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A conserved hairpin structure in *Alfamovirus* and *Bromovirus* subgenomic promoters is required for efficient RNA synthesis in vitro

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ABSTRACT

The coat protein gene in RNA 3 of alfalfa mosaic virus (AMV; genus *Alfamovirus,* family *Bromoviridae*) is translated from the subgenomic RNA 4. Analysis of the subgenomic promoter (sgp) in minus-strand RNA 3 showed that a sequence of 37 nt upstream of the RNA 4 start site (nt +1) was sufficient for full sgp activity in an in vitro assay with the purified viral RNA-dependent RNA-polymerase (RdRp). The sequence of nt –6 to –29 could be folded into a potential hairpin structure with a loop represented by nt –16, –17, and –18, and a bulge involving nt –23. By introducing mutations that disrupted base pairing and compensatory mutations that restored base pairing, it was shown that base pairing in the top half of the putative stem (between the loop and bulge) was essential for sgp activity, whereas base pairing in the bottom half of the stem was less stringently required. Deletion of the bulged residue A-23 or mutation of this residue into a C strongly reduced sgp activity, but mutation of A-23 into U or G had little effect on sgp activity. Mutation of loop residues A-16 and A-17 affected sgp activity, whereas mutation of U-18 did not. Using RNA templates corresponding to the sgp of brome mosaic virus (BMV; genus *Bromovirus*, family *Bromoviridae*) and purified BMV RdRp, evidence was obtained indicating that also in BMV RNA a triloop hairpin structure is required for sgp activity.

Keywords: alfalfa mosaic virus; brome mosaic virus; *Bromoviridae*; secondary RNA structure; subgenomic promoter; transcription

INTRODUCTION

The genomic RNAs of positive-strand viruses that belong to the alpha-like superfamily of RNA viruses are translated into protein(s) required for RNA replication, but downstream genes are frequently expressed through the synthesis of subgenomic messenger RNAs (sg mRNAs). For several viruses, it has been shown that in vitro sg mRNA synthesis can be initiated *de novo* by the purified viral RNA-dependent RNA-polymerase (RdRp) on internal subgenomic promoter (sgp) sequences in viral minus-strand RNAs (Miller et al., 1985; van der Kuyl et al., 1990). For viruses from the genera *Coronavirus* and *Arterivirus*, sg mRNA synthesis is believed to be primed by leader sequences corresponding to the 5′ terminus of the genomic RNA or to involve the synthesis of a set of sg minus-strand RNAs (Lai & Cavanagh, 1997; Sawicki & Sawicki, 1998; van Marle et al., 1999). The use of sg minus-strands as templates for sg mRNA synthesis has also been proposed for a virus from the genus *Nodavirus* (Zhong & Rueckert, 1993). For red clover necrotic mosaic virus, a bipartite virus from the genus *Dianthovirus*, base pairing between 8-nt sequences in RNAs 1 and 2 has been proposed to trigger the synthesis of a sg minus-strand intermediate in the production of a RNA 1-derived sg mRNA (Sit et al., 1998).

Sgp sequences have been studied for plant viruses from different genera, including beet necrotic yellow vein virus (*Benyvirus*; Balmori et al., 1993), cucumber necrosis virus (*Tombusvirus*; Johnston & Rochon, 1995), turnip crinkle virus (TCV; *Carmovirus*; Wang & Simon, 1997), barley yellow dwarf virus (BYDV; *Luteovirus*; Koev et al., 1999), brome mosaic virus (BMV; *Bromovirus*; French & Ahlquist, 1988), cowpea chlorotic mottle virus (CCMV; *Bromovirus*; Adkins & Kao, 1998), cucumber mosaic virus (CMV; *Cucumovirus*; Boccard & Baulcombe, 1993), and alfalfa mosaic virus (AMV; *Alfamovirus*; van der Kuyl et al., 1990, 1991; van der Vossen et al., 1995). The sgp of BMV has been studied most extensively (French & Ahlquist, 1987, 1988; Marsh et al., 1988; Adkins et al., 1997; Siegel et al., 1997, 1998; Adkins & Kao, 1998). The

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sgp in BMV minus-strand RNA 3 consists of several domains: a downstream A/U-rich sequence, a core promoter, a polyuridylate tract, and an upstream A/U-rich sequence. The core promoter is sufficient for a basal level of transcription in vitro and is recognized by the RdRp in a sequence specific manner by interaction with four essential nucleotides upstream of the transcription start site (+1): G-17, A-14, C-13, and G-11 (Siegel et al., 1997; Adkins & Kao, 1998). Recognition in vitro of the CCMV sgp by the BMV or CCMV RdRp required the same nucleotides and four additional nucleotides at positions −20, −16, −15, and −10 in the CCMV sgp (Adkins & Kao, 1998).

Stem-loop structures in the promoter region have been shown to be important for sgp activity of TCV (Wang et al., 1999) and BYDV (Koev et al., 1999), but secondary RNA structure was considered not to be important for BMV or CCMV sgp activity (Adkins & Kao, 1998). We have analyzed the possible requirement of a potential stem-loop structure in the AMV sgp for sgp activity in vitro. AMV, BMV, and CCMV are all members of the family *Bromoviridae* (Rybicki, 1995). AMV RNAs 1 and 2 encode the replicase proteins P1 and P2, respectively, whereas RNA 3 encodes the movement protein P3 and the coat protein (CP), which is translated from the subgenomic RNA 4 (reviewed in Bol, 1999). Binding of CP to the 3′ termini of the plus-strand AMV RNAs induces a conformational switch that shuts off recognition of the minus-strand promoter by the RdRp (Olsthoorn et al., 1999). On the other hand, CP strongly stimulated plus-strand RNA 4 synthesis on a minus-strand RNA 3 template by a purified transgenically expressed RdRp that is free of CP (de Graaff et al., 1995a). Studies in vivo revealed that the sequence in minus-strand RNA 3 between the RNA 4 start site (nt +1) and the upstream nt −26 was sufficient for a low level of sgp activity. Wild-type (wt) levels of sgp activity required sequence elements located between positions −94 and −136 (van der Vossen et al., 1995).

In the present study, we analyzed the nucleotides in the AMV sgp that are important for interactions with the AMV RdRp in vitro. Because the results indicated that a small hairpin structure in the AMV sgp is essential for sgp activity, we tested the possibility that a similar hairpin structure is required for BMV sgp activity. The results support the hypothesis that a hairpin structure in viral minus-strand RNA is required for sgp activity of viruses from at least two, and possibly all five, genera in the family of *Bromoviridae* (Jaspars, 1998).

RESULTS

AMV sgp sequences required for promoter activity in vitro

Figure 1 shows a schematic representation of AMV minus-strand RNA 3. The intercistronic region consists of nt −13/+36 when the transcription start site for RNA 4 synthesis is taken as +1. The sequence of nt −26/+1 was found to have a basal level of sgp activity in vivo

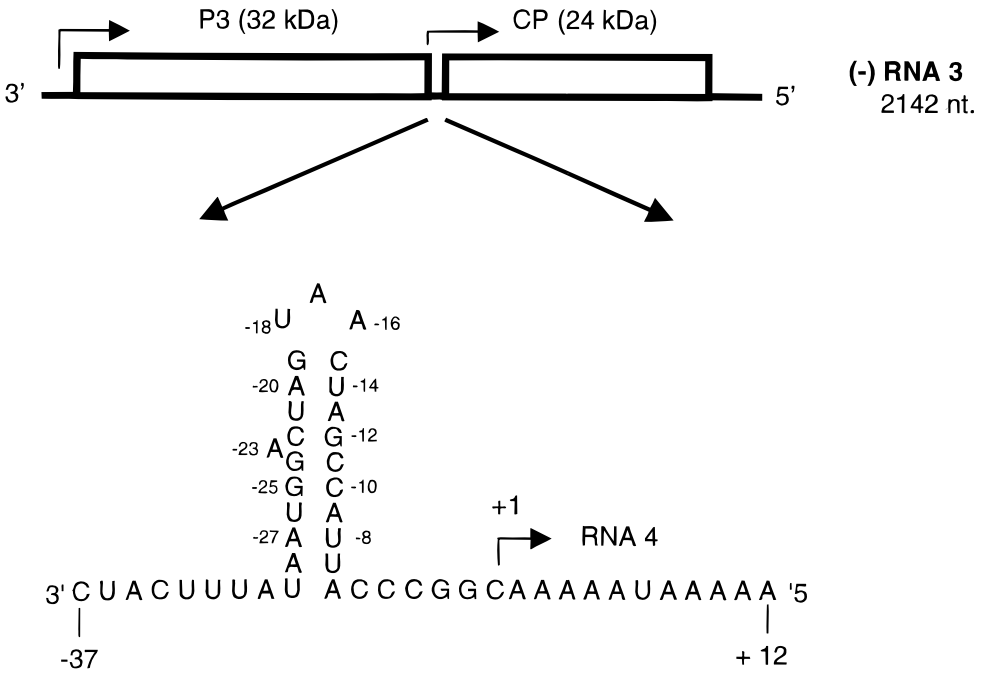

FIGURE 1. Schematic representation of AMV minus-strand RNA 3. A hairpin structure with a proposed role in subgenomic promoter activity is indicated. Nucleotides are numbered with the start site of RNA 4 transcription taken as +1. The UGA termination codon of the MP gene corresponds to nt −14/−16.

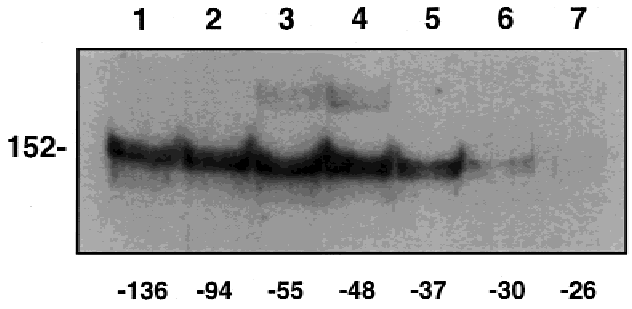

FIGURE 2. Determination of the 3′ endpoint of AMV minus-strand RNA 3 fragments with subgenomic promoter activity. Lane 1 shows the RNA product of 152 nt synthesized by AMV RdRp on a template corresponding to nt −136/+152. In lanes 2–7 the 3′ end of the template was truncated to position −94 (lane 2), −55 (lane 3), −48 (lane 4), −37 (lane 5), −30 (lane 6), or −26 (lane 7). The position of the 152-nt product synthesized by all templates with sgp activity is indicated in the left margin.

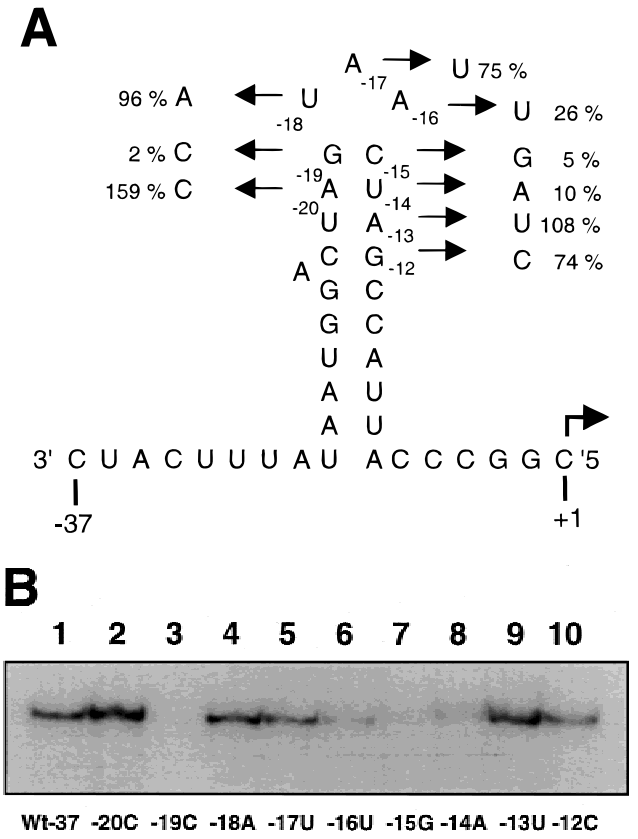

FIGURE 3. Mutational analysis of the region of the AMV subgenomic promoter that contains nucleotides conserved in putative subgenomic promoters of viruses from the family *Bromoviridae*. **A**: Diagram of the introduced point mutations. Transcription levels relative to the wild type (100%) are indicated. **B**: RNA products synthesized in vitro by the AMV RdRp on a wild-type template (nt −37/+152; lane 1) and the indicated mutant templates (lanes 2–10). Mutant −20C: nt −20 changed into C, and so forth.

(van der Vossen et al., 1995). This sequence is part of a stem-loop structure that has been predicted to occur in plus-strand RNA 3 (Koper-Zwarthoff et al., 1980) as well as in minus-strand RNA 3 (Fig. 1; Jaspars, 1998). To delineate the minimal sequence required for sgp activity in vitro, minus-strand RNA fragments were synthesized with 3′ termini corresponding to positions −136, −94, −55, −37, −30, and −26, and 5′ termini all corresponding to position +152. Figure 2 shows the synthesis of the plus-stranded product of 152 nt when these minus-strand RNAs were used as templates for the purified AMV RdRp in an in vitro assay. Template activity remained at an approximately constant level when the 3′ endpoint of the template was reduced from position −136 to −37. Truncation of the transcript to position −30 resulted in a sharp drop in template activity, and no significant activity was observed with a template ending at position −26. The template RNA −37/+152 was used in subsequent studies to analyze a putative role in sgp activity of the stem-loop structure shown in Figure 1. Modeling by the MFOLD program (Zuker, 1989; Mathews et al., 1999) did not predict an interaction between the sequences located 3′ of position +1 and the +1/+152 sequence in any of the templates used in this study.

Analysis of the –12 to –20 region of the AMV sgp

Nucleotides G-12, C-15, A-16, and G-19 of the AMV sgp correspond to nucleotides that are conserved in sgp sequences of viruses from the *Bromoviridae* and that are essential for BMV sgp activity (Siegel et al., 1997). To analyze the role of these nucleotides in the AMV sgp, all nucleotides at positions −12 to −20 were mutated as indicated in Figure 3A. The template activity in the RdRp assay of −37/+152 RNA fragments carrying these mutations is shown in Figure 3B. Mutation of the conserved positions −15, −16, and −19 reduced template activity to 5%, 26%, and 2%, respectively, compared to the activity of the wt-sgp sequence. Also, mutation of the nonconserved nucleotide at position −14 reduced template activity to 10%. The small reduction in template activity induced by mutation of nt A-17 and the conserved nt G-12 was not reproducibly observed in other experiments. In addition, mutation of nt −13, −18, or −20 had little effect on template activity (Fig. 3B). These results indicate that sequence elements important for AMV sgp activity partially differ from elements reported to be essential for BMV sgp activity. All experiments reported in this study were done at least twice. As an example of the variation observed, an analysis of mutants −12C (74–92%), −15G (2–5%), and −19C (1–2%) is also shown in Figure 4, and a duplicate analysis of mutants −16U (26–39%), −17U (75–111%), and −18A (96–139%) is included in Figure 6. Because of these variations, our major conclusions will focus on mutations that result in a template

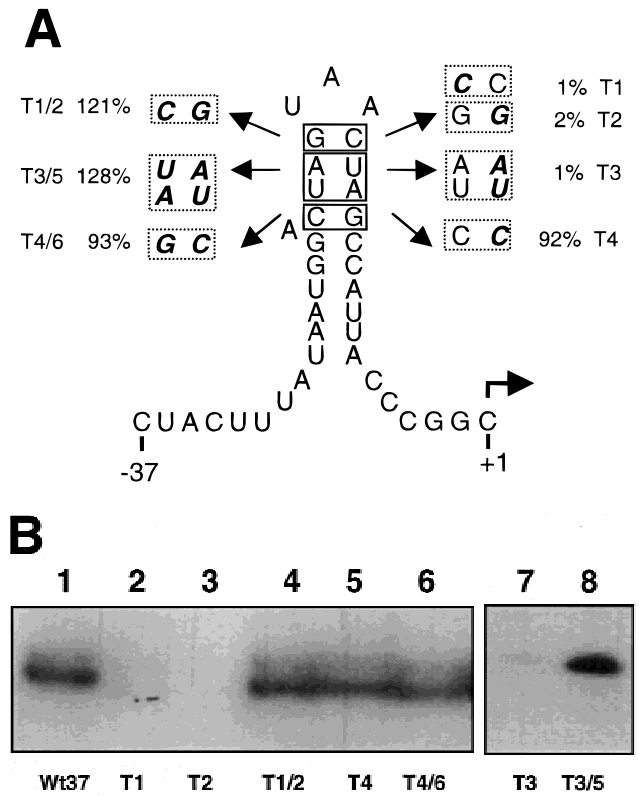

FIGURE 4. Mutational analysis of the top-stem region of the hairpin structure in the AMV subgenomic promoter. **A**: Diagram of the introduced mutations. Transcription levels relative to the wild type (100%) are indicated. **B**: RNA products synthesized in vitro by the AMV RdRp on a wild-type template (nt −37/+152; lane 1) and the indicated mutant templates (lanes 2–8).

activity that is more than twofold different from the wild type.

Requirement for base pairing in the upper stem of the AMV sgp hairpin

To determine whether the secondary structure shown in Figure 1 is important for sgp activity, mutations were made in the top half of the stem (base pairing between nt −12/−15 and −22/−19), the bottom half of the stem (base pairing between nt −6/−11 and −29/−24), the bulged nt A-23, and the loop nt −16/−18. Mutations in the top half of the stem are numbered with the prefix "T", whereas the prefix "B" is used for mutations in the bottom half of the stem. Because of this, a few mutants analyzed in Figure 3 had to be renamed.

Figure 4A shows the single and double mutations that were introduced in the upper stem of the predicted hairpin; Figure 4B shows the template activity of these mutants in the RdRp assay. In mutants T1 and T2, G-19 and C-15 are mutated into a C and G, respectively. Template activity of these single mutants is reduced to 1–2% of the wild type. When the two mutations were combined in the double mutant T1/2, possible base pairing between nt −15 and −19 was restored, and template activity increased to 121%. Similarly, when A-13 and U-14 were mutated in mutant T3 to disrupt possible base pairing, template activity dropped to 1%. However, when the compensatory mutations were made at positions −21 and −20 to restore base pairing in mutant T3/5, template activity increased to 128%. These data support the notion that base pairing in the upper stem of the hairpin is essential for sgp activity. Mutation of G-12 into a C-residue in mutant T4 would disrupt the putative bp −12/−22, but sgp activity is maintained at 92%. As base pairing is restored by a compensating mutation in mutant T4/6, sgp activity remains at 93%.

In Figure 3, it was shown that disruption of bp A-13/U-21 or U-14/A-20 had little effect on template activity. However, when both these base pairs were disrupted in mutant T3, sgp activity dropped 100-fold. Together, the data from Figures 3 and 4 indicate that the top base pair of the upper stem of the sgp hairpin is essential for sgp activity, whereas disruption of 1 of the other 3 bp has little effect on template activity. Moreover, the primary sequence in this upper stem appears not to be important for sgp activity.

Analysis of the lower stem of the AMV sgp hairpin

The lower stem of the sgp hairpin consists of six putative base pairs. Figure 5A shows the mutations introduced in this lower stem; Figure 5B shows the template activity of these mutants in the RdRp assay. Disruption of the upper 2 bp in mutant B1 reduced sgp activity to 5% of the wild type. When base pairing was restored by compensating mutations in mutant B1/5, sgp activity increased to 55%, indicating that bp C-10/G-25 and/or C-11/G-24 played a role in template activity. Disruption of the central base pair in the lower stem in mutant B2 reduced sgp activity to 24%, whereas compensating mutations in mutant B2/6 restored template activity to 57%. When base pairing of the three bottom base pairs was disrupted either by mutating A-6, U-7, and U-8 (mutant B3), or by mutating U-29, A-28, and A-27 (mutant B4), sgp activity was reduced to 47% and 54%, respectively. When base pairing was restored by combining the two mutations in mutant B3/4, template activity remained at 54%. In summary, we conclude that the top 1 or 2 bp of the lower stem are important for sgp activity, but the bottom 3 to 4 bp are not. The activity levels of 54–57% of mutants B1/5, B2/6, and B3/4 indicate that the primary sequence of the lower stem may have an effect on sgp activity.

Analysis of bulged and loop nucleotides in the AMV sgp hairpin

Figure 6A shows the mutations that were engineered in the bulge and loop of the predicted AMV sgp hairpin;

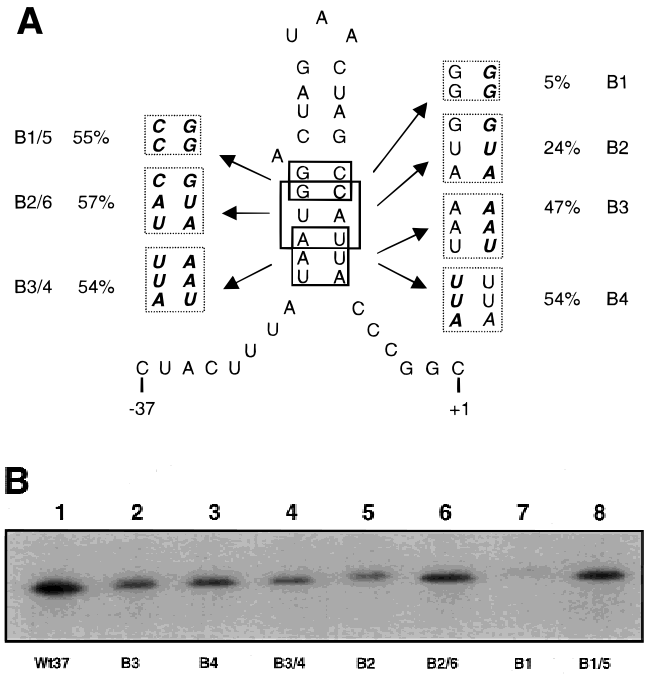

FIGURE 5. Mutational analysis of the bottom-stem region of the hairpin structure in the AMV subgenomic promoter. **A**: Diagram of the introduced mutations. Transcription levels relative to the wild type (100%) are indicated. **B**: RNA products synthesized in vitro by the AMV RdRp on a wild-type template (nt −37/+152; lane 1) and the indicated mutant templates (lanes 2–8).

template activity of the loop mutants in the in vitro RdRp assay is shown in Figure 6B, whereas template activity of the bulge mutants is shown in Figure 6C. Mutation of loop nt U-18 to A, C, or G resulted in an increase of template activity. Also, mutation of A-17 to G or U did not interfere with template activity, but mutation of this loop nucleotide to C reduced template activity to 48%. Requirements for the identity of the loop nucleotide at position −16 appeared to be even more stringent. Replacement of A-16 by a G-residue did not reduce sgp activity, but replacement by U or C reduced sgp activity to 39% and 47%, respectively.

Deletion of the bulged A-23 reduced template activity to 5%, indicating that this bulge is an important element of the sgp. Replacement of A-23 by U, C, or G reduced template activity to 54, 15, or 70%, respectively, indicating that the identity of the bulged nucleotide is recognized by the RdRp.

Evidence for the role of a hairpin structure in BMV sgp activity

The conserved nucleotides that are essential for BMV sgp activity are believed to be primarily involved in interactions with RdRp proteins, rather than to play a role in hairpin formation (Adkins & Kao, 1998). We noticed

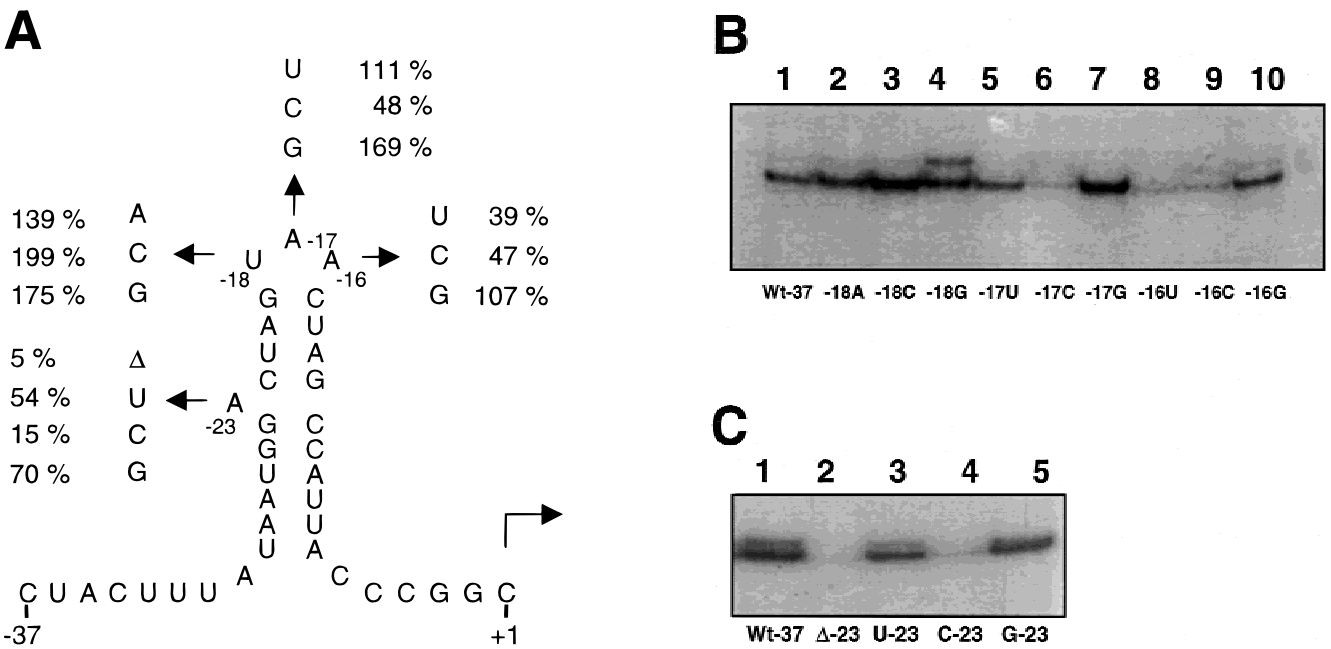

FIGURE 6. Mutational analysis of the loop nucleotides and the bulged adenosine in the hairpin structure in the AMV subgenomic promoter. **A**: Diagram of the introduced mutations. Transcription levels relative to the wild type (100%) are indicated. **B,C**: RNA products synthesized in vitro by the AMV RdRp on a wild-type template (nt −37/+152; lanes 1) and the indicated mutant templates with mutations in the loop (**B**) or bulge (**C**). Mutant −18A: nt −18 changed into A, and so forth.

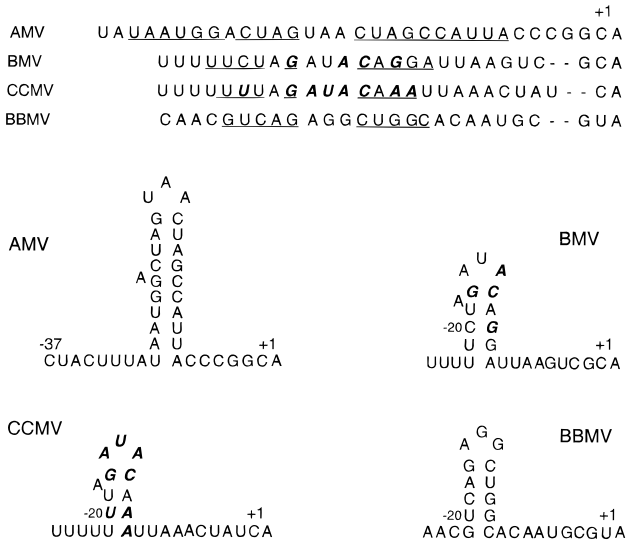

FIGURE 7. Proposed secondary structures and alignment of primary sequences of the core subgenomic promoters of AMV, BMV, CCMV, and BBMV. Nucleotides that have been shown to be essential for BMV and CCMV promoter activity are indicated in bold italics. Sequences proposed to be involved in base pairing are underlined.

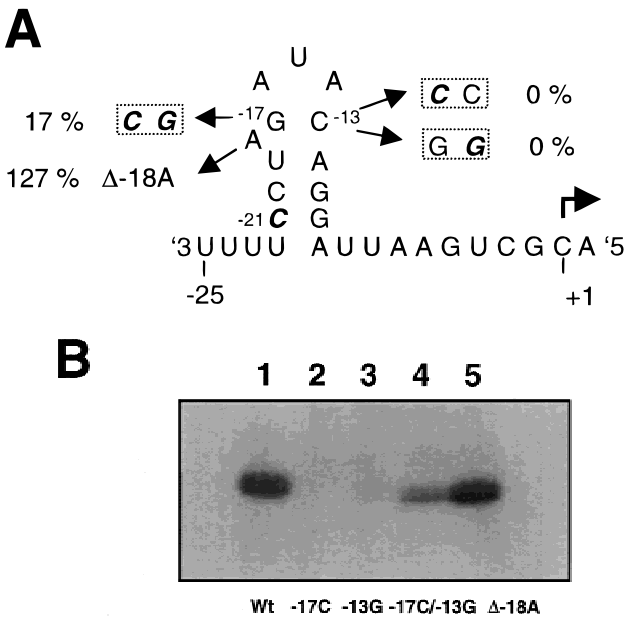

FIGURE 8. Mutational analysis of the BMV subgenomic promoter. **A**: Diagram of the proposed hairpin in the BMV subgenomic promoter and mutations that were introduced. Nucleotides are numbered with the start site of RNA 4 transcription taken as +1. In all templates, the U-residue at position −21 was changed into a C-residue. Transcription levels relative to the wild type (100%) are indicated. **B**: RNA products synthesized in vitro by the BMV RdRp on a wild-type template (nt −25/+17; lane 1) and the indicated mutant templates (lanes 2–5). Mutant −17C: nt −17 was changed into C, and so forth.

that the nt −9 to −22 of the BMV sgp, which include the conserved nucleotides, could be folded into a stem-loop structure with limited stability. This structure is shown in Figure 7, together with a similar structure in the sgp of the closely related bromovirus CCMV and the secondary structure of the sgp of broad bean mottle virus (BBMV) proposed by Jaspars (1998). In addition, an alignment of the primary sgp sequences is shown. Evidence for the importance of base pairing between G-11 and C-20 in BMV-sgp activity was already presented by Adkins and Kao (1998). We analyzed a possible role of base pairing between C-13 and G-17 by making the single mutants −13G and −17C and the double mutant −17C/−13G (Fig. 8A) in RNA transcripts corresponding to the BMV sgp sequence of nt −25 to +17. We noticed that mutant −13G and the double mutant could adopt an alternative secondary structure that involved base pairing between nt −9/−15 and nt −23/−8. To freeze the structure in the conformation that is most stable in the wild type, the U-residue at position −21 in the wild-type sequence was changed into a C-residue (mutant −21C). This mutation changed the putative U-21/G-10 bp into a C-G bp, but did not affect the template activity of the −25/+17 fragment in an in vitro replicase assay with the purified BMV RdRp (result not shown). When mutations −17C or −13G were introduced in the −21C mutant, possible base pairing between nt −17 and −13 was disrupted and no sgp activity with the BMV RdRp was detectable (Fig. 8B, lanes 2 and 3). This is in agreement with the results reported by Siegel et al. (1997) and Adkins and Kao (1998). However, when the G-17/C-13 bp was reversed to a C-G bp in the double mutant, sgp activity was restored to a level of 17% of the wild-type control (Fig. 8B, lane 4). This supports the notion that base pairing between nt −17 and −13 plays a role in BMV sgp activity. Deletion of the bulged A-18 (mutant Δ-18A) increased the stability of the proposed hairpin structure. The template activity of this mutant in the RdRp assay was slightly higher (127%) than wild-type (Fig. 8B, lane 5). Apparently, the bulged A-18 in the BMV sgp is not functionally equivalent to the bulged A-23 in the AMV sgp. We propose that the BMV sgp hairpin shown in Figure 7 is equivalent to the upper stem and loop of the AMV sgp hairpin. The secondary structure proposed for the CCMV sgp is discussed below.

DISCUSSION

Our results further support the notion that sg RNA synthesis by viruses from the *Bromoviridae* is directed by a sgp in genome-sized minus-strand RNA and does not involve sg minus-strand templates implicated in mRNA synthesis of several virus genera (see Introduction). In infected tobacco plants, maximum activity of a duplicated sgp sequence in AMV RNA 3 required the core promoter sequence −26/+1

and an upstream positive regulatory element that mapped to the sequence −136/−94 (van der Vossen et al., 1995). In our in vitro assays with AMV RdRp purified from infected *Nicotiana benthamiana* plants, no clear effect of deletion of the −136/−94 sequence from the template was observed (Fig. 2). Possibly, the activity of the −136/−94 sequence in vivo involves long-distance interactions between sequences in minus-strand or plus-strand RNA 3. The observation that several mutations in the CP-binding site at the 3′ end of plus-strand RNA 3 affected RNA 4 accumulation in vivo but not RNA 3 accumulation points to such long-distance interactions (Reusken et al., 1997). Moreover, it has been shown that oxidation of the 3′ terminal ribose of AMV minus-strand RNA 3 inhibits plus-strand RNA 4 synthesis in vitro (de Graaff et al., 1995b). Long-distance interactions may also explain the position effects observed as multiple copies of a sgp sequence are inserted into a single minus-strand template (French & Ahlquist, 1988; Lehto et al., 1990; Boccard & Baulcombe, 1993; Kim & Hemenway, 1999; Zhang et al., 1999). In our in vitro studies with the −37/+152 fragment, possible long-distance interactions will not be effective, and the sgp activity in this fragment may be considered to represent a core promoter.

The data presented in Figures 3–6 support a role of a hairpin structure in AMV sgp activity. Particularly, the results shown in Figures 3 and 4 indicate that bp G-19/C-15 is essential for sgp activity, whereas only one of the three other base pairs in the upper stem of the hairpin could be disrupted without loss of activity. Disruption of a single base pair at positions −12/−22, −13/−21, or −14/−20 had little effect on sgp activity, but as the base pair at positions −13/−21 and −14/−20 were both disrupted, sgp activity was virtually abolished. Currently, we are investigating whether the combined disruption of bp −12/−22 and −13/−21 also interferes with sgp activity. Base pairing in the lower stem of the hairpin appeared to be less critical for sgp activity. The results with mutants B1, B2, B1/5, and B2/6 point to a role of bp G-24/C-11 and G-25/C-10 in sgp activity (Fig. 5). Disruption of base pairs in the bottom half of the lower stem (mutants B3, B4) reduced sgp activity, but the activity was not enhanced by compensating mutations that restored base pairing (mutant B3/4). In agreement with these observations in vitro, it was shown that mutation of U-8 into a C-residue did not affect sgp activity in vivo (van der Vossen et al., 1995). However, deletion of the sequence AUUA from position −6 to −9 abolished CP synthesis and strongly reduced viral RNA accumulation in protoplasts (van der Vossen et al., 1995), indicating that sequence elements of the lower stem are important for sgp activity. In addition, base pairing in the plus-strand RNA 3 sequence corresponding to this lower stem may play a role in RNA 3 replication (Koper-Zwarthoff et al., 1980).

The loop nt A-16 corresponds to the U-residue in the UGA termination codon of the MP gene. Mutation of A-16 to C or U (Fig. 6) reduced sgp activity by more than 50%, indicating that this nucleotide has a dual role in translation and sg mRNA synthesis. The results presented in Figure 6 indicate that A-16 and A-17 are involved in sequence-specific contacts with the RdRp. The sequence of nt −17 to −37 of the core promoter analyzed in our study has a dual function in sgp activity and in coding the C-terminus of the MP. The bulged A-23 corresponds to the wobble position of a proline codon and is replaced by a G-residue in minus-strand RNA 3 of the Madison isolate of AMV (Barker et al., 1983). We observed that replacement of A-23 by G or U moderately reduced sgp activity, whereas replacement by C or deletion of A-23 affected sgp activity more severely (Fig. 6C). When A-23 and G-24 were deleted in full-length RNA 3, a low but significant level of RNA 4 accumulation was detectable in infected protoplasts (van der Vossen et al., 1995). Possibly, the bulged A-23 contributes to sgp activity by affecting the three-dimensional structure of the promoter.

The small hairpin structure in the BMV sgp shown in Figure 7 was not recognized in previous studies on sgp sequences of viruses from the family *Bromoviridae* (Adkins & Kao, 1998; Jaspars, 1998). However, the evidence of Adkins and Kao (1998) for a role of base pairing between G-11 and C-20 in BMV sgp activity, and our evidence for a similar role of base pairing between C-13 and G-17 indicates that the proposed secondary structure is essential for BMV sgp activity. Mutation of BMV G-11 to C reduced sgp activity to 1% of wild type (Siegel et al., 1997), whereas in the AMV sgp, 1 of the 3 bp involving G-12, A-13, and U-14 could be disrupted without loss of activity. This may reflect the relatively low stability of the proposed BMV secondary structure. However, there are examples of even weaker structures that are vital for RNA virus replication. The 2-bp stem in the hook structure in the 5′ arm of the genomic RNA of orthomyxoviruses is essential for interaction of the RNA with the viral RdRp and for viral mRNA synthesis (Leahy et al., 1998). Moreover, CP of phage MS2 binds specifically to a hairpin structure with a 2-bp stem derived from the viral RNA (Grahn et al., 1999). Possibly, formation of thermodynamically unstable hairpins is promoted by RNA–protein interactions. We propose that the weak hairpin structure that can be folded in the CCMV sgp (Fig. 7) may also be functional. Some of the mutations studied by Adkins and Kao (1998) support this notion. For instance, mutations that disrupted the putative bp A-11/U-20 strongly reduced RdRp activity, whereas replacement by a G-C bp increased sgp activity threefold over that of the wild-type sequence. In addition, the threefold increase of

transcription of CCMV mutants A-18/U and A-12/U can be explained by the possible base pairing of the bulge nt −18 with nt −12 that stabilizes the top G-C bp. Together, the data support the hypothesis that hairpin structures are functional components of sgps of all viruses from the *Bromoviridae* (Jaspars, 1998).

The hairpin structure in the BMV sgp is part of the domain that is involved in specific binding of the RdRp (Stawicki & Kao, 1999). Also for BYDV (genus *Luteovirus*), it has been proposed that a stem-loop structure is involved in positioning of the RdRp in close proximity to the initiation site for sg RNA synthesis (Koev et al., 1999). The hairpin structure in the AMV sgp is probably not sufficient for positioning of the RdRp on the template. The deletion analysis shown in Figure 2 indicates that the sequence of nt −30 to −37 that flanks the sgp hairpin is also involved in sgp activity. The role of these flanking sequences will be further investigated.

MATERIALS AND METHODS

Preparation of template RNAs

Template RNAs with AMV sgp sequences were transcribed with T7 RNA polymerase from DNA fragments amplified by PCR from clone pAL3. This clone contains a DNA copy corresponding to full-length RNA 3 of AMV strain 425 (Leiden isolate; Neeleman et al., 1991). In all PCR reactions, the downstream primer contained the T7 RNA polymerase promoter fused to a sequence complementary to nt +152/+134 of RNA 3, taking the transcription start site of RNA 4 as +1. Template RNAs with 3′ termini ending at positions −136, −94, −55, −37, or −26 were amplified with upstream primers corresponding to nt −136/−114, −94/−73, −55/−24, −37/−11, or −26/−5, respectively. Mutations in the template RNA corresponding to the sequence −37/+152 were introduced in an upstream primer corresponding to nt −37/+1.

Template RNAs with BMV-sgp sequences were transcribed from DNA fragments amplified by PCR from the BMV cDNA 3 clone pB3TP7 (Janda et al., 1987). The wt-sgp sequence was amplified with a downstream primer containing the T7 RNA polymerase promoter fused to the sequence complementary to nt +17/−4 (taking the transcription start site of RNA 4 as +1) and an upstream primer corresponding to nt −25/+11. Mutations in the sgp were introduced in this upstream primer.

Transcripts made with T7 RNA polymerase were purified by extraction with phenol/chloroform, precipitated two times with isopropanol in the presence of ammonium acetate, analyzed on ethidium bromide-stained agarose gels and quantified by UV absorbance.

RdRp assay

The RdRp was purified from *N. benthamiana* plants infected with AMV (Quadt et al., 1991). In vitro RNA synthesis was performed in a 50-μL mixture containing 10 μL AMV RdRp, 50 mM Tris-HCl, pH 8.2, 10 mM $MgCl_2$, 10 mM DTT, 1 mM ATP, GTP, CTP, 10 μM UTP, and 5 μCi [α-^{32}P]UTP (400 Ci/mmol). The mixtures were incubated at 28 °C for 60 min. BMV RdRp was kindly provided by Dr. E.M.J. Jaspars. The enzyme was isolated from BMV-infected barley leaves, and purified as described by Bujarski et al. (1982) through sucrose gradient centrifugation, using dodecyl-β-D-maltoside as detergent. Five microliters of the sucrose gradient fraction were treated with micrococcal nuclease and used in the RdRp assay as described above.

Labeled RNAs were purified by phenol/chloroform extraction and isopropanol precipitation, in the presence of 0.6 M ammonium acetate. AMV-specific RNA products were treated with nuclease S1 and run on a 2% agarose gel or an 8% polyacrylamide gel. BMV-specific RNA products were run on a 20% polyacrylamide gel containing 8 M urea. The RNAs were visualized by autoradiography and quantified with a Phosphor-Imager.

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