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Minimal Sequence and Structural Requirements of a Subgenomic
RNA Promoter for Turnip Crinkle Virus

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Infection of plants or protoplasts with turnip crinkle virus (TCV) results in the synthesis of the genomic RNA and two subgenomic (sg) RNAs of 1.7 kb and 1.45 kb, respectively. Both of the sgRNA promoters were characterized previously and their secondary structures predicted by computer analysis [J. Wang and A. E. Simon (1997). *Virology* 232, 174–186]. Secondary structure-sensitive chemical and enzymatic probes have now been used to determine the structure of the promoter directing synthesis of the 1.45-kb sgRNA, namely the 1.45-kb sgRNA promoter, in solution. The newly obtained structure conforms with the previously predicted hairpin structure except for the hairpin base: four CG base pairs and a CA bulge are present instead of an A bulge. Studies of deletions within the 96-nucleotide (nt) 1.45-kb sgRNA promoter defined a minimal 30-nt core sequence as essential for promoter activity: a 21-nt hairpin and a 9-nt flanking single-stranded sequence. Mutational analysis in the stem section of the core promoter supported a role for the primary sequence and secondary structure in promoter activity. Sequence alterations in the flanking single-stranded region further suggest that the sequence CCCAUUA, encompassing the transcription start site, is required for efficient transcription of the 1.45-kb sgRNA by the TCV RNA-dependent RNA polymerase *in vivo*.   © 1999 Academic Press

Key Words: turnip crinkle virus; RNA replication; RNA-dependent RNA polymerase; subgenomic RNAs; plant RNA viruses.

INTRODUCTION

Subgenomic RNA (sgRNA) synthesis is a common strategy employed by many plus-strand RNA viruses for expression of downstream open reading frames (ORFs). sgRNAs, which are 3′ coterminal with the genomic RNA, are synthesized by the viral replicase from internal promoters located on the complementary minus strands (Miller *et al.,* 1985; Gargouri *et al.,* 1989; van der Kuyl *et al.,* 1990). The boundaries of several sgRNA promoters have been delineated by deletion analysis either *in vivo* and/or *in vitro*. For example, taking the transcription start site as +1, the core promoter for the sgRNA of alfalfa mosaic virus was mapped to positions −8 to −55 *in vitro* with an upstream enhancer element from −55 to −136 (van der Kuyl *et al.,* 1990). Additional sequences between positions +12 and −136 were required for full activity *in vivo* (van der Kuyl *et al.,* 1991; van der Vossen *et al.,* 1995). The sgRNA promoter of beet necrotic yellow vein virus was mapped to positions from −16 to between +100 and +208 *in vivo* (Balmori *et al.,* 1993). *In vivo* studies also localized the sgRNA promoter of cucumber mosaic virus to between positions −70 and +20, which encompass an ICR2-like motif upstream of the transcription start site (Boccard and Baulcombe, 1993). This is in contrast to a

similar ICR2 motif in brome mosaic virus (BMV) that is outside the sgRNA promoter (French and Ahlquist, 1988; Pogue *et al.,* 1992).

The promoter for the coat protein mRNA of BMV is the best-characterized sgRNA promoter. Using an *in vitro* approach, four sgRNA promoter elements were defined: the upstream AU-rich region, the polyuridylate tract, the core promoter, and the downstream AU-rich region (Marsh *et al.,* 1988). The upstream AU-rich region has been implicated in high-level sgRNA synthesis both *in vitro* (Marsh *et al.,* 1988) and *in vivo* (French and Ahlquist, 1988), whereas the downstream AU-rich region is involved primarily in determining or allowing correct initiation of sgRNA synthesis (Marsh *et al.,* 1988). The most accepted role of the polyuridylate tract is that of a spacer, which allows the RdRp better access to the actual subgenomic promoter elements. Deletion studies have demonstrated that loss of the polyuridylate tract leads to a substantial reduction in sgRNA synthesis (French and Ahlquist, 1988). Recent *in vitro* analyses using proscripts, short regions of (−)-strand BMV RNA3, which contain the sgRNA promoter and a minimal transcribed region, indicate that the polyuridylate tract binds to some component(s) required for RdRp activity and acts as a sequence-specific and position-dependent upregulator for correct initiation of BMV sgRNA synthesis. Successive 3′ and 5′ deletions further demonstrated that the minimal elements required for accurate initiation of sgRNA syn-

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thesis are within a proscript of 22 nucleotides (nt), reducing the previously reported size of the functional core promoter by 12 nt (Adkins et al., 1997).

We are studying the sgRNA promoters of turnip crinkle virus (TCV), a member of the flavivirus-like superfamily of plus-strand RNA viruses (Koonin and Dolja, 1993). TCV has a monopartite genome of 4054 nt containing five ORFs that encode the following products: p28 and its readthrough product p88, which comprise the viral RNA-dependent RNA polymerase (RdRp) (Hacker et al., 1992; White et al., 1995); p8 and p9, which are viral movement proteins (Hacker et al., 1992; Li et al., 1998), and p38, the viral coat protein (CP) (Carrington et al., 1987). The TCV RdRp subunits are translated from the genomic RNA, while two sgRNAs (1.7 and 1.45 kb) are required for translation of the movement proteins and CP, respectively (Carrington and Morris, 1986; Carrington et al., 1989; Oh et al., 1995; Li et al., 1998). Analysis of these two sgRNA promoters in vivo defined a sequence from −90 to +6 for the 1.45-kb sgRNA promoter and from −90 to +4 for the 1.7-kb sgRNA promoter. Both sequences were predicted by computer modeling to form large hairpin structures. The 1.45-kb sgRNA promoter, but not the 1.7-kb sgRNA promoter, was active in an in vitro RdRp transcription assay (Wang and Simon, 1997).

In this paper we report the use of chemical and enzymatic probes to modify the computer-generated secondary structure of the 1.45-kb sgRNA promoter. In addition, we have determined that the 30 nt that comprise the base of the previously identified 96-nt hairpin are sufficient for wild-type (wt) levels of sgRNA synthesis in protoplasts. Mutational studies on the minimal functional promoter as well as the wt promoter suggest that both primary sequence and secondary structure of the hairpin contribute to promoter activity in vivo.

RESULTS

Probing the secondary structure of the 1.45-kb sgRNA promoter

To characterize the potential structure-specific elements required for promoter activity, the secondary structure of the 1.45-kb sgRNA promoter was determined by chemical and enzymatic modifications followed by reverse transcriptase-mediated extension from oligonucleotides as described under Materials and Methods. Diethylpyrocarbonate (DEPC), which modifies the N-7 positions of A residues, and dimethylsulfate (DMS), which reacts specifically with the N-1 positions of A residues and N-3 positions of C residues, were used to identify single-stranded bases. RNase T1 is an endoribonuclease that hydrolyzes RNA chains on the 3′ side of G residues in single-stranded RNA. RNase T2 is a nonspecific endoribonuclease that hydrolyzes single-stranded RNA and shows a preference for phosphodiester bonds on the 3′ side of A residues (Ehresmann et

al., 1987). Endonuclease V1 digests phosphodiester bonds at double-stranded or stacked single-stranded regions (Lowman and Draper, 1986).

Sample gels obtained by subjecting full-length transcripts of minus-strand TCV genomic RNA to chemical and enzymatic modifications are shown in Fig. 1A, and a summary of the data are displayed in Fig. 1B. The structure of the previously delineated 1.45-kb sgRNA promoter as determined by computer predictions (Wang and Simon, 1997) was similar to the structure shown in Fig. 1B. Differences between the two structures were at the base of the hairpin, where the computer-generated model predicted four CG base pairs (positions 2518–2521 paired with positions 2600–2603) and a small asymmetrical bulge comprising A2522 (Wang and Simon, 1997). The new data obtained by structural probing suggest that the four CG base pairs are formed between C2517–2520 and G2600–2603, with C2521 and A2522 forming the asymmetrical bulge (Fig. 1B). This CA bulge was defined by the modification of C2521 by DMS and A2522 by both DMS and RNase T2 (Fig. 1A, left; note that the sequence ladder has a +1 lag with respect to the primer extension products). The decreasing RNase V1 modification was accompanied by the increasing DMS modification of C2517–2520, and C2520 and G2600 were modified by both DMS and RNase V1 (Fig. 1A). These results suggest a "breathing effect" caused by the small CA bulge that may result in the accessibility of DMS to the labile base-paired region. The involvement of these four C residues in base pairing was further confirmed by RNase V1 modification of G2600–2603 (Fig. 1A, right). In addition, 3 bp between G2516/U2515/G2514 and C2604/A2605/C2606 possibly form at the very base of the stem (Figs. 1A and 1B). However, G2516/U2515/G2514 is not required for promoter activity as assayed by deletion analysis (Wang and Simon, 1997). The nt that G2516/U2515/G2514 are base-paired with (C2604/A2605/C2606) are required and are modified by both DMS and RNase V1. This suggests that these three promoter-required residues participate in more than a single structure (Figs. 1A and 1B) and, for the sake of simplicity, are drawn single-stranded in subsequent figures. Similar dual modification patterns were also found for the upper small stem formed between U2523–G2524 and C2598–A2599 and in the upper part of the hairpin (Figs. 1A and 1B), suggesting the existence of other regions of alternative local structures. Other minor differences between the computer modeling and biochemical structural probing in the upper part of the hairpin are also present but will not be addressed here since the upper hairpin is dispensable for promoter activity (see Fig. 2).

The existence of a number of unspecific signals in the parallel treatments with different probes (e.g., G2524 has a strong unspecific signal in DMS treatment; Fig. 1A) may reflect chemical fragility of the RNA molecule or may be due to peculiarities of the reaction conditions (Felden
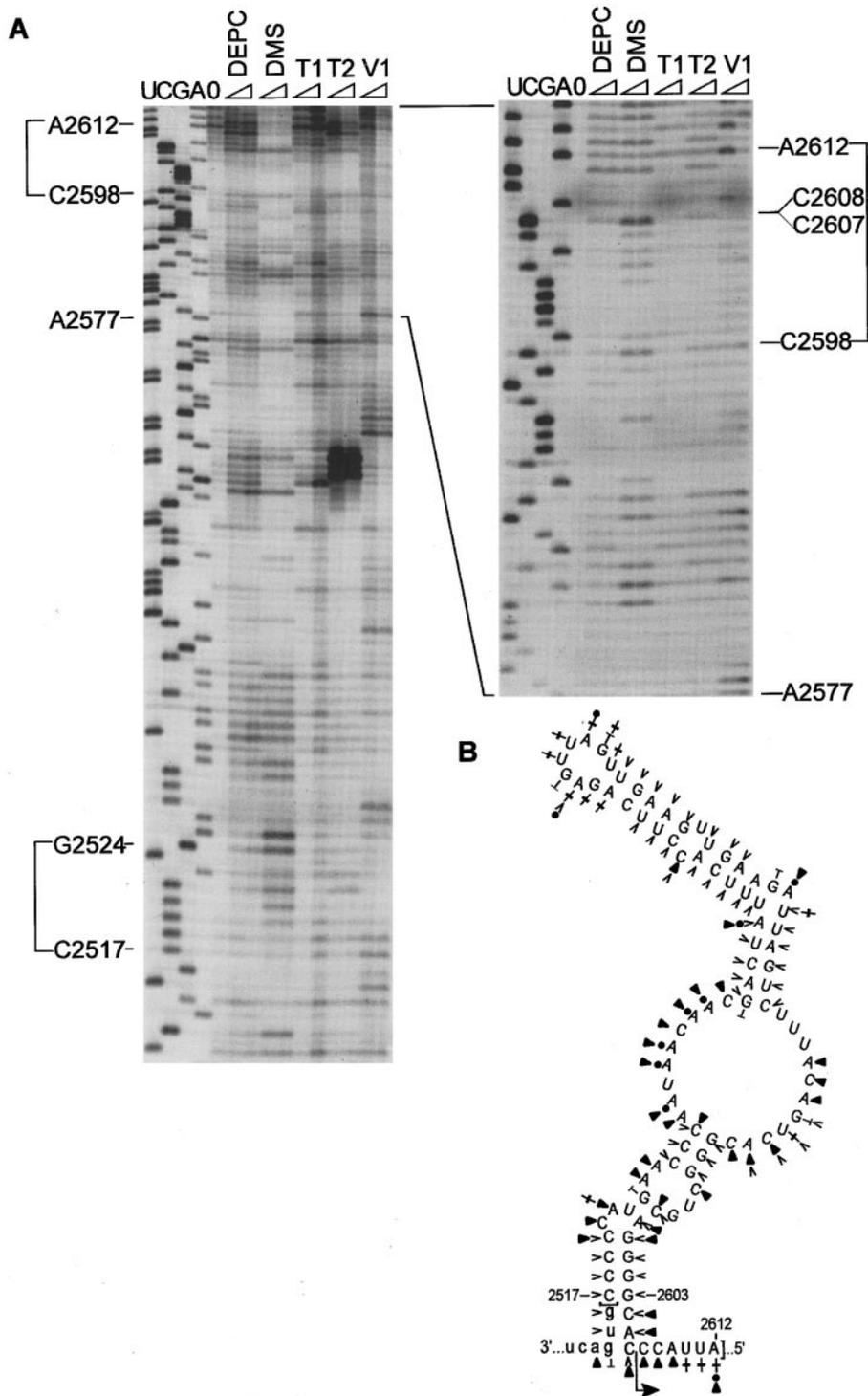
FIG. 1. Chemical and enzymatic structural analysis of the 1.45-kb sgRNA promoter. (A) Representative autoradiogram of a 7% denaturing acrylamide gel containing the primer extension products obtained with primer OL2464C(−) (Table 1) (left) or primer OL2546C(−) (Table 1) (right) following 10- or 20-min treatment of full-length minus-strand TCV genomic RNA with DEPC or DMS or 5- and 10-min treatments with nucleases T1, T2, or V1. "0" lane is the control reaction without any treatment. Triangles surpassing two lanes indicate the increased incubation time. The DNA sequences (5′AGCT3′) have been converted to the corresponding RNA sequences (3′UCGA5′) as shown in (B). Note that the sequence ladder is offset by one base (+1) relative to the primer extension products of the modified or cleaved RNAs. The nucleotides later defined as the core promoter sequence (Fig. 2) are indicated by brackets. The compressed bands containing C2607 and C2608 are indicated (right). (B) Putative secondary structure of the 1.45-kb sgRNA promoter. Nucleotides that have been modified are labeled as follows: ·, DEPC; ▲, DMS; T, RNase T1; +, RNase T2; v, RNase V1. The promoter sequence is bracketed and the nonpromoter nucleotides including those base paired with promoter sequences are shown in lowercase letters. Arrow denotes the transcription start site.
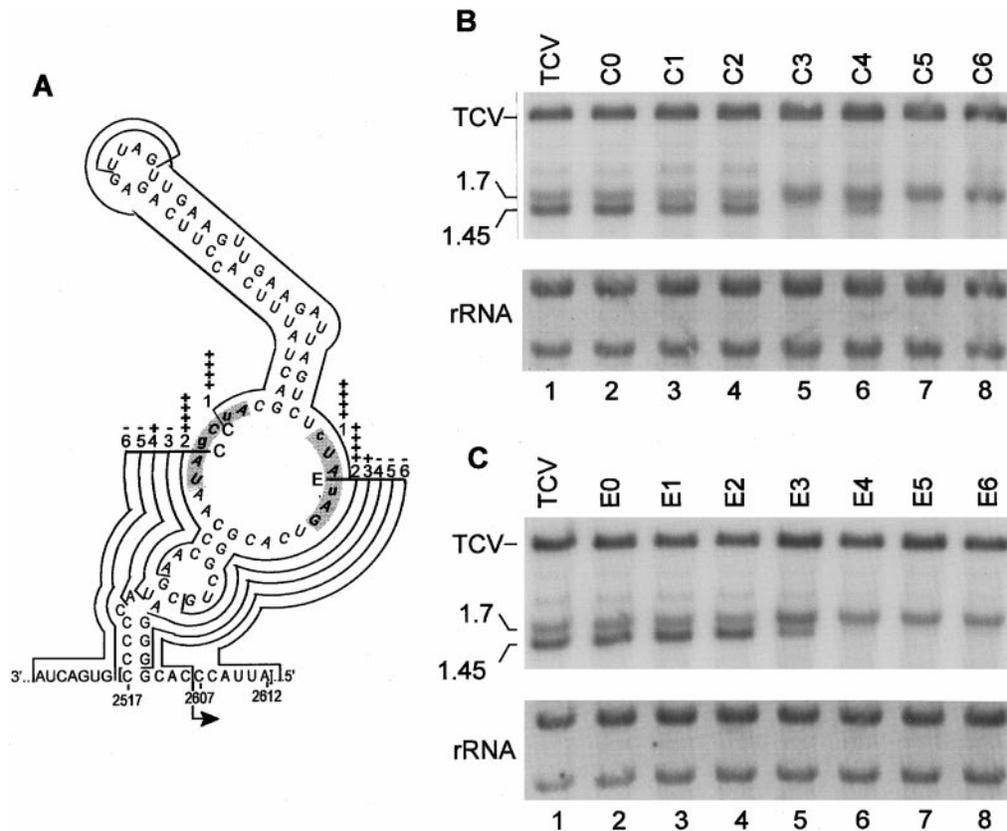
FIG. 2. Defining essential elements of the 1.45-kb sgRNA promoter. (A) Schematic representation of the deletions within the promoter. The sequences corresponding to the new *Cla*I (C) and *Eco*RV (E) sites are shaded. Lowercase letters denote the nt altered to generate the new restriction sites. Deletions, denoted by lines, were generated in both directions from the restriction sites as indicated. The names of the deletion mutants (C or E, 1–6) are indicated. Relative accumulation of the 1.45-kb sgRNA in protoplasts inoculated with the mutant transcripts are indicated above each mutant name: ++++, accumulation to wt levels; +, 10–20% of wt accumulation; −, no detectable accumulation. (B) Representative RNA gel blot analysis of mutants C0–C6. C0 denotes the mutant contained only the altered restriction site (*Cla*I). *Arabidopsis* protoplasts (5 × 10⁶) were inoculated with 20 μg of either wt TCV transcripts or mutant transcripts. Four micrograms of total RNA extracted 24 h p.i. were subjected to electrophoresis through a 1.2% nondenaturing agarose gel and analyzed by RNA gel blot hybridization using probes specific for TCV genomic RNA (see Table 1) and ribosomal RNA (rRNA). Species corresponding to TCV genomic RNA and the 1.45- and 1.7-kb sgRNAs are indicated. (C) Representative RNA gel blot analysis of the mutants E0 to E6. E0 denotes the mutant contained only the altered restriction site (*Eco*RV). Protoplast inoculation and RNA gel blot analysis were performed as described in (B).

et al., 1994). Furthermore, the nonhomogeneously distributed signals within a single treatment (e.g., RNase V1 digestion; Fig. 1A) might be due to steric impediments during the interaction between the probe and the RNA molecule (Bernal and Garcia-Arenal, 1997).

Defining the core sequence for the 1.45-kb sgRNA promoter

The 1.45-kb sgRNA promoter was initially determined to encompass 96 nt by deletions extending from positions 3′ and 5′ of the hairpin toward the promoter (Wang and Simon, 1997). To determine what portions of the hairpin are important for promoter activity, *Eco*RV and *Cla*I restriction sites were constructed within the corresponding hairpin sequence in a full-length TCV genomic cDNA sequence (shown in the corresponding RNA structure for the promoter; Fig. 2A). Transcripts synthesized *in vitro* containing the mutations (the 2 alterations required

to generate each restriction site are shown in lowercase letters) were inoculated onto *Arabidopsis* Col-0 protoplasts. Total RNA extracted at 24 h p.i. was subjected to RNA gel blot analysis. As shown in Figs. 2B and 2C, alterations leading to the generation of the new restriction sites (constructs C0 and E0) had no effect on the levels of 1.45-kb sgRNA synthesized.

A series of deletions upstream and downstream of the *Eco*RV and *Cla*I restriction sites was generated in the sequence corresponding to the hairpin region of the TCV cDNA. Transcripts containing the deletions were assayed for promoter activity along with wt TCV transcripts. The experiment was repeated three times, and representative autoradiograms are shown in Figs. 2B and 2C. Additional unidentified RNA bands of ca. 2 kb were not consistently observed and may denote genomic RNA degradation products. The amounts of 1.45-kb sgRNA synthesized were normalized to the internal ribosomal

RNA standard following densitometry. The relative levels of activity are summarized above each mutant in Fig. 2A.

Protoplasts inoculated with transcripts containing deletions of the long upper stem region (C1 and E1) had no effect on the levels of 1.45-kb sgRNA synthesized, indicating that these sequences are dispensable for promoter activity. Deletions that extended into the small bulge located just below the large middle bulge (C2 and E2) also did not reduce the levels of 1.45-kb sgRNA. However, mutants containing deletions that extended further into the base of the hairpin had either only 10–20% of wt promoter activity (C4 and E3) or no detectable promoter activity (C3, C5, C6, E4, E5, E6). The activity regained by construct C4, which contains a deletion of one additional base compared with the inactive construct C3, could be due to reformation of sequence or structure-specific elements required for promoter activity. These results, in combination with our previously obtained results (Wang and Simon, 1997), define a 23-nt sequence between positions 2517–2524 and 2598–2612 as essential for promoter activity.

Sequence and structure-specific requirements for 1.45-kb sgRNA promoter activity

The results described above indicate that only the lower stem region of the hairpin is required for promoter activity (Fig. 3A). To confirm this result and explore possible structural elements, two constructs were generated. Construct 1.45mini contains a deletion from positions 2528–2593, leaving only 30 nt within the previously defined promoter including 7 nt that form a small loop to maintain the hairpin structure (Fig. 3B). The second construct, 1.45miniΔ, contains a deletion of the entire upper stem region (positions 2525–2597), leaving only the defined 23 nt, which form an altered structure (Fig. 3B). Inoculation of *Arabidopsis* protoplasts with transcripts of 1.45mini followed by RNA gel blot analysis revealed that wt levels of 1.45-kb sgRNA were synthesized (Fig. 3D; compare lanes 1 and 2), confirming that the majority of the natural hairpin is dispensable for promoter activity. However, only very low levels of the 1.45-kb sgRNA were detected in 1.45miniΔ-infected protoplasts (Fig. 3D; compare lanes 6 and 7), suggesting that the structure in the upper stem is also important for promoter activity.

To elucidate the importance of the primary sequence and further explore the structural elements of the promoter, mutations were introduced into the bottom stem to alter base pairing (1.45cm1; Fig. 3B). In addition, compensatory mutations were generated in 1.45mini that exchanged the GGGG and CCCC in the lower stem (1.45cm3; Fig. 3B) or the AC and UG in the upper stem (1.45cm2; Fig. 3B). While the mutations in 1.45cm1 and 1.45cm2 eliminated detectable promoter activity (Fig. 3D, lanes 4 and 5), the compensatory exchange in 1.45cm3 maintained 12% of wt promoter activity (Fig. 3D, lane 8).

These results suggest that both the primary sequence and the secondary structure are important for promoter activity (see Discussion).

To further confirm the sequence- and structure-specific requirements of the promoter, point mutations were introduced into the two lower stems of the wt TCV cDNA to disrupt primary and secondary structure (Fig. 3C). Mutations in either the upper stem (stm1 and stm2) or the lower stem (stm4 and stm5) or both (stm3) eliminated detectable 1.45-kb sgRNA synthesis (Fig. 3E, lanes 2–6).

To elucidate the importance of the 9-nt single-stranded region located 5′ of the hairpin for promoter activity, an insertion and various base changes were introduced in either 1.45mini or wt TCV cDNA. This experiment was repeated three times with similar results. Transcripts containing an insertion of a single U residue 3 nt upstream of the transcription start site in 1.45mini (construct 1.45mini+U; Fig. 3B) did not generate detectable 1.45-kb sgRNA (Fig. 3D, lane 3). The multiple point mutations introduced into the 9-nt single-stranded sequence in the wt TCV cDNA (Fig. 4A) strongly reduced promoter activity if they affected one (ssm5) or more (ssm6, ssm7, ssm8, ssm9, ssm10, ssm11) of the three C residues at positions 2606–2608 (Fig. 4B, lanes 6–12). Constructs with single (ssm1 and ssm2) or double (ssm3) mutations in the remaining six positions (Fig. 4A) had no disruptive affect on the promoter (Fig. 4B, lanes 2–4). These results demonstrate the importance of the three C residues that surround the transcription start site for promoter activity. Furthermore, ssm4 with three mutations in the AU-rich positions from 2609 to 2612 (Fig. 4A) reduced the amount of 1.45-kb sgRNA synthesized by 20% (Fig. 4B, lane 5), suggesting that the A/U residues instead of G/C residues in this region are also required for full promoter activity.

DISCUSSION

The 1.45-kb sgRNA promoter in TCV was delineated previously to consist of a 96-nt sequence located between positions 2517 and 2612. To determine whether the entire 96-nt sequence was required for promoter activity, internal deletions were generated within the sequence and production of the 1.45-kb sgRNA assayed in protoplasts (Fig. 2A). Results demonstrate that a substantial portion of the interior of the 96-nt sequence could be deleted without loss of promoter activity (Figs. 2B and 2C). The remaining elements required for promoter function consist of two distantly located sequences between positions 2517–2524 and 2598–2612, present at the borders of the previously defined 96-nt sequence (Fig. 1A). These 23 nt form a hairpin, consistent with secondary structural elements contributing to the activity of the 1.45-kb sgRNA promoter (Fig. 3A).

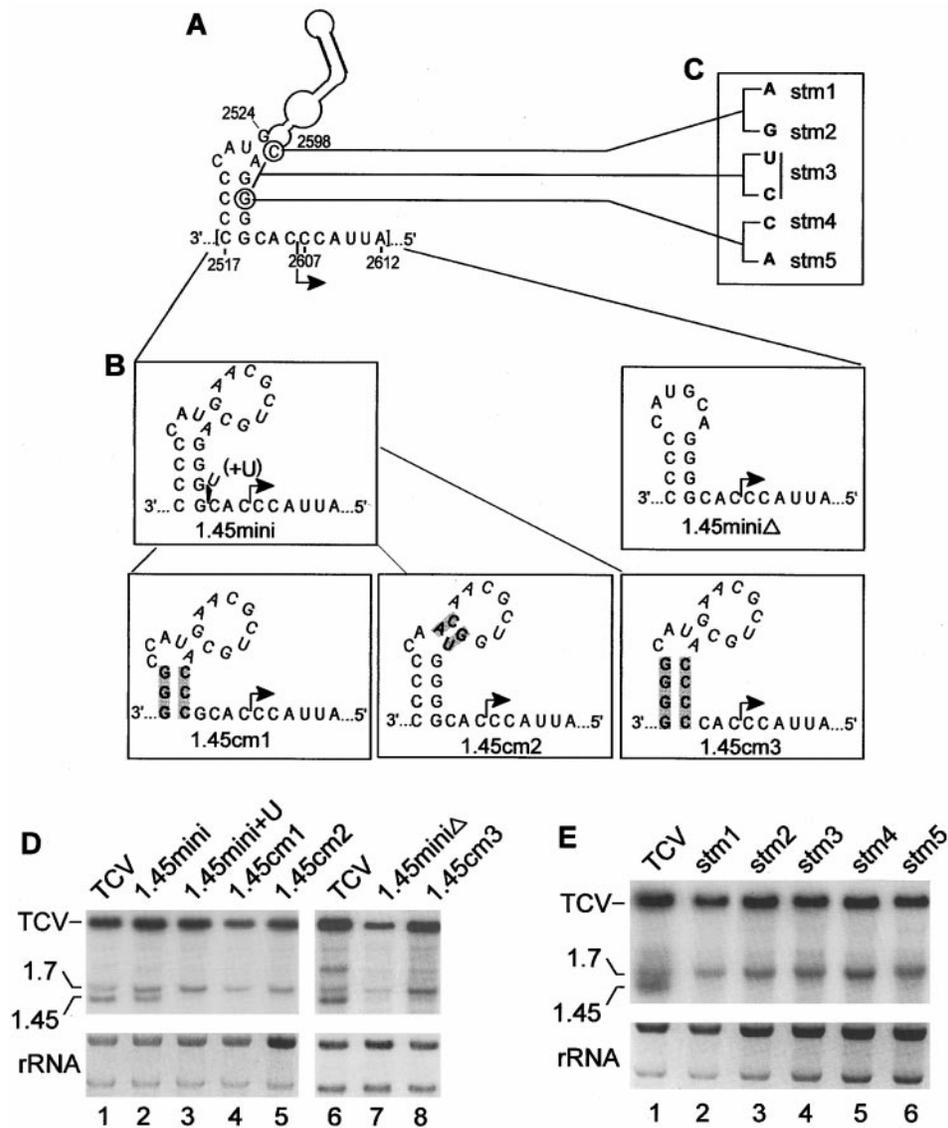Computer analysis of the structures of the promoter-competent deletion mutants C0, C1, C2, E0, E1, and E2

FIG. 3. Sequence and structure-specific requirements of the 1.45-kb sgRNA promoter. (A) Summary of the essential regions of the promoter defined in Fig. 2 and from previous deletion studies (Wang and Simon, 1997). The loop-containing lines denote the dispensable nucleotides within the previously defined promoter. (B) Schematic representation of the mutants constructed from wt TCV (1.45mini and 1.45miniΔ) and the mutants constructed within the minimal promoter construct 1.45mini (1.45cm1–3). Altered residues are shaded. An insertion of a U residue in 1.45mini is indicated as +U. (C) Site-directed mutagenesis of the two distinct stems within the defined promoter. (D) RNA gel blot analysis of the mutants shown in (B). Lanes 1–5 and lanes 6 and 7 are from two independent experiments. (E) RNA gel blot analysis of the mutants shown in (C). Infection of protoplasts and RNA blot hybridization were performed as described in Fig. 2.

(Fig. 2A) revealed that the 23-nt core promoter within each forms the same secondary structure at the base of the hairpin as wt TCV (data not shown). This result further supports the importance of the lower two short stems of the hairpin for promoter activity. For mutant E3, 2 nt were deleted that participated in base pairing in the short upper stem (Fig. 2A). According to the computer-derived structure of mutant E3, a structure similar to wt is still generated by replacement of these 2 nt with U2583 and A2584. Replacing the wt GC base pair with a weaker GU base pair in the upper stem probably contributed to the reduced promoter activity of E3 (Fig. 2C, lane 5). The deletion in mutant C3 eliminates the short upper stem

and results in nondetectable promoter activity (Fig. 2B, lane 5). However, an additional base deletion in mutant C4 recovers some promoter activity (Fig. 2B, lane 6), which may be due to regaining some unknown specific structural elements.

The importance of sequence and structure in the 30-nt core promoter of 1.45mini was studied by altering the base pairing (mutant 1.45cm1) or by exchanging bases in the two putative stems (mutants 1.45cm2 and 1.45cm3; Fig. 3B). While 1.45cm3 maintained ~12% of wt promoter activity (Fig. 3D; compare lanes 6 and 8), neither 1.45cm1 nor 1.45cm2 was able to generate any detectable 1.45-kb sgRNA (Fig. 3D, lanes 4 and 5). Computer analysis re-
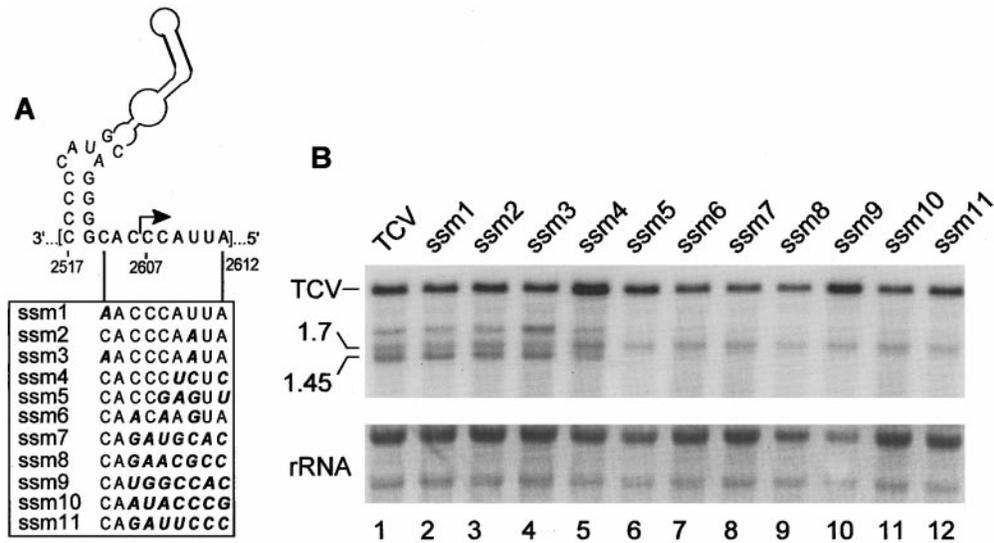
FIG. 4. Site-directed mutagenesis of the single-stranded region within the 1.45-kb sgRNA promoter. (A) Schematic representation of the introduced mutations. Mutated nucleotides are shown in bold italic and aligned with the wt promoter sequences. (B) RNA gel blot analysis of the mutants. Infection of protoplasts and RNA blot hybridization were performed as described in Fig. 3.

vealed that while 1.45cm2 maintains a similar structure as 1.45mini, 1.45cm1 forms a different structure and 1.45cm3 forms a 1.45miniΔ-like structure without the short upper stem present in 1.45mini (data not shown). This result further suggests the importance of the two short lower stems for full promoter activity. Additional single-base alterations in the upper and lower stem (mutants stm1 to stm5; Figs. 3C and 3E, lanes 2–6) abolished detectable 1.45-kb sgRNA synthesis, further demonstrating the importance of the sequence and/or structure of the core 30-nt promoter for 1.45-kb sgRNA synthesis.

Sequence specific requirements were studied for the single-stranded region 5′ of the hairpin (minus-strand orientation). Previous (Wang and Simon, 1997) and current results indicate that a 9-nt sequence is important for sgRNA promoter function. Within this sequence is the transcription start site at position 2607 (Wang and Simon, 1997). Encompassing the transcription start site is the sequence CCCAUUA, which is identical to the consensus sequence (C)CC A/U A/U A/U at the 3′ end of minus-strand TCV genomic and satellite (sat-) RNAs and other carmovirus genomic RNAs (Guan et al., 1997). All promoters for the TCV RdRp analyzed to date including plus- and minus-strand promoters for TCV sat-RNA C (Song and Simon, 1995; Guan et al., 1997), plus-strand promoter for sat-RNA D (Carpenter and Simon, 1996a,b), and the sgRNA promoters (Wang and Simon, 1997), contain the residues CCC at the transcription initiation site. Any alterations within the three C residues surrounding the transcription start site (mutants ssm5 to ssm11) abolished detectable promoter activity. This result, combined with similar results for other TCV RdRp promoters (H. Guan and A. E. Simon, in preparation), indicates that

three C residues play an important role in transcription initiation by the TCV RdRp.

The minus-strand promoter of sat-RNA C, like the 1.45-kb sgRNA promoter, consists of a hairpin and adjacent single-stranded region (Song and Simon, 1995; Stupina and Simon, 1997; Nagy et al., 1997; Carpenter and Simon, 1998). The TCV RdRp can also use single-stranded sequences as promoters: two promoters located on minus-strands of sat-RNA C are sequence specific and have no obvious structure (Guan et al., 1997; H. Guan and A. E. Simon, in preparation). These results indicate the versatility of the TCV RdRp in promoter recognition, which may be responsible for the high-frequency RNA recombination and multiple sat-RNAs and defective interfering (DI) RNAs associated with this virus (Simon and Nagy, 1996).

The BMV RdRp can also recognize diverse hairpin and linear promoters. The promoter at the 3′ end of plus-strands is a well-characterized tRNA-like structure (Dreher and Hall, 1988). In contrast, in vitro studies using the 33-nt BMV subgenomic promoter revealed a sequence-specific, not secondary-structure-dependent, interaction between the BMV RdRp and the promoter. In addition, positions −17G, −14A, −13C, and −11G relative to the transcription start site must be maintained for the interaction with BMV RdRp (Siegal et al., 1997). More recently mutational analysis of the subgenomic promoter in vitro further revealed that the +1C and +2A are important for BMV sgRNA synthesis (Adkins et al., 1998). Although secondary structure may facilitate efficient subgenomic synthesis in full-length minus-strand RNA3, the natural template for sgRNA synthesis, several lines of evidence suggest that it plays a minor role in the BMV RdRp

TABLE 1

Summary of the Oligonucleotides Used in This Study

Application/ Construct	Name	Position in TCVms[a]	Sequence[b]	Polarity[c]
Primer extension	OL2464C(−)	2464–2483	5′ ACGCGGCTAGCTCTGTTTTA 3′	+
	OL2546C(−)	2546–2562	5′ AGTGGAAGTCTCAATCA 3′	+
Site-directed	OL-ClaIC(−)	2524–2549	5′ CTTGGGTTATCGATGCTGATAAAGTG3′	+
mutagenesis	OL-EcoRVC(−)	2570–2594	5′ CTTCTAATCAGAGATATCAGTGCCC 3′	+
RNA gel blots	OL3892C(+)	3893–3913	5′ CCGTTTTTGGTCCCTAACACA 3′	−
1.45mini	1.45SmaIC(+)		5′ GGGGACGTCGCAAGTACCCCCGTGACTAGCAGAAC 3′	−
1.45cm1	1.45Cm1C(+)	⎡2503–2603	5′ GCCCACGTCGCAAGTACCGGGGTGACTAGCAGAAC 3′	−
1.45cm2	1.45Cm2C(+)	Δ2528–2593⎤	5′ NGGGTGGTCGCAACAACCCCGTGACTAGCAGAAC 3′	−
1.45cm3	1.45Cm3C(+)		5′ CCCCACGTCGCAAGTACGGGGGTGACTAGCAGAAC 3′	−
1.45miniΔ	1.45MmC(+)	⎡2503–2603 Δ2525–2597⎤	5′ GGGGACGTACCCCCGTGACTAGCAGAAC 3′	−
ssmX	SsmXC(−)	2604–2623	5′ NNNNNNNNNNATATGCTTTCT 3′	+
	OL2604C(−)	2604–2623	5′ GTGGGTAATATATGCTTTCT 3′	+
	OL3270C(+)	3270–3287	5′ TCCAGGGCACGCTAGATA 3′	−
	RV-1C(−)	2095–2114	5′ GATATCTTGCCTGAAGAGGAATTT 3′	+
	Dope-0C(+)	2584–2603	5′ GGGGACGTCGGGCACTGACA 3′	−

[a] ⎡2503–2603 Δ2528–2593⎤ indicates the deletions between positions 2528 and 2593 within the oligonucleotides designed between positions 2503 and 2603.

[b] Shaded sequences are mutated sequences either to introduce the restriction sites (underlined) or create the specific mutations.

[c] Polarity refers to homology (+) or complementarity (−) with plus-strands of TCV genomic RNA.

recognition of the subgenomic core promoter (Siegel *et al.,* 1997).

It is interesting that minus-strand synthesis of TCV genomic and subviral RNAs begins at the 3′ end of the plus-strand template and passes through the required hairpin, thus allowing the promoter to be included in the complementary strands of full-length genomic RNA for subsequent replication cycles. In contrast, synthesis of the 1.45-kb sgRNA begins 5′ of the hairpin and does not include the hairpin, resulting in the absence of the promoter from the sgRNA transcripts. The lack of sgRNA promoters in the sgRNA transcripts would likely preclude the replication of TCV sgRNAs, though there is some evidence to support replication of sgRNAs of coronaviruses (Sawicki and Sawicki, 1990; Schaad and Baric, 1994).

MATERIALS AND METHODS

RNA structure probing

Minus-strand transcripts of TCV genomic RNA were synthesized from plasmid pT7TCV7, which contains a full-length cDNA of TCV downstream from a T7 RNA polymerase promoter. The plasmid was digested with *Xba*I prior to transcription using T7 RNA polymerase as previously described (Carpenter *et al.,* 1995). The transcripts were treated with DNaseI (Gibco BRL) to remove template DNA followed by phenol/chloroform extraction and ammonium acetate/isopropanol precipitation.

Chemical and enzymatic modifications were performed as described (Song and Simon, 1995) with mod-

ifications. Eleven micrograms of purified minus-strand TCV genomic RNA plus 110 μg of crude yeast tRNA were added to 675 μl of modification buffer [70 mM HEPES (pH 7.5), 10 mM MgCl$_2$, 0.1 mM EDTA, 100 mM KCl], heated to 60°C, slowly cooled to 35°C, and then incubated at 25°C for 20 min. Fifty-microliter samples of the RNA were added to equal volumes of modification buffer containing either no additions or one of the following: 10% (v/v) DEPC (Sigma), 1% (v/v) DMS (Kodak), 0.05 units of RNase T1, 0.03 units of RNase T2, and 0.05 units of RNase V1 (Gibco BRL). DEPC and DMS reactions were incubated at 30°C for 10 and 20 min. Samples were incubated with RNases T1, T2, and V1 for 5 and 10 min at 30°C. The control sample with no chemicals or enzymes added was incubated at 30°C for 10 min. The control and modified RNA samples were extracted with phenol/chloroform, precipitated with ammonium acetate/isopropanol, and then dissolved in 5 μl of distilled water.

For primer extension reactions, 1 μl (6 μM) of oligonucleotide OL2464C(−) or OL2546C(−) (Table 1), complementary to minus-strand positions 2464–2483 and 2546–2562, respectively was mixed with 2 μl of the modified RNA, heated briefly at 90°C, and then slowly cooled to 40°C. Reverse transcription was performed in a 5 μl volume by the addition of 0.05 mM each of dGTP, dCTP, and dTTP, 2.5 μCi of [α-^{35}S]dATP (New England Nuclear, 1398 Ci/mM), 5 units of AMV reverse transcriptase (Amersham), and buffer recommended by the manufacturer. After incubation at 42°C for 5 min, 1 μl containing 0.6 mM each of dATP, dGTP, dCTP, and dTTP and 50 mM NaCl was added. After an additional 15 min

incubation, reactions were terminated by the addition of 3 μl of 95% (v/v) formamide, 20 mM EDTA, 0.05% (w/v) bromphenol blue, and 0.05% (w/v) xylene cyanol. The DNA sequence ladder was generated using the same primer on plasmid pT7TCV7 and a DNA sequenase kit (USB). Samples were subjected to electrophoresis on a 7% Long-Ranger sequencing gel (FMC BioProducts) and exposed to x-ray film overnight.

Plasmid constructions

To introduce deletions into the 1.45-kb sgRNA promoter, ClaI and EcoRV sites were engineered into pT7TCVms (Oh et al., 1995) at positions 2532–2537 (to generate construct C0) and 2882–2887 (to generate construct E0) by site-directed mutagenesis as described (Kunkel, 1985) using oligonucleotides OL-ClaIC(−) and OL-EcoRVC(−), respectively (Table 1). Mutants C1–C6 were constructed in the plasmid containing the ClaI site (construct C0) by linearization of the plasmid with ClaI and treatment with Bal 31 (New England Biolabs). This was followed by DNA polymerase large fragment (Klenow) treatment and digestion with XbaI, which digests once within the vector. Depending on the desired direction of deletion, either the larger or smaller resultant fragment was gel-purified and ligated to the appropriate fragment of the same untreated plasmid. Mutants E1–E6 were generated by a similar strategy using the engineered pT7TCVms clone containing the EcoRV site (construct E0).

For construction of plasmid 1.45mini, oligonucleotide 1.45SmaIC(+), containing a deletion from position 2528 to 2593, was used with oligonucleotide RV-1C(−) in a polymerase chain reaction (PCR). The PCR product was digested with EcoRI, and the fragment containing the partial promoter sequence was combined with a second fragment containing an additional partial promoter sequence. This second fragment was purified from a BsmI-digested product that was amplified by PCR using oligonucleotides OL2604C(−) and OL3270C(+). The mixture was then ligated to pT7TCVms, which had been previously digested with EcoRI and BsmI. Constructs 1.45miniΔ, 1.45cm1, 1.45cm2, and 1.45cm3 were generated in a similar fashion except that the internal primer 1.45SmaIC(+) was replaced with 1.45MmC(+), 1.45Cm1C(+), 1.45Cm2C(+), and 1.45Cm3C(+), respectively. 1.45mini+U is a fortuitous mutant that was generated during the cloning.

The ssmX series of mutants, which contain mutations in the single-stranded region of the 1.45-kb sgRNA promoter, were also constructed in a similar fashion except that primers 1.45SmaIC(+) and OL2604C(−) were replaced with Dope-0C(+) and SsmXC(−), respectively.

Inoculation of protoplasts

Plasmids were linearized by SmaI digestion (unless otherwise noted) and subjected to in vitro transcription as described previously (Carpenter et al., 1995). Such transcripts contained wt 5′ and 3′ ends. Protoplasts (5 × 10⁶) prepared from callus cultures of Arabidopsis ecotype Col-0 were inoculated with 20 μg of TCV genomic RNA transcripts, either wt or mutant, as described (Kong et al., 1997).

RNA gel blot analysis

Total RNA was isolated 24 h p.i. of protoplasts and subjected to electrophoresis through nondenaturing 1.2% agarose gels as previously described (Wang and Simon, 1997).

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