

RNA elements required for RNA recombination function as replication enhancers *in vitro* and *in vivo* in a plus-strand RNA virus

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RNA replication requires *cis*-acting elements to recruit the viral RNA-dependent RNA polymerase (RdRp) and facilitate *de novo* initiation of complementary strand synthesis. Hairpins that are hot spots for recombination in the genomic RNA of turnip crinkle virus (TCV) and satellite (sat)-RNA C, a parasitic RNA associated with TCV infections, stimulate RNA synthesis 10-fold from a downstream promoter sequence in an *in vitro* assay using partially purified TCV RdRp. Artificial hairpins had an inhibitory effect on transcription. RNA accumulation in single cells was enhanced 5- to 10-fold when the natural stem-loop structures were inserted into a poorly accumulating sat-RNA. The effect of the stem-loop structures on RNA replication was additive, with insertion of three stem-loop RNA elements increasing sat-RNA accumulation to the greatest extent (25-fold). These stem-loop structures do not influence the stability of the RNAs *in vivo*, but may serve to recruit the RdRp to the template.

Keywords: RNA-dependent RNA polymerase/RNA enhancers/RNA recombination /RNA replication/satellite RNAs

Introduction

Most of the pathogenic viruses of animals and plants are positive-stranded RNA viruses. Despite vast differences in virion morphology, host ranges, symptoms, and organization and expression of genomic RNAs, positive-stranded RNA viruses show striking similarities in replication strategies such as the amino acid sequences of virus-encoded replicases, RNA-dependent RNA polymerases (RdRps). Positive-stranded RNA viruses replicate efficiently in infected cells by a two-step process mediated by the viral RdRp. First, a complementary RNA strand is made from the invading positive-strand RNA template. Secondly, the new complementary (minus) strand serves as a template to produce large quantities of progeny positive-strand RNAs. The replication process is usually asymmetric, leading to a 20- to 100-fold excess of positive strands over minus strands. Despite the importance of RNA replication in the viral life cycle and pathogenesis, biochemical studies on the process of viral replication are still in their earliest stages.

To recognize and then replicate faithfully only the cognate RNA, the viral RdRp must require specific sequences, termed *cis*-acting elements, which are often located at the ends of the RNA (de Graaff and Jaspars, 1994; Buck, 1996). The best known *cis*-acting elements are the viral replication and transcription promoters that are required for initiation of RNA synthesis by specific RdRps. Replication and transcription promoters have been characterized for many viruses including bacterial, fungal, animal and plant viruses (reviewed by de Graaff 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 high-order structures. Another characteristic feature of most viral RdRps is the ability to initiate RNA synthesis *de novo* (i.e. without the need for an RNA primer). Therefore, transcription promoters must have at least two functions: (i) to recruit (bind to) the RdRp; and (ii) to promote complementary RNA synthesis from the initiating nucleotide.

Turnip crinkle virus (TCV) is one of the best characterized positive-stranded RNA viruses (reviewed by Buck, 1996; Simon and Nagy, 1996). It has a small genome (4 kb) with five genes, of which two are 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). sat-RNA D is the smallest sat-RNA at 194 nucleotides (nt) and shares little contiguous sequence similarity with the TCV genomic RNA (Figure 1). An unusual TCV sat-RNA is sat-RNA C, which is formed naturally by recombination between sat-RNA D and two short non-adjacent regions in the 3' region of the TCV genomic RNA (Figure 1).

sat-RNAs provide excellent models for studies on replication, recombination and symptom production due to their small size, lack of open reading frames and plasticity. *In vitro* and *in vivo* analyses of sequences required for minus-strand synthesis of sat-RNA C revealed that the promoter is contained within the 3'-terminal 29 bases of the plus strand (see Figure 1; Song and Simon, 1995; Stupina and Simon, 1997; Carpenter and Simon, 1998). Two separate sequences have been identified in sat-RNA C minus strands that are able to function as independent promoters *in vitro* (Guan *et al.*, 1997). The 3'-proximal element is located 11 bases from the 3' end of the minus strand; a second sequence is located 41 bases from the 5' end (see Figure 1; Guan *et al.*, 1997).

In addition to template-directed complementary RNA synthesis during standard replication, many viral RdRps are capable of template switching leading to the generation of recombinant RNA molecules (Lai, 1992; Nagy and Simon, 1997). *In vitro* and *in vivo* analyses revealed a role for a stable hairpin (termed the motif1-hairpin) located

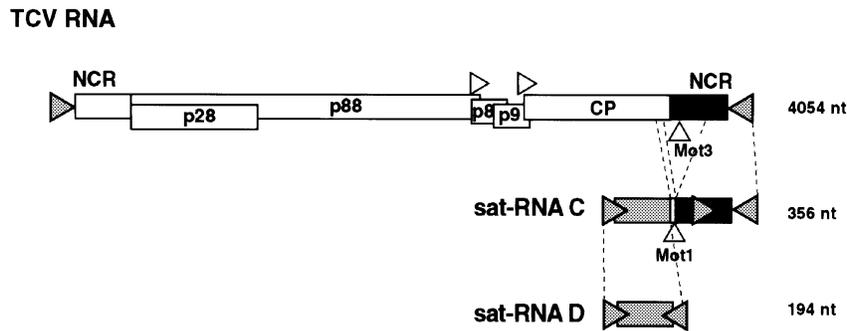


Fig. 1. Location of putative and defined *cis*-acting elements in TCV and its associated sat-RNAs. Promoters involved in minus-strand synthesis are indicated by triangles pointing to the left. Elements important for RdRp-mediated synthesis of plus strands *in vitro* and/or *in vivo* are denoted by shaded triangles pointing to the right. Elements important for plus-strand synthesis of sat-RNA C were defined by an *in vitro* deletion analysis (Guan *et al.*, 1997) and confirmed *in vivo* by site-specific mutagenesis and *in vivo* genetic selection (H.Guan and A.E.Simon, manuscript submitted). Either of the two elements was sufficient to direct complementary strand synthesis of sat-RNA C *in vitro*. sat-RNA D-related regions are shaded gray and TCV-related 3' end regions are in black. The origins of three non-contiguous regions in the chimeric sat-RNA C are enclosed by dotted lines. Subgenomic RNA synthesis promoters on the TCV genomic RNA are depicted by open triangles pointing to the right. Two RNA elements that facilitate RNA recombination, termed motif1-hairpin (indicated as mot1) and motif3-hairpin (mot3) are denoted by triangles pointing upwards. TCV codes for five proteins: p28 and p88 are required for replication; p8 and p9 are required for virus movement; and CP is the 38 kDa coat protein. sat-RNA C and sat-RNA D consist of non-coding sequences.

in minus strands of sat-RNA C in the formation of sat-RNA D/sat-RNA C recombinants (Cascone *et al.*, 1993; Nagy *et al.*, 1998). The possible role of the motif1-hairpin is recruitment of the RdRp to the acceptor minus-stranded sat-RNA C. Binding of the RdRp to the motif1-hairpin may occur, since competition experiments using an *in vitro* (cell-free) system that mimics *in vivo* RNA recombination demonstrated that the wild-type (wt) motif1-hairpin was a better competitor than two mutated motif1-hairpins or unrelated tRNA (Nagy *et al.*, 1998).

A second well characterized RNA recombination system involves sat-RNA D and the TCV genomic RNA (Carpenter *et al.*, 1995; subsequent references to 'TCV' refer to the genomic RNA). A hot spot for recombination is located in the 3' non-coding region of TCV at the base of a stem-loop element, termed the motif3-hairpin. The motif3-hairpin contains two imperfect 24-base tandem repeats that are similar in sequence to the 5' ends of the two TCV subgenomic RNAs. In addition to targeting recombination, the motif3-hairpin is important for viability of the genomic RNA, since deletions that eliminate either of the tandem repeats and extend into the second, either abolish or greatly decrease the accumulation of TCV in plants and protoplasts (Carpenter *et al.*, 1995).

The central role of the motif1- and motif3-hairpins in RNA recombination, and their possible interaction with the TCV RdRp, raise the question of whether these hairpins play *cis*-acting roles in standard replication. We have determined that both the motif1- and motif3-hairpins stimulate RNA synthesis from downstream promoters in an *in vitro* assay that makes use of a partially purified TCV RdRp preparation. In addition, deletion of the motif1-hairpin from sat-RNA C reduced its accumulation by >10-fold in protoplasts of the host plant *Arabidopsis thaliana*. Insertion of the above hairpins into a poorly replicating sat-RNA molecule demonstrated that RNA replication is stimulated by both the motif1- and motif3-hairpins without significantly affecting the stability of the corresponding RNAs in single-cell plant protoplasts. The motif1-hairpin is shown to function in both forward and reverse orientation and its activity is not strictly position-dependent. Based on these results, we propose that these

recombination hot spot elements are RNA replication enhancers that play vital roles in the biology of TCV and its associated RNAs.

Results

A stem-loop RNA element essential for RNA recombination is involved in accumulation of a sat-RNA associated with TCV infections

Previous *in vivo* and *in vitro* studies revealed an essential role for two unrelated hairpin structures (the motif1-hairpin present in sat-RNA C and the motif3-hairpin found in TCV) in facilitating high-frequency recombination between sat-RNA D and either sat-RNA C or TCV (Cascone *et al.*, 1993; Carpenter *et al.*, 1995; Nagy and Simon, 1998a,b; Nagy *et al.*, 1998). All junction sites in sat-RNA D/sat-RNA C recombinants mapped to the base of the motif1-hairpin (Cascone *et al.*, 1990, 1993) and most sat-RNA D/TCV recombinants contained junction sites within or close to the motif3-hairpin (Carpenter *et al.*, 1995). These observations suggested a role for the above hairpin structures in recruitment of the TCV RdRp to the sites of crossovers in the acceptor minus-stranded RNAs (Nagy and Simon, 1997), with recombination occurring during plus-strand synthesis. If the hairpins are involved in recruitment of the RdRp and/or other replication factors, then these hairpins may also play *cis*-acting roles in the replication of sat-RNA C and TCV. In support of this model, extensive deletions within the motif3-hairpin rendered TCV non-infectious in protoplasts and whole turnip plants (Carpenter *et al.*, 1995). However, the latter study did not exclude the possibilities that deletions in the 3' non-coding region of TCV altered its translatability or stability *in vivo*.

To characterize the putative *cis*-acting role of the motif1-hairpin in sat-RNA C accumulation, the hairpin sequence was deleted from wt sat-RNA C, producing Δ mot1 (Figure 2A). Monomeric plus-strand Δ mot1 accumulated at only 8.0% of the wt level of sat-RNA C at 44 h post-inoculation (h.p.i.) (Figure 2B and C). Since minus strands of Δ mot1 were produced in detectable amounts (Figure 2C, right panel) and increased between 16 and 44 h.p.i., Δ mot1

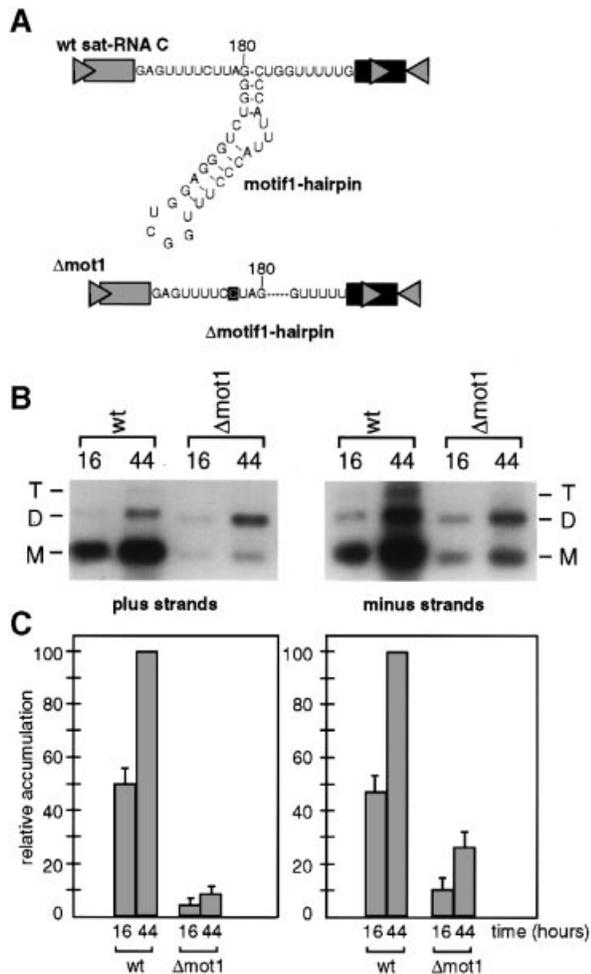


Fig. 2. The motif1-hairpin stimulates RNA accumulation. (A) The sequence and structure of the motif1-hairpin and its flanking regions in minus strands of sat-RNA C (Carpenter *et al.*, 1995) and in Δ mot1. The sequences are shown in 3' to 5' orientation and nucleotide positions were calculated from the 3' end of the minus strand. Symbols are as described in the legend to Figure 1. The shaded nucleotide is an alteration introduced in the cloning of Δ mot1. (B) RNA gel blot analysis of total RNA from *Arabidopsis* protoplasts inoculated with TCV and sat-RNA C or Δ mot1 and incubated for either 16 or 44 h. M indicates the position of the template-(monomer)-sized sat-RNAs, while D and T denote dimers and trimers that are generated during infection (Carpenter *et al.*, 1991). (C) Graphical presentation of the relative RNA levels from experiments such as that shown in (B). The left panel shows the relative accumulation levels of monomeric plus strands, while the right panel shows the relative accumulation levels of monomeric minus strands. The left and right graphs represent data from seven or two independent experiments, respectively.

was replication-competent *in vivo*. Interestingly, the level of sat-RNA dimers in Δ mot1 infections was not affected substantially by the loss of the motif1-hairpin (Figure 2B), suggesting that replication of dimers does not have the same *cis*-sequence requirements as replication of monomers.

To test whether deletion of the motif1-hairpin altered the stability of Δ mot1 compared with wt sat-RNA C, the turnover rates of the two sat-RNAs were examined in protoplasts in the absence of TCV. The results shown in Figure 3A indicate that the two sat-RNAs have similar stabilities in the absence of replication. To exclude the possibility that the remaining undegraded sat-RNAs

survived in the culture media and not inside the protoplasts (the degradation rate may be different inside versus outside the cells), polyethylene glycol (PEG) was omitted during the inoculation step in a control experiment. Omission of PEG resulted in undetectable levels of both sat-RNAs at all time points except the 0 h time point (data not shown). Therefore, the levels of sat-RNAs shown in Figure 3A (between 2 and 12 h.p.i.) reflect undegraded RNAs inside the cells. Altogether, these experiments demonstrate that the *in vivo* survival rates (stability) of wt sat-RNA C and Δ mot1 are similar in the absence of replication. Therefore, the motif1-hairpin does not play a major role in RNA stabilization, but rather may be directly involved in RNA replication.

To examine the effect of the motif1-hairpin on plus-versus minus-strand synthesis during virus replication in protoplasts, the levels of plus and minus strands of wt sat-RNA C and Δ mot1 were measured in the presence of TCV over a period of 12 h. Very low levels of minus strands for both monomeric wt sat-RNAs and Δ mot1 were detected at 2 and 4 h.p.i. (Figure 3B). The most dramatic increase in the level of minus strands occurred between 6 and 9 h.p.i. when the increase was 5- and 4-fold for monomeric wt sat-RNA C and Δ mot1, respectively.

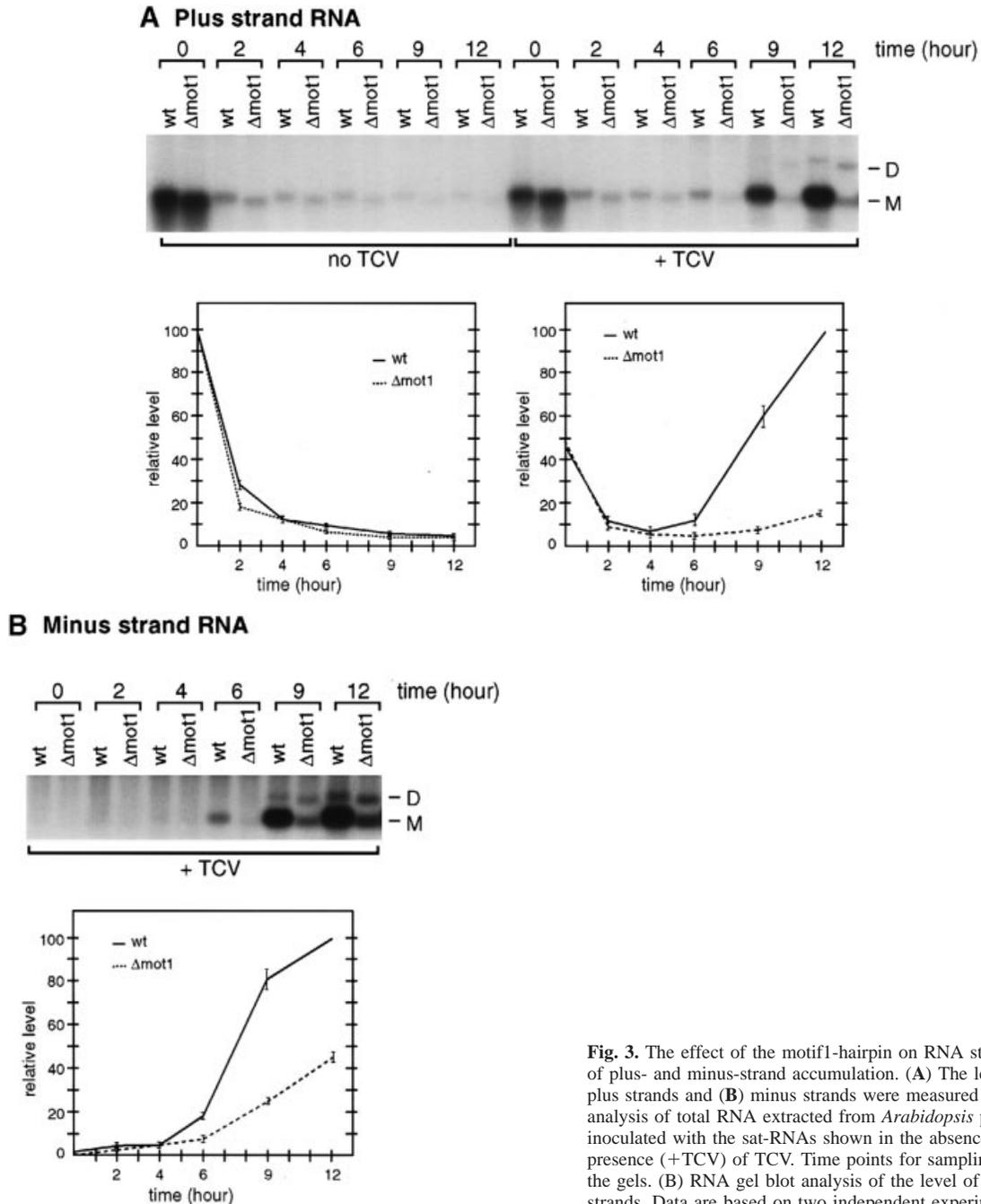
The level of plus strands decreased between 0 and 4 h.p.i. (Figure 3A, right) at a rate similar to that of sat-RNA degradation in the absence of TCV. At 6 h.p.i., the amount of monomeric wt sat-RNA C plus strands increased by 50% over basal levels in repeated experiments. In contrast, the level of Δ mot1 did not increase until 9 h.p.i. Throughout the remainder of the experiment, the absence of motif1-hairpin had a greater effect on the level of plus strands than minus strands, suggesting that the hairpin may affect plus-strand synthesis more than minus-strand synthesis.

The motif1-hairpin can stimulate RNA replication of sat-RNA C *in vivo* when present in forward or reverse orientations and at an alternative location

The above experiments demonstrated that the motif1-hairpin plays a significant role in the accumulation of sat-RNA C but is not absolutely required. This finding suggests that the motif1-hairpin may function like an enhancer of RNA replication, rather than being an essential transcription initiation element. To determine if the motif1-hairpin has properties similar to DNA transcription enhancers, a sat-RNA C mutant with the motif1-hairpin in the reverse orientation was generated (rev/mot1; Figure 4A). rev/mot1 accumulated only slightly less than wt sat-RNA C in protoplasts (66 and 80% of wt levels at 16 and 44 h.p.i., respectively; Figure 4B and C), indicating that the motif1-hairpin functions in either orientation, analogous to DNA transcription enhancers.

To test if the motif1-hairpin can function at locations other than wt, the motif1-hairpin with short single-stranded flanking sequences (required for the functioning of the hairpin; Nagy and Simon, 1998) was repositioned 73 nt 5' of the original location (Mot1-Nco; Figure 5A). While Mot1-Nco accumulated to only 35% of wt sat-RNA C in protoplasts, levels of accumulation were 4-fold higher than for Δ mot1 (Figure 5B and C).

To test the dosage effect of the motif1-hairpin on sat-



RNA C accumulation, a construct with two motif1-hairpins was generated (2xmot1; Figure 5A). While transcripts of wt sat-RNA C and 2xmot1 accumulated to similar levels at 16 h.p.i., wt sat-RNA C exceeded the level of 2xmot1 at 44 h.p.i. (Figure 5B and C). These results suggest that additional copies of the motif1-hairpin do not further enhance the accumulation of sat-RNA C.

Stimulative and additive effects of recombination hot spot hairpins on the replication of sat-RNA in vivo

Since sat-RNA C accumulates to a level comparable to that of 5S rRNA in plants and protoplasts, it may already be replicating at maximal efficiency. Therefore, to examine whether multiple hairpins have an additive or synergistic

effect on replication, a sat-RNA must be used that normally accumulates much more poorly.

To determine whether the motif1-hairpin and two TCV hairpins that are also recombination hot spots (motif3-hairpin and hairpin4) can stimulate the accumulation of a natural but poorly viable sat-RNA, each was inserted into the central portion of sat-RNA CX, a sat-RNA formed by a single recombination event between sat-RNA D and TCV (Carpenter *et al.*, 1995). sat-RNA CX contains the TCV 3' end, with sequences in the promoter region that are similar but not identical to those defined for sat-RNA C (Song and Simon, 1995). sat-RNA CX, which accumulates poorly in protoplasts, also differs from sat-RNA C by lacking the motif1-hairpin (Figure 6A). sat-RNA CX containing either the motif1-hairpin

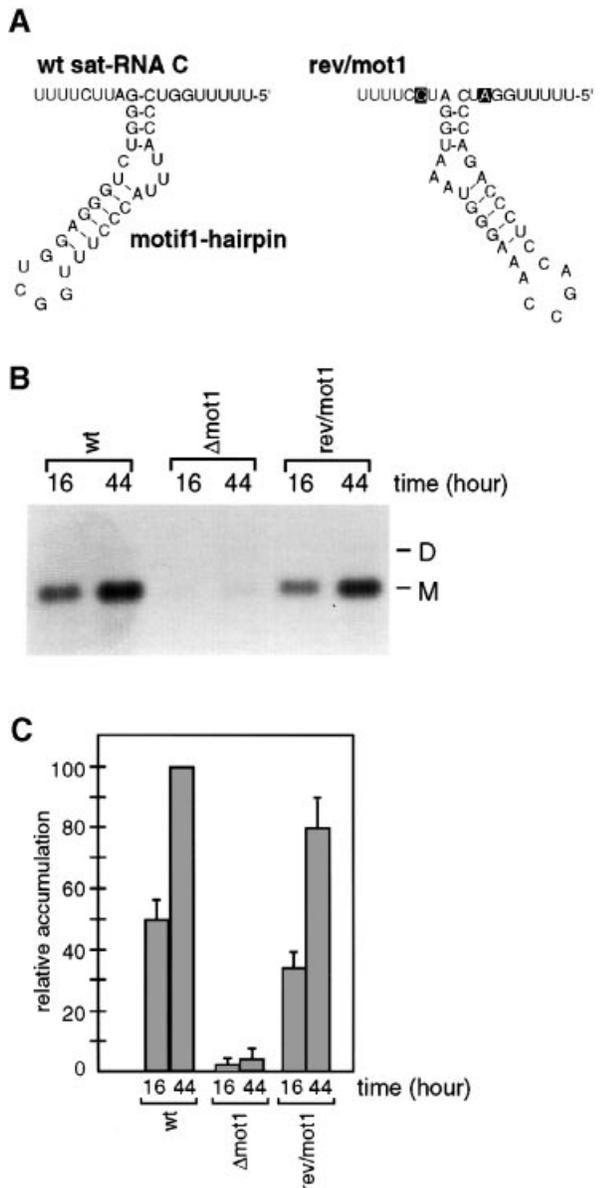


Fig. 4. Motif1-hairpin can stimulate sat-RNA C accumulation when present in either orientation. (A) Sequences and predicted structures of the motif1-hairpin in both forward (wt) and reverse orientations (rev/mot1) in sat-RNA C. Nucleotides shown in black boxes in the flanking regions differ from wt. (B) The level of sat-RNA C plus strands was measured by RNA gel blot analysis of total RNA extracted from protoplasts inoculated with the sat-RNAs shown in the presence of TCV. Time points for sampling are shown above the lanes. (C) Graphical presentation of the relative RNA levels from the gel in (B) and a second independent experiment.

(construct CXM1), motif3-hairpin (construct CXM3) or hairpin4 (construct CXH4) reached levels between 5- and 10-fold higher than sat-RNA CX alone (Figure 6B and C), indicating that all three recombination hot spot hairpins can stimulate the accumulation of a poorly viable sat-RNA.

To test the effect of multiple hairpins on the accumulation of sat-RNA CX, the motif3-hairpin and hairpin4 were introduced into sat-RNA CX to generate CXM3+H4 (Figure 6A). CXM3+H4 accumulated 15-fold better than sat-RNA CX in protoplasts. Insertion of all three hairpins (construct CXM1+M3+H4) supported the highest level of accumulation, 25-fold greater than that of sat-RNA CX

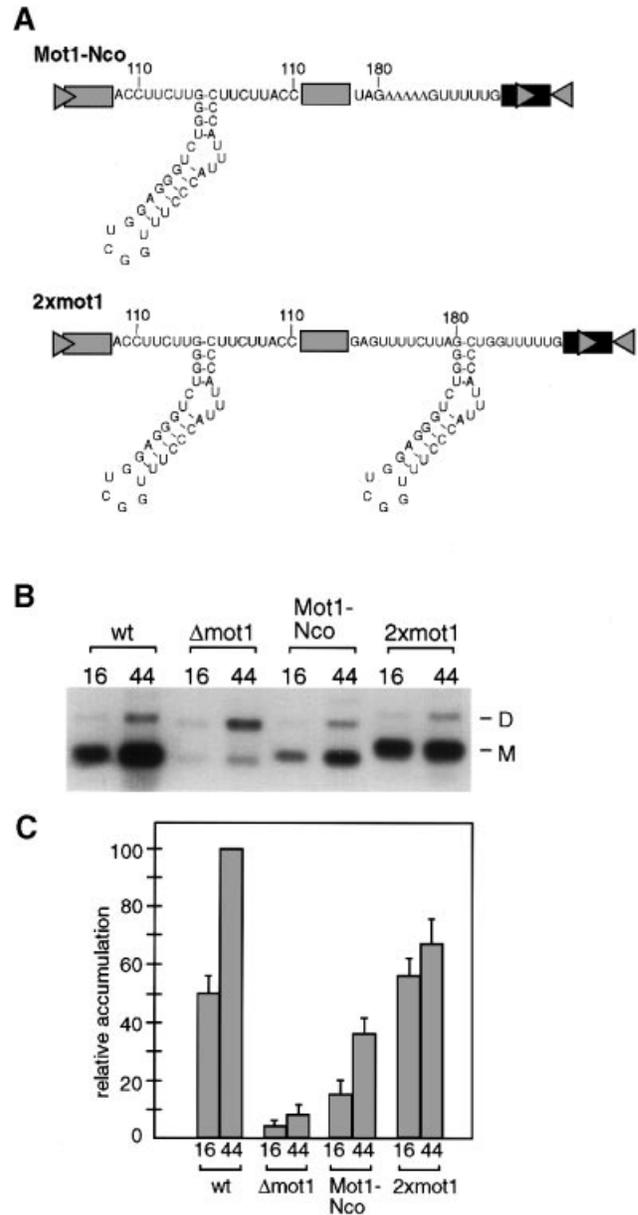


Fig. 5. Motif1-hairpin can stimulate sat-RNA C accumulation when present in a new location. (A) Schematic representation of the site of insertion of the motif1-hairpin and short single-stranded flanking regions into sat-RNA C. The deleted motif1-hairpin at the wt location is shown by five Δ symbols. The sequences are shown in 3' to 5' orientation and nucleotide positions were calculated from the 3' end of the minus strand. Coordinates representing the wt positions in sat-RNA C are shown on top of the constructs. Descriptions of the shading and symbols are given in the legend to Figure 1. (B) The level of monomeric sat-RNA C plus strands was measured by RNA gel blot analysis of total RNA extracted from protoplasts inoculated with the sat-RNAs shown in the presence of TCV. Time points for sampling are shown above the lanes (in h.p.i.). (C) Graphical presentation of the relative RNA levels from the gel in (B) and a second independent experiment.

(Figure 6B and C). These results indicate an additive effect of the hairpins when present in sat-RNA CX. None of the constructs showed increased stability when compared with sat-RNA CX in protoplasts in the absence of the TCV helper virus (data not shown). Altogether, the above experiments suggest that the motif1-hairpin, motif3-hairpin and hairpin4 increase sat-RNA accumulation

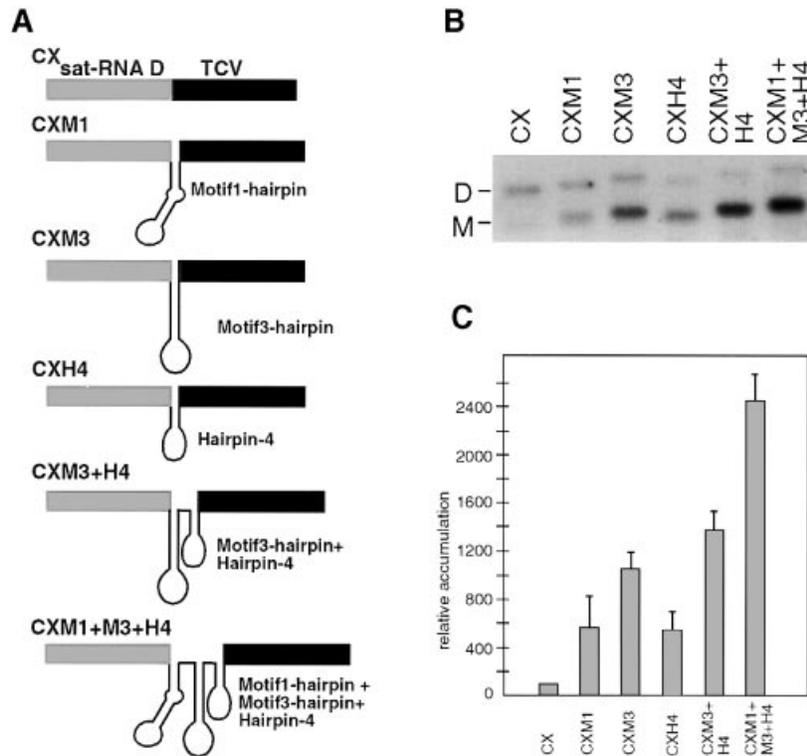


Fig. 6. Stimulative effect of motif1-hairpin, motif3-hairpin and hairpin4 on sat-RNA accumulation *in vivo*. **(A)** Schematic representation of a natural recombinant sat-RNA (termed sat-RNA CX; Carpenter *et al.*, 1995) and its derivatives generated by insertion of the RNA elements shown. **(B)** Levels of sat-RNA CX plus strands accumulating in protoplasts were measured by RNA gel blot analysis of total RNA extracted from protoplasts at 44 h.p.i. **(C)** Graphical presentation of the relative RNA levels from the gel in (B) and two additional independent experiments.

through roles in RNA transcription (replication) rather than by altering the rate of RNA turnover.

Stimulation of RNA synthesis by the motif1- and motif3-hairpins in vitro

In vitro and *in vivo* studies on recombination between sat-RNA D and sat-RNA C suggested a role for the motif1-hairpin in recruitment of the RdRp to the acceptor minus-stranded sat-RNA C (Cascone *et al.*, 1993; Nagy *et al.*, 1998). Putative binding of the TCV RdRp to the minus-stranded motif1-hairpin (Nagy *et al.*, 1998), and the enhancement of sat-RNA replication by the motif1- and motif3-hairpins in protoplasts (see above), suggest that these hairpins may act as general transcription enhancers in the replication of sat-RNAs and TCV, respectively. This, however, cannot be tested *in vitro* using full-length sat-RNA templates, since the partially purified TCV RdRp preparation prefers 3'-terminal extension (self-priming) over *de novo* initiation for full-length constructs (Song and Simon, 1994; P.D.Nagy and A.E.Simon, unpublished results). To circumvent this problem, short RNA templates were constructed that direct efficient *de novo* initiation of RNA synthesis. All these RNA templates contain a core linear plus-strand initiation promoter from sat-RNA C minus strands (12 nt; Guan *et al.*, 1997) at the 3' end and sequences of interest at the 5' end (Figure 7A). Minus-strand sequences representing a promoter for plus-strand synthesis and the motif1-hairpin in the minus-strand orientation were chosen for these studies, since previous results on recombination (Cascone *et al.*, 1993; Nagy and Simon, 1998a; Nagy *et al.*, 1998) and kinetic studies on sat-RNA C accumulation (see Figure 3B) indicate a more

significant role for the motif1-hairpin present on minus-strand sat-RNA C.

Identical amounts of gel-purified template RNAs were used to program an *in vitro* (cell-free) system that makes use of a partially purified, template-dependent TCV RdRp preparation (Song and Simon, 1994). Half of the RNA products were treated with S1 nuclease (data not shown) to differentiate between *de novo* initiation and 3'-terminal extension (Nagy *et al.*, 1998). Comparison of the template-sized and S1-resistant, radiolabeled RNA products in 5% PAGE-urea gels indicated that the motif1-hairpin present in minus-strand orientation supported a 10-fold higher level of complementary RNA synthesis than control constructs lacking the motif1-hairpin (Figure 7A and B, compare mot1+pr and Control1+pr).

To test the effect of the motif3-hairpin on RNA synthesis, its minus-strand sequence alone (construct mot3+pr, Figure 7A) or motif3-hairpin in combination with hairpin4 (construct mot3hairpin4+pr) were introduced 5' of the 12 nt promoter. The resulting constructs (mot3+pr and mot3hairpin4+pr) supported 11- and 13-fold increased transcription, respectively, when compared with Control1+pr (Figure 7A and B). Taken together, these results support a direct role for the motif1-hairpin, motif3-hairpin and hairpin4 in RNA transcription.

To test whether hairpins in general enhance complementary RNA synthesis, five different hairpins were introduced 5' of the promoter in Control1+pr RNA as shown in Figure 7A. The motif1-hairpin in the plus-strand orientation (construct mot1forw+pr) stimulated RNA synthesis by 6-fold over the level obtained with Control1+pr (Figure 7B), supporting previous findings that while the

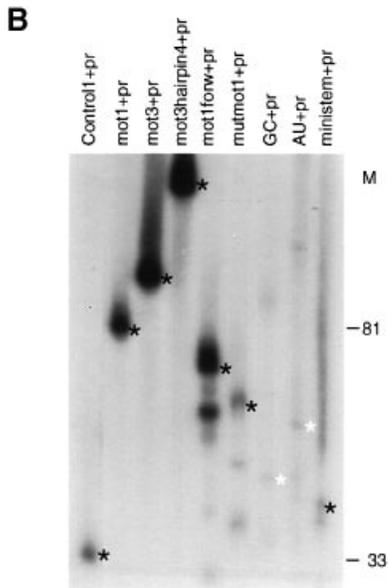
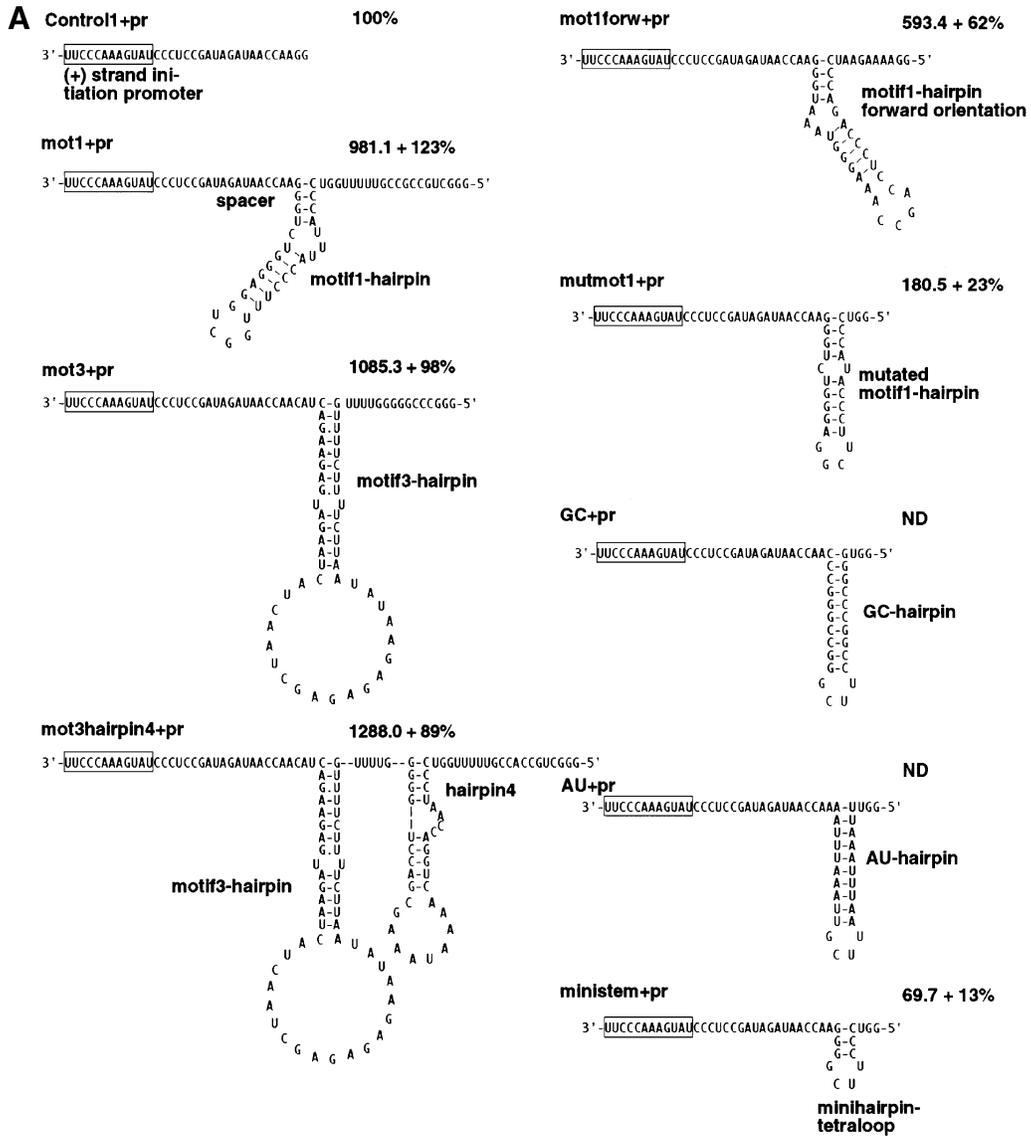


Fig. 7. Stimulative effect of the motif1-hairpin and motif3-hairpin on plus-strand RNA synthesis *in vitro*. **(A)** Schematic representation of RNA constructs used. Sequences and predicted structures are shown in the 3' to 5' orientation since they include a minimal plus-strand initiation promoter derived from sat-RNA C minus strands (Guan *et al.*, 1997). The linear 12 nt promoter sequence is boxed. The relative normalized activities of constructs, which were based on analysis of denaturing PAGE, followed by autoradiography and densitometry, are shown to the right of each construct. The data were normalized based on the number of template-directed radioactive UTP incorporated and the molar amounts of templates used. ND, not determined [due to aberrant RdRp reaction, such as premature termination, see (B)]. **(B)** A representative denaturing gel of radiolabeled RNA products synthesized by *in vitro* transcription with TCV RdRp. M, single-stranded RNA marker (in bases). Template-sized products are denoted by black asterisks. RNAs that migrate aberrantly (much faster than the template-sized RNAs in denaturing gels) are indicated by white asterisks.

hairpin is active in both orientations, activity is greater when present in the minus-sense orientation (Nagy and Simon, 1998; Figure 4).

Construct *mutmot1+pr* contains a hairpin similar to the *motif1*-hairpin in minus-strand orientation, except that the normal six-member loop is replaced by a tetraloop and a deletion of two bases results in a symmetrical internal loop. This construct supported complementary RNA synthesis at a level 2-fold higher than that of *Control1+pr* RNA (Figure 7B). The third construct (*GC+pr*) contains an unusually stable hairpin with 10 G–C base pairs and a UCGG tetraloop. This construct supported RNA synthesis at levels lower than the *Control1+pr* template (Figure 7B). In addition, the RNA products were S1 nuclease-resistant (not shown), but shorter than template-sized. While it is possible that the smaller than expected products were due to premature termination, full-length products were synthesized from a template containing a different promoter sequence and the same GC hairpin (P.D.Nagy and A.E.Simon, unpublished results). The fourth construct (*AU+pr*; Figure 7A) has a stem-loop structure with 10 A–U pairs, which is stabilized by a UCGG tetraloop. This construct also produced a lower level of products than the control construct with no hairpin. Although the products were shorter than template-sized, as with the GC hairpin construct, full-sized products were obtained using a different promoter (P.D.Nagy and A.E.Simon, unpublished results). Since the exact size of the products could not be determined due to the aberrant migration of RNAs with high AU or GC contents on PAGE–urea gels, the level of RNA synthesis could not be measured accurately for these constructs. Nevertheless, the very low amounts of radiolabeled products that were detectable for these constructs suggest that the GC and AU hairpins had an inhibitory effect on transcription (Figure 7B). Construct *ministem+pr*, which contains only three G–C pairs with the UCGG tetraloop (Figure 7A), was also less active than *Control1+pr* RNA (Figure 7B). Altogether, these experiments demonstrate (i) that the *motif1*-hairpin in either orientation and the *motif3*-hairpin are able to stimulate RNA synthesis from a downstream promoter, and (ii) that artificial hairpins inhibit transcription from the same promoter.

Possible mechanisms of stimulation of RNA synthesis by the *motif1*-hairpin

Sequence comparison between known and putative TCV promoter sequences and the *motif1*-hairpin reveals that portions of the *motif1*-hairpin are similar to the 3' end of minus-strand TCV and to a 5'-proximal sequence in minus strands of *sat*-RNA C, which is known to function as a positive-strand initiation promoter *in vitro* (Cascone *et al.*, 1990; Guan *et al.*, 1997; H.Guan and A.E.Simon, unpublished results). Portions of the *motif3*-hairpin sequence and its upstream flanking region on the left side are similar to the two subgenomic RNA promoters located on TCV minus strands (Zhang *et al.*, 1991). Previous studies on the *motif1*-hairpin revealed that it facilitates 3'-terminal extension ('self'-priming-dependent reaction), while in contrast to promoters, it does not support *de novo* initiation (primer-independent reaction) (Nagy and Simon, 1998a,b; Nagy *et al.*, 1998). It is possible that the *motif1*-hairpin and flanking sequences cannot function as an

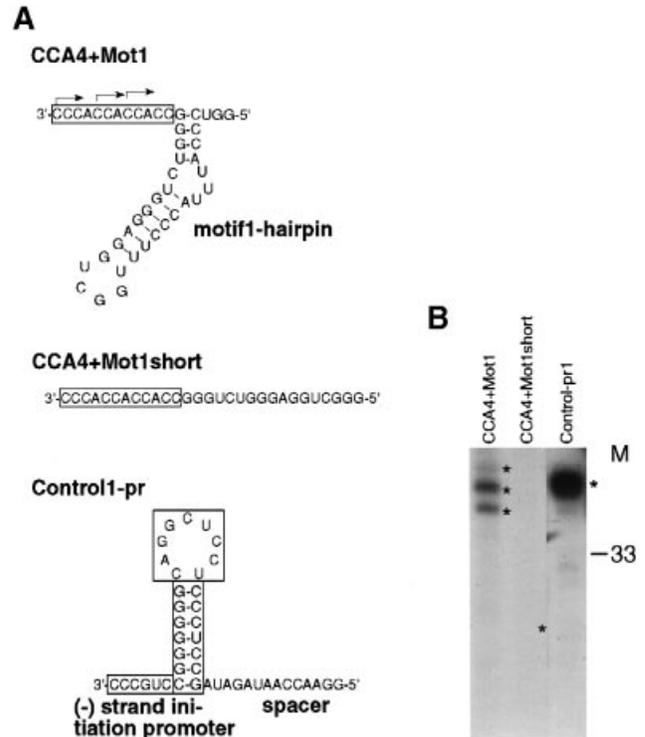


Fig. 8. *De novo* initiation of RNA synthesis in the presence of initiating sequences and the *motif1*-hairpin. (A) Sequences and predicted structures are shown in 3' to 5' orientation since the *motif1*-hairpin is present in minus-strand orientation. Arrows indicate sites of initiation for CCA4+Mot1. Boxed sequences in CCA4+Mot1 and CCA4+Mot1short represent putative initiator sequences, such as CCC and CC. The 29 nt stem-loop promoter derived from plus strands of *sat*-RNA C (Song and Simon, 1995) is boxed in Control1-pr. (B) A representative denaturing gel analysis of radiolabeled RNA products synthesized by *in vitro* transcription with TCV RdRp. Bands depicted by asterisks represent *de novo* initiated RNA products. M, single-stranded RNA marker (in bases).

independent promoter because it lacks sequences capable of directing *de novo* initiation of RNA synthesis.

TCV and its *sat*-RNAs have three C residues at all initiation start sites that are usually not base paired (Song and Simon, 1995; Guan *et al.*, 1997; Wang and Simon, 1997). To test whether the *motif1*-hairpin can stimulate RNA synthesis that starts from 3'-located single-stranded CCC or CC sites (in the absence of known TCV promoters), construct CCA4+Mot1 with four possible start sites was generated and tested for activity *in vitro* (Figure 8A). Three S1-resistant RNA products were obtained (Figure 8B) that correspond to initiation at three out of the four possible CC or CCC sites based on the size of the products, as indicated schematically in Figure 8A. A control construct that carried the same four possible start sites at the 3' terminus that are present in CCA4+Mot1, plus a short 5' flanking sequence, did not direct detectable RNA synthesis (construct CCA4+Mot1short; Figure 8B). The second control construct was Control1-pr that contains the wt *sat*-RNA C promoter for minus-strand synthesis along with the natural CCC start site (Figure 8A). This RNA directed the synthesis of a single complementary RNA product that was present in a 4-fold higher amount than the combined amounts of products obtained with CCA4+Mot1. Altogether, these experiments demonstrate that the *motif1*-hairpin can function like a promoter

if there are single-stranded initiating sequences 3' of the hairpin.

Discussion

The discovery of novel *cis*-acting elements, termed RNA replication enhancers, in the TCV system has major implications for TCV in particular, and possibly for RNA viruses in general. First, the presence of RNA replication enhancers in TCV and sat-RNA C suggests that RNA replication promoters are organized in a 'modular fashion' that consists of an RNA replication enhancer and an initiation sequence. Secondly, RNA replication enhancers may play central roles in RNA recombination, viral evolution and adaptation. These points are discussed separately below.

Hairpins required for RNA recombination are involved in replication

We established previously that the motif1-hairpin, motif3-hairpin and hairpin4 play important roles in targeting RNA recombination *in vivo* (Cascone *et al.*, 1990; Carpenter *et al.*, 1995) and *in vitro* (Nagy *et al.*, 1998). That these hairpins can also affect sat-RNA replication is supported by the 5- to 10-fold higher levels of sat-RNA CX accumulation in protoplasts when these elements are present (Figure 6). In addition, deletion of the motif1-hairpin in sat-RNA C reduces its accumulation by >10-fold in protoplasts (Figures 2 and 3), while deletion of large portions of the motif3-hairpin in TCV makes the RNA non-viable in turnip plants and reduces its accumulation to non-detectable levels in protoplasts (Carpenter *et al.*, 1995). Since accumulation in protoplasts is also a function of the stability of the templates, the possibility existed that the hairpins helped stabilize the sat-RNAs. However, the rate of RNA degradation of the input RNAs that carried or lacked the motif1-hairpin was similar in the absence of the TCV helper virus, suggesting that the hairpins are involved in enhancing replication and not stability (Figure 3A; data not shown). However, because the intercellular location of sat-RNA C may differ in cells containing TCV RdRp and in cells with no replicase present, we cannot exclude the possibility that the motif1-hairpin plays a role in RNA stabilization by influencing template selection during replication. A *cis*-acting element for brome mosaic virus (BMV) was demonstrated to increase RNA stability only in the presence of the 1a protein (Sullivan and Ahlquist, 1999).

A direct role for the motif1- and motif3-hairpins in RNA synthesis would explain the increased amount of RdRp products observed *in vitro* using a partially purified TCV RdRp preparation. Both hairpins enhanced transcription from a TCV RdRp promoter sequence by ~10-fold. The elevated level of RdRp products obtained *in vitro* in the presence of these hairpins can result from either an increased rate of initiation of RNA synthesis, increased processivity of the RdRp, increased rate of termination of RNA synthesis followed by reuse of released RdRps or a combination of these processes. Our data also suggest that the motif1-hairpin plays a strand-specific role in RNA replication. The coupled nature of plus- and minus-strand synthesis in RNA viruses, however, complicates the analysis, since a decreased level of newly synthesized

plus strands will also reduce the level of minus strands in subsequent rounds of replication. Nevertheless, minus-strand levels decreased by 2-fold, while plus-strand levels decreased by 6-fold in the absence of the motif1-hairpin at 12 h.p.i. (Figure 3). This can be explained if the motif1-hairpin functions to a greater extent when present on the minus strands (i.e. during plus-strand synthesis) than on the plus strands. This model is supported by the *in vitro* data; the motif1-hairpin in minus-strand orientation was 40% more effective than in plus-strand orientation (Figure 7A). In addition, when combined with the promoter at the 3' end of sat-RNA C plus strands, the motif1-hairpin only enhanced transcription by 2-fold (P.D.Nagy and A.E.Simon, manuscript in preparation), much less than the 10-fold enhancement achieved using the sat-RNA C minus-strand promoter sequence (Figure 7).

Recognition of the motif1-hairpin by the TCV RdRp is not highly specific since several variants of the motif1-hairpin were found to support 3'-terminal RNA extension almost as efficiently as the wt hairpin (Nagy and Simon, 1998a; Nagy *et al.*, 1998). More extensive modification of the motif1-hairpin, however, resulted in reduced RNA accumulation in protoplasts (J.Pogany and A.E.Simon, unpublished results) and a decreased level of complementary RNA synthesis by the TCV RdRp *in vitro* (Figure 7B). Short single-stranded sequences around the motif1-hairpin were also required for full enhancement of replication by the motif1-hairpin in protoplasts (J.Pogany and A.E.Simon, unpublished results), suggesting that these sequences are part of the enhancer.

Similarities between promoters and RNA replication enhancers in TCV

Comparison of the sequences and secondary structures of the three characterized RNA replication enhancers reveals that the motif3-hairpin differs from the motif1-hairpin and hairpin4. The partial similarity between the motif1-hairpin and hairpin4 is due to a portion of the motif1-hairpin and its 5' flanking sequence (minus-strand orientation) being derived from the corresponding portion of TCV during the formation of sat-RNA C. Although these hairpins have different overall sequences, simply having a stem-loop structure is not sufficient to enhance transcription *in vitro*. All three artificial hairpins placed downstream of a natural initiation sequence interfered with the synthesis of complementary strands (Figure 7). It is not yet known if the interference involves initiation, elongation or termination of transcription.

Comparison of motif1-hairpin, motif3-hairpin and hairpin4 with known TCV or sat-RNA promoter sequences reveals that portions of the motif1-hairpin are similar to the 3' end of TCV and to a 5'-proximal sequence in sat-RNA C (minus-strand orientation) that is known to function as a positive-strand initiation promoter *in vitro* (Cascone *et al.*, 1990; Zhang *et al.*, 1991; Guan *et al.*, 1997). Portions of the motif3-hairpin sequence (minus-strand orientation) and its 3' flanking region are also similar to the minus-strand subgenomic RNA promoters (Cascone *et al.*, 1990; Zhang *et al.*, 1991). The sequence similarities between the motif1- and motif3-hairpins and known replication promoters for the TCV RdRp suggest that these *cis*-acting elements may have similar functions, such as binding to the TCV RdRp.

Based on the sequence described above and structural similarities between TCV RdRp promoters and the motif1- and motif3-hairpins, the question remains as to why the motif1-hairpin can direct complementary RNA synthesis efficiently using a primer in a 3'-terminal extension reaction *in vitro*, while, by itself, the motif1-hairpin can not support *de novo* initiation (Nagy *et al.*, 1998; similar studies have not been conducted for the motif3-hairpin). One possibility is that TCV promoters are composed of two, not necessarily contiguous, components: (i) a hairpin enhancer that recruits the RdRp and/or other replication factors; and (ii) linear sequences, termed initiator sequences, which are used by the RdRp to initiate complementary RNA synthesis in a primer-independent manner. This hypothesis is supported by the observation that the motif1-hairpin can direct *de novo* synthesis when single-stranded sequences similar to initiator sequences (i.e. sequences located at the start site for transcription) such as CCC and CC are placed 3' of the hairpin (Figure 8B). The role of the hairpin as an attractor for the viral RdRp is supported by previous studies indicating that additional copies of the motif1-hairpin inhibited 3'-terminal extension by the TCV RdRp *in vitro* to a greater extent than other sequences. The modular nature of promoters may be advantageous for RNA viruses, since they can quickly delete or duplicate these *cis*-acting sequences in order to increase their competitiveness or adapt better to their hosts.

Similarities between RNA replication enhancers and DNA-based transcription enhancers

Motif1-hairpin, motif3-hairpin and hairpin4 have properties similar to DNA-based transcriptional enhancers and pre-mRNA splicing enhancers. Transcriptional enhancers and pre-mRNA splicing enhancers are *cis*-acting DNA and RNA sequences that promote transcription and RNA splicing, respectively (Hertel *et al.*, 1997). The similarities include (i) upregulation of the basal level of activities; (ii) functioning *in cis* and at a distance from the site of transcription initiation or splicing; (iii) functioning in both orientations (not yet shown for the motif3-hairpin and hairpin4); and (iv) increased product levels with multiple enhancers. While an additional motif1-hairpin did not increase the accumulation of sat-RNA C (which may already be replicating at maximal efficiency), the presence of multiple RNA replication enhancers had an additive effect on the accumulation of poorly replicating sat-RNA CX (Figure 6).

The use of DNA-based transcription enhancers is widespread in biological systems. Indeed, the concept of RNA-based replication enhancers has been indicated in several viral systems (Lai, 1998). The best studied example is the transcription of human immunodeficiency virus (HIV) which requires a *cis*-acting RNA element (TAR RNA enhancer) and a protein factor (the *trans*-activator protein, tat) (Karn *et al.*, 1994). The TAR is located in the 5' viral long terminal repeat and contains a stem-loop structure with three bulged nucleotides. The sequence and structure of the TAR RNA are important for tat binding and *trans*-activation of RNA polymerase II-mediated transcription (Karn *et al.*, 1994). Putative RNA replication enhancers also function in replication of the double-stranded L-A virus of yeast (Esteban *et al.*, 1989), plus-strand alfalfa mosaic virus (van Rossum *et al.*, 1997), Q β bacteriophage

(Barrera *et al.*, 1993; Schuppli *et al.*, 1998) and tomato bushy stunt virus (Ray and White, 1999).

Internal *cis*-acting sequences that may function as RNA replication or transcription enhancers have been found in many viral systems. For example, animal and human coronaviruses (Hsue and Masters, 1997) and their associated defective interfering RNAs (Kim *et al.*, 1993) contain *cis*-acting sequences from both internal and 3'-proximal regions of the genomic RNA. Animal alphaviruses have a conserved 51 nt sequence within the coding region (P123/4ORF) that is important in virus and defective interfering RNA accumulation (Niesters and Strauss, 1990). In addition, a 3'-proximal sequence in the P1 capsid gene of human rhinovirus 14 RNA is required for efficient RNA replication (McKnight and Lemon, 1996). A similar element may exist within the P2-P3 region of poliovirus (McKnight and Lemon, 1996). Flock house virus (Ball and Li, 1993), BMV (French and Ahlquist, 1988) and hepatitis delta virus (Wang *et al.*, 1997) also contain internal *cis*-acting sequences. For BMV, a 150-nt-long sequence in the central portion of the RNA3 genome segment influences the extent of asymmetry in RNA replication (the ratio of plus versus minus strands). In addition, the same region has been proposed to facilitate the assembly of the replicase components into a functional RdRp complex (Quadt *et al.*, 1995). Further studies are needed to determine whether these viruses also have modular promoters similar to TCV-associated RNAs.

Role of RNA replication enhancers in RNA recombination and viral evolution

In addition to the role in RNA replication discussed above, RNA replication enhancers play a central role in RNA recombination, virus evolution and adaptation in the TCV system and possibly in other virus systems as well. The RNA replication enhancers may promote RNA recombination directly by constituting recombination hot spots through binding of the replicase-aborted nascent strand complex during the crossover event (Nagy and Simon, 1997; Nagy *et al.*, 1998). Thus, it is possible that RNA replication enhancers are central elements used to reassemble functional viral 'modules' around *cis*-acting elements in genomes, as predicted by the theory of the modular evolution of viruses (Gibbs, 1987; Goldbach *et al.*, 1991; Dolja and Carrington, 1992). Accordingly, non-viable sat-RNA C mutants can frequently generate viable (i.e. repaired) sat-RNAs through recombination between sat-RNA D and the mutated sat-RNA C with the help of the motif1-hairpin replication enhancer (Cascone *et al.*, 1993). In addition, many novel recombinants are generated between sat-RNA D and TCV around the motif3/hairpin4 RNA replication enhancer (Carpenter *et al.*, 1995). In addition to TCV, *cis*-acting elements may play a role in RNA recombination in other viral systems as well. For example, some of the junction sites in flock house virus, an animal nodavirus, resemble a replication origin located at the extreme 3' terminus of RNA2 (Ball, 1997). The similarity between the junction site sequences and the 3' replication origin suggests that internal sequences may guide the polymerase during template switching (Ball, 1997). Also, subgenomic RNA promoters or related sequences are frequently found as recombination sites in BMV (Allison *et al.*, 1990), Sindbis virus (Weiss

and Schlesinger, 1991), tobacco mosaic virus (Beck and Dawson, 1990), citrus tristeza virus (Bar-Joseph *et al.*, 1997) and TCV (C.D.Carpenter and A.E.Simon, unpublished results). These internal *cis*-acting sequences may have played a role in recombination events by recruiting the RdRp–nascent strand complex.

A second possible role of RNA replication enhancers in virus evolution and adaptation is indirect; they can increase the fitness and competitiveness of the resulting recombinants by stimulating RNA replication of the recipient RNA. For example, the double-recombination event between sat-RNA D and TCV that occurred during the formation of sat-RNA C generated the motif1-hairpin RNA replication enhancer (Simon and Nagy, 1996; Figure 1). Owing to the presence of the novel RNA replication enhancer, sat-RNA C was able to become the most competitive and successful subviral RNA in the TCV system. The formation of sat-RNA C demonstrates that novel *cis*-acting sequences can be generated during recombination events. This, in turn, gives support to the significance of studying RNA replication enhancers that may confer a 'competitive edge' to viruses and subviral pathogens.

Materials and methods

RNA template construction

For protoplast inoculation, RNA templates were obtained by *in vitro* transcription with T7 RNA polymerase using pTCV66 (a full-length cDNA of TCV) and pT7C(+) (a full-length cDNA construct of wt sat-RNA C and its derivatives) (Song and Simon, 1994). Δ mot1 and rev/mot1 were generated by polymerase chain reaction (PCR) using primers T7C5' and C3' (Song and Simon, 1994) and either pCAMdiAB or pCMABr (Cascone *et al.*, 1993) as DNA templates. The resulting PCR products were cloned into pUC19 at the *Sma*I site. Construct 2xmot1 was generated by a three-step PCR method. First, a 3' fragment was obtained by PCR using primers Mot1-Nco (5'-CATGCCATGGA-AGAACCACAGACCTCCAGCCAAAGGGTAAATGGGAAGAATG GTGGGTTTTAAAGGCGG-3') and C3' on T7C(+) template, followed by treatment with *Nco*I and gel purification. Secondly, a 5' PCR fragment was obtained by PCR using primers oligo 13 (5'-AGAGCACTAGTTTTCCACCCT-3') and T7C5', followed by treatment with *Nco*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-cut pUC19. Construct Mot1-Nco was generated like 2xmot1, except that the template used for the 3' PCR fragment was Δ mot1.

Constructs CX [described as CX-10 in Carpenter *et al.* (1995)] and CXM3+H4 (described as CX-9) were generated by PCR using primers T7D5' and oligo 8, and cloned into *Sma*I-digested pUC19. CXM1 was obtained by a three-step PCR-based method. First, the 5' portion of CX with the added motif1-hairpin was generated with primers T7D5' (Song and Simon, 1994) and CX10-1 (5'-TTTTGGGCCCATTTACCCTT-TGGCTGGAGGGTCTGGGATTCTTCGAGTGGGATACTGC-3') on the CX template. Secondly, the 3' portion of CX was generated with primers CX10-2 (5'-AAATGGGCCCAAAAACGGTGGCAGCAC-3') and oligo 8 (Song and Simon, 1994) on the CX template. Both the 3' and 5' PCR fragments were digested with *Apa*I, gel purified, ligated together and then used as templates to amplify the full-length cDNA with PCR using the end primers (T7C5' and oligo 8). The full-length CXM1 PCR product was then ligated into *Sma*I-digested pUC19. CXM3 was obtained by replacing the *Nco*I–*Apa*I segment of CXM1 with the PCR product generated with primers CX10mot3 (5'-GTAATACGACTCACTATAGGGCCCGGGGTTTTGTTTTCTTTCT-3') and T7D5' on the CXM3+H4 template (Carpenter *et al.*, 1995), and treated with *Nco*I and *Apa*I. CXH4 was obtained by replacing the *Nco*I–*Apa*I segment of CXM1 with the PCR product generated with primers CXhairpin4 (5'-CAGTGGGCCCTAACACAGGTCAAAAATAAAGCGACCTGGG-GGTTTTGTTTTCTTTCCAGTGGGATACTGC-3') and T7D5' on CXM3+H4 template (Carpenter *et al.*, 1995), and treated with *Nco*I and *Apa*I. CXM1+M3+H4 was obtained by replacing the *Apa*I–*Spe*I segment

of CXM1 with the PCR product generated with primers CX9mot1 (5'-CTGAGGGCCCCGTAGTCTTCTCATC-3') and oligo 8 on TCVSnaB I (Carpenter *et al.*, 1995) and treated with *Apa*I and *Spe*I. All constructs were sequenced to verify the presence of correct sequences.

For the *in vitro* experiments, RNA templates were obtained by *in vitro* transcription reaction 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 full-length RNA transcripts were isolated from 5% PAGE–urea gels. After ethanol precipitation, the RNA transcripts obtained were dissolved in sterile water and their amount and size were measured by a UV spectrophotometer and 5% polyacrylamide–8 M urea gel (denaturing PAGE) analysis (Song and Simon, 1994; Nagy *et al.*, 1997).

Constructs Controll+pr, mot1+pr, mot1forw+pr, mutmot1+pr, GC+pr, AU+pr and ministem+pr DNAs were generated by two sequential rounds of PCR using one of the following templates: Controll-pr, mot1-pr, mot1forw-pr, mutmot1-pr, GC-pr, AU-pr and ministem-pr DNAs (P.D.Nagy and A.E.Simon, manuscript in preparation). In the first round PCR, the same 3' end primer C-prom (5'-GGGATACTAAGGGTTTCATAGGGAGGCATCTATTGG-3') and one of the following 5' primers were used: T7+SATC PROM, T7-MOT1+C, T7+MOT1+SATC, T7+AU/GC+SATC, T7+GC+SATC, T7+AU+SATC and T7+MINITETRA+SATC, respectively. In the second round of PCR, the same 3' end primer C(d9)-prom (5'-AAGGGTTTCATAGGGAGGC-3') and one of the following 5' primers were used: T7+SATC PROM, T7-MOT1+C, T7+MOT1+SATC, T7+AU/GC+SATC, T7+GC+SATC, T7+AU+SATC and T7+MINITETRA+SATC, respectively, on the templates obtained in the first round.

Constructs mot3+pr and mot3hairpin4+pr were generated by two sequential rounds of PCR, first using the same 3' end primer NEW/C-MOT3 (5'-GGGAGGCTATCTATTGGTTGTAGTCTTCTCATCTTAGTAG-3') and either T7Mot3/B (5'-GTAATACGACTCACTATAGGGCTGCCACCGTTTTTGGTCCC-3') or CX10mot3 5' primers on CXM3+H4 DNA template. In the second round of PCR, the same 3' end primer C(d9)-prom and either T7Mot3/B or CX10mot3 5' primers were used on the templates obtained in the first round. CCA4+Mot1 and CCA4+Mot1short DNAs were generated by PCR using the same 3' primer CCA4 (5'-GGGTGGTGGTGGCCCCAGACCCTCCAGCC-3') and either T7motif1 or T7motif1-short (Nagy *et al.*, 1998) primers on pT7C(+) template.

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 of the sat-RNAs and either 20 μ g of TCV genomic RNA transcripts for replication studies or no TCV genomic RNA transcripts for *in vivo* stability studies. To study the degradation rate of sat-RNAs inside versus outside the protoplast cells, the PEG–CaCl₂ step was omitted during protoplast inoculation to inhibit RNA uptake of the cells in one set of experiments. In another set of experiments, the protoplasts were treated with 3.3 μ g/ml RNase A to destroy residual sat-RNAs outside the cells. Neither the omission of the PEG–CaCl₂ step nor the RNase A treatment influenced the level of survival sat-RNAs from 2 to 44 h.p.i. when compared with the standard samples (data not shown), demonstrating that the survival RNAs were located inside the cells.

Total RNA extraction from protoplasts, RNA denaturation and gel blotting were conducted as described previously (Kong *et al.*, 1997). Plus strands of sat-RNAs were detected with an oligonucleotide C/D (5'-CTTGACTGATGACCCCTACG-3') labeled using polynucleotide kinase and [γ -³²P]ATP. The ribosomal RNA probe used as a loading control (not shown) has been described previously (Simon *et al.*, 1992). Minus strands of sat-RNAs were detected using an [α -³²P]UTP-labeled riboprobe obtained from *Dra*I-digested pT7C(+) by transcription with T7 RNA polymerase. To remove the excess amounts of plus strands of sat-RNAs, the total RNA samples obtained from protoplasts were treated with RNase A after annealing the plus and minus strands as described by Ishikawa *et al.* (1991). The RNase treatment did not increase the sensitivity of minus-strand detection for the sat-RNAs when compared with the untreated samples (not shown).

TCV RdRp assay

Preparation of template-dependent RdRp from TCV-infected turnip plants, *in vitro* transcription reactions, and product analysis were carried out as described previously (Song and Simon, 1994; Nagy *et al.*, 1997,

1998) using 20 μ l RdRp reaction mixtures 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 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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