed, labeled with phosphorus-32, and used as probe in AL1–DNA binding assays containing whole-cell extracts from baculovirus-infected insect cells expressing AL1. The bound complexes were resolved by electrophoresis and visualized by autoradiography. Lane 1 shows the probe alone. Lane 2 shows the probe plus protein extract in the absence of complex competitor DNA. In lanes 3 to 17, increasing amounts of complex competitor DNAs (triangles) were included in the assays: lanes 3 to 5, wt-CR (pNSB161); lanes 6 to 8, 3′ mutant (pNSB158); lanes 9 to 11, 5′ mutant (pNSB159); lanes 12 to 14, 5′-3′ mutant (pNSB271); and lanes 15 to 17, nonspecific competitor (pZmc802). The molar ratios of wt-CR, 3′ mutant, 5′ mutant, and 5′-3′ mutant competitors to probe DNA were 30- (lanes 3, 6, 9, and 12), 60- (lanes 4, 7, 10, and 13), or 90-fold (lanes 5, 8, 11, and 14). The molar ratio of the nonspecific competitor to probe DNA was 10-, 20-, or 30-fold (lanes 15, 16, and 17, respectively). All binding reactions (lanes 2 to 17) included 100 ng of poly(dI-dC). The shifted complexes corresponding AL1 binding to double-stranded (ds) or single-stranded (ss) DNA are marked. A third complex (•, lane 2) that is competed by low levels of double-stranded DNA is also indicated.

and analyzed their impact on repression. The AL1 binding site consists of a directly repeated motif that contains GG dinucleotides at positions 72 to 73 and 80 to 81 in TGMV A DNA; these are evolutionarily conserved among related geminiviruses. (The GG residues correspond to the CC nucleotides in Figure 3A, which is diagrammed 5′ to 3′ relative to AL1 transcription and is in the opposite orientation of the TGMV A genome.) Transversion mutations were introduced into each of the conserved dinucleotides, and the mutant TGMV A sequences (positions 28 to 326) were cloned upstream of the *luc* reporter gene to generate 5′, 3′, and 5′-3′ mutant versions of wt-CR.

All of the AL1 binding site mutants retained the ability to direct expression of the *luc* reporter gene when transfected alone into NB protoplasts. The wt-CR, 5′ mutant, 3′ mutant, and 5′-3′ mutant constructs supported relative Luc activities of 1.0, 1.3, 0.9, and 1.4, respectively, indicating that the mutations did not alter the activity of the AL61 promoter region. As shown in Figure 3B, the activities of the 5′, 3′, and 5′-3′ mutant CR constructs were repressed when cotransfected with the AL1 expression cassette, showing 11-, 4-, and 5-fold repression, respectively. These levels of repression were significantly less than the 33-fold repression of wt-CR activity in these experiments. (The difference in the level of wt-CR repression in Figure 3B as compared to that observed in Figure 2 most likely reflected experiment-to-experiment variation between protoplast preparations.) These results indicated that although both the 3′ and 5′ conserved dinucleotides of the AL1 binding site are important for AL1-mediated repression, mutation of the 3′ motif affects repression more severely than mutation of the 5′ motif. In contrast, a fourth mutant, Δ-mutant, in which nucleotides at TGMV A positions 96, 97, and 101 were deleted (Figure 3A), resulted in 1.4-fold Luc activity relative to wt-CR when transfected alone and in 48-fold repression when cotransfected with the AL1 expression cassette. This level of repression is similar to that observed for wt-CR ($P > 0.1$), indicating that the deletions in Δ-mutant do not impact repression and, thus, that the AL1 binding site mutations specifically reduce repression.

The failure to fully abolish repression could reflect low-level binding of AL1 to the mutant sites. We examined this possibility by assessing the capacity of DNA fragments that contained either wild-type, mutant, or no AL1 binding site to compete for binding by AL1 to the wild-type site (Figure 3C). Electrophoretic mobility shift assays were performed using protein extracts from recombinant, baculovirus-infected insect cells expressing AL1 (Fontes et al., 1992). Three major and several minor complexes were detected in a binding reaction (Figure 3C, lane 2) containing probe DNA, AL1-containing extract, and 100 ng of poly(dI-dC). The major complexes were not detected using an equivalent protein extract from infected insect cells expressing β-galactosidase, whereas the minor complexes were detected with this protein extract (data not shown). These results establish that only the major complexes are dependent on the presence of AL1. The major complex of fastest mobility (Figure 3C, labeled ss) was also observed when the probe DNA was denatured prior to binding (data not shown), indicating that it reflects AL1 binding to single-stranded DNA. This complex was not competed by increasing amounts of double-stranded competitor DNA (Figure 3C, lanes 3 to 17), which is consistent with the high affinity of AL1 for single-stranded DNA (Thommes et al., 1993). The other major complexes (Figure 3C, labeled with dot and ds) were not detected using denatured probe and were sensitive to competition by double-stranded DNA. Low levels of competitor DNA, including a 10-fold molar excess of the nonspecific competitor (lane 15), interfered with formation of the complex of intermediate mobility (Figure 3C, labeled with dot), suggesting that it corresponds to nonspecific binding of AL1 to double-stranded DNA.

The major complex of slowest mobility (ds) displayed differential sensitivity to the various competitor DNAs in Figure 3C, thereby indicating that it corresponds to specific binding of AL1 to double-stranded DNA. In lanes 3 to 5, competitor DNA containing a wild-type AL1 binding site (wt-CR, pNSB161) competed efficiently for AL1-DNA binding, with reduced or no detectable binding at 30-, 60-, or 90-fold molar excess to probe DNA. In contrast, significant levels of AL1-DNA binding were seen in the presence of equivalent levels of competitor DNAs containing the 3′ mutant (pNSB158, lanes 6 to 8), the 5′ mutant (pNSB159, lanes 9 to 11), or the 5′-3′ mutant (pNSB271, lanes 12 to 14) AL1 binding sites. However, the levels of AL1-DNA binding in the presence of the 5′-3′ mutant competitor were greater than in the presence of the 3′ and 5′ mutant competitors. Furthermore, the levels of AL1-DNA binding in the presence of the 5′-3′ mutant competitor at 30-, 60-, or 90-fold molar excess were similar to those seen using a nonspecific competitor of complex sequence (pZmc802) at 10-, 20-, or 30-fold molar excess (cf. lanes 12 to 14 and 15 to 17). These results show that AL1 does not bind the 5′-3′ mutant binding site in vitro. The fivefold repression seen with this mutant in the Luc activity assay (Figure 3B) is not likely to be the result of a low level of AL1 binding to the 5′-3′ mutant site, and instead, suggests that a second *cis*-acting element is involved in repression.

The AL1 Binding Site Can Confer AL1-Mediated Repression to a Heterologous Promoter

We asked if the AL1 binding site is sufficient for AL1-dependent repression by introducing the site into a heterologous promoter. The CaMV E35S promoter was mutated to replace sequences between its TATA box and transcription initiation site with the 13-bp AL1 high-affinity binding site and 6 bp of the flanking TGMV A sequence (mutant E35S; Figure 4A). The additional TGMV A sequences were included because an earlier report suggested that flanking sequences may enhance AL1-DNA interaction (Fontes et al., 1994a). The position and spacing of the AL1 binding site in the mutant 35S promoter were maintained relative to the TGMV AL61 promoter. A *luc* reporter construct under the control of the mutant E35S promoter was

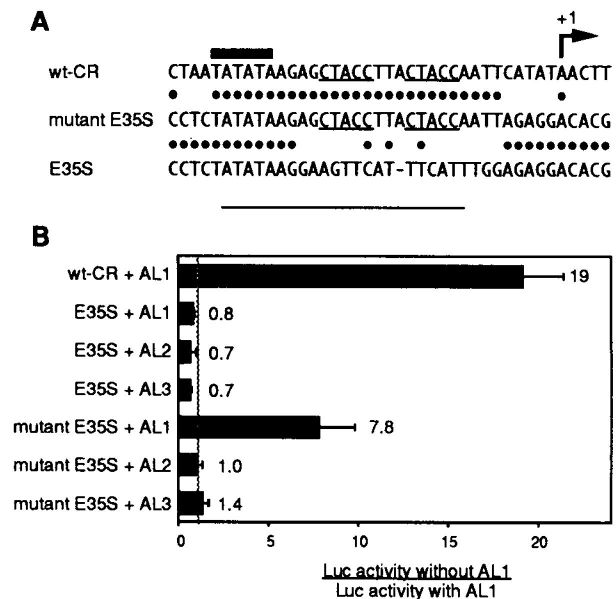

Figure 4. The AL1 Binding Site Confers Repression to a Heterologous Promoter.

(A) The sequences of the promoter regions of wt-CR (pNSB114), mutant E35S (pNSB283), and E35S (pMON8796) constructs are aligned. Nucleotide identity is shown by closed circles. The transcription start sites (+1) and TATA boxes (solid bar) are marked. The repeated motifs in the AL1 binding site are underlined. The sequence of the AL61 promoter region is oriented opposite of the TGMV A genome such that transcription is shown 5′ to 3′.

(B) NB protoplasts were electroporated with 10 μg of a *luc* reporter construct and 10 μg of the AL1 (pMON1549), AL2 (pNSB28), or AL3 (pNSB46) plant expression cassette indicated at left. The *luc* reporter constructs are wt-CR (pNSB114), E35S (pMON8796), and mutant E35S (pNSB283). Repression was measured by the ratio of the activity from either the AL61, E35S, or mutant E35S promoter in the absence versus the presence of the AL expression cassettes. A ratio >1 is repression, <1 is activation, and of 1 is no change (dotted line). The lines indicate two standard errors.

transfected into NB protoplasts in the presence and absence of plant expression cassettes for the TGMV AL proteins (Figure 4B). In the absence of viral proteins, the mutant E35S promoter supported 1.6-fold Luc activity relative to the wild-type E35S promoter, indicating that the mutation did not significantly alter promoter function. When cotransfected with the TGMV expression cassettes, the activity of the wild-type E35S promoter was not affected by the AL1, AL2, or AL3 protein. In contrast, the activity of the mutant E35S promoter containing the AL1 binding site was repressed 7.8-fold when cotransfected in the presence of the AL1 expression cassette. Expression from the mutant E35S promoter was not altered by the presence of the AL2 or the AL3 expression cassette, thereby establishing that AL1 specifically represses the activity of the mutant promoter. However, the level of repression of the mutant E35S promoter (7.8-fold) was lower than that of wt-CR (19-fold). This result, which was seen in seven independent experiments and was significant at $P < 0.01$ in a two-tailed Student's *t* test, is consistent with the hypothesis that a second *cis*-acting element in the AL61 promoter region is required for full repression. Alternatively, because the E35S promoter is threefold more active than the AL1 promoter (Figure 2), AL1 repression may be partially attenuated by other elements in the E35S promoter.

AL1 Mutant Proteins That Are Deficient for DNA Replication Retain Repression Function

TGMV AL1 is a multifunctional protein that is required for viral DNA replication (Elmer et al., 1988) and is involved in the regulation of its own expression (Figure 2; Sunter et al., 1993). To determine if the role of AL1 in replication is independent from its function in repression, AL1 proteins that are deficient for TGMV replication were examined in repression assays. We analyzed repression using two TGMV AL1 variants (pMON1670 and pMON1671) that contained mutations in a predicted helicase motif (Figure 5A; Gorbalenya and Koonin, 1989) and were unable to complement TGMV genomes with AL1 frameshift mutations in leaf disc replication assays (J.S. Elmer and S.G. Rogers, manuscript in preparation). We confirmed that these TGMV AL1 mutants also failed to support TGMV replication in protoplasts (data not shown). As shown in Figure 5B, cotransfection of pMON1670 or pMON1671 with wt-CR resulted in repression levels of 19- and 26-fold, respectively. These levels are similar ($P > 0.1$) to the 25-fold repression that resulted from cotransfection of wt-CR with the wild-type AL1 expression cassette (pMON1549). These results show that at least one region of the AL1 protein that is required for DNA replication is not essential for repression.

The AL1 protein from a related geminivirus, bean golden mosaic virus (BGMV), is also unable to support TGMV replication in vivo (Fontes et al., 1994b). We tested the ability of BGMV AL1 to repress expression from the TGMV AL61 promoter region by cotransfecting protoplasts with a BGMV AL1 expression cassette (pNSB152) and wt-CR. In contrast to the

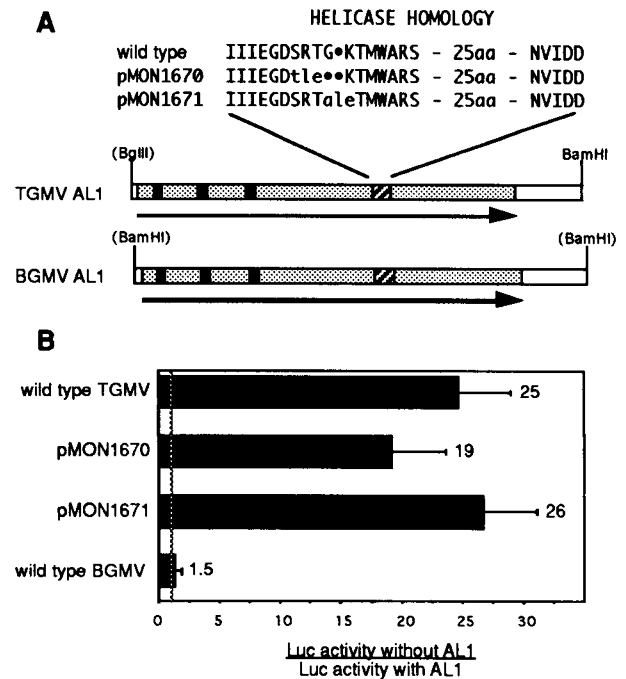

Figure 5. Repression Analysis of AL1 Proteins That Are Deficient for TGMV Replication.

(A) The viral DNA inserts of the TGMV and BGMV AL1 expression cassettes are shown. Restriction sites used in the construction are shown. Engineered sites are enclosed by parentheses. The AL1 open reading frames are marked by arrows; the open boxes indicate viral sequences that do not encode AL1. Regions of the AL1 protein that show sequence identity to motifs in proteins known to act as site-specific endonucleases during rolling-circle replication (solid boxes; Ilyina and Koonin, 1992) or as DNA helicases (hatched boxes; Gorbalenya and Koonin, 1989) are shown. The amino acid sequences of the putative helicase regions of wild-type and mutant TGMV AL1 proteins are shown. The lowercase letters indicate amino acids that differ from the wild-type AL1 protein, and the closed circles mark deleted residues. aa, amino acid.

(B) NB protoplasts were electroporated with 10 μg of the *luc* reporter construct, wt-CR (pNSB114), and 10 μg of plasmids expressing either wild-type TGMV AL1 (pMON1549), wild-type BGMV AL1 (pNSB152), or the TGMV AL1 proteins with mutations in the putative helicase domain (pMON1670 and pMON1671). The AL1 expression cassettes are indicated at left. Repression was measured by the ratio of the AL61 promoter activity in the absence versus the presence of the different AL1 expression cassettes. A ratio >1 is repression, <1 is activation, and of 1 is no change (dotted line). The lines indicate two standard errors. The relative Luc activities were compared using a two-tailed Student's *t* test. The repression levels conferred by wild-type TGMV AL1, pMON1670, and pMON1671 were not statistically different ($P > 0.1$).

TGMV AL1 mutants, no repression was detected for BGMV AL1 (Figure 5B). This result cannot be attributed to a failure to express BGMV AL1 because the same expression cassette can support replication of BGMV B in protoplasts (E.P.B. Fontes, H.J. Gladfelter, and L. Hanley-Bowdoin, manuscript

in preparation). The lack of repression is most likely a result of the inability of BGMV AL1 to interact with the TGMV AL1 binding site (Fontes et al., 1994b).

Repression of the AL61 Promoter Region Does Not Require a Functional Origin

Because the AL1 promoter region and the TGMV origin of replication overlap, we asked if AL1-mediated repression is dependent on a competent replication origin. This hypothesis was addressed by analyzing the SspI-CR and wt-CR constructs, which are repressed by AL1 to similar levels (Figure 2), in transient DNA replication assays. The wt-CR construct contains the entire TGMV common region, whereas the SspI-CR construct includes only 107 bp of the common region (Figure 1B). The wt-CR or the SspI-CR plasmid was transfected into NB protoplasts in the presence of either the AL1 expression cassette (pMON1549) or the AL1,2,3 expression cassette (pMON1548). Total DNA was harvested 48 hr post-transfection, digested with DpnI and BglII, and analyzed by DNA gel blot hybridization. The newly synthesized, double-stranded DNA, which is resistant to DpnI digestion and linearized by BglII, was detected as a 5.9-kb product. In the absence of an expression cassette, no DpnI-resistant DNA was detected (data not shown). Transfection of wt-CR in the presence of the AL1 (Figure 6A, lane 2) or the AL1,2,3 cassette (lane 1) resulted in the 5.9-kb product corresponding to replicated, double-stranded DNA. In contrast, no replicated DNA corresponding to SspI-CR was detected in the presence of either expression cassette (Figure 6A, lanes 3 and 4). Because SspI-CR is fully repressed by AL1 (Figure 2) and is unable to replicate to detectable levels, these results show that a functional replication origin is not required for AL1-mediated repression of the AL61 promoter.

AL1-Mediated Repression Differs among Independent *Nicotiana* Cell Lines

We examined AL1-mediated repression of the AL61 promoter in protoplasts prepared from an *N. tabacum* cell line (NT-1). Initial experiments indicated that repression levels were less than threefold in NT-1 protoplasts cotransfected with 10 μg of wt-CR and 10 μg of either the AL1 or the AL1,2,3 expression cassette. This result was in strong contrast to the ~30-fold repression observed in NB protoplasts transfected with equivalent amounts of the plasmids (Figure 3). The discrepancy in repression levels could not be explained by differences in the activity of the AL61 promoter region, which displayed relative activities of 1 and 1.3 in NB and NT-1 protoplasts, respectively, in transfections lacking the AL1 expression cassette. To further verify that repression levels in the two cell lines were different, transient expression assays were performed in the presence of increasing amounts of the AL1 expression cassette (0.5 to 40 μg) and a constant amount of wt-CR (10 μg;

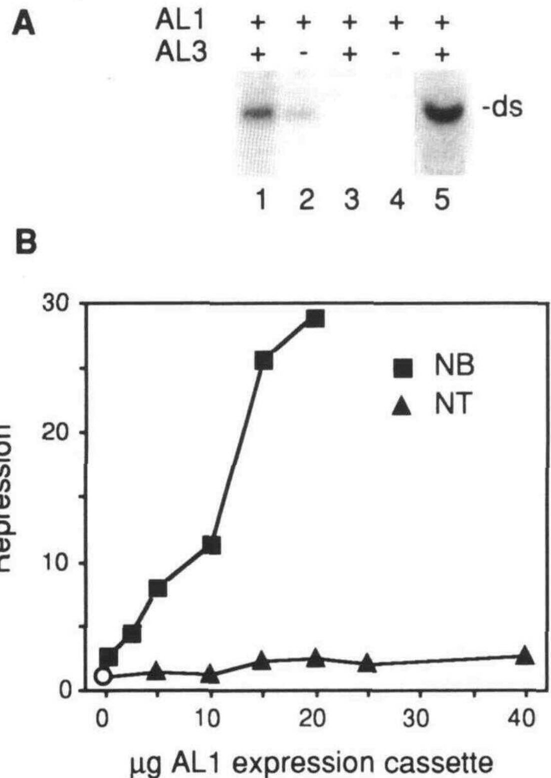

Figure 6. AL1-Mediated Replication and Repression in Two *Nicotiana* Cell Lines.

(A) NB (lanes 1 to 4) or NT-1 (lane 5) protoplasts (1.6×10^6) were electroporated with 15 μg of the wt-CR (pNSB114) or the SspI-CR (pNSB115) construct in the presence of 10 μg of the AL1 expression cassette (pMON1549) or the AL1,2,3 expression cassette (pMON1548). The presence (+) or absence (−) of the AL1 or the AL3 protein is indicated at the top. Total DNA was isolated 2 days post-transfection, digested with BglII-DpnI, resolved on agarose gels, and analyzed by DNA gel blot hybridization using a ^{32}P-labeled *luc* probe. Newly synthesized, double-stranded DNA is indicated (ds). Lanes 1 and 2 show a 5.9-kb product corresponding to wt-CR that replicated in the presence of the AL1,2,3 (lane 1) or the AL1 (lane 2) expression cassette in NB protoplasts. Lanes 3 and 4 show the absence of a product corresponding to SspI-CR in the presence of the AL1,2,3 (lane 3) or the AL1 (lane 4) expression cassette. The predicted product corresponding to replicated SspI-CR would be 183 bp smaller than the 5.9-kb product seen for wt-CR and, thus, would have a similar migration in the agarose gel. Lane 5 shows a 5.9-kb product corresponding to wt-CR that replicated in the presence of the AL1,2,3 expression cassette in NT-1 protoplasts.
(B) NB or NT-1 (NT) protoplasts were electroporated with 10 μg of wt-CR (pNSB114) and different concentrations of the AL1 plant expression cassette (pMON1549). For transfection of NB protoplasts (■), 0.25, 0.5, 1.0, 2.5, 5.0, 10, 15, or 20 μg of the AL1 expression cassette was used. For the NT-1 protoplasts (▲), 1.0, 5.0, 10, 15, 20, 25, or 40 μg of the AL1 expression cassette was used. Repression was measured as the ratio of the AL61 promoter activity in the absence versus the presence of the AL1 expression cassette.

Figure 6B). Less than threefold repression was observed in NT-1 protoplasts transfected with 40 μg of the AL1 expression cassette, whereas less than 0.5 μg of the cassette was necessary to achieve the same level of repression in NB protoplasts. In addition, ~30-fold repression was observed in NB protoplasts transfected with 20 μg of the expression cassette as compared to less than threefold repression in NT-1 protoplasts also transfected with 20 μg of the cassette. These results show that the ability of the AL1 expression cassette to support repression is significantly reduced in NT-1 cells.

The different levels of repression in the two cell lines may reflect differential production of functional AL1 protein. It is not likely that expression of AL1 is impaired in the NT-1 cells because the E35S promoter was equally active in the two cell lines, as assessed by its capacity to support *luc* expression (data not shown). Alternatively, AL1 protein produced by NT-1 cells may not be active. We addressed this possibility by examining the ability of an AL1,2,3 expression cassette to support replication of wt-CR in NT-1 protoplasts. The AL1,2,3 cassette was used in the replication assays because earlier studies established that both AL1 and AL3 are expressed from this cassette (Hanley-Bowdoin et al., 1989). Sunter et al. (1990) showed that AL3 can enhance TGMV replication in protoplasts. A 5.9-kb product corresponding to newly synthesized, double-stranded DNA was detected in the NT-1 protoplasts (Figure 6A, lane 5), as previously described for NB protoplasts (Figure 6, lane 1). In these experiments, NT-1 and NB protoplasts transfected with identical amounts of wt-CR and the AL1,2,3 expression cassette supported similar levels of wt-CR replication. These results show that the different repression levels in the two cell lines are not a result of a failure to produce functional AL1 in NT-1 cells.

DISCUSSION

Studies of eukaryotes and their viruses have identified proteins that function both in DNA replication and transcription. The large T antigen of simian virus 40 (Fanning and Knippers, 1992) and the E1 and E2 proteins of papillomaviruses (Dostatni et al., 1991; Sandler et al., 1993) interact with their respective viral origins to initiate replication and with viral promoters to regulate gene expression. A protein complex that interacts with chromosomal origins and transcriptional silencers has been identified in yeast (Bell and Stillman, 1992; Bell et al., 1993). In earlier studies, we have reported that the AL1 protein of the geminivirus TGMV binds specifically to its origin and that this interaction is necessary for viral replication (Fontes et al., 1994a). We show here that AL1 also functions as a transcriptional regulator by binding to the same site and repressing its own transcription. These studies identify AL1 as a plant viral protein that can be classified with other eukaryotic proteins known to have dual roles in transcription and DNA replication.

The TGMV AL1 binding site is located between the TATA box and transcriptional start site of the AL61 promoter (Fontes et al., 1994a). This position is highly reminiscent of negative transcriptional regulatory elements in other eukaryotic genes (Roberts et al., 1988; Jupp et al., 1993). We provide three lines of evidence demonstrating that this site mediates AL1 repression. First, a 107-bp fragment (SspI-CR) of the TGMV common region that includes the AL1 binding site contains all of the *cis*-acting sequences required for repression (Figure 2). Second, mutations in the AL1 binding site greatly reduced the level of repression. Last, a mutant E35S promoter that contains the AL1 binding site in the same position as the AL61 promoter was repressed by AL1 (Figure 3). Interestingly, mutation of the AL1 binding site did not fully abolish repression, and the mutant E35S promoter containing this site was not repressed to the same extent as the AL61 promoter (Figure 4). These data suggest that a second *cis*-acting element, in addition to the AL1 binding site, is required for full repression. Analysis of the 5′-3′ mutant, which displayed fivefold repression but was unable to compete for AL1/DNA binding in vitro, strongly supports the existence of a second *cis*-acting element. An earlier study showed that AL1 binds with reduced affinity to other sequences in the TGMV A common region (Fontes et al., 1994a). There is also a sequence in the 5′ leader region of the AL1 gene that is identical to the AL1 binding site at 8 of 13 positions. Both sequences are potential candidates for a second AL1-responsive element that might contribute to repression. Alternatively, the second *cis* element may interact with the proposed product of the AL4 open reading frame, which is located internal to the AL1 coding sequence, as suggested recently by Groning et al. (1994).

There is strong correlation between repression and the interaction between AL1 and its DNA binding site. The TGMV AL1 binding site consists of a repeated pentanucleotide sequence (GGTAG) separated by a 3-bp central core to give 5′-GGTAGTAAGGTAG (Fontes et al., 1994a). Mutation of either the 5′ or 3′ GG dinucleotides in the repeated motifs significantly reduced AL1-mediated repression (Figure 3B). However, the 3′ mutation reduced repression ninefold, whereas the 5′ mutation reduced repression only threefold. These results show that the repeated motifs make different contributions to repression and that the 3′ motif is the more critical *cis* element. Analogous results have been obtained in DNA binding studies that demonstrated that AL1 binds the 3′ repeat plus core sequence with a fourfold higher affinity than the 5′ repeat plus core sequence (Fontes et al., 1994a). The correlation between repression and AL1–DNA binding is also supported by the inability of BGMV AL1 to repress the TGMV AL61 promoter and to bind to the TGMV AL1 recognition sequence (Fontes et al., 1994b).

The precise mechanism whereby AL1 represses its promoter remains to be elucidated. Because the AL1 binding site is located only five nucleotides downstream of the TATA box, the AL1 protein could repress transcription by interfering with the assembly or activity of the transcription preinitiation complex (for review, see Herschbach and Johnson, 1993). The general transcription factors TFIID and TFIIB are good candidates for AL1 targets because their probable binding sites within the

AL61 promoter would position them close to AL1 (for review, see Zawel and Reinberg, 1993). AL1 could repress transcription by direct interaction with these transcription factors (Horikoshi et al., 1991; Baniahmad et al., 1993) or by competing for their binding sites in the promoter (Ohkuma et al., 1990; Dostatni et al., 1991; Dong et al., 1994). However, the differences in repression levels in the NT-1 and NB cell lines (Figure 6) suggest that the mechanism of AL1-mediated repression may be more complex, possibly involving a host factor that could enhance the repression activity of AL1 in NB cells or interfere with its activity in NT-1 cells via protein–protein interactions (Nevins, 1992) or by differential modification of AL1 (Virshup et al., 1992).

AL1 functions in transcription and replication by binding to the same TGMV sequence. Analysis of AL1 binding site mutants in transient assays showed that the 3′ repeated motif of the binding site makes a significant contribution to repression and is essential for replication (Fontes et al., 1994a). In both cases, the 5′ repeated motif makes a smaller contribution. These results indicate that AL1–DNA interactions are similar in repression and replication. However, four lines of evidence suggest that the AL1 protein has independent roles in transcription and replication. First, the AL1 promoter can be repressed to the same level from a replication-competent (wt-CR) or replication-deficient (SspI-CR) template (Figure 2), thereby showing that repression is not simply the consequence of replication disrupting transcription initiation at the AL61 promoter. Second, the AL1 binding site conferred repression to the E35S promoter in the absence of other TGMV origin sequences (Figure 4). Third, mutant TGMV AL1 proteins that are deficient for replication repressed the AL61 promoter to the same level as the wild-type AL1 protein (Figure 5). Last, the ability of AL1 to repress its promoter in the NT-1 cell line was greatly reduced compared to the NB cell line even though replication from the TGMV origin was similar in both lines (Figure 6). The low level of repression versus efficient replication in NT-1 cells suggests that repression is not essential for viral replication. However, repression may be important for some other aspect of virus infection in intact plants.

Several possible mechanisms could regulate the activity of the AL1–DNA complex in replication and transcription. Interaction with other viral or cellular proteins (Nevins, 1992; Sandler et al., 1993; Dong et al., 1994) or modification of the AL1 protein (Virshup et al., 1992) might modulate its replication and repression activities. For example, the TGMV AL3 protein, which greatly enhances viral DNA accumulation (Sunter et al., 1990; Etessami et al., 1991; Morris et al., 1991), may function as a replication accessory factor by interacting with AL1 and increasing its activity in replication. This idea is supported by recent studies showing that AL3 can help AL1 overcome a mutation in its binding site to support replication (Fontes et al., 1994a). Other *cis*-acting elements contained within the viral origin and/or promoter may also contribute to the regulation of the two processes (DePamphilis, 1988). Experiments are in progress to address these possibilities and the mechanism of AL1-mediated repression.

METHODS

Plasmid Constructs and Site-Directed Mutagenesis

The position numbers used to describe the following clones refer to the nucleotide coordinates of the tomato golden mosaic virus (TGMV) A sequence determined by Hamilton et al. (1984). In this numbering scheme, the TGMV common region is delimited by positions 1 to 210. The coordinates for bean golden mosaic virus (BGMV) refer to the sequence of the Guatemalan isolate (BGMV-GA, GenBank accession number M91604). The plasmids described in this section are summarized in Table 1.

The bacterial cloning vector pUC18 (Vieira and Messing, 1982) was modified by insertion of a BglII linker into the SmaI site and by subsequent insertion of a NotI linker into the SspI site to create pNSB109. The plasmid pMON477 (Elmer et al., 1988) was digested with BglII and BamHI to yield a 298-bp fragment corresponding to TGMV A positions 28 to 326, and this fragment was cloned into pNSB109, previously digested with BglII and BamHI, to yield pNSB110. The open reading frame of the firefly luciferase gene (*luc*; Ow et al., 1986) fused to the 3′ end of the nopaline synthetase (*nos*) gene was isolated from pMON8796 by digestion with BglII and SmaI and cloned into pNSB110 previously digested with EcoRI, repaired with *Escherichia coli* DNA polymerase (Klenow fragment), and then digested with BglII. The resulting wild-type (wt) common region (CR) construct (wt-CR; pNSB114) contains the AL61 transcription start site (Hanley-Bowdoin et al., 1989), 32 bp of transcribed TGMV A DNA, and 266 bp of 5′ nontranscribed DNA driving expression of the *luc* reporter gene. Plasmid pNSB110 was digested with SspI and XbaI, repaired with the Klenow fragment, and religated. The resulting clone, pNSB112, was digested with EcoRI, repaired with the Klenow fragment, and digested with BglII prior to insertion of the *luc* open reading frame to create SspI-CR (pNSB115). The plasmid pMON8796 contains the cauliflower mosaic virus (CaMV) 35S promoter with a duplicated enhancer region (E35S; Kay et al., 1987) driving expression of the *luc* open reading frame. The plasmid pZmc802 contains a 191-bp EcoRI fragment of the maize chloroplast genome that includes the promoter and 5′ end of the ribulose bisphosphate carboxylase large subunit gene (*rbcL*; Hanley-Bowdoin et al., 1985).

The expression cassette for TGMV AL1, pMON1549, which contains a 1.2-kb BamHI-BglII fragment of TGMV A (positions 1357 to 28) under the control of the E35S promoter and the 3′ end of the pea *rbcS* E9 gene (Coruzzi et al., 1984), has been described previously (Fontes et al., 1994a). The corresponding 1.2-kb fragment containing the BGMV AL1 open reading frame was generated from BGMV A (positions 1420 to 26) using the polymerase chain reaction. The reaction product, which included engineered BamHI sites, was cloned into the plant expression cassette pMON921, which was digested with BglII (Fontes et al., 1994a), to create pNSB152. In pNSB152, expression of the BGMV AL1 protein was also under the control of the E35S promoter and the pea *rbcS* E9 3′ end. The expression cassette for TGMV AL3 (pNSB46) placed the AL3 open reading frame under the control of the E35S promoter and the pea *rbcS* E9 3′ end (Fontes et al., 1994a). The expression cassette for TGMV AL2 (pNSB28) was constructed by digesting pMON428 (Elmer et al., 1988) with NheI and repairing with the Klenow fragment, which was followed by digestion with XbaI to liberate a 450-bp TGMV A fragment (1210 to 1660). This fragment was cloned into pMON999 digested with BglII, repaired with the Klenow fragment, and then digested with XbaI. Plasmid pMON999 is a pUC-based plant expression cassette that includes the E35S promoter and the *nos* 3′ end.

Table 1. Plasmids Used in This Study

Name	DNA Insert (Restriction Sites[a])	Description[b]	Genotype	Vector
Vectors				
pMON921		E35S promoter and *rbcS* E9 3′		pUC118
pMON999		E35S promoter and *nos* 3′		pUC118
pNSB69		NdeI and BglII linkers inserted		pBSII SK+
pNSB109		BglII and NotI linkers inserted		pUC18
General Plasmids				
pMON428	TGMV A (EcoRI/EcoRI)	2251 to 2251,[c] −88 bp from 1123 to 1211		pUC119
pMON477	TGMV A (EcoRI/EcoRI)	2251 to 2251,[c] linker mutations at 28 and 326		pUC119
pNSB273	luciferase	Promoterless *luc*-E9 3′		pNSB109
pZmc802	maize *rbcL*	*rbcL* promoter and 5′ end		pUC13
TGMV A CR Plasmids				
pNSB110	(BglII/BamHI)	28 to 326		pNSB109
pNSB112	(BglII/SspI)	28 to 135		pNSB109
pNSB117	(BglII/BamHI)	28 to 326		pUC118
pNSB158	(BglII/AluI)	28 to 84, mutated at 80 to 81	3′ mutant	pNSB69
pNSB159	(BglII/AluI)	28 to 84, mutated at 72 to 73	5′ mutant	pNSB69
pNSB161	(BglII/AluI)	28 to 84		pNSB69
pNSB186	(BglII/BamHI)	28 to 326, mutated at 72 to 73	5′ mutant	pUC118
pNSB188	(BglII/BamHI)	28 to 326, deleted at 96, 97, 101	Δ-mutant	pUC118
pNSB246	(BglII/BamHI)	28 to 326, mutated at 72 to 73, and 80 to 81	5′,3′ mutant	pUC118
pNSB247	(BglII/BamHI)	28 to 326, mutated at 80 to 81	3′ mutant	pUC118
pNSB271	(BglII/AluI)	28 to 84, mutated at 72 to 73, and 80 to 81	5′,3′ mutant	pNSB69
Expression Plasmids[d]				
pMON1548	TGMV AL1,2,3 (BstBI/BglII)	1038 to 28	AL1,2,3	pMON921
pMON1549	TGMV AL1 (BamHI/BglII)	1357 to 28	AL1	pMON921
pMON1670	TGMV AL1 (BamHI/BglII)	1357 to 28, replaced from 1918 to 1929	AL1 mutant	pMON921
pMON1671	TGMV AL1 (BamHI/BglII)	1357 to 28, replaced from 1918 to 1920	AL1 mutant	pMON921
pNSB28	TGMV AL2 (XbaI/NcoI)	1660 to 1210	AL2	pMON999
pNSB46	TGMV AL3 (BstBI/NcoI)	1465 to 1038	AL3	pMON921
pNSB152	BGMV AL1 (BamHI/BamHI)	BGMV A 1420 to 26	BGMV AL1	pMON921
Reporter Plasmids				
pMON8796			E35S-*luc-nos*	pMON999
pNSB114	TGMV A CR	28 to 326	(wt-CR)-*luc*-E9	pNSB273
pNSB115	TGMV A CR	28 to 135	(SspI-CR)-*luc*-E9	pNSB273
pNSB189	TGMV A CR	28 to 326, deleted at 96, 97, 101	(Δ-mutant)-*luc*-E9	pNSB273
pNSB218	TGMV A CR	28 to 326, mutated at 72 to 73	(5′ mutant)-*luc*-E9	pNSB273
pNSB255	TGMV A CR	28 to 326, mutated at 72 to 73 and 80 to 81	(5′-3′ mutant)-*luc*-E9	pNSB273
pNSB256	TGMV A CR	28 to 326, mutated at 80 to 81	(3′ mutant)-*luc*-E9	pNSB273
pNSB283	E35S	68 to 86	(E35S mutant)-*luc-nos*	pMON999

[a] Underlined restriction sites represent engineered sites in TGMV A.
[b] Numbers refer to TGMV A nucleotide positions according to the sequence by Hayes et al. (1984).
[c] Circular TGMV A genome linearized and cloned at a given position number.
[d] The fragments were cloned in the sense orientation of the viral open reading frame.

The AL1,2,3 expression cassette, pMON1548, was constructed by BstBI digestion of the TGMV A insert of pMON477 (Elmer et al., 1988), repair with the Klenow fragment, digestion with BglII, and subsequent cloning into pMON921 digested with BglII and StuI. The TGMV AL1,2,3 plant expression gene in pMON1548 is identical to the gene used previously to express functional AL1 and AL3 proteins in transgenic plants (Hanley-Bowdoin et al., 1989).

Site-directed mutagenesis was performed according to Kunkel (1985). To generate a template for mutagenesis, pMON477 (Elmer et al., 1988) was digested with BglII and BamHI to yield a 298-bp TGMV A fragment that was then inserted into the corresponding sites of a pUC118 derivative with a BglII linker in the SmaI site to create pNSB117. The plasmid pNSB117 was mutated at TGMV A positions 72 to 73 or 80 to 81 to give pNSB186 (5′ mutant; 72-ccTAGTAAGGTAG-84) and pNSB247 (3′ mutant; 72-GGTAGTAAccTAG-84), respectively, as described by Fontes et al. (1994a). Plasmid pNSB186 was further mutated at positions 80 to 81 to create pNSB246 (5′-3′ mutant; ccTAGTAAccTAG). Plasmid pNSB117 was also deleted at TGMV positions 96, 97, and 101 to create pNSB188 (Δ-mutant; 91-TATATGAATTCC-105). The inserts of the mutant clones were sequenced using a Sequenase II kit (U.S. Biochemicals). Following mutagenesis, pNSB186, pNSB247, pNSB188, and pNSB246 were digested with BglII and BamHI to liberate their TGMV A DNA inserts, which were then subcloned into pNSB273 also digested with BglII. Plasmid pNSB273 was generated from pNSB114 by digestion with SalI and BglII to remove the wild-type TGMV A DNA insert, repaired with the Klenow fragment, and ligated to restore the BglII site. Insertion of the mutant BglII-BamHI fragments, which were oriented such that *luc* expression was under the control of the AL61 promoter region, into pNSB273 resulted in pNSB218 (5′ mutant), pNSB256 (3′ mutant), pNSB255 (5′-3′ mutant), and pNSB189 (Δ-mutant). The 56-bp BglII-AluI fragments (positions 28 to 84) of pNSB117, pNSB186, pNSB247, and pNSB246 were subcloned into a pBSII SK+ derivative (pNSB69), as described previously by Fontes et al. (1994a), to give pNSB161 (wild type), pNSB159 (5′ mutant), pNSB158 (3′ mutant), and pNSB271 (5′-3′ mutant), respectively.

The mutant E35S reporter construct (pNSB283) was generated by replacement mutagenesis of pMON8796 using the primer 5′-CCTCCTCTATATAAGAGCTACCTTACTACCAATTAGAGGACACGCTGA containing the TGMV AL1 binding site. The replacement sequence in the E35S promoter was positioned between positions −7 to −24 relative to the transcription start site with the loss of a nucleotide at −23. The mutation in pNSB283 was verified by DNA sequencing.

The mutant TGMV AL1 expression cassettes pMON1670 and pMON1671 were generated by site-directed mutagenesis of the AL1 open reading frame in the helicase homology region of the coding sequence (J.S. Elmer and S.G. Rogers, manuscript in preparation). In pMON1670, TGMV A positions 1918 to 1929 were replaced with GTCTAGAGT. In pMON1671, TGMV A positions 1918 to 1920 were replaced with CTCTAGAGC.

Repression Assays

Protoplasts were prepared from a *Nicotiana benthamiana* leaf-derived, suspension cell line (NB) cultured in MSO media (400 mM mannitol, 87 mM sucrose, 0.43% Murashige and Skoog salt mixture [Gibco-BRL], and 0.002% B-5 vitamin stock containing 0.5% *myo*-inositol, 0.5% thiamine-HCl, 0.05% nicotinic acid, and 0.05% pyridoxine-HCl) containing 0.1 μg/mL 2,4-dichlorophenoxyacetic acid. Protoplasts were isolated by digestion with 0.5% cellulase R-10, 0.5% BSA, 0.5% Rhozyme, 0.02% Y-23 pectolyase, and 7 mM β-mercaptoethanol in PIM buffer (250 mM mannitol, 50 mM CaCl, 7 mM NaOAc, pH 5.8) for 60 min rotating at 60 rpm. The protoplasts were washed twice with PIM buffer and resuspended in electroporation buffer (200 mM mannitol, 0.75% NaCl, 0.002% KH_2PO_4, 0.011% Na_2HPO_4, pH 7.2) at a concentration of 4×10^6 per mL. Protoplasts were prepared from an *N. tabacum* cell line (NT-1) as described previously (Fontes et al., 1994a).

Repression assays were performed by electroporation (250 V, 500 μF) of 10 μg of each expression cassette, 10 μg of each reporter cassette, and 40 μg of sheared salmon sperm DNA, except for the titration experiment (Figure 6). Prior to electroporation, the DNAs were diluted to a final volume of 400 μL with electroporation buffer and mixed with 400 μL of protoplasts. After electroporation, the protoplasts were diluted into 7 mL of MSO media supplemented with 0.1 μg/mL 2,4-dichlorophenoxyacetic acid and 400 mM mannitol. Total protein was harvested from the protoplasts 36 hr post-transfection by resuspension in extraction buffer (100 mM KH_2PO_4, 1 mM EDTA, 10 mM DTT, 8 mM phenylmethylsulfonyl fluoride, 0.5% glycerol, pH 7.8) followed by sonication. Luciferase assays were performed in the following reaction buffer: 25 mM Tricine, pH 7.8, 15 mM $MgCl_2$, 50 mg/mL BSA, and 5 mM ATP with 0.5 mM D-luciferin (Analytical Luminesence Laboratory, San Diego, CA), as described by Ow et al. (1986). Protein concentrations were determined using a Bradford protein assay kit (Bio-Rad), and Luc activity was corrected for variations in protein concentration. Repression is represented as the ratio of Luc activity with no AL1 versus Luc activity in the presence of AL1. Each construct was assayed in duplicate in at least three independent experiments. A two-tailed Student's *t* test was used to compare the mean Luc activities or repression levels of the various constructs and combinations.

Replication Assays

NB and NT-1 protoplasts were prepared and electroporated as described above. Protoplasts were harvested 48 hr post-transfection, and total DNA was isolated (Junghans and Metzlaff, 1990). The DNA was digested with DpnI and BglII, resolved on a 1% agarose gel, and transferred and UV cross-linked to a MagnaGraph nylon membrane (MSI, Westboro, MA). The blots were analyzed by DNA gel blot hybridization according to the protocol of Thomashow et al. (1980), except that 0.2% sodium pyrophosphate was included in all solutions. The probe was a 2.1-kb BglII-SmaI, *luc*-containing fragment isolated from pMON8796 and randomly labeled using ^{32}P-dATP and the Klenow fragment (Sambrook et al., 1989).

DNA Binding Assays

AL1–DNA binding was examined using electrophoretic mobility shift assays (Fried and Crother, 1981). Radiolabeled probe DNA and competitor DNA were incubated with 3.2 μg of whole-cell extract isolated from recombinant baculovirus-infected, *Spodoptera frugiperda* Sf9 cells expressing AL1 (Fontes et al., 1992). Each binding reaction contained 100 ng of poly(dI-dC), 40 fmol of probe DNA, and 0.5 to 3.5 pmol of competitor DNA in 15 μL of buffer (25 mM Hepes, pH 7.5, 40 mM KCl, 5 mM $MgCl_2$, 1 mM EDTA, 1 mM DTT, 2.5 mM ATP, and 5% glycerol). After a 20-min incubation at 25°C, the bound complexes were resolved by electrophoresis in a 4% polyacrylamide gel containing 50 mM Tris-borate, pH 8.3, and 1 mM EDTA. The gel was dried by vacuum onto Whatman DE81 paper prior to autoradiography.

Probe DNA was prepared using the synthetic complementary oligonucleotides 5′-agcttcccAATTGGATGTAAGGATGgggg and

5′-gatccccCATCCTTACATCCAATTggga, which included TGMV A positions 68 to 84 in uppercase letters and flanking non-TGMV sequence in lowercase letters. The oligonucleotides were annealed and radiolabeled using ^{32}P-dATP and the Klenow fragment. The 86-bp specific competitor DNA fragments were isolated from pNSB161 (wild type), pNSB159 (5′ mutant), pNSB158 (3′ mutant), and pNSB271 (5′-3′ mutant) after BglII-HindIII digestion, as described previously (Fontes et al., 1994a). The 191-bp nonspecific competitor DNA fragment was isolated from pZmc802 after EcoRI digestion (Hanley-Bowdoin et al., 1985).

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