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Requirement of a 5′-Proximal Linear Sequence on Minus Strands for Plus-Strand Synthesis of a Satellite RNA Associated with Turnip Crinkle Virus

Hancheng Guan, Clifford D. Carpenter, and Anne E. Simon[1]

Department of Biochemistry and Molecular Biology and Program in Molecular and Cellular Biology, University of Massachusetts, Amherst, Massachusetts 01003

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Viral RNA replication begins with specific recognition of *cis*-acting RNA elements by the viral RNA-dependent RNA polymerase (RdRp) and/or associated host factors. A short RNA element (3′-AACCCCUGGGAGGC) located 41 bases from the 5′ end of minus strands of satellite RNA C (satC), a 356-base subviral RNA naturally associated with turnip crinkle virus (TCV), was previously identified as important for plus-strand synthesis using an *in vitro* RdRp assay (H. Guan, C. Song, A. E. Simon, 1997, *RNA* 3, 1401–1412). To examine the functional significance of this element in RNA replication, mutations were introduced into the consecutive C residues in the element. A single mutation of the 3′-most C residue resulted in undetectable levels of satC plus strands when transcripts were assayed in protoplasts and suppressed transcription directed by the element *in vitro.* However, satC minus strands were detectable at 6 h postinoculation (hpi) of protoplasts, accumulating to about 10% of wild-type levels at 24 hpi. This mutation, when in the plus-sense orientation, had little or no effect on minus-strand synthesis from full-length satC plus strands *in vitro,* suggesting that the 5′-proximal RNA element is required for satC plus-strand synthesis. In addition, *in vivo* genetic selection revealed a strict requirement for 10 of the 14 nucleotides of the element, indicating that the primary sequence is essential for RNA accumulation.

INTRODUCTION

Replication of positive-sense RNA viruses proceeds through complementary minus-strand intermediates, which in turn serve as templates for plus-strand RNA synthesis. This process requires both *cis*-acting signals located on viral RNA molecules and *trans*-acting factors such as virus-encoded RNA-dependent RNA polymerase (RdRp) and host proteins (Buck, 1996; Lai, 1998).

A wide variety of *cis*-acting promoter elements, ranging from short linear sequences to extensive multiple stem-loop structures, are recognized by RdRp. For example, brome mosaic virus (BMV) has three different types of promoters: a tRNA-like structure at the 3′ end of the genomic RNAs that contains elements required for minus-strand synthesis, a promoter located at the 3′ terminus of minus-strand intermediates for genomic plus-strand RNA synthesis, and an internal subgenomic RNA promoter (Dreher and Hall, 1988; Marsh *et al.,* 1988; Pogue *et al.,* 1992; Pogue and Hall, 1992; Duggal *et al.,* 1994). Recognition of at least the subgenomic RNA core promoter by the BMV RdRp *in vitro* is through a sequence-specific mechanism (Siegel *et al.,* 1997, 1998). How a single RdRp recognizes diverse linear and hairpin elements is not known, but may involve different RNA-binding sites in the RdRp or different host factors. Studies with Qβ replicase indicate the presence of two different RNA-binding sites responsible for the recognition of pseudoknot structures and pyrimidine-rich sequences (Brown and Gold, 1995a,b). Furthermore, hairpins found in promoter regions may be present for reasons other than directing RdRp recognition. Singh and Dreher (1998) have demonstrated that specific recognition of a 3′-CCA triplet by the turnip yellow mosaic virus RdRp requires only an adjacent nonspecific secondary structure, indicating that the function of the structure is to keep the specific sequence sterically accessible to the RdRp and/or associated factors.

While *cis*-acting signals required for RNA replication have generally been localized to the ends of virus genomic RNAs (Duggal *et al.,* 1994; Buck, 1996), internal *cis*-acting elements are necessary for replication of some RNA viruses including BMV (French and Ahlquist, 1987; Pogue *et al.,* 1992). For bacteriophage Qβ, two internal sites are required for the synthesis of minus strands (Barrera *et al.,* 1993) while an internal region consisting of a stem-loop structure is involved in plus-strand synthesis of a defective-interfering (DI) RNA of mouse hepatitis virus (Lin and Lai, 1993; Kim and Makino, 1995). An internal region in potato virus X genomic RNA participates in a long-distance interaction with terminal sequences, which is required for efficient plus-strand synthesis (Kim and Hemenway, 1999). The

[1] To whom correspondence and reprint requests should be addressed at Department of Biochemistry and Molecular Biology, University of Massachusetts, Amherst, MA 01003. Fax: (413) 545-4529. E-mail: simon@biochem.umass.edu.

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function of internal *cis*-acting elements is not well known, but roles in RNA folding, host factor binding, and replicase assembly have been proposed (Buck, 1996; Klovins *et al.,* 1998; Kim and Hemenway, 1999).

Defining a complete set of elements required for replication of a viral RNA is complicated by the large size of most viral RNAs. Identification of internal sequences required for replication has mainly come from studies where random, limited deletions were generated, making it likely that the majority of specific virus regions involved in replication for any given genomic RNA remain to be determined. Also complicating studies on virus replication is the link between replication and translation; sequences required for translation of many viral proteins can have a substantial, but indirect, impact on replication.

Turnip crinkle virus (TCV), a single-stranded positive-sense RNA virus, is naturally associated with several small subviral RNAs (Simon and Howell, 1986). Since these viral-associated RNAs are not translated and are replicated by the viral RdRp, they have been very useful in studying sequences required for RNA replication (Song and Simon, 1995; Guan *et al.,* 1997; Stupina and Simon, 1997; Carpenter and Simon, 1998). One of the subviral RNAs, satC, is a chimeric RNA containing the sequence of a second TCV subviral RNA at its 5′ end and two regions from TCV genomic RNA at its 3′ end (Simon and Howell, 1986). The promoter for minus-strand synthesis of satC is a hairpin with a single-stranded tail contained within a 29-base sequence at the 3′ end of the RNA (Song and Simon, 1995a; Stupina and Simon, 1997; Carpenter and Simon, 1998). Two short elements on satC minus strands, located 11 bases from the 3′ end (3′-UCCCAAAGUAU) and 41 bases from the 5′ end (3′-AACCCCUGGGAGGC), were previously identified by deletion analysis as having a role in complementary strand synthesis *in vitro* (Guan *et al.,* 1997). Deletion of both elements was required to eliminate detectable complementary strand synthesis, suggesting that the elements play a redundant role under *in vitro* conditions (Guan *et al.,* 1997). When joined to the 3′ end of a Qβ bacteriophage-associated midivariant (MDV) RNA, which alone is not a template for the TCV RdRp, either element can direct the TCV RdRp to synthesize complementary strands *in vitro* with transcription initiating internally in the vicinity of the multiple C residues within the elements. We now report that the 5′-proximal element is sequence-specific and essential for satC plus-strand synthesis *in vivo.*

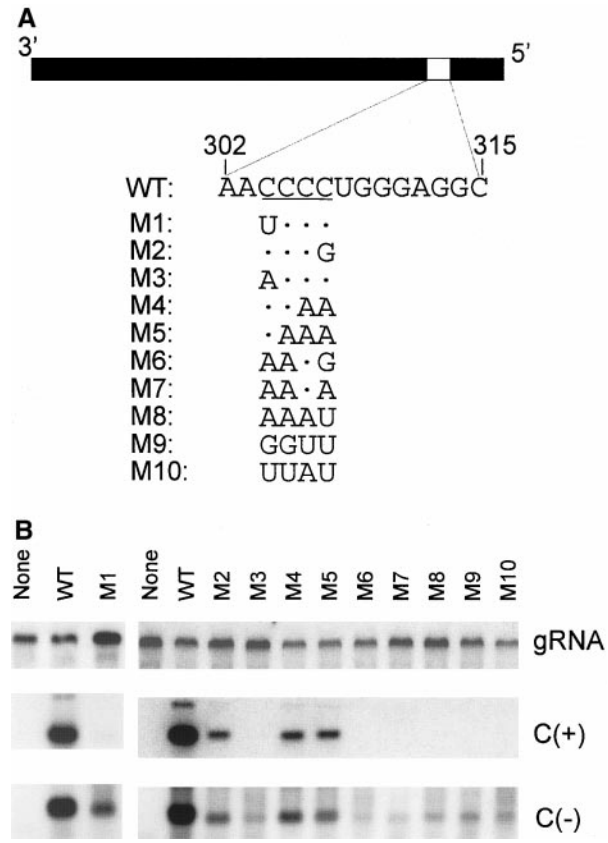

FIG. 1. Mutational analysis of the 5′-proximal element. (A) Mutations introduced into the consecutive C residues (underlined) of the element. SatC minus strand is represented by a black bar. The 5′-proximal element is denoted by a small open box. The 5′-proximal sequence is shown under the bar and positions with respect to location in the minus strand are indicated. Dots indicate no change of base. Names of the constructs are shown to the left. WT, wild-type satC. (B) RNA gel-blot analysis of TCV genomic RNA (gRNA), satC plus strand [C(+)], and minus strand [C(−)]. To probe gRNA and C(+), equal amounts of total RNA isolated from 2×10^5 protoplasts at 24 hpi were subjected to agarose gel electrophoresis. Double-stranded RNA obtained from 8×10^5 protoplasts was used to detect satC minus strands. None, no sat-RNA added.

RESULTS AND DISCUSSION

The 5′-proximal element is required for replication *in vivo*

The 5′-proximal element and other sequences that can serve as TCV promoters, including the promoter for satC minus-strand synthesis and two subgenomic RNA promoters located on the viral genomic RNA, contain multiple consecutive C residues (Song and Simon, 1995a; Wang and Simon, 1997; Wang *et al.,* 1999). In addition, multiple consecutive C residues are present in a hairpin replication enhancer element located on satC minus strands, which is thought to aid in attracting the RdRp to the template (Nagy *et al.,* 1998, 1999). To determine if the *in vitro* identified 5′-proximal element is important for satC accumulation in protoplasts, mutations were introduced into positions 304 to 307 containing four consecutive C residues (Fig. 1A). Conversion of the C residue at position 304 (C^{304}) to A, U, or G, either singly or in context with other alterations, eliminated detection of satC plus strands at 24 h postinoculation (hpi) (constructs M1, M3, and M6-10, Fig. 1B) while minus strands accumulated at 10 to 35% of wild-type. Single-, double-,

and triple-base mutations introduced into the other three C residues at positions 305 to 307 reduced plus-strand accumulation of satC to 20% of wild-type at 24 hpi (constructs M2, M4, and M5, Fig. 1B). Minus strands of these three mutants accumulated at 20 to 30% of wild-type at this time point (Fig. 1B).

Since minus-strand levels at 24 hpi are also a reflection of the level of nascent plus strands, we tested the effect of the mutations in the 5′-proximal element region on minus-strand synthesis of satC in protoplasts at early times postinoculation (6, 9, and 12 hpi). As shown in Fig. 2, alteration of the C residue at position 307 to G (construct M2), which reduced plus-strand accumulation to 20% of wild-type at 24 hpi (Fig. 1), resulted in almost undetectable plus-strand accumulation at 6 hpi, a time point where wild-type satC plus strands were clearly detectable. Plus-strand levels of this mutant reached 2.5 and 10% of wild-type at 9 and 12 hpi, respectively (Figs. 2A and 2B). In contrast to the barely detectable accumulation of plus strands at 6 hpi, minus strands of M2 mutant accumulated to 2% of wild-type at 6 hpi (Figs. 2A and 2C). At 9 and 12 hpi, minus-strand levels increased to 4 and 12% of wild-type, respectively. No plus strands of construct M3 were detected at 6, 9, or 12 hpi (Fig. 2A). In contrast, minus strands of M3 accumulated at 2.5, 3, and 3.5% of wild-type levels at the three time points, respectively (Figs. 2A and 2C). Altogether, these results suggest that minus strands of template containing an A at position 304 (M3) are synthesized at a very low rate from basal levels of transfected plus strands, while further synthesis of nascent plus strands from minus-strand template is inhibited.

Alteration of C^{304} abolishes transcription directed by the 5′-proximal element *in vitro*

To further explore whether alteration of C^{304} mainly affects plus-strand synthesis, plus- and minus-strand templates containing mutations at position 304 were subjected to *in vitro* transcription using the TCV RdRp. Since full-length satC minus strands contain the 3′-proximal element and either element is sufficient to direct complementary strand synthesis *in vitro* (Guan *et al.,* 1997), chimeric RNAs were constructed containing the 5′-proximal element and downstream sequences (positions 284 to 356) of satC minus strands joined to nontemplate Qβ bacteriophage-associated MDV RNA generating construct MC5′(−) (Fig. 3A). Initiation of transcription for this construct is not at the 3′ end of the transcript but internally within the multiple consecutive C residues of the 5′-proximal element, producing a complementary product shorter than template length (Guan *et al.,* 1997). Since the conversion of C^{304} to A in satC minus strands eliminated detectable plus-strand synthesis *in vivo* (Fig. 1B and Fig. 2A, M3), the same mutation was introduced into the chimeric construct to generate

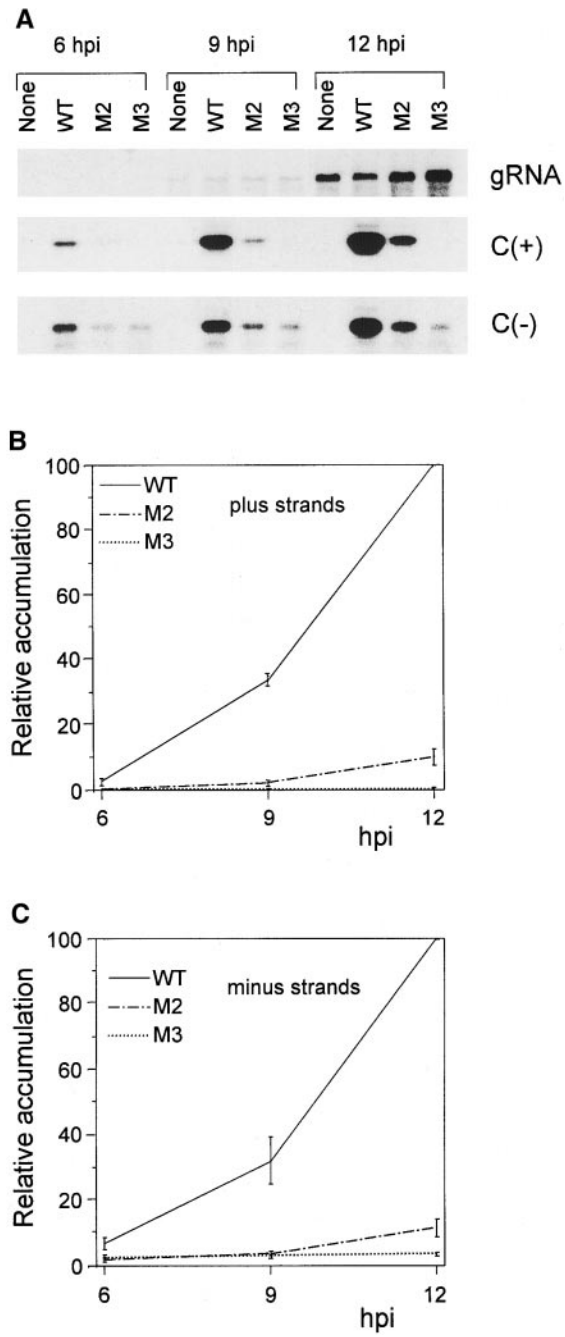

FIG. 2. Effect of mutations in the 5′-proximal element on satC accumulation in protoplasts at early times postinoculation. (A) RNA gel-blot analysis. Time points of the sampling are shown above the blots. See legend to Fig. 1 for other details. The relative levels of satC plus (B) and minus strands (C) were measured from the blots shown in (A) and another independent experiment. Error bars indicate standard error.

MM5′(−) (Fig. 3A). Results obtained from *in vitro* transcription reactions indicate that the C to A change when the 5′-proximal element is in minus-sense orientation caused a 95% reduction in activity (Fig. 3B). To test the mutation's effect when in the plus-sense orientation, a second construct was generated containing positions 284 to 356 of satC plus strands, which contains the

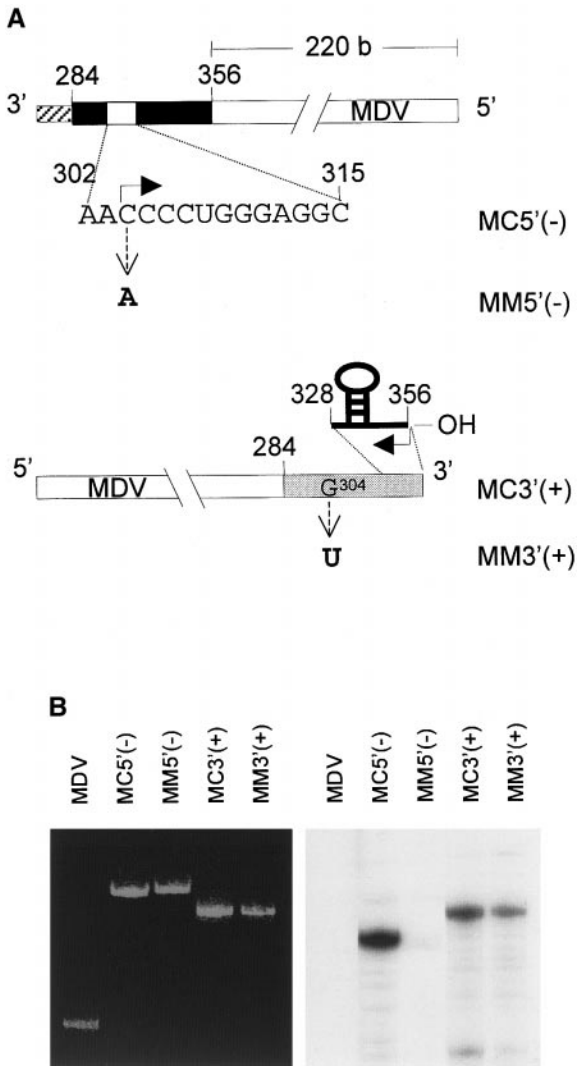

FIG. 3. *In vitro* transcription using partially purified RdRp and chimeric RNA templates containing a mutation at position 304 of plus- and minus-strand satC. (A) Schematic representation of the chimeric RNAs derived from MDV RNA and the 5′-end sequence of satC minus strand or the 3′-end complementary sequence of satC plus strand. Black and shaded bars represent satC minus- and plus-strand sequences, respectively. Long open bar denotes MDV RNA (220 bases). Small open box represents the 5′-proximal element. Hatched bar indicates the 18 plasmid-derived nucleotides. The 5′-proximal element and the hairpin promoter for satC minus-strand synthesis are shown. Numbers indicate positions with respect to location in satC. Mutations introduced into position 304 are indicated. Names of constructs are shown to the right. Bent arrows denote the putative transcription start sites. (B) Denaturing gel analysis of ^{32}P-labeled products synthesized *in vitro*. The ethidium bromide-stained gel showing the migration positions and the relative levels of the templates is shown to the left of the autoradiogram.

hairpin promoter for minus-strand synthesis, connected to the 3′ end of MDV RNA (construct MC3′(+), Fig. 3A). Transcription initiating from this construct is at the 3′ end, generating a template-length product (Fig. 3B). Alteration of the analogous G residue at position 304 to U reduced activity of the hairpin promoter by 30% (construct MM3′(+), Fig. 3B). These results suggest that the ability of the 5′-proximal element in the minus-sense orientation to serve as a promoter *in vitro* is connected to its function *in vivo* and that the element functions primarily, but possibly not exclusively, in the minus-sense orientation.

The effect of the mutations at position 304 on full-length template activity *in vitro* was also assayed. The TCV RdRp generated both template-sized products (T-RNA), which are synthesized by transcription initiation at the 3′ terminus of the template, and larger-than-template-length products (L-RNA) (C(−), Fig. 4), which are synthesized by primer extension from the template 3′ end at an internal location (Song and Simon, 1995b). Conversion of C^{304} to A in the 5′-proximal element had little or no effect on T-RNA synthesis *in vitro* (M3(−), Fig. 4). This is likely because the minus-strand template contains the 3′-proximal element that can also direct complementary strand synthesis *in vitro* (Guan *et al.,* 1997). However, synthesis of L-RNA and other larger-than-full-length products was markedly reduced by this mutation, suggesting that the mutation interferes with priming of RNA synthesis at internal locations. Transcription of full-length plus-strand satC template *in vitro* produced only template-length products (C(+), Fig. 4). Alteration of the G residue at position 304 to U in the plus-strand RNA did not reduce template activity *in vitro* (M3(+), Fig. 4). While it is not clear what role the ability to generate L-RNA has on normal replication *in vivo,* these results further demonstrate that position 304 in satC functions mainly on minus strands of satC.

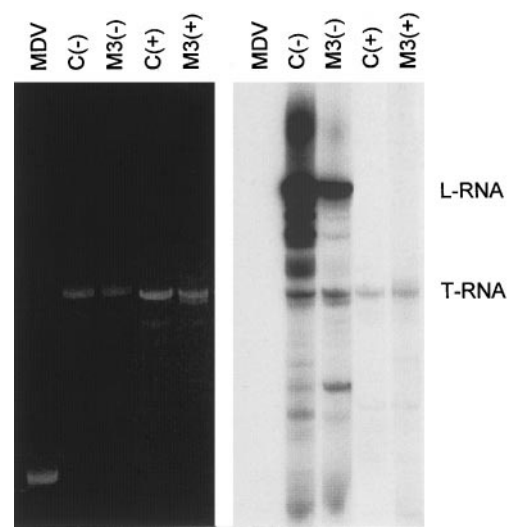

FIG. 4. *In vitro* transcription of full-length satC plus- and minus-strand templates using TCV RdRp. M3(−) denotes satC minus strand with a C to A change at position 304. M3(+) is satC plus strand containing an alteration of G to U at the same position. T-RNA, template-sized products; L-RNA, larger-than-template-length products. See legend to Fig. 3.

TABLE 1

First-Round *in Vivo* Selection

Name	Sequence[a]	Plant[b] 1	2	3	4	6	7	8	10	11	19	20	26
WT	3′-A**ACCC**C**UGGGAGG**C							3					
1-a	A**ACCG**C**UGGGAGG**U	5											
1-b	Ag**CCG**C**UGGGAGG**U	1											
2-a	A**AUCCA**C**UGGGAGG**		2										
2-b	A**AUCCA**CUGaGAGG		2										
3-a	C**ACCUCUGGC**G**AGG**			1									
3-b	C**AC**uc**CUGGC**G**AGG**			3									
3-c	C**AC**uU**CUGGC**G**AGG**			1									
4-a	G**ACCACUGGAUAGG**				2	2	5	1		1		2	2
4-b	a**ACCACUGGAUAGG**											1	
4-c	c**ACCACUGGAUAGG**												3
5-a	A**ACCUCUGGGAAGG**						1	1	3				
5-b	A**ACCUg**U**GGGAAGG**											4	
6-a	C**CUGCCACGGUGGA**										2		
Total clones sequenced								48					

[a] Only sequences in the 5′-proximal element region are shown. Lowercase letters denote differences from an arbitrarily selected "parental" molecule. All the sequences, except sequence 6-a from plant 19, contain the consensus sequence, 3′–(A/C/G)**ACCNCUGGN**$_{1–2}$**AGG**(C/U)$_{0–1}$, in the 5′-proximal element region. The most conserved bases are shown in bold.

[b] The number of clones of each sequence found in the plants is indicated.

The primary sequence of the 5′-proximal element is important for accumulation of satC in plants

To further identify sequence requirements of the 5′-proximal element for satC accumulation *in vivo,* the sequence of the element was randomized and *in vivo* genetic selection, also known as SELEX (Systematic Evolution of Ligands by Exponential enrichment) (Ellington and Szostak, 1990; Tuerk and Gold, 1990), was carried out as described under Materials and Methods. Transcripts of satC containing 14 randomized bases in the 5′-proximal element were coinoculated onto 42 turnip seedlings along with the TCV helper virus. Total RNA was isolated from uninoculated leaves at 21 days postinoculation (dpi). Only 2 of the 42 infected plants (plants 7 and 11) generated satC-sized RNAs that were visible in ethidium bromide-stained agarose gels (data not shown; satC normally accumulates to a level similar to that of 5S ribosomal RNA). Although sat-RNA species were not detectable in the other plants, satC species could be cloned from all infected plants. All cloned satC-like species accumulating in 12 randomly selected plants contained similar sequences in the 5′-proximal element region (Table 1). The wild-type sequence was also recovered from one of the analyzed plants (plant 8), indicating that sufficient complexity existed in the initial randomized population to recover the wild-type sequence. This is in contrast with the results obtained from *in vivo* SELEX of the 3′ end of satC minus strands, including the 3′-proximal element, in which no wild-type or identical sequences were generated in different plants from the first round (Guan *et al.,* 2000). However, unlike the sequence-specific nature of the 5′-proximal element (see below), little sequence conservation was found within the 3′-proximal element.

Comparison of the recovered molecules revealed the presence of the consensus sequence 3′-(A/C/G)ACCNCUGGN$_{1–2}$AGG(C/U)$_{0–1}$ in the 5′-proximal region of all cloned RNAs except those obtained from plant 19 (Table 1). The bases at position N$_{1–2}$ in the consensus sequence were not completely random; if one base was present at this position, it was usually a G residue. If two bases were present, they were usually a combination of AU, CG, or GA (3′ to 5′ orientation). In addition, the least conserved sequence (found in plant 19) still contained multiple C residues followed by multiple purines, which is the hallmark of the 5′- and 3′-proximal elements (Guan *et al.,* 1997). These results suggest that alterations at only limited positions are tolerated in the 5′-proximal element.

To subject the sat-RNA sequences to further competition, equal portions of total RNA from the 12 analyzed plants (pool 1) or all 42 infected plants (pool 2) from the first round were combined and inoculated onto 8 new plants. Three weeks after inoculation, sat-RNA species were cloned and sequenced. Clone 1-a, which differed from the wild-type sequence at only two positions in the 5′-proximal element and was previously identified in plant 1 from the first round, was recovered from all 8 plants inoculated with RNA of pool 1 (Table 2). This sequence was the only species found in 7 of the 8 plants.

TABLE 2

Second-Round *in Vivo* Selection

Name	Sequence[a]	Pool 1[b] 1	2	3	4	5	6	7	8	Pool 2[c] 1	2	3	4	5	6	7	8
WT	3′-AACCCCUGGGAGGC									6	5	6	6	6	4	6	5
1-a	AACCGCUGGGAGGU	6	5	6	3	6	3	5	4						2		
1-c	AACCGCcGGGAGGU						1										
3-b	CACUCCUGGCGAGG						1										
Total clones sequenced					40								46				

[a] See the legend to Table 1.
[b] RNA used for infection was pooled from the 12 plants analyzed in the first round.
[c] RNA used for infection was pooled from all 42 infected plants from the first round.

Two other RNA species (1-c, which differed from sequence 1-a at one position, and 3-b, which was previously identified in plant 3 of the first round) were cloned from one of the plants (plant 6). No clones containing wild-type sequence were recovered, although wild-type satC was contained in pool 1 from plant 8 (Table 1). In contrast, almost all RNA molecules obtained from inoculation of pool 2 were wild-type (Table 2), suggesting that additional wild-type satC was accumulating in unanalyzed plants of the first round.

Although a single-base change of C^{304} to U abolished plus-strand synthesis in protoplasts (M1, Fig. 1), several of the SELEX sequences such as 2-a, 2-b, and 6-a contained a U residue at this position. Comparison of sequences 2-a, 2-b, and 6-a indicates that all contained "CCNC" immediately or 1 base 5′ of the U residue at position 304. This suggests that a C residue at position 304 or "CCNC" in a nearby position relative to other essential bases in the 5′-proximal element is important for satC plus-strand synthesis.

Bases in the unconserved positions of the 5′-proximal element may also affect template activity, since a single purine in position N_{1-2} was preferred. Sequence 1-a and wild-type satC contained a G residue in this position, while the other recovered sequences had 2 bases at this site. An extra base in this position may affect the recognition of the element by the TCV RdRp and/or associated factors (see below), thus resulting in a reduction in RNA competitiveness for accumulation. Nucleotide insertions or deletions between the four key nucleotides in the BMV subgenomic RNA core promoter decrease promoter activity *in vitro* (Stawicki and Kao, 1999).

To assess whether sequence 1-a or wild-type satC was more fit for accumulation, competition assays were conducted. Equal amounts of transcripts of sequence 1-a and wild-type satC were used to inoculate nine plants along with TCV helper virus. Sat-RNA species were cloned at 10 or 21 dpi. These two sequences, which differ by only 2 bases at nonconserved positions 306 and 315 in the element, were similarly competent for accumulation in plants (Table 3), indicating that strict nucleotide conservation is not required at positions 306 or 315.

Computer secondary structure analysis using an algorithm that predicts optimal and suboptimal RNA structures (Zuker *et al.,* 1991) indicates that the 5′-proximal element might form secondary structures with other sequences on satC minus strands. However, replacement of C^{304} with U or A did not affect the predicted structures. In addition, mutations disrupting the predicted structures had no effect on template activity *in vitro* (data not shown). Altogether, these results suggest that the primary sequence, especially the 10 most conserved bases, of the 5′-proximal element are important for RNA replication and/or RNA stability, possibly from recognition by the RdRp or other replication factors. The presence of a linear RNA element is reminiscent of the BMV subgenomic RNA core promoter, in which 4 of 22 nucleotides (at positions −17, −14, −13, and −11) are essential for recognition by the BMV RdRp *in vitro* (Siegel *et al.,* 1997, 1998).

Possible functions of the 5′-proximal element

In addition to the 5′-proximal element and possibly the 3′-proximal element (Guan *et al.,* 2000), two additional

TABLE 3

Competition between Wild-Type satC and Sequence 1-a for Accumulation *in Vivo*

Name	Sequence[a]	Plant[b] 1–3 10 dpi	1–9 21 dpi
WT	3′-AACCCCUGGGAGGC	10	25
1-a	AACCgCUGGGAGGu	8	26
Total clones sequenced		18	51

[a] See the legend to Table 1.
[b] The number of clones for the sequence found in plants 1–3 at 10 dpi or plants 1–9 at 21 dpi is shown.

sequences are known to be required for efficient accumulation of plus-strand satC *in vivo:* the 3′-terminal sequence (3′-CCCUAU) of satC minus strands known as the carmovirus consensus sequence (Guan *et al.,* 2000) and a 30-base hairpin structure called the motif1-hairpin. The motif1-hairpin is located between the 3′ end and the 5′-proximal element in satC minus strands and is required for recombination between a second TCV subviral RNA and satC (Cascone *et al.,* 1993; Nagy and Simon, 1998; Nagy *et al.,* 1998). In addition, the motif1-hairpin enhances satC plus-strand accumulation in protoplasts and increases transcription from the linear 3′-proximal element by almost 10-fold *in vitro* (Nagy *et al.,* 1999). Based on competition experiments, the motif1-hairpin is thought to bind either the RdRp or a factor that bridges the connection between the RdRp and the RNA template (Nagy *et al.,* 1998). The motif1-hairpin contains 8 bases (3′-CUGGGAGG) also found in the 5′-proximal element, and thus it is possible that the 5′-proximal element also serves as an attractor for the TCV RdRp.

While the 5′-proximal element can function as an independent promoter *in vitro,* transcription only initiates internally at the multiple consecutive C residues within the element (Guan *et al.,* 1997; Fig. 3 in this paper). It is unlikely that such initiation occurs to any great extent *in vivo,* since the RNAs generated would be inviable. Therefore, if the 5′-proximal element serves as a promoter element *in vivo,* it must do so only within the context of sequences that help target the RdRp to the 3′ end of the template. It is possible, however, that the ability of the 5′-proximal element to attract the RdRp, and thus serve as a promoter *in vitro,* is necessary to fulfill a role other than as a promoter *in vivo.* For example, the RdRp and/or host factors may serve as RNA chaperones to assist in folding the newly synthesized minus-strand RNA and eliminate the formation of kinetically trapped intermediates (Herschlag, 1995). This function would be similar to one of the two internal replicase-binding sites (M site) in Qβ RNA that is involved in a long-range interaction with the 3′ end of the bacteriophage RNA, which brings the 3′ end to the bound replicase at the M site (Klovines *et al.,* 1998). Further studies are currently underway to characterize the relationship between the structure of satC minus strands and the functions of the various elements required for satC plus-strand synthesis.

MATERIALS AND METHODS

Site-directed mutagenesis of the 5′-proximal element

Site-specific mutations were introduced into the multiple C residues (positions 304 to 307) in the 5′-proximal element using polymerase chain reaction (PCR). The primers used in the reaction were the −20 sequencing primer (Biolabs) and a 29-mer oligonucleotide, which contains positions 294 to 322 of satC with randomized bases in positions 304 to 307 (5′-GGTGGGCTTTNNNNACCCTCCGAACCAAT, the randomized bases are denoted by "N"). The template was plasmid pT7C(+), which contains a full-length satC cDNA immediately downstream from a T7 RNA polymerase promoter (Song and Simon, 1995a). The PCR product was treated with T4 DNA polymerase, followed by digestion with *Eco*RI, and then ligated into *Eco*RI/*Hin*dIII-digested pUC19 with a second PCR product. The second PCR product was amplified using oligo 293 (5′-CTTTCGGGATTTTAGTGGTT, complementary to positions 274 to 293) and the −48 reverse sequencing primer (Biolabs). Before ligation, this product was treated with T4 DNA polymerase and digested with *Hin*dIII. All the clones contained a T7 RNA polymerase promoter upstream of full-length satC containing site-specific mutations in positions 304 to 307.

To construct minus-sense satC containing mutations in positions 304 to 307, the plus-sense satC cDNA mutants as described above were used as templates for PCR. The primers used were C5′ (5′-GGGATAACTAAGGGTTTCA, homologous to positions 1 to 19 of satC) and T7C3′ (5′-GTAATACGACTCACTATAGGGCAGGCCCCCCGTCCGA, complementary to positions 338 to 356 of satC; the 5′ 18 nucleotides are the T7 RNA polymerase promoter sequence). The PCR products were cloned into the *Sma*I site of pUC19 and mutations were verified by sequencing.

In vitro transcription and inoculation of *Arabidopsis* protoplasts

Plasmids were linearized with *Sma*I (unless otherwise noted) and subjected to *in vitro* transcription using T7 RNA polymerase (Carpenter *et al.,* 1995). The *Sma*I-linearized plasmids generated RNA transcripts containing the wild-type 3′ and 5′ ends. Protoplasts (5×10^6) prepared from Col-0 callus cultures were inoculated with 20 μg of TCV genomic RNA transcripts and 2.0 μg of either wild-type or mutant satC transcripts as described previously (Kong *et al.,* 1997).

RNA gel-blot analysis

Total RNA extracted from protoplasts at 6, 9, 12, or 24 hpi was used for RNA gel-blot analysis as previously described (Guan *et al.,* 2000).

Construction of RNA chimeras

To construct RNA chimeras, positions 284 to 356 of wild-type or mutant satC in either plus- or minus-strand orientation were joined to the 3′ end of MDV RNA. cDNA fragments containing positions 284 to 356 were amplified by PCR from pT7C(+) or a mutant satC clone (pM2), which contains an A/T basepair in position 304 in place of the wild-type G/C pair. The primers used were oligo 284 (5′-ATCCCGAAAGGGTGGGCT, homologous to positions 284 to 301) and oligo 7 (5′-GGGCAGGCCCCCCGTCCGA, complementary to positions 338 to

356). The PCR products were treated with T4 DNA polymerase and cloned into the *Sma*I site of pT7MDV (Axelrod *et al.,* 1991). Plasmids containing positions 284 to 356 of satC in plus and minus orientations were selected and linearized with *Sma*I and *Eco*RI, respectively. *Eco*RI-digested plasmids produced T7 transcripts with 18 additional plasmid-derived bases at the 3′ end.

In vitro transcription using the TCV RdRp

In vitro transcription using partially purified TCV RdRp was carried out as described previously (Guan *et al.,* 1997). Autoradiograms were scanned with a GS-700 densitometer and the relative template activities were calculated after being normalized to the template levels.

In vivo genetic selection

In vivo genetic selection was performed as previously described (Carpenter and Simon, 1998; Guan *et al.,* 2000). A cDNA product containing a T7 RNA polymerase promoter upstream of positions 1 to 301 of satC was first amplified by PCR from pT7C(+) using primers T7C5′ (5′-GTAATACGACTCACTATAGGGATAACTAAGGG) and $C_{287–301}$ (5′-AGCCCACCCTTTCGG). The PCR product was purified through an agarose gel and then used as template in a second PCR with primers T7C5′ and 302-315SEL, which contains positions 287 to 356 of satC minus strand with positions 302 to 315 randomized. Products of the second PCR contained a T7 RNA polymerase promoter upstream of full-length satC with randomized bases in positions 302 to 315.

For the first-round selection, 42 two-week-old turnip seedlings were inoculated with transcripts (~5 μg/plant) synthesized from the second PCR product along with TCV helper virus. SatC-sized species from 12 randomly selected plants at 21 dpi were amplified by RT-PCR using primers T7C5′ and oligo 7 and cloned into the *Sma*I site of pUC 19. This clone contained full-length satC sequence immediately downstream from a T7 RNA polymerase promoter. All the clones were sequenced using a primer homologous to positions 206 to 223.

For the second-round selection, total RNA from the 12 analyzed plants or all 42 infected plants of the first round were pooled, and each RNA pool was then reinoculated onto 8 turnip seedlings (~5 μg/plant). SatC-sized species at 21 dpi were assayed as described above.

Competition assays between wild-type satC and sequence 1-a

Equal amounts of transcripts (0.2 μg/plant) of wild-type satC and sequence 1-a were used to inoculate nine turnip seedlings along with TCV genomic RNA transcripts (2 μg/plant). SatC-like molecules were assayed at 10 or 21 dpi as described above.

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REFERENCES

Axelrod, V. D., Brown, E., Priano, C., and Mills, D. R. (1991). Coliphage Qβ RNA replication: RNA catalytic for single-strand release. *Virology* **184,** 595–608.

Barrera, I., Schuppli, D., Sogo, J. M., and Weber, H. (1993). Different mechanisms of recognition of bacteriophage Qβ plus and minus strand RNAs by Qβ replicase. *J. Mol. Biol.* **232,** 512–521.

Brown, D., and Gold, L. (1995a). Template recognition by an RNA-dependent RNA polymerase: Identification and characterization of two RNA binding sites on Qβ replicase. *Biochemistry* **34,** 14765–14774.

Brown, D., and Gold, L. (1995b). Selection and characterization of RNAs replicated by Qβ replicase. *Biochemistry* **34,** 14775–14782.

Buck, K. W. (1996). Comparison of the replication of positive-stranded RNA virus of plants and animals. *Adv. Virus Res.* **47,** 159–251.

Carpenter, C. D., Oh, J. W., Zhang, C., and Simon, A. E. (1995). Involvement of a stem-loop structure in the location of junction sites in viral RNA recombination. *J. Mol. Biol.* **245,** 608–622.

Carpenter, C. D., and Simon, A. E. (1998). Analysis of sequences and predicted structures required for viral satellite RNA accumulation by *in vivo* genetic selection. *Nucleic Acids Res.* **26,** 2426–2432.

Cascone, P. J., Haydar, T. F., and Simon, A. E. (1993). Sequences and structures required for recombination between virus-associated RNAs. *Science* **260,** 801–805.

Dreher, T. W., and Hall, T. C. (1988). Mutational analysis of the sequence and structural requirements in brome mosaic virus RNA for minus strand promoter activity. *J. Mol. Biol.* **201,** 31–40.

Duggal, R., Lahser, F. C., and Hall, T. C. (1994). *Cis*-acting sequences in the replication of plant viruses with plus-sense RNA genomes. *Annu. Rev. Phytopathol.* **32,** 287–309.

Ellington, A. D., and Szostak, J. W. (1990). *In vitro* selection of RNA molecules that bind specific ligands. *Nature* **346,** 818–822.

French, R., and Ahlquist, P. (1987). Intercistronic as well as terminal sequences are required for efficient amplification of brome mosaic virus RNA3. *J. Virol.* **61,** 1457–1465.

Guan, H., Song, C., and Simon, A. E. (1997). RNA promoters located on minus-strands of a subviral RNA associated with turnip crinkle virus. *RNA* **3,** 1401–1412.

Guan, H., Carpenter, C. D., and Simon, A. E. (2000). Analysis of *cis*-acting sequences involved in plus-strand synthesis of a turnip crinkle virus-associated satellite RNA identifies a new carmovirus replication element. *Virology* **268,** 345–354.

Herschlag, D. (1995). RNA chaperones and the RNA folding problem. *J. Biol. Chem.* **270,** 20871–20874.

Kim, Y. N., Jeong, Y. S., and Makino, S. (1993). Analysis of cis-acting sequences essential for coronavirus defective interfering RNA replication. *Virology* **197,** 53–63.

Kim, K. H., and Hemenway, C. L. (1999). Long-distance RNA-RNA interactions and conserved sequence elements affect potato virus X plus-strand RNA accumulation. *RNA* **5,** 636–645.

Klovins, J., Berzins, V., and van Duin, J. (1998). A long-range interaction in Qβ RNA that bridges the thousand nucleotides between the M-site and the 3′ end is required for replication. *RNA* **4,** 948–957.

Lai, M. M. (1998). Cellular factors in the transcription and replication of viral RNA genomes: A parallel to DNA-dependent RNA transcription. *Virology* **244,** 1–12.

Lin, Y. J., and Lai, M. M. (1993). Deletion mapping of a mouse hepatitis virus defective interfering RNA reveals the requirement of an internal and discontiguous sequence for replication. *J. Virol.* **67,** 6110–6118.

Marsh, L. E., Dreher, T. W., and Hall, T. C. (1988). Mutational analysis of the core and modulator sequences of the BMV RNA3 subgenomic promoter. *Nucleic Acids Res.* **16,** 981–995.

Miller, E. D., Plante, C. A., Kim, K. H., Brown, J. W., and Hemenway, C. (1998). Stem-loop structure in the 5′ region of potato virus X genome required for plus-strand RNA accumulation. *J. Mol. Biol.* **284,** 591–608.

Nagy, P. D., and Simon, A. E. (1998). *In vitro* characterization of late steps of RNA recombination in turnip crinkle virus. I. Role of motif1-hairpin structure. *Virology* **249,** 379–392.

Nagy, P. D., Zhang, C., and Simon, A. E. (1998). Dissecting RNA recombination *in vitro:* Role of RNA sequences and the viral replicase. *EMBO J.* **17,** 2392–2403.

Nagy, P. D., Pogany, J., and Simon, A. E. (1999). RNA elements required for RNA recombination function as replication enhancers *in vitro* and *in vivo* in a plus strand RNA virus. *EMBO J.* **18,** 5653–5665.

Pogue, G. P., and Hall, T. C. (1992). The requirement for a 5′ stem-loop structure in brome mosaic virus replication supports a new model for viral positive-strand RNA initiation. *J. Virol.* **66,** 674–684.

Pogue, G. P., Marsh, L. E., Connell, J. P., and Hall, T. C. (1992). Requirement for ICR-like sequences in the replication of brome mosaic virus genomic RNA. *Virology* **188,** 742–753.

Siegel, R. W., Adkins, S., and Kao, C. C. (1997). Sequence-specific recognition of a subgenomic RNA promoter by a viral RNA polymerase. *Proc. Natl. Acad. Sci. USA* **94,** 11238–11243.

Siegel, R. W., Bellon, L., Beigelman, L., and Kao, C. C. (1998). Moieties in an RNA promoter specifically recognized by a viral RNA-dependent RNA polymerase. *Proc. Natl. Acad. Sci. USA* **95,** 11613–11618.

Simon, A. E., and Howell, S. H. (1986). The virulent satellite RNA of turnip crinkle virus has a major domain homologous to the 3′ end of the helper virus genome. *EMBO J.* **5,** 3423–3428.

Singh, R. N., and Dreher, T. W. (1997). Turnip yellow mosaic virus RNA-dependent RNA polymerase: Initiation of minus strand synthesis *in vitro*. *Virology* **233,** 430–439.

Singh, R. N., and Dreher, T. W. (1998). Specific site selection in RNA resulting from a combination of nonspecific secondary structure and -CCR- boxes: Initiation of minus strand synthesis by turnip yellow mosaic virus RNA-dependent RNA polymerase. *RNA* **4,** 1083–1095.

Song, C., and Simon, A. E. (1995a). Requirement of a 3′-terminal stem-loop in *in vitro* transcription by an RNA-dependent RNA polymerase. *J. Mol. Biol.* **254,** 6–14.

Song, C., and Simon, A. E. (1995b). Synthesis of novel products *in vitro* by an RNA-dependent RNA polymerase. *J. Virol.* **69,** 4020–4028.

Stawicki, S. S., and Kao, C. C. (1999). Spatial perturbations within an RNA promoter specifically recognized by a viral RNA-dependent RNA polymerase (RdRp) reveal that RdRp can adjust its promoter binding sites. *J. Virol.* **73,** 198–204.

Stupina, V., and Simon, A. E. (1997). Analysis *in vivo* of turnip crinkle virus satellite RNA C variants with mutations in the 3′-terminal minus-strand promoter. *Virology* **238,** 470–477.

Tuerk, G., and Gold, L. (1990). Systematic evolution of ligands by exponential enrichment: RNA ligands to bacteriophage T4 DNA polymerase. *Science* **249,** 505–510.

Wang, J., and Simon, A. E. (1997). Analysis of the two subgenomic RNA promoters for turnip crinkle virus *in vivo* and *in vitro*. *Virology* **232,** 174–186.

Wang, J., Carpenter, C. D., and Simon, A. E. (1999). Minimal sequence and structural requirements of a subgenomic RNA promoter for turnip crinkle virus. *Virology* **253,** 327–336.

Zuker, M., Jaeger, J. A., and Turner, D. H. (1991). A comparison of optimal and suboptimal RNA secondary structures predicted by free energy minimization with structures determined by phylogenetic comparison. *Nucleic Acids Res.* **19,** 2707–2714.