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cis Elements That Contribute to Geminivirus Transcriptional Regulation and the Efficiency of DNA Replication

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The A genomic component of the geminivirus tomato golden mosaic virus (TGMV) contains a 5′ intergenic sequence that includes the overlapping AL61 promoter and positive-strand origin of DNA replication. The TGMV AL1 protein negatively regulates its own transcription and mediates origin recognition by binding to a repeated motif shared by the AL61 promoter and the viral origin. We examined a series of truncated or mutated 5′ intergenic regions in transient expression and replication assays to identify other DNA sequences that contribute to TGMV promoter and origin function. These experiments revealed that negative regulation of the AL61 promoter is complex, involving multiple *cis*-acting sequences and the AL1 and AL4 proteins, which acted through different DNA elements. We also found that mutation of the TATA box motif in the AL61 promoter reduced overall transcriptional activity and AL1-mediated repression, confirming the importance of this sequence in promoter function. Mutation of a G-box consensus sequence was highly detrimental to AL61 transcription and abolished AL1 sensitivity, suggesting that AL1 interferes with transcriptional activation. Cotransfection experiments showed that the TATA box and G-box motif mutations also impaired viral DNA replication in the presence of a wild-type origin but had no effect in its absence, demonstrating that these transcriptional motifs also function as replication efficiency elements.

Geminiviruses are a family of plant viruses with twin icosahedral particles and single-stranded DNA genomes (37, 59). They replicate their small, circular genomes through double-stranded DNA intermediates by using a rolling-circle mechanism (27, 49, 52). The double-stranded forms also assemble into nucleosomes (44) and act as transcription templates for viral gene expression (25, 54). Geminiviruses encode only a few proteins for their replication and transcription and depend on host polymerases for these processes (14, 24, 41). Hence, geminiviruses represent excellent systems with which to study the mechanisms and coordination of DNA replication and transcription in plant cells.

The geminivirus tomato golden mosaic virus (TGMV) has a bipartite genome, with the two components designated A and B (4). The DNA components are configured similarly, with divergent open reading frames separated by a highly conserved 5′ intergenic sequence or common region (23) that includes both replication and transcription elements (Fig. 1A) (16, 25, 42, 54, 55). TGMV A contains five open reading frames that encode polypeptides larger than 10 kDa. The rightward or virion-sense coding sequence corresponds to the coat protein, AR1 (32). The four overlapping, leftward or complementary-sense open reading frames (AL1, AL2, AL3, and AL4) encode viral replication and transcription factors (13, 14, 19, 53, 57). The TGMV movement proteins are specified by the B component (7).

mRNAs corresponding to both the virion- and complementary-sense open reading frames have been identified in TGMV-infected plants (25, 54, 55). The RNAs are polyadenylated and initiate downstream of either consensus TATA box motifs (6) or initiator elements (60), indicating that they are transcribed by host RNA polymerase II. A single AR1 RNA, AR319, results from virion-sense transcription of TGMV A (25). (The A-component RNAs are designated by their polarity and the genomic positions of their 5′ ends.) In contrast, complementary-sense transcription is complex, leading to five overlapping RNAs with different 5′ ends and a common 3′ end (25). The AL61 RNA is the largest complementary-sense transcript and encodes the entire left side of TGMV A. It is the only RNA that includes the 5′ end of the AL1 coding sequence and, thus, the only species that can be translated to produce the full-length AL1 protein, which is essential for viral replication. The AL2540 and AL2515 RNAs may be translated to give AL4, which is represented by the first of three open reading frames found on these RNAs. The role of the AL4 protein during infection is unclear (14, 31, 50), but it may act as a transcriptional regulator (19). The AL1935 and AL1629 RNAs specify both AL2 and AL3, which function in transcription (53) and replication (14, 57), respectively.

Geminivirus transcription and DNA replication are closely linked. Several studies demonstrated that the TGMV AL1 protein is involved in both viral processes (13, 15, 24, 41, 56). AL1 binds double- and single-stranded DNA (18, 58), with double-stranded binding mediating virus-specific transcriptional repression (13) and origin recognition (16, 17). AL1 also hydrolyzes ATP (11, 26, 43) and catalyzes sequence-specific DNA cleavage and ligation (36, 42). All three activities are necessary for replication (11, 26), with DNA cleavage and ligation involved in initiation and termination of positive-strand DNA synthesis. Recent experiments showed that AL1 oligomerizes with itself and interacts with AL3 (47). The significance of these protein complexes for replication and transcription is not known. However, recent experiments mapped the AL1 DNA binding, DNA cleavage, and oligomerization domains to the N terminus of the protein and suggested that oligomerization is a prerequisite for DNA binding but not DNA cleavage in vitro (43).

There is also overlap between the *cis*-acting regions involved in TGMV transcription and replication. The origin for positive-strand DNA replication has been mapped to a ca. 100-bp common region fragment that contains the AL61 transcription

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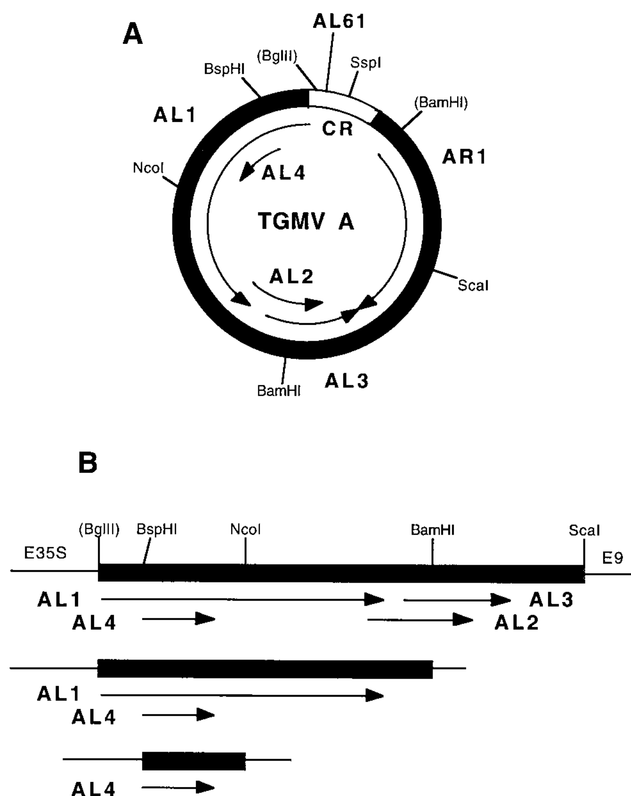

FIG. 1. (A) The A component of the TGMV genome. The AL1, AL2, AL3, AL4, and AR1 open reading frames are indicated by arrows. The open box represents the common region (CR). The position of the AL61 promoter is indicated. Significant restriction sites are shown, with parentheses indicating engineered sites. (B) Schematics of the AL1,2,3,4 (pMON1548), AL1 (pMON1549), and AL4 (pNSB366) expression cassettes. The black boxes mark TGMV A sequences, and the arrows indicate open reading frames. The restriction sites correspond to those shown in panel A. The CaMV 35S promoter with duplicated enhancer sequences (E35S [33]) directs expression of the TGMV A open reading frames. The 3′ end is from the pea *rbcS* E9 gene (8).

start site (38). The origin includes two essential elements, a 30-bp sequence that forms a hairpin structure (42) and an AL1 binding site comprised of a 17-bp sequence with directly repeated motifs (16). The cleavage site for initiation of positive-strand DNA synthesis is located in the loop of the hairpin (36, 42). The AL1 binding site, which is between the TATA box and transcription start site of the AL61 promoter, acts as the origin recognition sequence (17) and as a negative regulatory element for AL61 transcription (13). Autoregulation of the AL61 promoter does not require a functional viral origin, suggesting that the AL1 binding site acts independently during transcription and replication (13). However, the *cis*-acting sequences necessary for AL61 promoter activity and their significance for viral replication have not been studied in detail. In this study, we identified DNA sequences that contribute to AL61 promoter activity and examined their roles in TGMV replication. Our results established that the TATA box and G-box motifs function in both transcriptional regulation and DNA synthesis.

MATERIALS AND METHODS

Plasmid constructs and site-directed mutagenesis. Table 1 provides a description of the plasmids used in transcription and replication assays.

(i) AL61 reporter plasmids. The luciferase (*luc*) reporter plasmids $A_{28\text{-}326}$-*luc* and $A_{28\text{-}135}$-*luc*, which include the AL61 transcription start site, 32 bp of transcribed TGMV A DNA, and 266 and 75 bp, respectively, of 5′ nontranscribed viral DNA, have been described previously (13). Plasmid pMON477 (14) was digested with *Bgl*II/*Hin*dIII to yield a 763-bp fragment corresponding to TGMV A positions 28 to 791. This fragment was cloned into the *Bgl*II and *Hin*dIII sites of the promoterless *luc* cassette, pNSB273 (13), to create $A_{28\text{-}791}$-*luc*. Plasmid pNSB110 (13) was digested with either *Bsa*A1/*Xba*I or *Bsu*36I/*Xba*I, repaired with *Escherichia coli* DNA polymerase (Klenow fragment), and religated. The resulting plasmids were digested with *Bgl*II/*Hin*dIII to yield fragments corresponding to TGMV A positions 28 to 115 or 28 to 108. These fragments were cloned into the promoterless *luc* cassette, pNSB273, also digested with *Bgl*II/*Hin*dIII to create $A_{28\text{-}115}$-*luc* and $A_{28\text{-}108}$-*luc*. Plasmid pNSB136, which included an engineered *Hpa*I site at the AL61 transcription start site (TGMV A position 64), was digested with *Hpa*I and *Ssp*I to yield a 71-bp fragment corresponding to TGMV A positions 64 to 135. This fragment was cloned into pMON772 digested with *Pst*I/*Stu*I and treated with T4 DNA polymerase. The resulting clone, $A_{64\text{-}135}$-*luc*, contains TGMV A positions 64 to 135 and 80 bp of E35S 5′ leader DNA.

Plasmid $mA_{28\text{-}326}$-*luc*, which includes a 298-bp AL61 promoter fragment with a modified AL1 binding site (5′-ccTAGTAAccTAG; the mutated nucleotides are in lowercase) has been described earlier (13). TATA-1 and TATA-2, with altered TATA consensus sequences as described below, were digested with *Bgl*II to generate mutant fragments corresponding to TGMV A positions 28 to 326. The 298-bp fragments were cloned into *Bgl*II-digested pNSB273 to give TATA-$1_{28\text{-}326}$-*luc* and TATA-$2_{28\text{-}326}$-*luc*. Plasmid $A_{28\text{-}326}$-*luc* served as template in a PCR with primers 5′-CTAGAAATATTAAACGAATGGCCGCtAtGTGCC and 5′-AGCGGATAACAATTTCACACAGGA. The PCR product, which was mutated at TGMV A positions 117 and 119 (as indicated by lowercase in the first primer sequence), was digested with *Bgl*II/*Ssp*I and cloned into pNSB273 previously digested with *Pst*I, treated with T4 DNA polymerase, and then digested with *Bgl*II. The resulting clone, G-$box_{28\text{-}135}$-*luc*, contained TGMV A positions 28 to 135 with a mutated G-box motif, as verified by DNA sequencing.

(ii) 35S reporter plasmids. Reporter plasmid m35S-*luc*, which has a modified E35S promoter with AL1 binding site sequences from positions −7 to −24, has been described before (13). Plasmid pMON999, a pUC-based plant expression cassette with the cauliflower mosaic virus (CaMV) 35S promoter with a duplicated enhancer (E35S [33]), was used as the template for site-directed mutagenesis using the primer 5′-CTTCCTCTATATAAGaattggtagtaaggtagctAGAGGA CACGCTGA (35). The mutation replaced sequences in the E35S promoter between positions −7 and −24 with an inverted copy of the AL1 binding site (lowercase). After verification of the mutation by DNA sequencing, the mutant plasmid was digested with *Bgl*II/*Hin*dIII, and the resulting 649-bp fragment was cloned into pMON8796 also digested with *Bgl*II/*Hin*dIII to create mr35S-*luc*.

(iii) Replicon plasmids. Plasmids pNSB110 and pNSB117 are based on pUC19 and pUC119, respectively, and contain identically inserted TGMV A positions 28 to 326 (13). Plasmid pNSB117 was mutated at TGMV A positions 90 and 91, using the oligonucleotide 5′-ACTTCTAATAggTAAGAGCTACCT, to give TATA-1. Plasmid pNSB117 was also modified at TGMV A positions 90 to 95, using the primer 5′-GGAACTTCTccatggTAAGAGCTACCTT, to generate TATA-2. The mutations were verified by DNA sequencing of the entire 298-bp TGMV A fragment. The *Bgl*II/*Ssp*I-digested PCR product described above for G-$box_{28\text{-}135}$-*luc* was cloned into pNSB110 also digested with *Bgl*II and *Ssp*I. The resulting plasmid, G-box, contained TGMV A sequences 28 to 326 and a mutated G-box motif.

(iv) Plant expression cassettes. Plasmids pMON1549, encoding the AL1 open reading frame, and pMON1548, encoding the AL1, -2, -3, and -4 open reading frames (referred to as the AL1,2,3,4 expression cassette), have been described elsewhere (13, 16). Plasmid pMON425, containing a single copy of TGMV A, was digested with *Nco*I/*Bsp*HI to release a 489-bp fragment containing the AL4 coding sequence. The AL4 expression cassette, pNSB366, was made by cloning the 489-bp fragment treated with Klenow enzyme into the plant expression cassette pMON921 digested with *Bgl*II and treated with Klenow enzyme.

Transient expression and replication assays. Protoplasts prepared from *Nicotiana benthamiana* suspension cells were transfected with 15 μg of *luc* reporter plasmid, 15 μg of expression cassette, and 40 μg of sheared salmon sperm DNA as described by Eagle et al. (13). Luciferase activity in total soluble protein extracts was measured 36 h posttransfection and standardized for protein concentration. Relative promoter activity was determined by normalizing Luc activity resulting from each construct against that determined for $A_{28\text{-}326}$-*luc* or the appropriate wild-type control. Repression was determined as the ratio of Luc activity in the absence versus the presence of AL1. Each reporter construct was assayed in duplicate in at least three independent experiments. A two-tailed Student t test was used to compare the relative promoter activities or repression levels of the various constructs and combinations.

For replication assays, protoplasts were isolated from *Nicotiana tabacum* suspension cells, electroporated, and cultured according to published methods (16). For standard assays, the transfection mixtures contained 20 μg of each replicon DNA, 15 μg of the AL1,2,3,4 expression cassette, and 15 μg of sheared salmon sperm DNA. For replication assays performed in the presence of competitor DNA, the transfection mixtures contained 20 μg of each replicon DNA, 15 μg of the AL1,2,3,4 expression cassette, and 5 μg of sheared salmon sperm DNA.

TABLE 1. Constructs used

Name	No.	Description
Luciferase reporter plasmids[a]		
A_{28-791}-*luc*	pNSB394	*Bgl*II/*Hin*dIII AL61 promoter
A_{28-326}-*luc*	pNSB114	*Bgl*II/*Bam*HI AL61 promoter
A_{28-135}-*luc*	pNSB115	*Bgl*II/*Ssp*I AL61 promoter
A_{28-115}-*luc*	pNSB364	*Bgl*II/*Bsa*AI AL61 promoter
A_{28-108}-*luc*	pNSB330	*Bgl*II/*Bsu*36I AL6 promoter
A_{64-135}-*luc*	pNSB367	*Hpa*I/*Ssp*I 35S leader in AL61 promoter
mA_{28-326}-*luc*	pNSB255	*Bgl*II/*Bam*HI, AL61 promoter mutated at 72–73 and 80–81
TATA-1_{28-326}-*luc*	pNSB351	*Bam*HI/*Bgl*II, AL61 promoter mutated at 92 and 93
TATA-2_{28-326}-*luc*	pNSB543	*Bam*HI/*Bgl*II, AL61 promoter mutated at 92–97
G Box_{28-135}-*luc*	pNSB588	*Ssp*I/*Bgl*II, AL61 promoter mutated at 117 and 119
m35S-*luc*	pNSB283	E35S promoter with AL1 BS
mr35S-*luc*	pNSB540	E35S promoter with reversed AL1 BS
Replicon plasmids		
TGMV A	pNSB110	Wild-type common region in pUC19
TGMV A	pNSB117	Wild-type common region in pUC119
G-box	pNSB437	Common region mutated at 117 and 119 in pUC19
TATA-1	pNSB401	Common region mutated at 92 and 93 in pUC119
TATA-2	pNSB415	Common region mutated at 92–97 in pUC119
Expression cassettes		
AL1	pMON1549	E35S-TGMV AL1-E9 3′
AL1,2,3,4	pMON1548	E35S-TGMV AL1, 2, 3-E9 3′
AL4	pNSB366	E35S-TGMV AL4-E9 3′

[a] TGMV A genome positions are indicated by subscripts.

RESULTS

The *cis* regulatory elements of the AL61 promoter. TGMV AL1 negatively regulates its own expression by binding to a directly repeated sequence, 5′-CTACCTTACTACC, between the predicted TATA box and the transcription start site of the AL61 promoter (Fig. 2A) (13). Previous results suggested that a second *cis* element in the AL61 promoter contributes to full transcriptional repression (13). To further delineate the *cis*-acting regulatory sequences of the AL61 promoter, a series of promoter constructs with 5′ and 3′ modifications was analyzed in transient expression assays. We used the reporter construct A_{28-326}-*luc* (see Materials and Methods) as the starting point for the analyses. A_{28-326}-*luc* supports efficient Luc expression in *N. benthamiana* protoplasts and is repressed in the presence of AL1 (13). We tested whether additional 5′ nontranscribed sequences contribute to AL61 promoter function by comparing the activity of A_{28-326}-*luc* with that of A_{28-791}-*luc* (Fig. 2A). When introduced alone into *N. benthamiana* protoplasts, A_{28-326}-*luc* and A_{28-791}-*luc* displayed relative Luc activities of 100 and 121, respectively (Fig. 2B). In the presence of an AL1 plant expression cassette, the activities of A_{28-326}-*luc* and A_{28-791}-*luc* were repressed 22- and 26-fold, respectively (Fig. 2C). The relative Luc activities and repression levels of A_{28-326}-*luc* and A_{28-791}-*luc* were not statistically different when compared by using a two-tailed Student *t* test ($P > 0.1$). These results demonstrated that TGMV A positions 327 to 791 do not significantly affect positive or negative regulation of the AL61 promoter in transient expression assays, suggesting that this upstream region does not contain any additional *cis* regulatory elements necessary for full AL61 promoter function.

The *cis* elements in the 5′ nontranscribed region of the AL61 promoter were also examined by deletion analysis. We constructed three reporter plasmids based on A_{28-326}-*luc* that contained progressive deletions of sequences upstream of the AL61 transcription start site. Each construct included 32 bp of 5′ transcribed viral DNA, the AL1 binding site between TGMV positions 72 and 84, and either 75 (A_{28-135}-*luc*), 54 (A_{28-115}-*luc*), or 47 (A_{28-108}-*luc*) bp of 5′ nontranscribed viral sequences (Fig. 2A). The A_{28-135}-*luc* construct showed about twofold less activity (Fig. 2B) and repression (Fig. 2C) than A_{28-326}-*luc* in *N. benthamiana* protoplasts. The twofold differences were significant at $P < 0.025$, suggesting that sequences between TGMV A positions 136 and 326 contribute weakly to AL61 promoter regulation. In contrast, A_{28-115}-*luc* and A_{28-108}-*luc* showed only 1 and 0.6%, respectively, of Luc activity compared to A_{28-326}-*luc* and were not repressed by AL1. The large reduction in the promoter activities of A_{28-115}-*luc* and A_{28-108}-*luc* indicated the presence of a major transcriptional activator element between positions TGMV A 115 and 135. The relative promoter activities of A_{28-115}-*luc* and A_{28-108}-*luc* were at least 10-fold above background levels in mock-transfected protoplasts (data not shown), indicating that AL1-mediated repression of these constructs was readily detectable.

We also examined the impact of 5′ leader sequences on TGMV AL1 expression by replacing the sequences downstream of the AL61 transcription start site in A_{28-135}-*luc* with the leader from the CaMV 35S gene in A_{64-135}-*luc* (Fig. 2A). The A_{64-135}-*luc* and A_{28-135}-*luc* constructs supported similar levels of Luc expression (Fig. 2B), as assessed in a two-tailed Student *t* test ($P > 0.1$). When cotransfected with the AL1 expression cassette (Fig. 2C), A_{64-135}-*luc* was repressed 6.5-fold whereas A_{28-135}-*luc* was repressed 13-fold. The twofold difference in repression was statistically significant at $P < 0.025$. Together, these results showed that the 5′ leader sequence does not alter the activity of the AL61 promoter in the absence of AL1 and, instead, may contribute to AL1-mediated repression.

AL4 represses the AL61 promoter independent of the AL1 binding site. The TGMV AL1 gene contains a second coding sequence, designated AL4, that is in a different frame and has the potential to encode a 10-kDa polypeptide (Fig. 1A). Transient expression experiments suggested that TGMV AL4 may

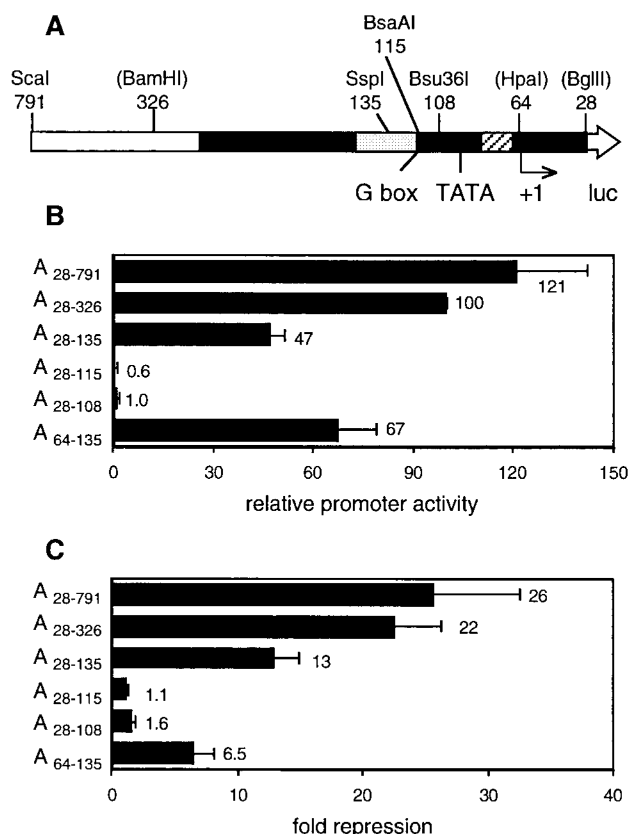

FIG. 2. Analysis of *cis* regulatory elements in the AL61 promoter. (A) Schematic of the AL61 promoter region cloned upstream of *luc*. The open area represents TGMV A coding sequence, while the shaded area indicates sequence within the common region. The common region includes a hairpin structure (dotted box) and the AL1 binding site (dashed box). The positions of TATA box and G-box consensus sequences are marked. The transcription initiation site is indicated as +1. The restriction sites are numbered according to the TGMV A sequence (23). The AL61 promoter region is oriented opposite the TGMV A genome such that transcription is shown 5′ to 3′. (B and C) *N. benthamiana* protoplasts were electroporated with each *luc* reporter construct on the left either alone (B) or in the presence of the AL1 expression cassette (pMON1549) (C). Luc specific activity resulting from transfection of $A_{28\text{-}326}$-*luc* was arbitrarily set at 100, and other Luc specificity activities were standardized against this value to give relative promoter activity. Fold repression was determined as the ratio of activity resulting from the *luc* reporter constructs in the absence versus the presence of AL1. The bars indicate 2 standard errors.

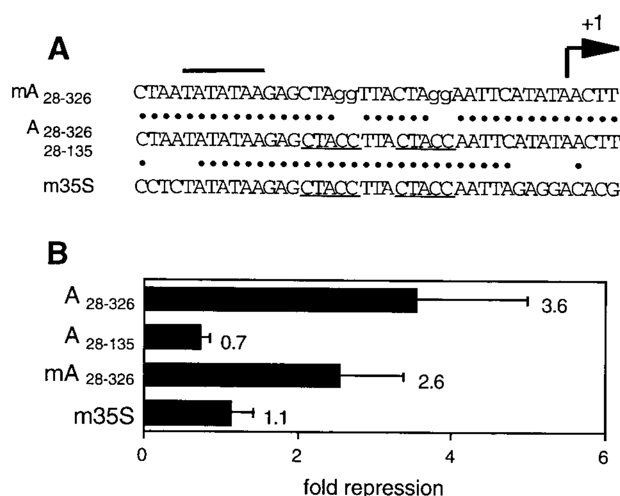

FIG. 3. AL4 represses the AL61 promoter. (A) Partial promoter sequences for $mA_{28\text{-}326}$-*luc*, $A_{28\text{-}326}$-*luc*, $A_{28\text{-}135}$-*luc*, and m35S-*luc* are aligned. Nucleotide identity is shown by dots. The transcription initiation site (+1) and TATA box (solid bar) are marked. The AL1 binding site is underlined. Mutations are represented by lowercase letters. The sequence of the AL61 promoter region is oriented opposite the TGMV A genome such that transcription is shown 5′ to 3′. (B) *N. benthamiana* protoplasts were electroporated with each *luc* reporter construct on the left and the AL4 expression cassette (pNSB366). Fold repression was determined by the ratio of activity resulting from the *luc* reporter constructs in the absence versus the presence of AL4. The bars indicate 2 standard errors.

contribute to negative regulation of the AL61 promoter (19). In these experiments, plant expression cassettes with frameshift mutations that disrupted the AL1 coding sequence but not the AL4 open reading frame repressed AL61 promoter activity about threefold. We examined whether the TGMV AL61 promoter was also repressed by a plant expression cassette that contained only the AL4 open reading frame and no flanking AL1 sequences. The $A_{28\text{-}326}$-*luc* construct was repressed 3.6-fold in the presence of the AL4 expression cassette (Fig. 3B), similar to the level reported previously (19). In contrast, no AL4-mediated repression was detected for $A_{28\text{-}135}$-*luc*. These results established that the TGMV A sequences necessary for AL4-mediated repression of the AL61 promoter are between genome positions 136 and 326 and do not overlap the AL1 binding site.

To further confirm that the AL1 binding site has no role in AL4-mediated repression, we examined whether AL4 repressed the activity of $mA_{28\text{-}326}$-*luc* (Fig. 3A), which was mutated in the conserved GG dinucleotides of both repeated motifs in the *cis* element (13). We detected 2.6-fold repression of $mA_{28\text{-}326}$-*luc* activity in the presence of the AL4 expression cassette (Fig. 3B). We also examined whether AL4 affected the activity of a heterologous promoter containing only the AL1 binding site from TGMV A. For these experiments, we used a mutant version of the CaMV 35S promoter (m35S) in which sequences between the TATA box and transcriptional start site were partially replaced by the 13-bp AL1 binding site and 6 bp of flanking TGMV A DNA (13). As shown in Fig. 4C, the m35S promoter was repressed 6.4-fold when cotransfected into protoplasts with the AL1 expression cassette. In contrast, when the m35S-*luc* construct was cotransfected with the AL4 expression cassette (Fig. 3B), no repression of the modified heterologous promoter was detected. These results supported our conclusion that AL4-mediated repression of the AL61 promoter is independent of the AL1 binding site.

AL1-mediated repression is dependent on the orientation of the AL1 binding site. The AL1 binding site is positioned between the TATA box and the transcription start site of the AL61 promoter. This location is likely to overlap the contact sites of the basal transcription machinery such that bound AL1 may interfere sterically with the recruitment or activities of host transcription factors. The m35S promoter described above contains the AL1 binding site in the equivalent position and orientation as the TGMV AL61 promoter (13). To determine if repression is dependent on the orientation of AL1 binding site relative to the TATA box, we constructed a second version of the 35S promoter, mr35S, in which the orientation of the AL1 binding site relative to the TATA box and transcriptional start site was reversed (Fig. 4A). Although both mutant promoters appeared more active than the wild-type 35S promoter (Fig. 4B), the differences were not statistically different ($P > 0.1$). The activity of the m35S promoter was repressed 6.4-fold by the presence of the AL1 expression cassette (Fig. 4C). In

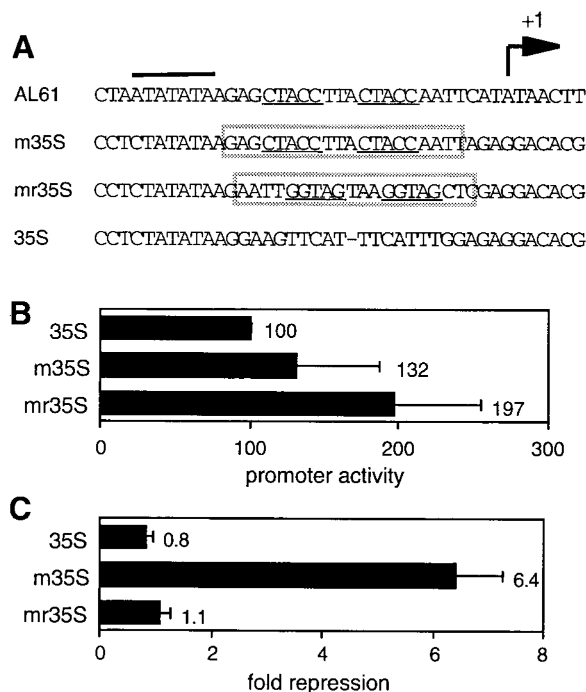

FIG. 4. Orientation of the AL1 binding site is important for repression. (A) Partial promoter sequences for 35S-*luc*, m35S-*luc*, and mr35S-*luc* constructs are compared to the AL61 promoter sequence by alignment at the TATA box (bar line). The boxes indicate nucleotide substitutions corresponding to the AL1 binding site. The direct repeats of the AL1 binding site are underlined. The transcription initiation site is shown (+1). The AL61 promoter region is oriented opposite the TGMV A genome such that transcription is shown 5′ to 3′. (B and C) *N. benthamiana* protoplasts were electroporated with each *luc* reporter construct on the left either alone (B) or in the presence of the AL1 expression cassette (pMON1549) (C). Luc specific activity resulting from transfection of 35S-*luc* was arbitrarily set at 100, and other Luc specificity activities were standardized against this value to give relative promoter activity. Fold repression was determined as for Fig. 2. The bars indicate 2 standard errors.

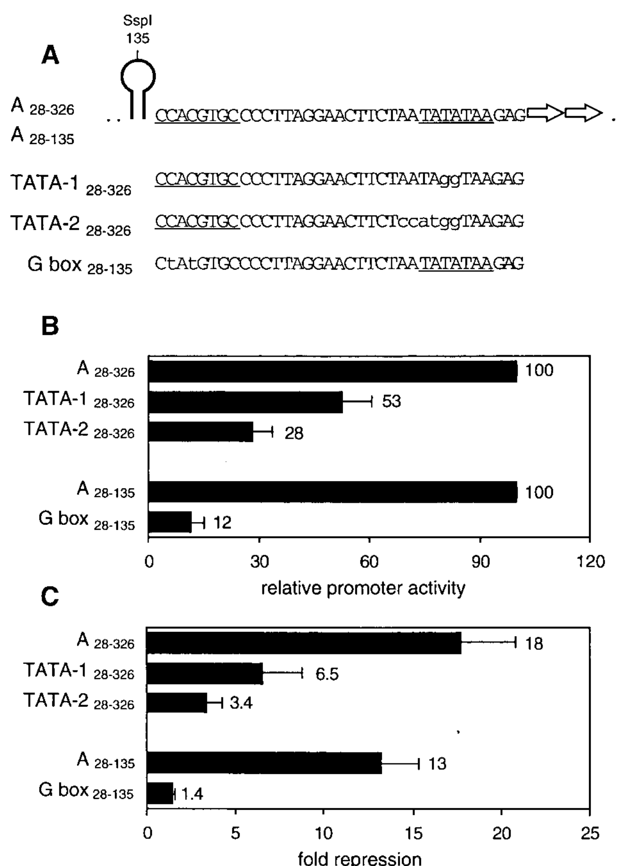

FIG. 5. Analysis of the TATA box and G-box elements in the AL61 promoter. (A) Partial promoter sequences for A_{28-326}-*luc*, A_{28-135}-*luc*, TATA-1_{28-326}-*luc*, TATA-2_{28-326}-*luc*, and G box_{28-135}-*luc* are aligned. The G-box and TATA box motifs are underlined. The hairpin structure included in the constructs that extended to TGMV position 326 is shown for A_{28-326}-*luc*. The lowercase letters indicate mutations. The arrows correspond to the directly repeated motifs in the AL1 binding site. (B and C) *N. benthamiana* protoplasts were electroporated with each *luc* reporter construct on the left either alone (B) or in the presence of the AL1 expression cassette (pMON1549) (C). Luc specific activity resulting from transfection of A_{28-326}-*luc* was arbitrarily set at 100, and Luc specificity activities resulting from TATA-1_{28-326}-*luc* and TATA-2_{28-326}-*luc* were standardized against this value to give relative promoter activity. The value for G-box $_{28-135}$-*luc* was standardized against that for A_{28-135}-*luc*, which was arbitrarily set at 100. Fold repression was determined as for Fig. 2. The bars indicate 2 standard errors.

contrast, the mr35S promoter was not repressed by AL1 (Fig. 4C). These results established that AL1-mediated repression is dependent on the orientation of the AL1 binding site relative to the TATA box of the heterologous promoter and might reflect the need for the correct alignment of the AL1 protein to block binding or to interact with a host factor during repression.

The AL61 promoter is activated by a G-box element. The 48-fold difference in Luc activities supported by the A_{28-115} and A_{28-135} promoters (Fig. 2C) suggested that a transcriptional activator element is between TGMV A positions 115 and 135. Sequence analysis of this region identified a consensus G-box motif (CACGTG) that is located 15 bp upstream of the TATA box and is conserved among related geminiviruses (1, 12). To determine whether TGMV utilizes a host G-box factor to activate transcription of the AL61 promoter, we mutated the G-box motif at the first and third positions of the hexameric core. Block et al. (5) showed that these positions are essential for G-box function during transcriptional activation. The G-box_{28-135}-*luc* construct (Fig. 5A) contains 107 bp of TGMV A DNA flanking the AL61 transcription start site and is identical to A_{28-135}-*luc* except for the mutated G box. Comparison of the promoter activities of the two constructs in protoplast transfection experiments revealed that the mutant G-box_{28-135} promoter is about eightfold less active than its A_{28-135} counterpart (Fig. 5B). The residual activity of G-box_{28-135}-*luc* construct, which was 25-fold above background (data not shown), was not repressed in the presence of the AL1 expression cassette, whereas A_{28-135}-*luc* displayed 13-fold repression (Fig. 5C). These results demonstrated that the G-box element functions as a transcriptional activator sequence in the AL61 promoter and suggested that AL1-mediated repression is dependent on transcriptional activation. This idea is further supported by the observation that the levels of Luc activity for G-box_{28-135}-*luc* in the absence of AL1 and for A_{28-135}-*luc* in the presence of AL1 were equivalent (data not shown).

The AL61 promoter also contains a sequence between TGMV A positions 88 and 94 that matches the consensus eukaryotic TATA box sequence, TATATAA (6). To determine if this sequence is important for AL61 promoter function and AL1-mediated repression, we constructed two TATA box mutants. The TATA-1_{28-326}-*luc* mutant contained the modified sequence TAggTAA, whereas TATA-2_{28-326}-*luc* included the sequence atggTAA (Fig. 5A). Both mutants contained 298 bp

of TGMV A DNA flanking the AL61 transcription start site and were identical to $A_{28\text{-}326}$-*luc* except for the TATA box mutations (indicated in lowercase). Although the TATA box mutations were predicted to disrupt transcription (30, 51, 61), the promoter activities of the TATA-$1_{28\text{-}326}$-*luc* and TATA-$2_{28\text{-}326}$-*luc* constructs were reduced only about two- and fourfold, respectively, relative to $A_{28\text{-}326}$-*luc* (Fig. 5B). In the presence of AL1, the TATA-$1_{28\text{-}326}$-*luc* and TATA-$2_{28\text{-}326}$-*luc* constructs were repressed 6.5 and 3.4-fold, respectively, while the control $A_{28\text{-}326}$-*luc* construct was repressed 18-fold. The levels of Luc activity detected for the TATA box mutants in the presence of AL1 were threefold greater than those seen for $A_{28\text{-}326}$-*luc* in repression assays (data not shown). Thus, the TATA box mutants displayed reduced promoter activity and were less sensitive to AL1-mediated repression.

AL61 promoter elements affect viral replication. The AL61 promoter overlaps the TGMV positive-strand origin and may affect initiation of viral DNA replication. To address this possibility, we examined whether the TATA box and G-box motifs of the AL61 promoter influence TGMV replication. Previous experiments showed that a TGMV A common region fragment from positions 28 to 326 supports episomal replication of bacterial plasmid DNA when the viral replication proteins, AL1 and AL3, are supplied in *trans* from a plant expression cassette (13). We cloned DNA fragments that included TGMV A positions 28 to 326 and the promoter mutations in Fig. 5A into pUC plasmids to create the TATA-1, TATA-2, and G-box mutant replicons. The replication properties of the mutant plasmids were compared to those of a clone, pNSB110, that contained the corresponding wild-type 304-bp TGMV A fragment. The replicon DNAs were cotransfected into *N. tabacum* protoplasts with an AL1,2,3,4 plant expression cassette, and total DNA was isolated 48 h posttransfection, digested with *Dpn*I to distinguish input from newly replicated DNA, linearized with *Hin*dIII, and analyzed by DNA gel blot hybridization. In Fig. 6A, bands corresponding to newly synthesized, double-stranded DNA were detected for the wild-type (lane 1), G-box (lane 2), TATA-1 (lane 3), and TATA-2 (lane 4) replicons. The hybridization products for the wild-type and G-box mutant products were ca. 600 bp smaller than those seen for the TATA-1 and TATA-2 mutants because the former were cloned by using pUC19 whereas the latter used the larger pUC119 vector. Similar levels of product were detected for the wild-type and mutant replicons (Fig. 6A; compare lane 1 with lanes 2 to 4), suggesting that the mutant replicons were as efficient as the wild-type control at supporting initiation of DNA replication in standard assays.

Competition assays (42) were also performed to determine if the replicons with mutations in the transcription elements could compete effectively with a wild-type origin. The strategy for these assays was to cotransfect replicons of different sizes that could be readily distinguished by DNA gel blot hybridization. Equal amounts of the two wild-type replicons (pNSB110 and the larger pNSB117), the G-box mutant and pNSB117, the TATA-1 mutant and pNSB110, or the TATA-2 mutant and pNSB110 were cotransfected into *N. tabacum* cells with the AL1,2,3,4 expression cassette and assayed for replication as described above. When the two wild-type replicons were cotransfected (Fig. 6B, lane 1), two double-stranded DNA products of 2.9 and 3.5 kb, corresponding to pNSB110 and pNSB117, respectively, were detected. In contrast, a single replicating DNA was detected in all of the cotransfection assays containing a mutant and a wild-type replicon (Fig. 6B, lanes 2 to 4). In each case, the product correlated in size to the wild-type replicon, and no replicated DNA corresponding to the G-box (Fig. 6B, lane 2), TATA-1 (lane 3), or TATA-2 (lane 4) replicon was observed. These results demonstrated that replicons containing mutations in the G-box or the TATA box motif do not replicate to detectable levels in the presence of wild-type origin sequences and suggested that these transcription elements contribute to efficient origin function.

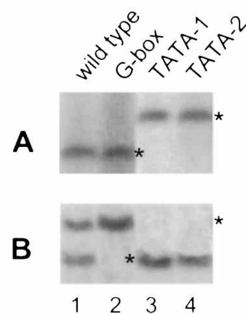

FIG. 6. AL61 promoter motifs affect the efficiency of viral replication. (A) NT-1 protoplasts were electroporated with either the wild-type TGMV A (pNSB110; lane 1), G-box (pNSB437; lane 2), TATA-1 (pNSB401; lane 3), or TATA-2 (pNSB415; lane 4) origin construct in the presence of the AL1,2,3,4 expression cassette (pMON1548). Replication was analyzed on DNA gel blots probed for TGMV A sequences. Newly synthesized, double-stranded DNA is indicated by the asterisks. Lanes 1 and 2 show 2.9-kb replication products corresponding to the wild-type TGMV A origin and the G-box mutant; lanes 3 and 4 show 3.5-kb products corresponding to TATA-1 and TATA-2 mutants. (B) NT-1 protoplasts were coelectroporated with either the wild-type TGMV A (pNSB110; lane 1), G-box (pNSB437; lane 2), TATA-1 (pNSB401; lane 3), or TATA-2 (pNSB415, lane 4) origin construct in the presence of competitor replicon DNA and the AL1,2,3,4 expression cassette (pMON1548) and analyzed for replication on DNA gel blots. The competitor DNAs were the wild-type TGMV A origin in a pUC119 (lanes 1 and 2) or a pUC19 (lanes 3 and 4) background. Lane 1 shows 2.9- and 3.5-kb products resulting from replication of both wild-type TGMV A origin constructs; lane 2 shows a 3.5-kb replication product corresponding to the wild-type origin in pUC119; lanes 3 and 4 show 2.9-kb replication product corresponding to the wild-type origin in pUC19. No bands indicated by the asterisks were detected for replicated G-box (lane 2), TATA-1 (lane 3), or TATA-2 (lane 4) DNA.

DISCUSSION

Although transcription and DNA replication are distinct biological processes, they frequently share *cis*- and *trans*-acting components. Replication origins from fungal and animal replicons contain transcription factor binding sites (9, 10, 39), and there is considerable evidence that proteins bound at these sites are involved in stimulation of DNA replication as well as transcriptional regulation (2, 3, 9, 21, 22, 29). In contrast, AL1 is the only example of a protein with dual roles in transcription and replication in plants. We previously showed that binding of TGMV AL1 to a DNA sequence shared by its AL61 promoter and the overlapping positive-strand origin contributes to transcriptional repression (13) and is essential for geminivirus DNA synthesis (16). We have now extended this relationship by showing that the TATA box and G-box motifs, which are involved in basal and activated transcription of many plant genes (9, 40, 61), are necessary for full AL61 promoter activity and efficient viral DNA replication.

Earlier observations showing that interaction between AL1 and its known DNA binding site cannot fully account for negative regulation of AL61 promoter activity (13) suggested that multiple *cis* elements are involved in AL1-mediated repression. Our current studies have identified two additional sequences that contribute to negative regulation of the AL61 promoter. One sequence was located to the 47-bp 5′ nontranscribed leader of the AL61 gene by showing that substituting

the CaMV 35S leader resulted in a twofold reduction in repression levels in the presence of AL1. The AL61 leader contains a partial inverted copy of the AL1 binding site (13 of 17 bp) that might interact with AL1 to enhance repression. Although no function has been attributed to this partial binding site in vivo or in vitro, analogous motifs are found in equivalent positions in the genomes of other Western hemisphere geminiviruses (1), indicative of functional conservation.

We found another negative regulatory sequence in experiments that examined the impact of the TGMV AL4 protein on AL61 promoter activity. Groning et al. (19) demonstrated that the AL61 promoter is repressed about threefold by the AL4 protein, whose open reading frame is internal to the AL1 coding sequence. We confirmed these results in our system and showed that AL4-mediated repression is independent of the AL1 binding site. This conclusion was based on the observations that m35S-*luc*, which contains only the AL1 binding site from the AL61 promoter, was not repressed by AL4, whereas $mA_{28\text{-}326}$-*luc*, which has a mutated AL1 binding site, was negatively regulated by AL4. The differential impact of AL4 on $A_{28\text{-}326}$-*luc* versus $mA_{28\text{-}135}$-*luc* activity located the AL4-responsive element between TGMV A positions 136 and 326 and distal from the AL1 binding site. However, no role for AL4 during TGMV infection has been found, and the protein has not been detected in infected plants (14, 31, 50).

The 5′ limit of the AL1 basal promoter region, as shown by the deletion mutant $AL61_{28\text{-}108}$, is at position −47 relative to the transcription start site. The AL61 TATA box motif is located between positions 88 and 94 in the basal promoter region and conforms to the eukaryotic consensus sequence TATA(T/A)A(T/A) (6). Mutations in the AL61 TATA motif were detrimental to transcription but resulted in only a fourfold reduction at best, even though equivalent changes in other promoters have strong negative effects (30, 51, 61). However, the AL61 TATA box is embedded in an AT-rich region, and it may have been difficult to eliminate its activity due to the use of cryptic TATA boxes (46). Because of low RNA levels, it was not technically feasible to determine if the transcription start sites of TATA box mutants shifted relative to the wild-type AL61 promoter as an indicator of alternative TATA box usage. The AL61 promoter also contains a G-box core motif, CACGTG, at −53 relative to the transcription start site. A combination of deletion and site-directed mutagenesis established that the G-box sequence acts as a strong positive regulator of the AL61 promoter. An interesting feature of the mutant G-box AL61 promoter was that it was not negatively regulated by AL1, suggesting that activated transcription may be the target of repression.

AL1 has many properties in common with ICP4, a well-studied transcriptional repressor of herpes simplex virus type 1. ICP4 negatively regulates its own expression by binding to a site that overlaps the transcription start site in an orientation-dependent manner (20, 45). The AL1 binding site is also located near the AL61 transcription start site and functions in an orientation-dependent fashion in the context of the E35S promoter. ICP4 repression is dependent on the spacing between its binding site and the TATA box (34). As discussed above, our observation that AL61 TATA box mutants display reduced repression may also reflect a change in the spacing between the AL1 binding site and the active TATA box. Finally, analogous to the dependence of AL1-mediated repression on an intact G-box element, ICP4 has no effect on transcription in the absence of binding sites corresponding to the Sp1 transcriptional activator (20). The precise mechanism of ICP4 repression is not known, but it is thought to involve a tripartite protein complex consisting of the TATA binding protein, TFIIB, and ICP4 that is unable to respond to Sp1 activation (34, 48). AL1-mediated repression may occur through similar protein interactions between AL1 and one or more basal transcription components that interfere with G-box factor activation.

Comparison of our results with those of Hong and Stanley (28), who analyzed the equivalent AC1 promoter from the Eastern hemisphere geminivirus African cassava mosaic virus (ACMV), suggested that TGMV and ACMV have evolved related but different mechanisms to regulate expression of their replication proteins. (The TGMV AL1 and AL4 homologs from ACMV are designated AC1 and AC4.) The TGMV AL61 promoter is activated primarily through a single G-box element. In contrast, the ACMV AC1 promoter does not contain a G-box motif, and its activation is thought to involve multiple *cis* elements. Both promoters are negatively regulated by their respective AL1 and AC1 proteins, but only the TGMV AL61 promoter is sensitive to negative regulation by AL4. Hong and Stanley (28) proposed that like TGMV AL1, the ACMV AC1 protein binds to a site between the transcription start site and TATA box to repress gene expression. The putative AC1 binding site lacks repeated motifs, suggesting that ACMV AC1 contacts its promoter differently than TGMV AL1. This difference may account for the ability of ACMV AC1, but not TGMV AL1, to repress a truncated promoter containing the TATA box and transcription start site. Nonetheless, like TGMV AL1, the ACMV AC1 protein cannot fully silence its promoter, suggesting that it also targets activated transcription.

The positive-strand origin of replication overlaps the AL61 promoter in the TGMV A genome. The TATA box is positioned 3 bp upstream of the origin recognition site, whereas the G box is located at the base of the hairpin structure that includes the DNA cleavage site for priming of positive-strand synthesis (36, 42). Replication analysis of mutant origins revealed that neither motif is essential for viral replication. However, competition assays in the presence of wild-type origins demonstrated that both motifs contribute to replication efficiency. TATA box sequences and TATA binding protein have been implicated in origin function in yeast (39) and may also be important for geminivirus replication. However, eukaryotic origins typically include an AT-rich element that facilitates duplex DNA unwinding during the initiation of replication (10). The TATA box mutations in the AL61 promoter altered a region of high A/T content and may have affected origin structure and function independently of the ability to recruit the TATA binding protein. In contrast, the effect of the G-box mutation cannot be attributed to changing an origin structural element and, instead, is likely to reflect reduced affinity for a putative G-box factor. A G-box factor could affect origin function by facilitating AL1 recruitment and binding to the origin, modulating chromatin assembly and origin accessibility, or stabilizing an origin conformation required for efficient replication. These models will be addressed in future experiments after identification and isolation of the relevant G-box factor.

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