

In Vivo and *In Vitro* Characterization of an RNA Replication Enhancer in a Satellite RNA Associated with *Turnip crinkle virus*

Peter D. Nagy,^{1,*} Judit Pogany,^{1,*} and Anne E. Simon^{2,†}

^{*}Department of Plant Pathology, University of Kentucky, Lexington, Kentucky 40546; and [†]Department of Cell Biology and Molecular Genetics, University of Maryland College Park, College Park, Maryland 20742

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RNA replication enhancers are *cis*-acting elements that can stimulate replication or transcription of RNA viruses. *Turnip crinkle virus* (TCV) and satC, a parasitic RNA associated with TCV infections, contain stem-loop structures that are RNA replication enhancers (P. Nagy, J. Pogany, and A. E. Simon, *EMBO J.* 1999, 18, 5653–5665). We have found that replacement of 28 nt of the satC enhancer, termed the motif1-hairpin, with 28 randomized bases reduced satC accumulation 8- to 13-fold in *Arabidopsis thaliana* protoplasts. Deletion of single-stranded flanking sequences at either side of the hairpin also affected RNA accumulation with combined alterations at both sides of the hairpin showing the most detrimental effect in protoplasts. *In vitro* analysis with a partially purified TCV RdRp preparation demonstrated that the motif1-hairpin in its minus-sense orientation was able to stimulate RNA synthesis from the satC hairpin promoter (located at the 3' end of plus strands) by almost twofold. This level of RNA synthesis stimulation is ~fivefold lower than that observed with a linear promoter, suggesting that a highly stable hairpin promoter is less responsive to the presence of the motif1-hairpin enhancer than a linear promoter. The motif1-hairpin in its plus-sense orientation was only 60% as active in enhancing transcription from the hairpin promoter. Since the motif1-hairpin is a hotspot for RNA recombination during plus-strand synthesis and since satC promoters located on the minus-strand are all short linear sequences, these findings support the hypothesis that the motif1-hairpin is primarily involved in enhancing plus-strand synthesis. © 2001 Academic Press

INTRODUCTION

Plus-strand RNA viruses replicate efficiently in infected cells by a two-step process mediated by viral RNA-dependent RNA polymerases (RdRp). First, minus-strand RNAs are synthesized using the plus-strand RNA as template. Second, the new minus strands serve as templates to produce large quantities of positive-strand RNAs. To recognize and then replicate faithfully only the cognate RNA, the viral RdRp must recognize specific sequences, termed *cis*-acting elements, which are often located at the ends of the RNA (de Graaf and Jaspars, 1994; Buck, 1996).

Cis-acting elements that are absolutely required for viral replication or transcription are called promoters. RNA promoters in most RNA viruses are required for positioning the polymerase such that initiation of RNA synthesis *de novo*, i.e., independent of oligonucleotide primers, can take place. Replication and transcription promoters have been characterized for many viruses, including bacterial, fungal, animal, and plant viruses (reviewed by de Graaf and Jaspars, 1994; Buck, 1996). Promoter sequences/structures for these viruses contain

either poly(A) tails, pseudoknots, tRNA-like structures, stem-loop structures, or short primary sequences without apparent high-order structures. In contrast to promoters, RNA replication enhancers are nonessential *cis*-acting elements that can modulate the level of transcription and replication in RNA viruses. RNA replication enhancers have been shown or suggested to play significant roles in the biology of several RNA viruses (Lai, 1998).

Turnip crinkle virus (TCV; genus *Carmovirus*) is one of the best characterized model plus-strand RNA virus systems (reviewed by Simon and Nagy, 1996 and in Buck, 1996). TCV has a small genome (4054 nt) with two of its five genes required for replication. In addition, TCV infections are associated with several small parasitic RNAs, such as defective interfering RNAs (Li *et al.*, 1989) and satellite (sat) RNAs (Simon and Howell, 1986). The smallest satRNA is designated satD (194 nt). An unusual satRNA is the recombinant satC (356 nt) with the 5' portion derived from satD and the 3' portion originating from two short noncontiguous regions from the 3' region of TCV genomic RNA (Fig. 1A). Their small size, lack of open reading frames, and ability to modulate viral symptoms make satRNAs excellent models for studies on replication, recombination, and symptom production by viral RNAs.

In vitro and *in vivo* analyses of sequences that affect

¹ These authors contributed equally to this work.

² To whom correspondence and reprint requests should be addressed. Fax: 301-314-7930. E-mail: Anne_Simon@umail.umd.edu.

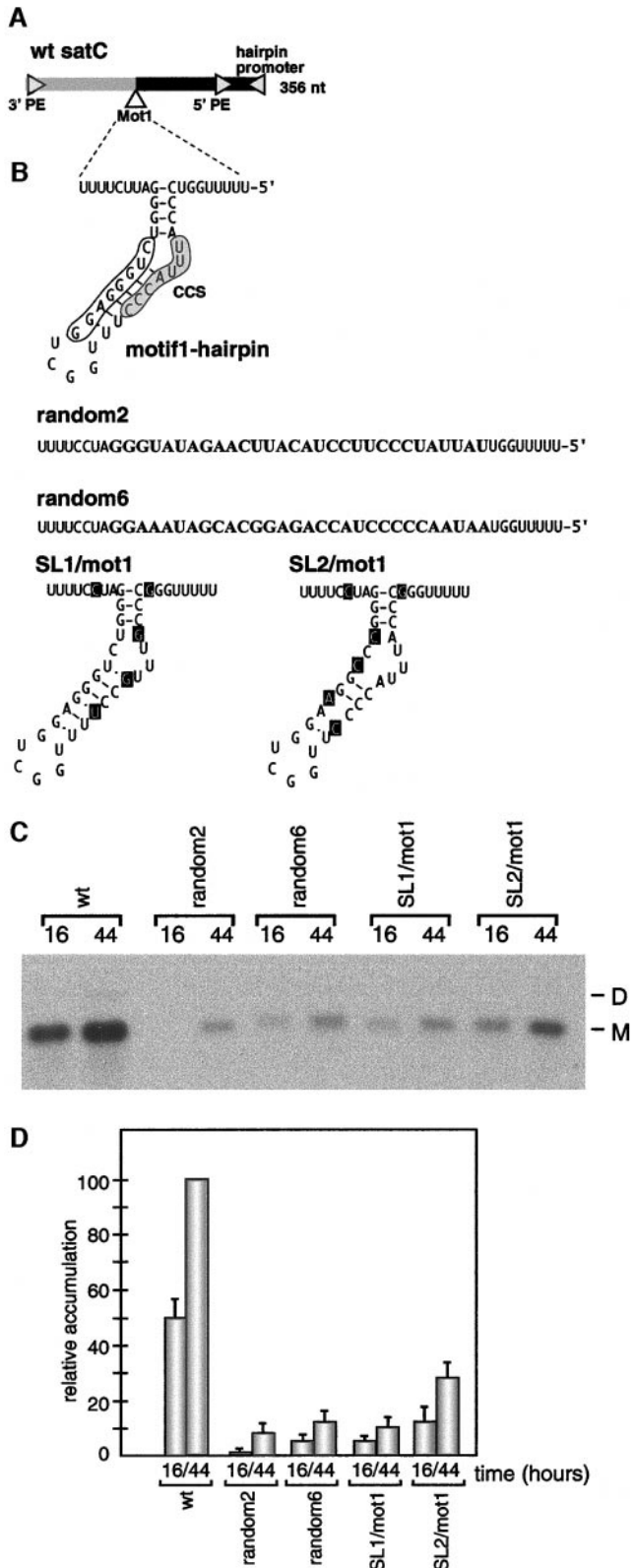


FIG. 1. Effect of the motif1-hairpin sequence and structure on satC accumulation. (A) Positions of defined *cis*-acting elements in satC. The hairpin promoter involved in minus-strand synthesis is indicated with a triangle pointing to the left. Two sequences, the 3' proximal element (3' PE) and 5' proximal element (5' PE) are involved in plus-strand synthesis and are represented by shaded triangles pointing to the right.

minus- and plus-strand synthesis revealed the presence of at least five elements that are required for or enhance accumulation of satC. The plus-strand contains a 3'-terminal 29-nt hairpin promoter that is sufficient for complementary strand synthesis *in vitro* (Song and Simon, 1995). Site-specific mutagenesis and *in vivo* genetic selection (SELEX) revealed a role for both sequence and structure of the hairpin in directing minus-strand synthesis *in vivo* (Stupina and Simon, 1997; Carpenter and Simon, 1998). Four elements on minus-strand satC have been identified as important for plus-strand synthesis. At the 3' end of minus strands is a 6-nt sequence [Carmovirus Consensus Sequence or CCS (C_{2-3} A/U A/U A/U)] that is conserved among viruses of the genus *Carmovirus* and is required for plus-strand satC synthesis *in vivo* (Guan *et al.*, 2000a). 5' of this sequence in satC minus strands (positions 11 to 21) is an 11-nt sequence called the 3' proximal element (3' PE) that also contains a CCS and can serve as an independent promoter for complementary-strand synthesis *in vitro* (Guan *et al.*, 1997, 2000a). A second sequence in satC minus strands called the 5' proximal element or 5' PE (positions 302 to 315) can also function as an independent promoter *in vitro* (Guan *et al.*, 1997), is highly sequence specific, and is required for plus-strand synthesis *in vivo* (Guan *et al.*, 2000b).

A fourth element on minus strands of satC important for plus-strand synthesis is a 30-nt hairpin (positions 180 to 209), denoted the motif1-hairpin, which was initially identified as a hot spot for recombination with satD during plus-strand synthesis *in vivo* (Cascone *et al.*, 1993). The generation of recombinant RNA molecules likely occurs by a template switching mechanism mediated by the presence of the motif1-hairpin (Cascone *et al.*, 1990, 1993). TCV RdRp is also capable of extension of self-primed templates *in vitro* if the 3' end is basepaired in the vicinity of the motif1-hairpin (Nagy *et al.*, 1998), a

The motif1-hairpin (indicated as mot1) is denoted by a triangle pointing upward. (B) Sequences used to replace the motif1-hairpin in satC. The sequences are all shown in 3' to 5' orientation since they are from minus strands of satC. The sequence and structure of the motif1-hairpin and its flanking regions in minus strands of satC (Carpenter *et al.*, 1995) are shown on the top. The motif1-hairpin contains a CCS element (gray-circled region) similar to the CCS at the 3' terminus of satC and TCV minus strands (Guan *et al.*, 2000a). In addition, the motif1-hairpin contains an 8-nt sequence that is also present in the 5' PE (circled). Nucleotides shown in black boxes in the mutant SL1/mot1 and SL2/mot1 sequences differ from wt. (C) Levels of satC plus strands were measured by RNA gel blot analysis of total RNA extracted from *Arabidopsis* protoplasts inoculated with the shown satRNAs in the presence of TCV. Time points (in hours postinoculation) for sampling are shown above the lanes. M indicates the position of the template (monomer)-sized satRNAs, while D denotes dimers that are generated during infection and are only weakly visible in this gel blot (Carpenter *et al.*, 1991). (D) Graphical presentation of the relative levels of monomeric-sized satC's from three independent experiments such as that shown in (C).

reaction analogous to recombination *in vivo*. Such *in vitro* and *in vivo* processes are thought to require the motif1-hairpin (Cascone *et al.*, 1993; Nagy *et al.*, 1998) to recruit the RdRp to the minus strand of satC. Binding of the RdRp to the motif1-hairpin may occur based on competition experiments between a template capable of being extended by the RdRp and wt and mutant motif1-hairpins. Such experiments demonstrated that the wt motif1-hairpin was a better competitor than two mutated motif1-hairpins or tRNA (Nagy *et al.*, 1998). More recently, the motif1-hairpin was found to function as an RNA replication enhancer for accumulation of satC monomers, but not head-to-tail dimers, as dimer levels were largely unaffected by deletion of the motif1-hairpin (Nagy *et al.*, 1999). The motif1-hairpin (all references to the motif1-hairpin refer to its natural minus-sense orientation except when noted) stimulated plus-strand RNA accumulation sixfold when inserted into a poorly accumulating satRNA in single cells of *Arabidopsis*, without affecting the stability of the RNAs. The motif1-hairpin also stimulated RNA synthesis by 10-fold from the 3' PE promoter in an *in vitro* assay using partially purified TCV RdRp (Nagy *et al.*, 1999). These data support a direct role for the motif1-hairpin in satC replication.

In this paper, we confirm a role for the motif1-hairpin in satC monomer accumulation *in vivo* and determine that flanking single-stranded sequences are also important for satRNA accumulation. In addition, we have determined that the motif1-hairpin in either orientation is only a weak enhancer of the hairpin promoter at the 3' end of satC plus strands *in vitro*, extending our previous suggestion that the motif1-hairpin functions mainly during plus-strand synthesis.

RESULTS

Replacement of the motif1-hairpin with random sequences or alteration of several nucleotides reduces the level of satC replication *in vivo*

Previous *in vivo* studies revealed that deletion of the motif1-hairpin reduced satC accumulation in protoplasts by 92% at 44 h postinoculation (hpi) (Nagy *et al.*, 1999). Deletion of the motif1-hairpin could have had an indirect effect on satC accumulation since previous studies indicated that the size of a subviral RNA is a significant factor in RNA accumulation (Li and Simon, 1991; Zhang and Simon, 1994). Therefore, satC derivatives were constructed that contained alterations within the motif1-hairpin without changing the overall size of the molecule. To this end, the motif1-hairpin was replaced with two different sequences of the same length (selected at random) or altered only at selective positions (Fig. 1). Accumulation of the resulting constructs in *Arabidopsis* protoplasts when coinoculated with the helper TCV was determined using RNA gel blot analysis. Replacing the motif1-hairpin with either of the two alternate sequences of the same

length (constructs random2 and random6; Fig. 1B) reduced the accumulation of satC by 8- to 13-fold at 44 hpi when compared to wt satC (Figs. 1C and 1D). Introduction of either four or five mismatch mutations within the motif1-hairpin and the flanking sequences, which altered both sequence and the predicted structure of the motif1-hairpin (constructs SL1/mot1 and SL2/mot1, Fig. 1B), reduced the accumulation of the satRNAs by 3- to 10-fold (Figs. 1C and 1D). Interestingly, mutations in the construct SL1/mot1 reduced satC RNA accumulation as much as replacing the motif1-hairpin with random sequences. Mutations in construct SL1/mot1 are predicted to have a less detrimental effect on the motif1-hairpin structure than mutations in SL2/mot1, yet SL1/mot1 has a lower level of accumulation than SL2/mot1. The SL1 mutations are within the CCS of the motif1-hairpin (Fig. 1B), suggesting that the CCS might be important for enhancer function. Several mutations in SL2/mot1 are within the region of 8-nt sequence similarity with the 5' PE, which appears to be of less importance. Together, these data support a role for the sequence of the motif1-hairpin in satC accumulation in protoplasts.

Sequences flanking the motif1-hairpin can affect satC accumulation *in vivo*

Chemical and enzymatic mapping of the solution structure of minus-strand satC in the vicinity of the motif1-hairpin revealed the existence of two short single-stranded regions on either side of the motif1-hairpin (Carpenter *et al.*, 1995). These single-stranded flanking regions together with the motif1-hairpin affect 3'-terminal extension reactions by partially purified TCV RdRp *in vitro* (Nagy and Simon, 1998b). One possibility is that these single-stranded regions facilitate the exposure of the motif1-hairpin to the RdRp. Alternatively, these flanking sequences may be directly involved along with the motif1-hairpin in putative RdRp binding. To test the role of the flanking sequences, termed left and right spacers (Fig. 2A), in the accumulation of satC *in vivo*, portions of these sequences were altered by deletion and mismatch mutations. One nucleotide mutation present at the base of the hairpin within both the right and the left spacer sequences produced construct CAMB (Fig. 2). These alterations are predicted to extend the lower (smaller) stem of the motif1-hairpin by three base pairs (following a bulged adenylate). CAMB supported satC accumulation at the 50% level of the WT (Fig. 2C). Deletion of 8 or 6 nt in the left and right spacers, respectively, in combination with the two single mismatch mutations present in CAMB (constructs Δ L-spacer and Δ R-spacer, respectively, Fig. 2A) reduced the accumulation of satC by 74 and 60% when compared to wt satC (Figs. 2B and 2C). SatC with Δ R-spacer mutations, which are predicted to maintain the motif1-hairpin structure similar to that of CAMB, accumulated just slightly less than CAMB. In

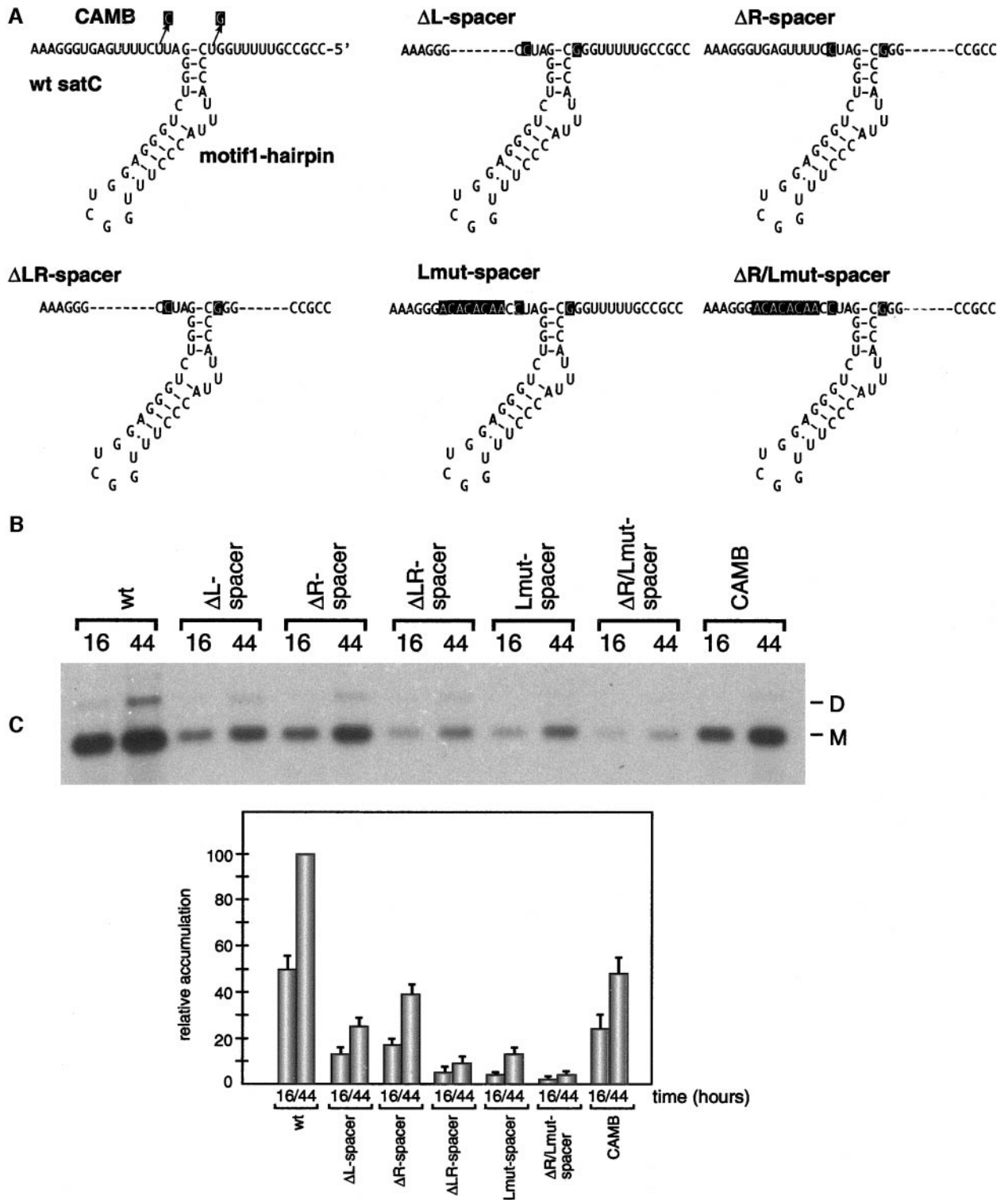


FIG. 2. Effect of the single-stranded sequences flanking the motif1-hairpin on satC accumulation *in vivo*. (A) Sequence deletions and alterations within the flanking sequences. Nucleotides shown in black boxes differ from wt satC. Deletions are denoted by dotted lines. The sequences are shown in 3' to 5' orientation. (B) The levels of satC plus strands were measured by RNA gel blot analysis of total RNA extracted from protoplasts inoculated with TCV and the satRNAs shown in (A). Time points for sampling are shown above the lanes (in hpi). (C) Graphical presentation of the relative monomeric-sized RNA levels from two independent experiments such as that shown in (B).

contrast, mutations in Δ L-spacer are predicted by the Zucker mFOLD RNA structure program (Mathews *et al.*, 1999; Zucker *et al.*, 1999) to shorten the lower stem to

three base pairs. Deleting both the left and the right spacers in construct Δ LR-spacer (Fig. 2A) decreased RNA accumulation to only 9% of wt (Figs. 2B and 2C).

These deletions, however, are predicted to change the structure of the entire motif1-hairpin (not shown). To make less dramatic changes in the structure of the motif1-hairpin, we introduced eight mismatch mutations that changed the U-rich left spacer to an A/C-rich region (construct Lmut-spacer, Fig. 2A) which maintained the folding of the motif1-hairpin similar to that of CAMB. Construct Lmut-spacer supported RNA accumulation at 13% relative to wt (Fig. 2C). When the same mismatch mutations were combined with deletion of the right spacer in construct ΔR /Lmut-spacer, the level of satC accumulation was reduced further ($\sim 4\%$ of wt levels, Figs. 2B and 2C). The structure of the motif1-hairpin in ΔR /Lmut-spacer is predicted to be similar to that present in CAMB (not shown), but the spacer sequences at both sides of the hairpin lack single-stranded U-rich stretches. These data support the possibility that the sequence of the spacers flanking the motif-hairpin are important for full enhancer activity. Taken together, these results demonstrate that the single-stranded sequences flanking the motif1-hairpin can significantly influence satC accumulation in protoplasts.

Testing strand-specificity of stimulation of RNA synthesis by the motif1-hairpin *in vitro*

The above experiments did not address whether the motif1-hairpin enhancer stimulated plus- or minus-strand RNA synthesis, or both. Previous *in vitro* work with partially purified TCV RdRp indicated that the motif1-hairpin when connected to the 11 nt, linear 3' PE (normally located near the 3' end of satC minus strands and able to serve as an independent promoter *in vitro*; Fig. 1A) increased the level of RNA synthesis directed by the 3' PE alone by 9.8-fold (Nagy *et al.*, 1999). To test whether the motif1-hairpin can also stimulate RNA synthesis from the 3'-terminal hairpin promoter on satC plus strands, the motif1-hairpin was connected to the hairpin promoter, as shown in Fig. 3A. This promoter contains a 21-nt stem-loop and a 6-nt single-stranded tail (Fig. 3A, see Song and Simon, 1995; Stupina and Simon, 1997; Carpenter and Simon, 1998). Identical amounts of PAGE/urea gel-isolated template RNAs were used to program an *in vitro* reaction with a partially purified, template-dependent TCV RdRp preparation (Song and Simon, 1994). Half of the RNA products were treated with S1 nuclease to differentiate between *de novo* initiation and 3'-terminal extension (Nagy *et al.*, 1998), which produces partially double-stranded products. Comparison of the template-sized and S1 resistant radiolabeled RNA products in 5% PAGE/urea gels indicated that the motif1-hairpin in its natural minus-sense orientation (construct mot1-pr) enhanced transcription by approximately twofold (normalized for radioactive nucleotide incorporation) compared with control constructs Control1-pr and Control2-pr, which lack the motif1-hairpin (Fig. 3B). To test

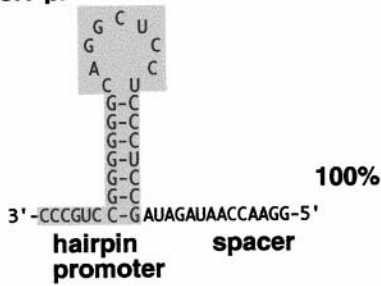
whether the approximately twofold increase in the amount of RNA synthesis with the motif1-hairpin can be achieved with other hairpins, five different stem-loop structures were connected individually to the above core hairpin promoter (Fig. 3A). Construct mot1forw-pr with the motif1-hairpin in the forward (plus-strand) orientation only stimulated RNA synthesis by 20% (Fig. 3A). In contrast, RNA synthesis was reduced by 15% when a mutated motif1-hairpin with a tetraloop and a symmetrical internal loop replaced the motif1-hairpin (see mutmot1-pr, Fig. 3A). Construct GC-pr containing an unusually stable hairpin with 10 C-G pairs and a UCGG tetraloop or construct AU-pr containing a stem-loop with 10 A-U pairs that is stabilized by a UCGG tetraloop also showed 9 to 31% less RNA synthesis when compared to Control1-pr (Fig. 3B). Construct ministem-pr with only three G-C pairs and a UCGG tetraloop showed a 25% increased level of RNA synthesis when compared to Control1-pr (Figs. 3A and 3B). Altogether, these experiments demonstrate that the motif1-hairpin in its natural minus-sense orientation has the greatest enhancing effect on transcription from the hairpin promoter normally found at the 3' end of plus-strand satC. Furthermore, the modest level of transcription enhancement by the motif1-hairpin, in either orientation, compared with the 9.8-fold stimulation of plus-strand synthesis by the motif1-hairpin from a linear promoter from satC minus strands (Nagy *et al.*, 1999) suggests that the motif1-hairpin naturally functions to enhance plus-strand synthesis of satC *in vivo*.

DISCUSSION

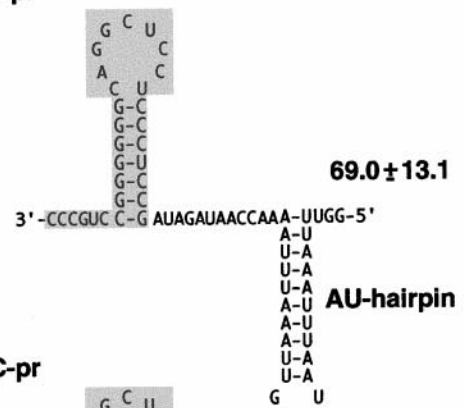
Current models of satC replication and recombination in the TCV system suggest a central role for the motif1-hairpin in both primer-independent and primer-dependent RNA synthesis. *In vivo*, primer-independent RNA synthesis results in the production of full-length complementary RNAs, while primer-dependent RNA synthesis can lead to the formation of recombinant RNAs. Mutations within the motif1-hairpin or its replacement with random sequences also inhibited both primer-independent (*de novo*) and primer-dependent (3'-terminal extension) RNA synthesis *in vitro* (Nagy *et al.*, 1999; this study). A mutated motif1-hairpin (e.g., mutmot1-pr, Fig. 3A) and three other heterologous hairpins failed to enhance RNA synthesis *in vitro*, suggesting a specific role for the motif1-hairpin. In addition, the hairpin contains motifs (CCS and 3' CUGGGAGG) found in the short linear 3' and 5' PEs, respectively. Since the 3' and 5' PEs can function as independent promoters *in vitro* (Guan *et al.*, 1997), they must contain recognition sequences for the RdRp (or for a factor that recruits the RdRp). Recent analysis of the motif1-hairpin using an *in vivo* genetic selection (*in vivo* SELEX) approach revealed that the hairpin could be replaced with multiple short motifs found in TCV replication-associated sequences (G. Zhang and A. E. Simon,

A

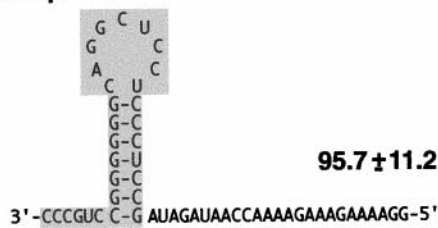
Control1-pr



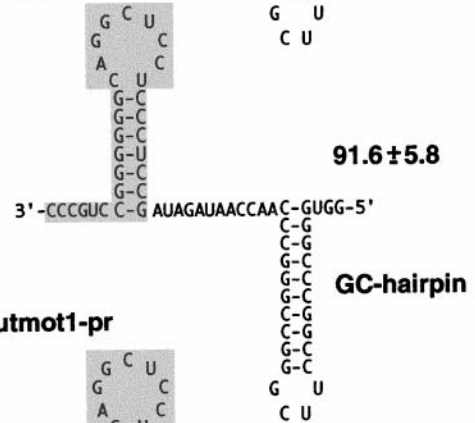
AU-pr



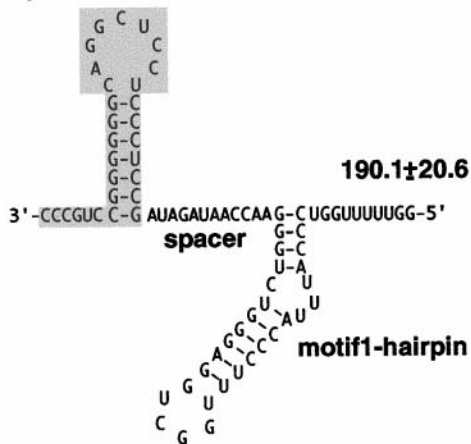
Control2-pr



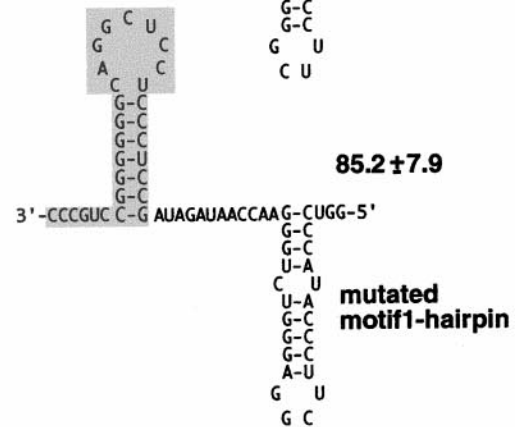
GC-pr



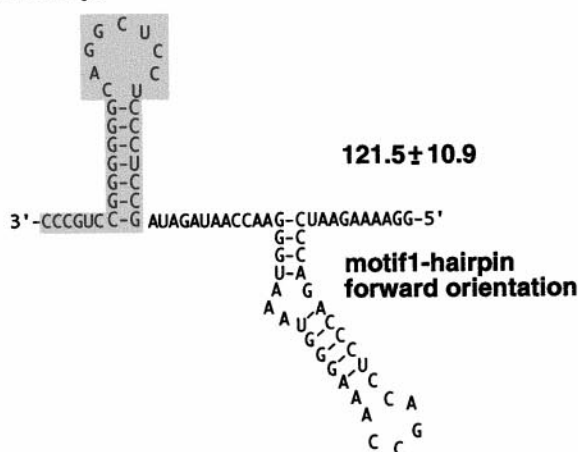
mot1-pr



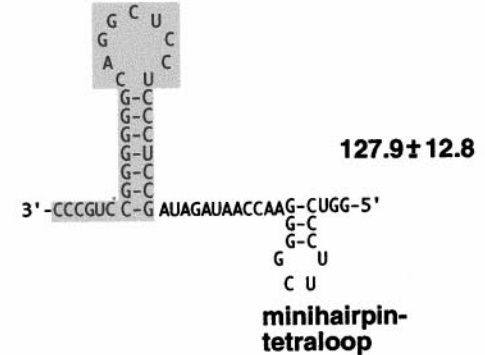
mutmot1-pr



mot1forw-pr



ministem-pr



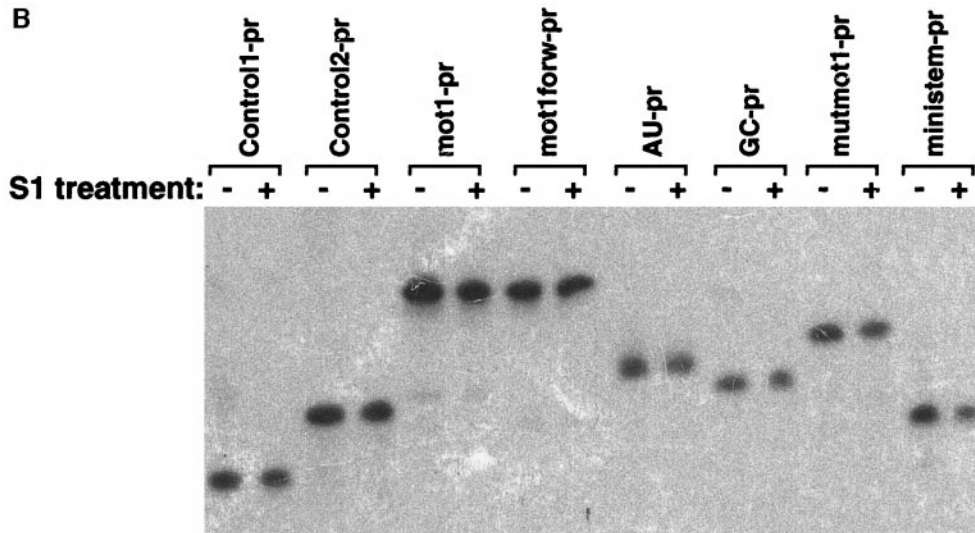


FIG. 3—Continued

unpublished data). The proposed role of the motif1-hairpin in the above processes therefore is likely in recruitment of the RdRp. The data presented here and in previous papers (Nagy *et al.*, 1998; Nagy and Simon, 1998a) are consistent with this model. Furthermore, a competitor RNA that contained the wt motif1-hairpin was the best inhibitor of 3'-terminal extension (Nagy *et al.*, 1998), supporting a role for the motif1-hairpin in RdRp binding (recruitment).

Internally located RNA elements important for virus replication are being discovered in an increasing number of RNA viruses. A 61-nt stem loop within the coding sequence of poliovirus serves as a template for VPg uridylylation *in vitro* and is required for replication *in vivo* (Goodfellow *et al.*, 2000; Paul *et al.*, 2000; Rieder *et al.*, 2000). This element, which is position independent and bound by the poliovirus polymerase precursor 3CD^{pro}, shares a conserved sequence motif with other recently discovered interior elements required for the replication of rhinovirus type 14 (McKnight and Lemon, 1996) and Theiler's virus (Lobert *et al.*, 1999). Intergenic sequences of BMV RNA 3 stimulate minus-strand synthesis by ~100-fold (Quadt *et al.*, 1995) by nucleating the assembly of a functional replicase complex and stabilizing the RNA, events probably connected with the switch from translation to transcription (Quadt *et al.*, 1995; Sullivan and Ahlquist, 1999). While the poliovirus and BMV inter-

nal elements function during minus-strand synthesis, other elements, such as the 68-nt hairpin in the 3' UTR of the coronavirus mouse hepatitis, appear to function during synthesis of plus-strand genomic, and possibly sub-genomic, RNAs (Hsue and Masters, 1997; Lui *et al.*, 1997). Putative RNA replication enhancers have also been characterized for the double-stranded L-A virus of yeast (Esteban *et al.*, 1989), alfalfa mosaic virus (van Rossum *et al.*, 1997), tomato bushy stunt virus (Ray and White, 1999), hepatitis delta virus (Wang *et al.*, 1997), flock house virus (Ball and Li, 1993), and Q β bacteriophage (Barrera *et al.*, 1993; Schuppli *et al.*, 1998).

The motif1-hairpin of satC appears to function during plus-strand synthesis. We previously showed that the motif1-hairpin's role in RNA recombination *in vivo* or primer extension *in vitro* was associated with its orientation in minus strands of satC (Cascone *et al.*, 1993; Nagy *et al.*, 1998, 1999). This determination led to the original hypothesis that, by attracting the RdRp, the hairpin might be an enhancer of plus-strand satC synthesis. Our current results support this hypothesis by revealing that the satC hairpin promoter located at the 3' end of plus strands is much less responsive (1.9-fold increase) to the presence of the motif1-hairpin in its plus-strand orientation than is a linear promoter element (the 3' PE) from satC minus strands when associated with the hairpin in its minus-sense orientation (9.8-fold increase).

FIG. 3. Effect of the motif1-hairpin on RNA synthesis from the satC minus-strand initiation promoter *in vitro*. (A) Schematic representation of RNA constructs used. Sequences and predicted structures are shown in the 3' to 5' orientation to match the presentation in earlier figures. Shaded sequence represents a previously characterized promoter located at the 3' end of satC plus strands (Song and Simon, 1995). The relative normalized activities of constructs, which were based on analysis of denaturing PAGE, followed by autoradiography and densitometry, are shown on the right. The data were normalized based on the amount of template-directed radioactive UTP incorporated and the molar amounts of templates used. (B) A representative denaturing gel analysis of radiolabeled RNA products synthesized by *in vitro* transcription with TCV RdRp. Lanes depicted by – and + denote products that were not treated, or treated by S1 nuclease, respectively.

Moreover, when associated with the hairpin promoter from satC plus strands, the plus-stranded motif1-hairpin stimulated RNA synthesis *in vitro* at only 60% of the level obtained with the minus-stranded motif1-hairpin (Fig. 3A). Similar results were obtained using the linear 3' PE promoter (Nagy *et al.*, 1999). Thus, the *in vitro* data support the hypothesis that the motif1-hairpin mainly functions during plus-strand synthesis. In addition, the ability of the motif1-hairpin to serve as a hotspot for recombination between satD and satC (which requires the fully functional replicase) suggests that the hairpin is not required for assembling a functional replicase, one role of the enhancer in the intergenic region of BMV RNA 3 (Quadt *et al.*, 1995). Furthermore, based on the lack of discernible degradation of any template RNAs in this study, deletion of the motif1-hairpin did not affect the stability of the RNA *in vitro*, unlike deletion of the intergenic region of BMV (Sullivan and Ahlquist, 1999).

It currently is not known why the linear promoter is more responsive to the presence of the motif1-hairpin enhancer than is the hairpin promoter. Our model suggests that the motif1-hairpin binds to the RdRp and then transfers the enzyme, in an unknown manner, to the 3' promoter. One possibility is that the hairpin promoter has a greater affinity for the RdRp than either the motif1-hairpin or the 3' PE promoter, thus relying less on a separate element to attract the RdRp to the template. Alternatively, the transfer of the RdRp from the enhancer to the promoter and subsequent alignment of the 3' hydroxyl with the enzyme's active site may be structurally constricted by the presence of the hairpin. Interestingly, the effect of artificial hairpins on transcription from the 3' PE (Nagy *et al.*, 1999) and the hairpin promoter (Fig. 3) differed considerably. Artificial hairpins connected to the 3' PE substantially reduced transcription and led to the synthesis of small, aberrantly sized products (Nagy *et al.*, 1999). The same artificial hairpins when associated with the hairpin promoter only marginally reduced transcription and the products were of the expected, full-length size (Fig. 3B). These results altogether suggest that recruitment of the RdRp by the hairpin and linear promoters in the presence of the motif1-hairpin or artificial hairpins differs in some fundamental fashion.

A role has now been found for sequences flanking the motif1-hairpin in RNA accumulation in protoplasts. The sequences flanking the hairpin are single-stranded in minus-stranded satC (Carpenter *et al.*, 1995). Deletion of either the left or the right flanking sequences inhibited satC accumulation in protoplasts with constructs containing deletions or sequence modifications of both flanking regions having a greater detrimental effect (Fig. 2B). This result supports previous results obtained from 3'-terminal extension reactions *in vitro* (Nagy and Simon, 1998b). The most detrimental effect on RNA accumulation was caused by deleting one spacer and changing the other to a A/C-rich sequence (construct Δ R/Lmut-

spacer, Fig. 2A). In this construct, the minus-stranded RNA is still predicted to form the motif1-hairpin (with a three base-pair extension of the smaller stem following an adenylate bulge, similar to that in CAMB) with the hairpin flanked by short single-stranded spacers. This construct, however, does not contain single-stranded U-rich stretches flanking the hairpin region. The fact that construct Δ R/Lmut-spacer accumulated as poorly as when random sequence replaced the motif1-hairpin (random 2 and random 6, Fig. 1A) suggests that the role of the flanking sequences may be equally important as that of the hairpin in satC accumulation. A single-stranded poly(U) spacer present on minus-stranded RNA3 of BMV, was also proposed to enhance subgenomic synthesis, possibly by facilitating access of the replicase to the promoter (French and Ahlquist, 1988; Marsh *et al.*, 1988). While these results support a role for the single-stranded U-rich stretches in satC accumulation, we cannot discount the possibility that the mutant satC folds differently *in vivo* (either plus or minus strands) from its computer-predicted structure, thus disrupting structural elements required for replication.

MATERIALS AND METHODS

RNA template construction

RNA templates for protoplast inoculation were obtained by *in vitro* transcription with T7 RNA polymerase using pTCV66, which contains a full-length cDNA of TCV, and pT7C(+) (Song and Simon, 1994), a full-length cDNA construct of wt satC, and its derivatives.

SL1/mot1 was generated by a three-step method. First, a 5' PCR fragment was obtained using primers T7C5' (Song and Simon, 1994) and mot1(+mut) (5'-CATGGGGCCCGTTTGCCTTTTGGCTGGAGG-3') on the pCAMB template (Cascone *et al.*, 1993), followed by treatment with *Apa*I and gel purification. Second, a 3' PCR fragment was obtained using primers T7C5' and C3' (Song and Simon, 1994), followed by treatment with *Apa*I and gel purification. The 3' and 5' PCR products were ligated together, followed by PCR amplification of the full-length cDNA with end primers T7C5' and C3'. The resulting PCR product was cloned into *Sma*I-digested pUC19. Construct SL2/mot1 was generated like SL1/mot1, except the primers used for the 5' PCR fragment were T7C5' and mot1(-mut) (5'-CATGGGGC-CATTACCCTTGGCTGGAAGGCCCGGGATCCTTTTG-3').

Construct Δ L-spacer was obtained as follows. First, a 5' PCR fragment was generated using primers T7C5' and 5'del-spacer (5'-TCTGGGATCCGGAAACAGCCAG-GTTTTAC-3') on the pCAMB template, followed by treatment with *Bam*HI. Second, a 3' fragment was obtained by digestion of pCAMB with *Bam*HI and *Sma*I. Both the 5' PCR fragment and the 3' fragment were gel purified, ligated together, and used as template to am-

plify full-length cDNA by PCR using end primers T7C5' and C3'. Δ R-spacer was obtained as follows. First, a 3' PCR fragment was generated with primers C3' and 3'del-spacer (5'-CATGGGGCCCGGCGGCAGCACCGTC-TAGCTG-3') on the pCAMB template, followed by treatment with *Apal* and *SpeI*. The resulting gel-purified 3' PCR fragment was used to replace the *Apal* and *SpeI* region in T7CAMB (pCAMB with a T7 promoter). Δ LR-spacer was obtained as Δ L-spacer, except the 3' fragment was obtained by digestion of Δ R-spacer with *Bam*HI and *Sma*I. Lmut-spacer was obtained using a three-step method. First, a 5' PCR fragment was generated with primers T7C5' and 5'spacer+AC (5'-TCTGGG-GATCCAACACACAGGGAAACAGCCAGGTTTTTCAC-3') on pCAMB template, followed by treatment with *Bam*HI. Second, a 3' fragment was obtained by digestion of pCAMB with *Bam*HI and *Sma*I. Both the 5' PCR fragment and the 3' fragment were gel purified, ligated together, and used as template to amplify the full-length cDNA by PCR with the end primers T7C5' and C3'. The resulting PCR product was cloned into *Sma*I-cut pUC19. Δ R/Lmut-spacer was obtained as was Lmut-spacer, except that the 3' fragment was generated by digestion of Δ R-spacer with *Bam*HI and *Sma*I.

For the *in vitro* experiments, RNA templates were obtained by *in vitro* transcription with T7 RNA polymerase using either PCR-amplified DNA templates or purified and linearized plasmid DNA (Song and Simon, 1994; Nagy *et al.*, 1997). After phenol/chloroform extraction, unincorporated nucleotides were removed by repeated ammonium acetate/isopropanol precipitation (Song and Simon, 1994; Nagy *et al.*, 1997). The obtained RNA transcripts were dissolved in sterile water and their amount and size measured using a UV spectrophotometer and 5% polyacrylamide/8 M urea gel (denaturing PAGE) analysis (Song and Simon, 1994; Nagy *et al.*, 1997).

Control1-pr, Control2-pr, mot1-pr, mot1forw-pr, mut-mot1-pr, GC-pr, AU-pr, and ministem-pr DNAs were obtained by PCR using the same 3' end primer C3' and one of the following 5' primers:

T7+SATC PROM (5'-GTAATACGACTCACTATAGGAACC-AATAGATAGCCTCCC-3'), T7+GA+SATC (5'-GTAATACGACTCACTATAGGAAAAGAAAGAAAACCAATAGATAGCCTCC-C-3'), T7-MOT1+C (5'-GTAATACGACTCACTATAGGTTTTT-GGTCCCATTTACCTTTTGG-CTGGAGGGTCTGGG AACCA-ATAGATAGCCTCCC-3'), T7+MOT1+SATC (5'-GTAATACGACTCACTATAGGAAAAGAATCCCAGA-CCCTCCAGCCAA-AGGGTAAATGGGAACCAATAGATAGCCTCCC-3'), T7+AU/GC+SATC (5'-GTAATACGACTCACTATAGGTCCCATACCC-TTCG-GAGGGTCTGGG AACCAATAGATAGCCTCCC-3'), T7+GC+SATC (5'-GTAATACGACTCACTATAGGTGGGC-CCGGCCTTCGGGCCGGG-CCC AACCAATAGATAGCCTCC-C-3'), T7+AU+SATC (5'-GTAATACGACTCACTATAGGTT-TAAATTTAATTCGTTAAATTTAA AACCAATAGATAGCCTCC-C-3'), T7+MINITETRA+SATC (5'-GTAATACGACTCACTAT-

AGGTCCTTCG-GGG AACCAATAGATAGCCTCCC-3'), respectively, on template T7C(+).

Isolation of *Arabidopsis* protoplasts, inoculation, and RNA gel blots

Protoplasts (5×10^6) prepared from callus cultures of *Arabidopsis* ecotype Col-0 (Kong *et al.*, 1997) were inoculated with 2 μ g satRNA and 20 μ g TCV genomic RNA transcripts. Total RNA extraction from protoplasts, RNA denaturation, and gel blotting were performed as described previously (Kong *et al.*, 1997). Plus strands of satRNAs were detected with oligonucleotide C/D (5'-CTTGACTGATGACCCCTACG-3') labeled with polynucleotide kinase and γ -[32 P]ATP. The ribosomal RNA probe used as a loading control (not shown) has been described previously (Simon *et al.*, 1992).

TCV RdRp assay

Preparation of template-dependent RdRp from TCV-infected turnip plants, *in vitro* transcription reactions, and product analysis was carried out as previously described (Song and Simon, 1994; Nagy *et al.*, 1997, 1998) using a 20- μ l RdRp reaction mixture that contained 3 μ g of template RNA.

After phenol/chloroform extraction and ammonium acetate/isopropanol precipitation, the products were analyzed on a 20-cm-long denaturing 5% PAGE/8 M urea gel, followed by autoradiography and densitometry (Nagy *et al.*, 1997). The RdRp products were treated with S1 nuclease as described previously (Nagy *et al.*, 1998). The data were normalized based on the number of template-directed radioactive UTP incorporated into the RdRp products and the molar amount of the template RNA in the RdRp reaction. For some experiments, the gels were stained with ethidium bromide, photographed, and dried, followed by analysis with a phosphorimager as described (Nagy *et al.*, 1997).

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