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Analysis of the Two Subgenomic RNA Promoters for Turnip Crinkle Virus *in Vivo* and *in Vitro*

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Infection of plants or protoplasts with turnip crinkle virus (TCV), a monopartite RNA virus, results in the synthesis of the genomic RNA and two subgenomic (sg) RNAs. The transcription start site for the 1.45-kb sgRNA was previously mapped to position 2606 (J. C. Carrington, T. J. Morris, P. G. Stockley, and S. C. Harrison, (1987). *J. Mol. Biol.* 194, 265–276) corresponding to position 2607 in the TCVms isolate and the start site for the 1.7-kb sgRNA has now been mapped to position 2333 in TCVms. A 96-base sequence (90 bases upstream and 6 bases downstream) encompassing the transcription start site for the 1.45-kb sgRNA was sufficient for full promoter activity. Similarly, a 94-base sequence (90 bases upstream and 4 bases downstream) encompassing the start site was required for full activity of the 1.7-kb sgRNA promoter. The 1.45-kb sgRNA promoter, but not the 1.7-kb sgRNA promoter, was able to direct synthesis of a nontemplate RNA *in vitro* using partially purified TCV RNA-dependent RNA polymerase. Computer generated secondary structures for the two sgRNA promoters revealed an extensive hairpin just upstream from the transcription start site. Comparisons of corresponding sequences from related viruses indicates higher sequence conservation for the 1.45-kb sgRNA promoter compared with the 1.7-kb sgRNA promoter, despite the latter's location within the RNA-dependent RNA polymerase open reading frame.

INTRODUCTION

Multicistronic RNA viruses with genomes of positive polarity translate open reading frames (ORFs) that are not 5′ proximal from subgenomic (sg) RNAs. SgRNAs, which are 3′ coterminal with the genomic RNA, are generally believed to be synthesized either via initiation by the viral replicase at internal, minus-strand promoters [e.g., brome mosaic virus (BMV), Miller *et al.,* 1985; alfalfa mosaic virus (AlMV), Gargouri *et al.,* 1989; turnip yellow mosaic virus, van der Kuyl *et al.,* 1990], or by discontinuous leader-primed transcription, as described for coronaviruses (Spaan *et al.,* 1983; Lai *et al.,* 1984; Baric *et al.,* 1985; Lai *et al.,* 1990; Zhang *et al.,* 1994).

Most studies using plant viruses have concentrated on defining the single sgRNA promoter associated with coat protein (CP) mRNA synthesis in viruses containing multipartite genomes. Delineations of the boundaries for the sgRNA promoters have depended on whether *in vivo* or *in vitro* assays were used. For example, the sgRNA promoter of AlMV was mapped *in vitro* to two regions: a core promoter from −8 to −55 (the transcription start site is +1) and an enhancer element from −55 to −136 (van der Kuyl *et al.,* 1990). *In vivo,* additional sequence between +12 and −136 is required for full activity (van der Vossen *et al.,* 1995). The core promoter for *in vitro* sgRNA synthesis in BMV is located between −20 and +16, with full activity requiring a repetitive UUA sequence between −40 and −54 and a poly (A)-stretch between −20 and −40 (Marsh *et al.,* 1988). Activity of the sgRNA promoter *in vivo,* however, required sequences extending to at least −74 but not beyond −95 (French and Ahlquist, 1988). *In vivo* studies have localized the sgRNA promoter of cucumber mosaic virus to between −70 and +20 (Boccard and Baulcombe, 1993). The sgRNA promoter on RNA3 of beet necrotic yellow vein virus differs from the sgRNA promoters described above as it is situated largely downstream of the transcription start site, extending from −16 to between +100 and +208 of the start site (Balmori *et al.,* 1993).

Viruses without divided genomes require the synthesis of several sgRNAs from the single genomic RNA for translation of all ORFs. Only the sgRNA promoter responsible for transcription of the cucumber necrosis virus (CNV) CP mRNA (0.9 kb) has been precisely mapped for a plant monopartite virus (Johnston and Rochon, 1995) or any member of virus supergroup II (the flavivirus-like supergroup; Koonin and Dolja, 1993). Sequences required for full activity of the 0.9-kb sgRNA promoter were localized to between −20 and +6 of the transcription start site. Little similarity was found between the 0.9-kb sgRNA promoter and the region surrounding the transcription start site for the CNV 2.1-kb sgRNA.

Turnip crinkle carmovirus (TCV), a member of supergroup II, is a single-stranded positive sense RNA virus containing five ORFs in its 4,054 base genome (Fig. 1A) (Carrington *et al.,* 1989; Oh *et al.,* 1995). The genomic RNA encodes the RNA-dependent RNA polymerase (RdRp) composed of p28 and its readthrough product,

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p88, which are necessary and sufficient for virus replication (Hacker *et al.,* 1992; White *et al.,* 1995). Two 3′ coterminal sgRNAs that are synthesized during infection encode three additional ORFs: a 1.7-kb sgRNA that directs the synthesis of two movement proteins (p8 and p9) (Hacker *et al.,* 1992), and a 1.45-kb sgRNA that serves as mRNA for the synthesis of viral coat protein (CP) (Fig.1A) (Carrington *et al.,* 1987). The transcription start site of the 1.45-kb sgRNA has been mapped to position 2606 in the TCV isolate TCV-B (Carrington *et al.,* 1987).

In this report, we have mapped the transcription start site of the 1.7-kb sgRNA and delineated the promoters for the 1.45- and 1.7-kb sgRNAs. Both promoters were able to direct the synthesis of new sgRNAs when ectopically expressed in the viral genome. The 1.45-kb sgRNA promoter, but not the 1.7-kb sgRNA promoter, was also able to serve as an independent promoter in an *in vitro* RdRp transcription assay.

MATERIALS AND METHODS

Plasmid constructions

Two sets of deletion constructs (NΔX and BΔX) were generated to delineate the 1.45-kb sgRNA promoter. pT7TCVms, a plasmid containing a full-length cDNA of TCV isolate TCVms downstream from a T7 RNA polymerase promoter (Oh *et al.,* 1995), was digested with *Nhe*I digestion (two sites at positions 2457 and 2469 in the TCVms sequence) and the large fragment was treated with an empirical amount of Exonuclease III (Gibco BRL) at 37°. Aliquots were removed at various times and treated with S1 nuclease and DNA polymerase large fragment as described (Ausubel *et al.,* 1995). Samples were digested with *Pst*I, which cleaves within the plasmid sequence, yielding fragments ranging from 1.3 to 1.6 kb. Fragments containing deletions were ligated to the large *Nhe*I–*Pst*I fragment of pT7TCVms following digestion of pT7TCVms with *Nhe*I, treatment with DNA polymerase large fragment to blunt the ends, then digestion with *Pst*I followed by isolation of the large *Nhe*I–*Pst*I pT7TCVms backbone fragment. The BΔX series was generated by digesting pT7TCVms with *Bsm*I (position 3200) and the ends were made blunt with mung bean nuclease (New England Biolabs) before treatment with Exonuclease III/S1 nuclease/DNA polymerase large fragement and subsequent *Pst*I digestion as described above. The larger fragments containing deletions were ligated to the small (0.86 kb) *Bsm*I–*Pst*I fragment of pT7TCVms following digestion of pT7TCVms with *Bsm*I, treatment with mung bean nuclease to blunt the ends, then digestion with *Pst*I and isolation of the small *Bsm*I–*Pst*I fragment.

To construct plasmids SG6, SG7, and SG8, the oligonucleotides listed in Table 1 were used to amplify fragments containing the 1.7-kb sgRNA promoter sequence by PCR. These fragments, two of which (SG6 and SG7) contained *Eco*RV sites introduced by primers RV-1 and RV-2 during PCR, were cloned into the *Bsm*I site in pT7TCVms. To characterize the 1.7-kb sgRNA promoter, two sets of deletions were generated in SG7. The EΔX series was generated by digesting SG7 with *Eco*RV, treating with Exonuclease III as described above and then self-ligation. The HΔX series was generated by digesting SG7 with *Hin*dIII (position 3356 in TCVms), followed by treatment with Exonuclease III as described above. Samples were then digested with *Nhe*I and the smaller fragments containing the deletions cloned into SG7 as replacements for the corresponding *Bsm*I–*Nhe*I fragment.

Plasmids containing the 1.45- or 1.7-kb sgRNA promoters in the forward and reverse orientations downstream from MDV cDNA sequence for analysis of activity *in vitro* were generated by cloning PCR-amplified sgRNA promoter sequences (using primers p9*Sac*I and OL 2613C(+) for the 1.45-kb sgRNA promoter and primers 1.7 LB and 1.7 RB for the 1.7-kb sgRNA promoter, see Table 1) into the *Sma*I site of pT7MDV (Axelrod *et al.,* 1991), generating plasmids pT7MDV1.45(+/−) and pT7MDV1.7(+/−). A *Sac*I site was introduced into pT7MDV1.45(+/−) just downstream from the promoter insert during PCR (Table 1).

In vitro transcription by T7 RNA polymerase and inoculation of protoplasts

Plasmids were linearized by *Sma*I digestion (unless otherwise noted) and subjected to *in vitro* transcription by T7 DNA-dependent RNA polymerase as described previously (Carpenter *et al.,* 1995). Such transcripts contained wild-type (wt) 5′ and 3′ ends. Protoplasts (5×10^6) prepared from callus cultures of *Arabidopsis* ecotype Col-0 were inoculated with 20 μg of TCV genomic RNA transcripts, either wt or containing deletions, as described elsewhere (Kong *et al.,* manuscript in preparation).

RNA gel blot analysis

Four micrograms of total RNA isolated from protoplasts (Simon *et al.,* 1992) was denatured by heating in 50 to 70% formamide and then subjected to electrophoresis through nondenaturing 1.5% agarose gels. RNA was then transferred to NitroPlus membranes (Micron Separations Inc., Westboro, MA) as previously described (Kong *et al.,* 1995). The RNA was crosslinked by placing the blot on a UV light box (310 nm, Fotodyne Inc.) for 2 min followed by drying the blot at 80° for 5 min. An oligonucleotide complementary to positions 3893 to 3913 of TCVms was labeled using polynucleotide kinase and γ-[^{32}P]ATP and then used for detecting genomic and subgenomic RNAs. The ribosomal RNA probe used as a loading control has been described previously (Simon *et al.,* 1992). Prehybridization, hybridization, and washing conditions were performed as described previously (Kong *et al.,* 1995).

Primer extension

The 5′ end of the TCV 1.7-kb sgRNA was mapped by primer extension using MMLV reverse transcriptase as described (Carrington and Morris, 1986; Carrington *et al.,* 1987) with modifications. Briefly, 1 μg of total RNA extracted from protoplasts at 24 hr postinoculation (PI) was annealed to 0.3 pmol of a γ-[^{32}P]ATP-labeled oligonucleotide complementary to positions 2400 to 2419 of TCVms. The RNAs were annealed by heating at 80° for 3 min followed by slowly cooling to 10° below the T_m of the oligonucleotide in the presence of reverse transcription buffer (50 m*M* Tris–HCl, pH 8.3, 75 m*M* KCl, 3 m*M* $MgCl_2$). Following the addition of 0.5 m*M* of each dNTP, 10 m*M* DTT, and 200 units of MMLV reverse transcriptase (Gibco-BRL), cDNA was generated at 37° for 30 min. Reaction products were analyzed on a 7% Long-Ranger sequencing gel (FMC BioProducts) in parallel with dideoxy-termination sequencing ladders (Sequenase, Amersham) generated with the same primer using plasmid pT7TCVms.

In vitro RdRp assay

RNA transcripts synthesized by T7 RNA polymerase were used as templates for transcription by partially purified TCV RdRp prepared from infected turnip leaves (Song and Simon, 1994). RdRp reactions were carried out as described previously in the presence of α-[^{32}P]UTP (Song and Simon, 1994). Radiolabeled products were subjected to electrophoresis through 42 cm, 5% polyacrylamide/8 *M* urea gels. After electrophoresis, gels were stained with ethidium bromide, photographed under UV illumination, dried, and exposed to X-ray film.

Oligonucleotide-directed RNase H digestion

Radiolabeled 550-base RNA, synthesized by the RdRp *in vitro* from the 1.45-kb sgRNA promoter, was gel purified and then hybridized to 0, 10, and 20 pmol of oligonucleotide homologous to the plus-strand of the 1.45-kb sgRNA promoter [positions −61 to −45 (+1 is the transcription start site)] in a reaction mixture previously described (Song and Simon, 1995b). The reactions were heated at 100° for 1 min and then slowly cooled to 40°. One unit of RNase H (BRL) was added, and the reactions incubated at 37° for 30 min. The products were phenol-extracted, precipitated, and then subjected to electrophoresis through 7% polyacrylamide/8 *M* urea gels.

RNA secondary structure predictions

The M-FOLD program in the GCG package (Genetics Computer Group, Inc., University of Wisconsin) was used for secondary structure predictions.

RESULTS

Accumulation of TCV RNA species in infected *Arabidopsis thaliana* protoplasts

Infection of *Arabidopsis* protoplasts with wt transcripts synthesized using a full-length TCV cDNA clone template (pT7TCVms; Oh *et al.,* 1995) resulted in the accumulation of genomic RNA as well as the two sgRNAs (1.45 and 1.7 kb) previously described (Carrington *et al.,* 1987). The rate of accumulation and relative abundance of these RNA species in *Arabidopsis* protoplasts at various times postinoculation (PI) were examined by RNA gel blot analysis using a 3′ TCV-specific probe (Table 1; Fig. 1B). Plus-strand TCV genomic RNA was detected by 12 hr PI and levels of the genomic RNA increased up to 48 hr PI. Accumulation of the sgRNAs paralleled the accumulation of the genomic RNA, with the 1.45-kb sgRNA present at approximately 2- to 3-fold the level of the 1.7-kb sgRNA. Additional unidentified RNA bands that migrated between the TCV genomic RNA and the 1.7-kb sgRNA were not consistently observed and may denote genomic RNA degradation products (compare Figs. 1B and 2B).

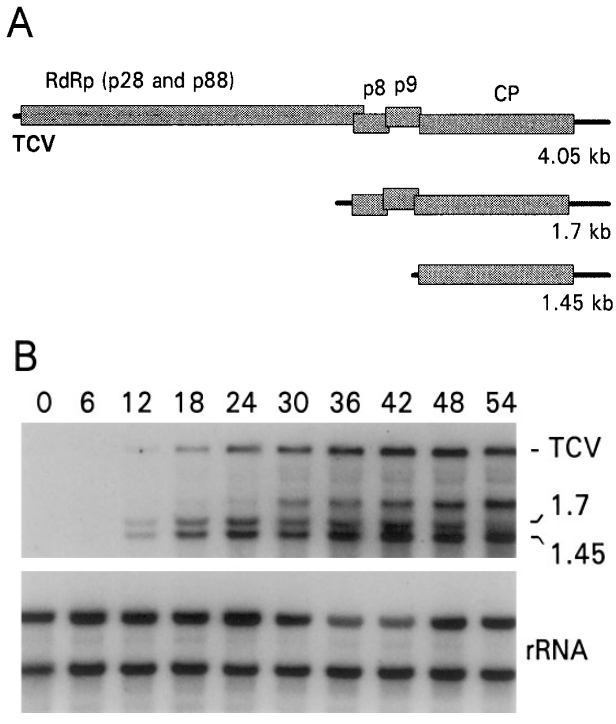

FIG. 1. TCV and its associated subgenomic RNAs. (A) Schematic representation of TCV genomic and sgRNAs. ORFs are denoted by boxes and the encoded products are indicated above the ORFs in the genomic RNA. (B) Accumulation of TCV genomic and sgRNAs in protoplasts over time. *Arabidopsis* protoplasts (5×10^6) were inoculated with 20 μg of wt TCV genomic RNA transcripts. Four micrograms of total RNA extracted at the times indicated (in hours PI) over each lane were subjected to electrophoresis through nondenaturing agarose gels 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.

Defining the boundaries of the 1.45-kb subgenomic RNA promoter

The transcription start site of the 1.45-kb sgRNA was mapped to the vicinity of position 2606 by Carrington *et al.* (1987) using a TCV isolate containing a 1-base dele-

TABLE 1

Summary of the Oligonucleotides Used in This Study

Application/ construct[a]	Name	Position in TCVms	Sequence[b]	Polarity[c]
RNA gel blots	OL 3892C(+)	3893–3913	5′ CCGTTTTTGGTCCCTAACACA 3′	−
Primer extension	OL 2420C(+)	2400–2419	5′ TTCCGTTTTCCTGTTGCGTC 3′	−
RNase H digestion	OL 2546C(−)	2546–2562	5′ AGTGGAAGTCTCAATCA 3′	+
SG6	RV-1	2095–2114	5′ GATATCTTGCCTGAAGAGGAATTT 3′	+
	OL 242OC(+)	2400–2419	5′ TTCCGTTTTCCTGTTGCGTC 3′	−
SG7	RV-2	2160–2178	5′ GATATCTTTGATAGTGGATTCTAC 3′	+
	OL 242OC(+)	2400–2419	5′ TTCCGTTTTCCTGTTGCGTC 3′	−
SG8	OL 2221C(−)	2220–2239	5′ CCAAGATGCTAGGTTCAGCT 3′	+
	OL 242OC(+)	2400–2419	5′ TTCCGTTTTCCTGTTGCGTC 3′	−
SG3(+/−) and	P9SacI	2517–2531	5′ TACGAGCTCGGGGGTACTTGGGTT 3′	+
pT7MDV1.45(+/−)	OL 2613C(+)	2596–2612	5′ ATTACCCACGGGGACGT 3′	−
pT7MDV1.7(+/−)	1.7 LB	2243–2260	5′ ACCGGGGGTTCGGCTACA 3′	+
	1.7 RB	2318–2336	5′TATCCCGTGGGGTCCCACT 3′	−

[a] (+/−) denotes that plasmids were generated with inserts in both forward and reverse orientations.
[b] Shaded sequences are not from TCV and were included to generate restriction sites *Eco*RV (RV-1 and RV-2) or *Sac*I (p9SacI) (underlined).
[c] Polarity refers to homology (+) or complementarity (−) with plus-strands of TCV genomic RNA.

tion in the 5′-noncoding region as compared with the TCVms strain used in our laboratory (Oh *et al.,* 1995). This start site therefore corresponds with position 2607 in our TCVms strain. The boundaries of the 1.45-kb sgRNA promoter were determined using a deletion strategy in which deletions with fixed end points were generated upstream and downstream from the transcription start site of the 1.45-kb sgRNA. Since p8, p9, and CP are dispensable for RNA accumulation in protoplasts (Hacker *et al.,* 1992; Oh *et al.,* 1995), deletions were generated from the upstream *Nhe*I site (within the p8 ORF) or the downstream *Bsm*I site (within the CP ORF) toward the transcription start site (Fig. 2A).

Transcripts synthesized *in vitro* containing these deletions were inoculated to *Arabidopsis* protoplasts and total RNA extracted at 40 hr PI was subjected to RNA gel blot analysis. Protoplasts inoculated with transcripts containing deletions from the *Nhe*I site to 90 bases upstream of the transcription start site (N-90) accumulated wt levels of the 1.45-kb sgRNA. However, protoplasts inoculated with transcripts containing deletions past −90, e.g., to −66 (N-66; +1 is the start site), did not accumulate 1.45-kb sgRNA, indicating that the 5′ border of the sgRNA promoter is located between −90 and −66. To delineate the 3′ border of the 1.45-kb sgRNA, protoplasts were inoculated with transcripts containing deletions from the downstream *Bsm*I site. TCV mutants containing deletions terminating at or downstream of the +6 position (e.g., B+6; +1 is the start site) were active in generating 1.45-kb sgRNA (Fig. 2C), while mutant TCV containing a deletion to the +4 position (B+4) generated substantially less 1.45-kb sgRNA. Mutant TCV with a deletion to +3 (B+3) did not produce any detectable 1.45-kb sgRNA. In this particular experiment, the genomic and 1.7-kb sgRNAs in most protoplast preparations were difficult to detect possibly due to degradation. However, two additional repetitions of the experiment using B+76, B+35, and B+3 transcripts did not show degradation of the genomic RNA and 1.7-kb sgRNA but did give identical results for production of the 1.45-kb sgRNA as shown in Fig. 2C (data not shown). Taken together, the 1.45-kb sgRNA promoter is encompassed by approximately 96 bases (positions 2517–2612), including 90 bases upstream and 6 bases downstream of the transcription start site.

Ability of the defined 1.45-kb sgRNA promoter to support ectopic synthesis of subgenomic RNA *in vivo*

The deletion analysis described above defined a core sequence required for synthesis of the 1.45-kb sgRNA but did not establish that this contiguous sequence was sufficient to promote sgRNA transcription. To address this issue, the 96-base sequence was duplicated and inserted in both orientations into the *Bsm*I site located in the middle of the CP ORF (Fig. 3A). If the 96-base sequence specified an active sgRNA promoter when located in the *Bsm*I site, then synthesis of a new 0.86-kb sgRNA should occur. Regardless of the activity of the sgRNA promoter, the sizes of the 1.7- and 1.45-kb sgRNAs should increase to 1.8 and 1.55-kb, respectively, due to the location of the inserted sequence downstream from the two wt sgRNA transcription start sites.

Protoplasts were inoculated with transcripts synthesized from SG3+ (sgRNA promoter in the forward orientation), SG3− (sgRNA promoter in the reverse orientation), and pT7TCVms (wt TCV) and total RNA was extracted at 36 hr PI. RNA gel blot analysis revealed that only inoculation of protoplasts with SG3+ transcripts resulted in the synthesis of an additional sgRNA of expected size (0.86 kb; Fig. 3B). This result demonstrates that the defined 96-base sgRNA promoter can direct syn-

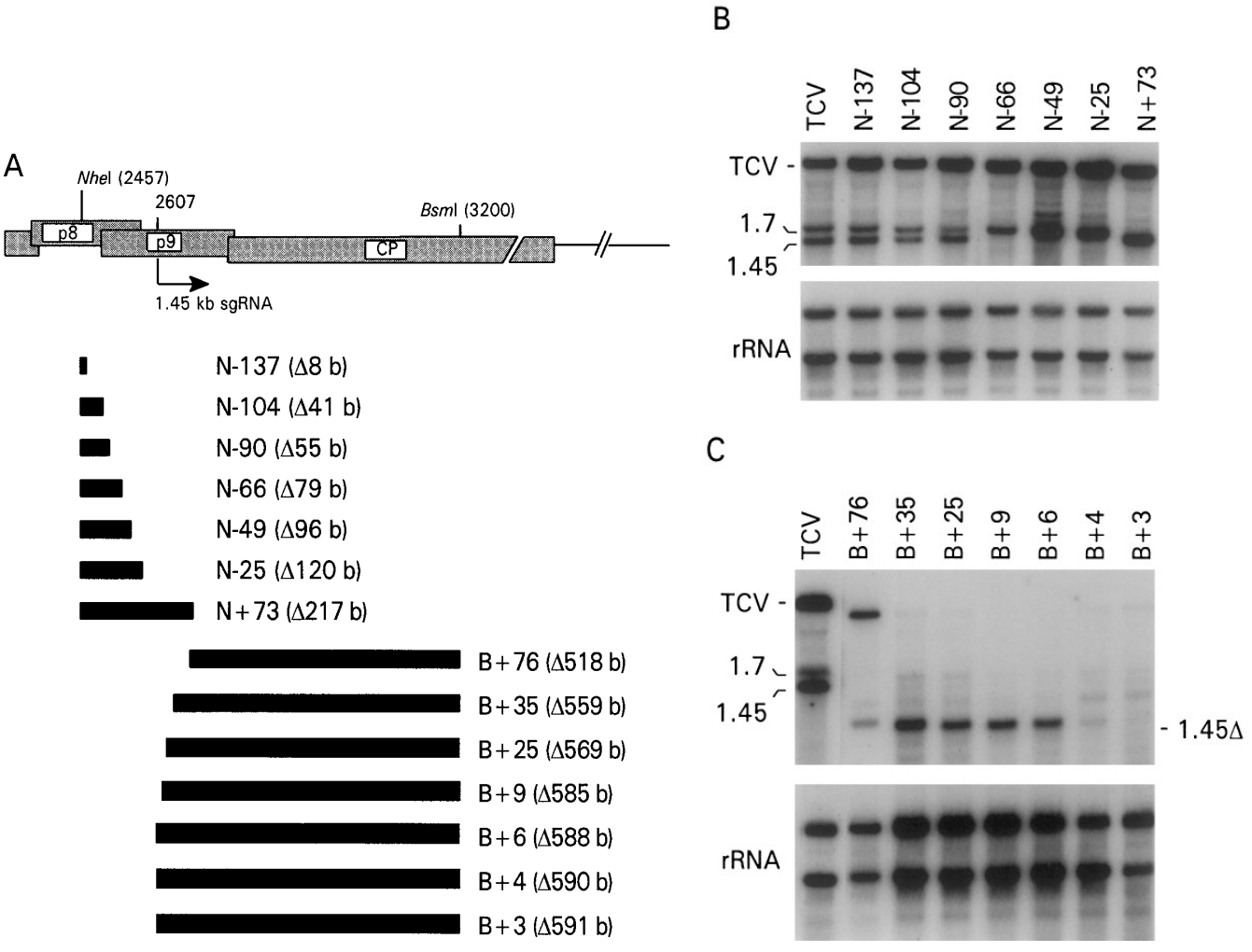

FIG. 2. Determining the boundaries of the 1.45-kb sgRNA promoter. (A) Schematic representation of deletion mutants. Black bars indicate the locations and extents of deleted region within individual mutants. Numbers in mutant names denote the deletion end points in relationship to the transcription start site (e.g., N-137 has a deletion end point 137 base pairs upstream of the transcription start site [+1, position 2607, in the TCVms genome]). − and + indicate upstream and downstream deletions, respectively. Numbers in parentheses indicate number of bases deleted in the mutant. (B) RNA gel blot analysis of the 1.45-kb sgRNA in protoplasts inoculated with transcripts containing upstream deletions. Names above each lane refer to the mutants listed in (A). (C) Accumulation of 1.45-kb sgRNA in protoplasts inoculated with downstream deletions. Species corresponding to TCV genomic RNA and 1.45- and 1.7-kb sgRNAs are indicated. The species corresponding to the deleted 1.45-kb sgRNA (1.45Δ) is indicated.

thesis of a new sgRNA when ectopically located in the TCV genome in the proper orientation.

Mapping the transcription start site for the 1.7-kb sgRNA

Since the transcription start site for the 1.7-kb sgRNA promoter had not been previously delineated, primer extension mapping was used to determine the start site in TCVms using a primer complementary to positions 2400–2419. The same primer was also used to generate a defined sequence ladder that was run in parallel with the product of the primer extension reaction. As shown in Fig. 4, two strong-stop products were synthesized in the primer-extension reaction that differed in size by a single base, corresponding to positions 2332 and 2333 in TCVms. These strong-stop signals were not detected in primer-extension reactions using gel-purified genomic RNA as template, excluding the possibility that they were the result of nonspecific termination due to strong local secondary structure (data not shown). The finding of two closely spaced extension products using viral RNA template commonly indicates either that the RdRp can initiate at either of the two positions, or the presence of a 5′ cap (m^7G), which can lead to the synthesis of a complementary cytosine residue by the MMLV reverse transcriptase (Ahlquist and Janda, 1984; Carrington and Morris, 1986). Alternatively, reverse transcriptase may exhibit cap- and template-independent activity on some completed transcripts as suggested by Shelness and Williams (1985). Consequently, we have designated the penultimate cytosine (position 2333) as the transcription start site of 1.7-kb sgRNA, corresponding to a sgRNA of 1720 bases.

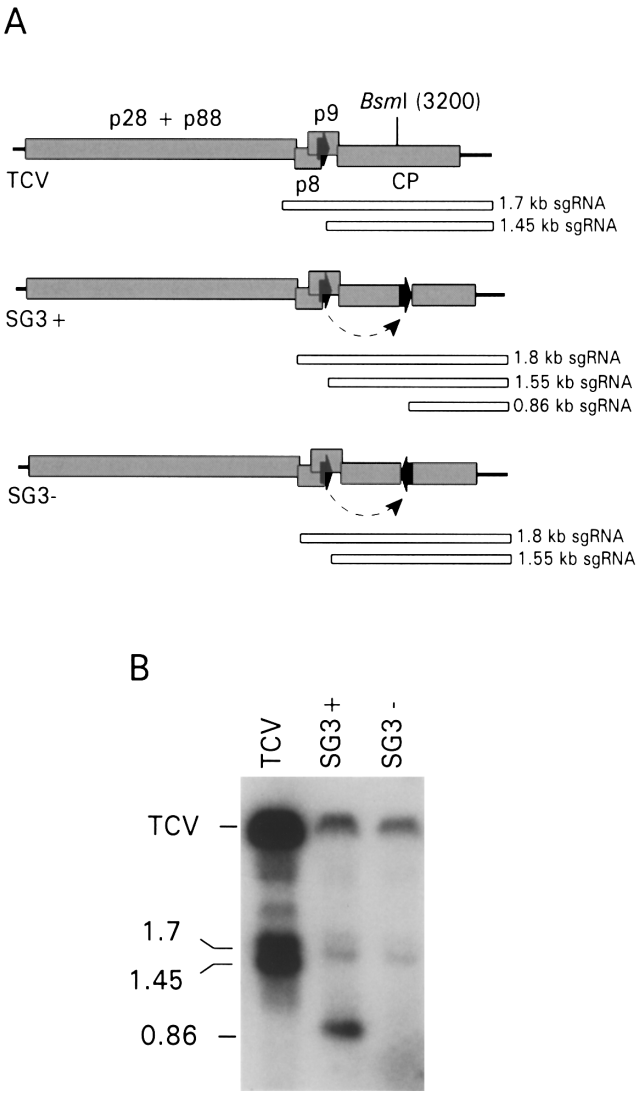

FIG. 3. Ectopic expression of the 96-base 1.45-kb sgRNA promoter. (A) Schematic representation of constructs that contain the 96-base promoter in the forward (SG3+) and reverse (SG3−) orientations in the *Bsm*I site of TCV. Thick black arrowheads denote the duplicated sequence in the natural location (TCV) and the transplanted locations (SG3+ and SG3−). The expected sizes of the sgRNAs are shown below each construct. (B) Accumulation of a new sgRNA transcribed from the 96-base insert in the forward orientation. Inoculation of protoplasts and RNA gel blot analysis were as described in the legend to Fig. 2 except that total RNA was extracted at 36 hr PI. Species corresponding to TCV genomic RNA, and the 1.45-, 1.7-, and 0.86-kb sgRNAs are indicated.

Defining the boundaries of the 1.7-kb subgenomic RNA promoter

Since the start site of the 1.7-kb sgRNA is within the p88 ORF, and the p88 product (the RdRp) is essential for replication in protoplasts, we employed a different strategy for determining the boundaries of the 1.7-kb sgRNA promoter. Three fragments (324 base pairs, 259 base pairs, and 199 base pairs), with identical 3′ ends and variable 5′ ends, and which contained the transcription start site were amplified by PCR and inserted into the *Bsm*I site in the CP ORF of pT7TCVms. If the fragments contained an active sgRNA promoter, then a new sgRNA of 0.94 kb should accumulate in infected protoplasts. Also, due to the location of the inserted fragments, the 1.45- and 1.7-kb sgRNAs should increase in size corresponding to the length of the inserted fragment (Fig. 5A). Protoplasts inoculated with transcripts synthesized from SG6 (fragment positions 2095–2419), SG7 (fragment positions 2160–2419), and SG8 (fragment positions 2220–2419) all directed the synthesis of equal levels of a new sgRNA of 0.94 kb. This result indicates that the smallest inserted fragment (199 base pairs; SG8) contained an active sgRNA promoter. SG7 (260 base pair fragment) was chosen for further delineation of the promoter boundaries because it contained a restriction site absent in SG8.

Figure 6A shows a schematic representation of the deletions made from restriction enzyme sites upstream and downstream of the start site for the relocated 1.7-kb sgRNA promoter in SG7. To define the 5′ border, bidirectional deletions were generated from an *Eco*RV restriction site engineered into the 5′ border of the inserted promoter fragment during the construction of SG7. Transcripts synthesized from E-90 (deletion end point 90

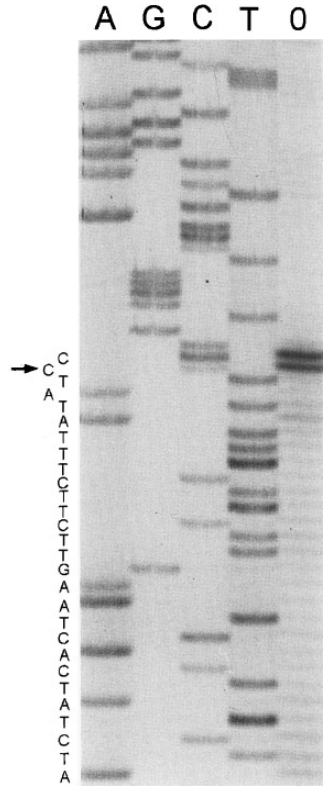

FIG. 4. Mapping the 5′ end of the 1.7-kb sgRNA by primer extension. ^{32}P-labeled primer OL 2420C(+) (Table 1) was used for primer-extension on total RNA extracted from TCV-infected protoplasts at 24 hr PI. Extension products (lane 0) and sequencing ladders (lanes A, G, C, T) were subjected to electrophoresing on a 7% sequencing gel followed by autoradiography. The penultimate C indicated with an arrow is designated the 5′ end of the 1.7-kb sgRNA, which corresponds to position 2333 in the TCVms genome.

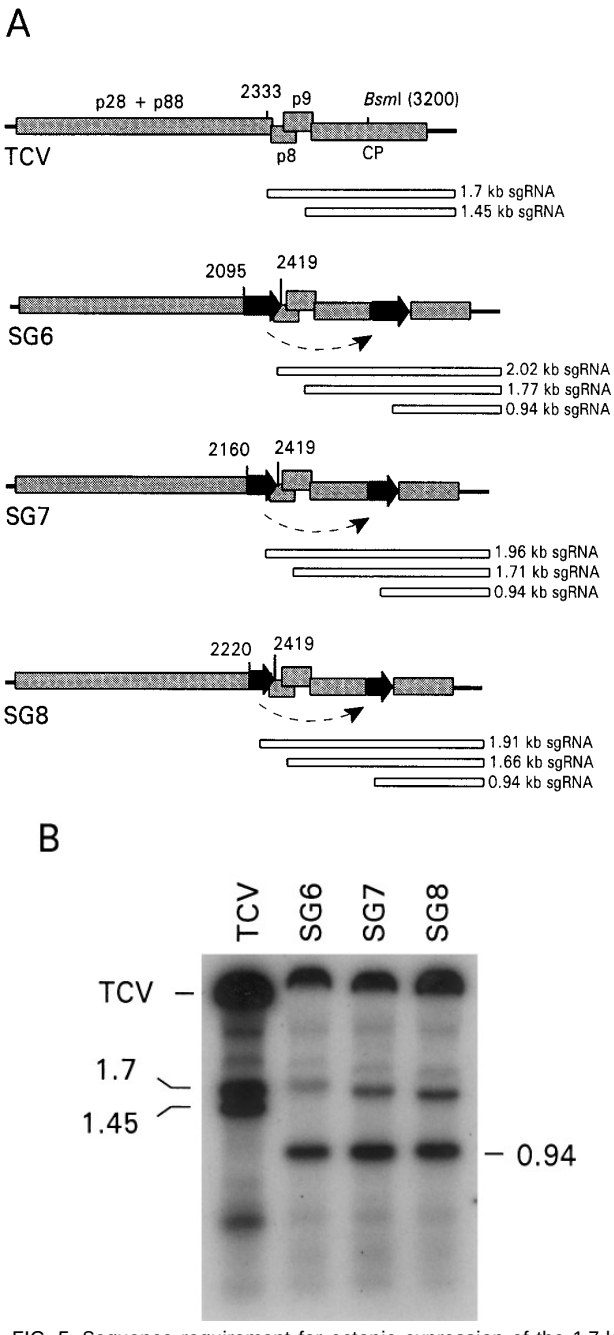

FIG. 5. Sequence requirement for ectopic expression of the 1.7-kb sgRNA promoter. (A) Diagrammatic representation of constructs in which sequence surrounding the 1.7-kb transcription start site was duplicated and inserted into the *Bsm*I site in the CP ORF. The position of the 1.7-kb sgRNA transcription start site is shown (position 2333). Filled, thick arrowheads denote the duplicated sequence in the transplanted locations in constructs SG6, SG7, and SG8. The end points of the duplicated sequences are indicated and the expected sizes of the sgRNAs are indicated below each construct. (B) RNA gel blot analysis of sgRNAs accumulating in protoplasts inoculated with the viral genomic RNAs shown in (A). Inoculation of protoplasts and RNA gel blot analysis were as described in the legend to Fig. 2 except that total RNA was extracted at 24 hr PI. Bands corresponding to TCV genomic RNA, and the 1.45-, 1.7-, and 0.94-kb sgRNAs are indicated.

bases upstream of the transcription start site) generated control (SG7) amounts of the new 0.94-kb sgRNA in protoplasts while a further deletion to −65 (E-65) resulted in a substantial decrease in the level of sgRNA (Fig. 6B). A low level of the 0.94-kb sgRNA was still present in protoplasts inoculated with transcripts containing deletion end points as close as 10 bases upstream of the transcription start site (e.g., E-10).

To delineate the 3′ border, deletions were generated from the 3′ border of the inserted sequence in SG7 toward the transcription start site. Deletions up to the +4 position (e.g., H+4; +1 is the start site) did not substantially affect the synthesis of the 0.94-kb sgRNA (Fig. 6C). However, deletion to the +1 position (H+1) eliminated detectable synthesis of the 0.94-kb sgRNA, indicating that the 3′ border of the 0.94-kb sgRNA promoter is between +1 and +4. Taken together, the 0.94-kb sgRNA promoter, and by analogy, the 1.7-kb sgRNA promoter, contains approximately 94 bases consisting of 90 bases upstream of the start site and 4 bases downstream of the start site, when assayed *in vivo.*

In vitro analysis of the 1.45- and 1.7-kb sgRNA promoters

Partially purified RdRp preparations from TCV-infected turnip plants are able to catalyze the synthesis of complementary strands of TCV-associated subviral RNA templates (satellite and defective interfering RNAs) of both plus- and minus-strand polarities (Song and Simon, 1994). To test for independent promoter activity, an assay was developed (Song and Simon, 1995a) whereby putative promoter sequences were placed upstream of the non-TCV RdRp template sequence, MDV, a 219-base RNA associated with Qβ bacteriophage. To test for the ability of the 1.45- and 1.7-kb sgRNA promoters to serve as independent promoters *in vitro,* the 96-base (1.45-kb sgRNA promoter) and 94-base (1.7-kb sgRNA promoter) promoter sequences were placed in forward and reverse orientations adjacent to the MDV sequence, as described in Fig. 7A. Initial experiments were performed using transcripts synthesized by T7 RNA polymerase using *Eco*RI-digested plasmids. Transcripts were then added to the TCV RdRp extracts and radiolabeled products analyzed by gel electrophoresis and autoradiography (Figs. 7B and 7C).

The 94-base 1.7-kb sgRNA promoter was not active in the *in vitro* RdRp assay in either orientation (Fig. 7C, lanes 3 and 4). In contrast, the 1.45-kb sgRNA promoter was able to direct the synthesis of three major products in the forward orientation but was inactive in the reverse orientation, using constructs digested with *Eco*RI (Fig. 7B, lanes 3 and 5). The most abundant product produced by the 1.45-kb sgRNA promoter in the forward orientation migrated at the position of 550 bases. A second product migrated at the position of 225 bases, the expected size for MDV RNA transcribed from the transcription start site of the 1.45-kb sgRNA promoter (219 base MDV + 6 base promoter sequence). The larger 550-base product was reminiscent of the so-called L-RNA product synthesized

by the RdRp *in vitro* using minus-strands of satellite (sat-) RNA C (a TCV subviral RNA) as template (Fig. 7B, lane 1). This L-RNA has a panhandle structure composed of the minus-stand template covalently linked to newly synthesized RNA complementary to the templates 5′ 190 bases (Song and Simon, 1995b). The 550-base product was confirmed as being a similar type product following gel purification, hybridization to an oligonucleotide (see Table 1), digestion with RNase H, and sizing by gel electrophoresis (data not shown).

Since synthesis of the panhandle product by the TCV RdRp could be influenced by base-pairing between 3′-terminal sequences and residues in the vicinity of the transcription start site (see Fig. 7A), a *Sac*I restriction site was introduced by PCR into the MDV plasmid harboring the 1.45-kb sgRNA promoter. Linearization of the plasmid using *Sac*I (instead of *Eco*RI) eliminated 15 plasmid-derived residues at the 3′ end of the transcript template. The major product synthesized by the RdRp using transcripts produced from plasmid digested with *Sac*I was the 225-base MDV product (Fig. 7B, lane 4). These results indicate that the 96-base 1.45-kb sgRNA promoter, but not the 94-base 1.7-kb sgRNA promoter is active as a promoter *in vitro* in the absence of additional genomic RNA sequences.

DISCUSSION

The 1.7- and 1.45-kb sgRNA promoters in TCV have been delineated using a deletion strategy. The 1.45-kb sgRNA promoter was analyzed in its natural location in protoplasts since the transcription start site was located in a region of the genome that could be deleted without substantially affecting genomic RNA synthesis. However, the 1.7-kb sgRNA start site was in the ORF for the RdRp, precluding direct deletion mapping and necessitating

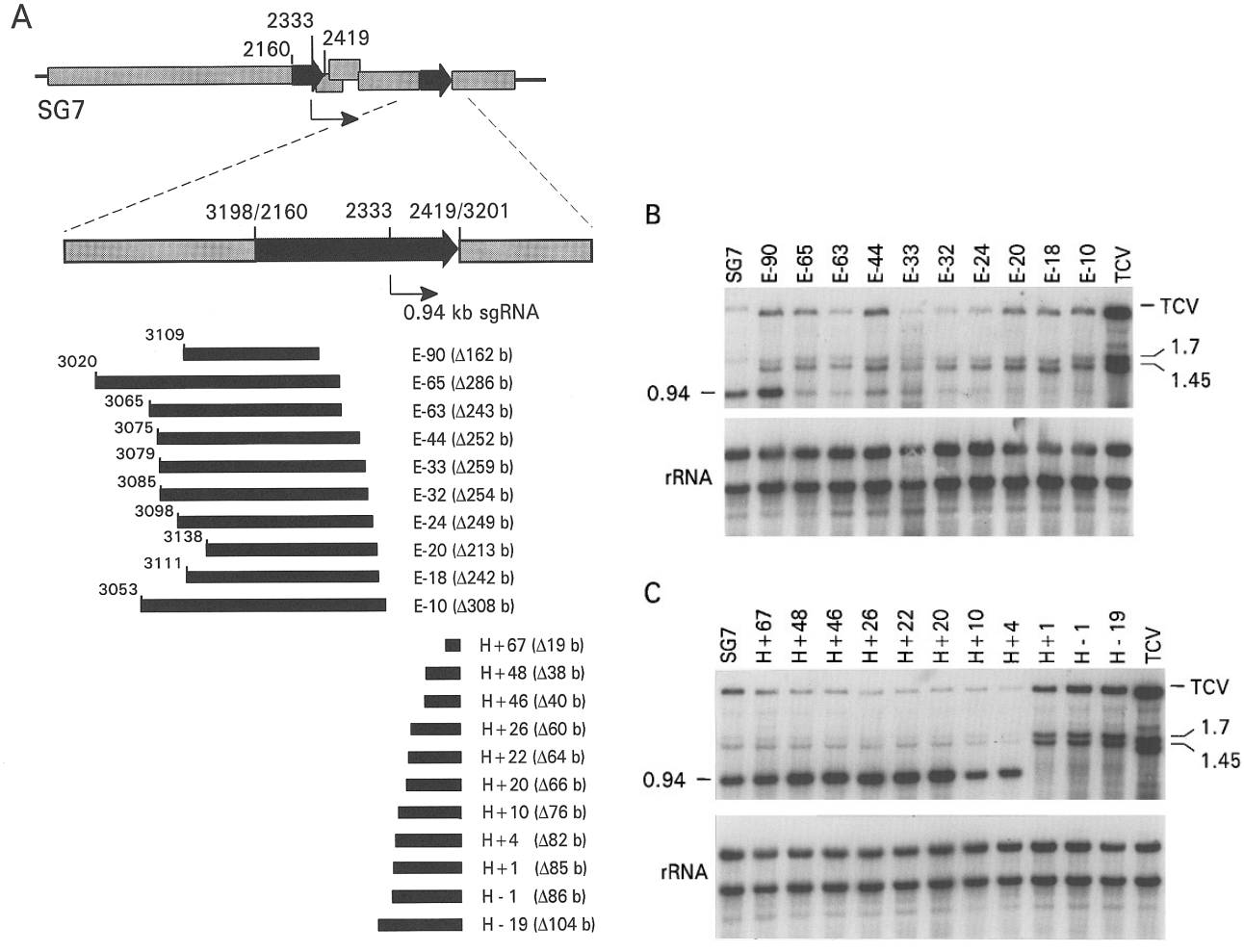

FIG. 6. Determining the boundaries of the duplicated 1.7-kb sgRNA promoter in SG7. (A) Schematic representation of deletion mutants. Black bars indicate the location and extent of deletions within individual mutants. The nomenclature for deletion mutants was as defined in the legend to Fig. 2. Upstream deletions were bidirectional and the positions of the 5′ end points are shown. Thick black arrowheads denote the duplicated sequence in the natural location and the transplanted, 3′ proximal location. (B) RNA gel blot analysis of the 0.94-kb sgRNA specified by the transplanted promoter in protoplasts inoculated with SG7 containing upstream deletions. (C) Accumulation of the 0.94-kb sgRNA using constructs containing downstream deletions. Inoculation of protoplasts was as described in the legend to Fig. 2. Species corresponding to TCV genomic RNA, and the 1.45-, 1.7-, and 0.94-kb sgRNAs are indicated.

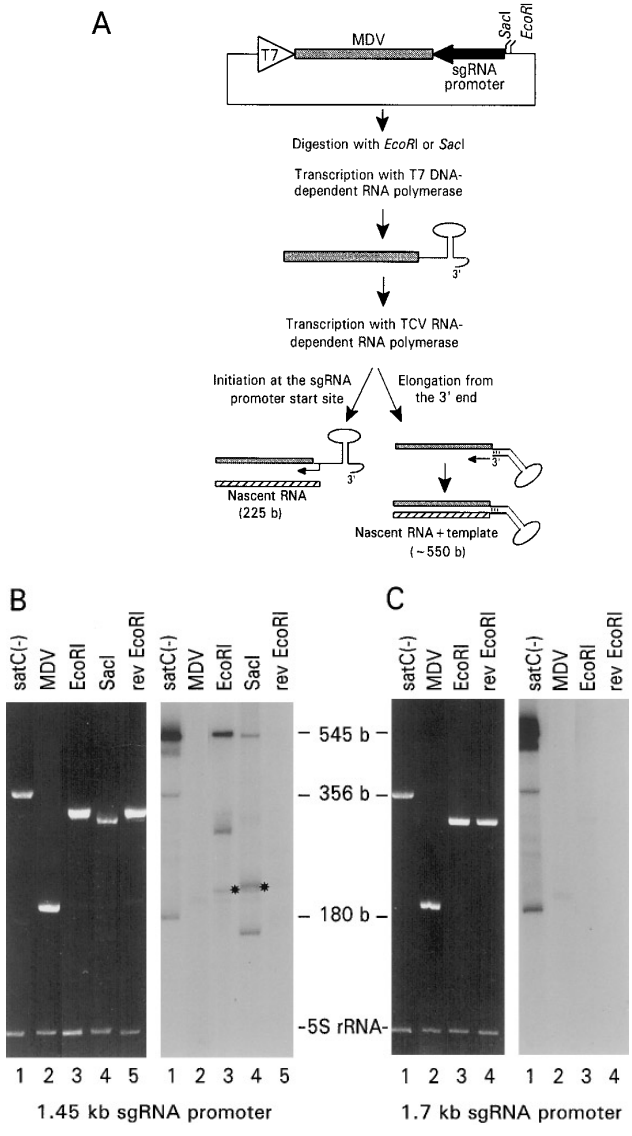

FIG. 7. *In vitro* activity of the 1.45- and 1.7-kb sgRNA promoters using partially purified TCV RdRp. (A) Schematic representation of the construct used to generate templates for the RdRp reactions. The 1.45-kb sgRNA promoter inserted into plasmid pT7MDV (in both orientations; only the forward orientation is shown) and the steps used to assay for sgRNA promoter activity are shown. The construct containing the 1.7-kb sgRNA promoter was identical except for the absence of the *Sac*I site. T7 triangle denotes the location of a T7 RNA polymerase promoter. The box labeled MDV is the cDNA for an RNA associated with Qβ bacteriophage. The sgRNA promoter is drawn as a stem–loop based on computer predictions (see Fig. 8). (B) Denaturing gel analysis of α-[^{32}P]UTP-labeled products synthesized using RdRp and templates containing the 1.45-kb sgRNA promoter. Ethidium bromide-stained gel showing the migration positions and input levels of templates and 5S rRNA is shown to the left of the autoradiogram. Transcription of minus-strand sat-RNA C [satC(−)] generated three products that migrate at positions of 545, 356, and 180 bases (lane 1; Song and Simon, 1994). Lanes 2–4 contain templates transcribed from *Sma*I-digested pT7MDV (generating full-length MDV RNA only), and *Eco*RI- and *Sac*I-linearized pT7MDV1.45 containing the 1.45-kb sgRNA promoter, respectively. Lane 5 template is transcribed from plasmid containing the 1.45-kb sgRNA promoter in reverse orientation, linearized with *Eco*RI. * denotes the product corresponding to a size of 225 bases. Lane 4 was underloaded and RNAs migrated slightly slower than other lanes, as indicated by the migration of 5S rRNA. (C) Same as (B), except that the plasmid contained the 1.7-kb sgRNA promoter with no *Sac*I site.

movement of the region surrounding the start site to an alternate position in the genome (the CP ORF) prior to analysis. The relative accumulation of the sgRNA directed by the relocated 1.7-kb sgRNA promoter (compared to the level of genomic RNA) was similar to the level of sgRNA produced by the 1.45-kb sgRNA promoter in the same CP ORF location (see Figs. 3 and 5). While additional contiguous sequences are not required for substantial activity of the 1.7-kb sgRNA promoter, it is still possible that additional sequences can influence the level of promoter activity.

The location of the promoters relative to the transcription start sites for the sgRNAs were similar, with the promoter for the 1.45-kb sgRNA located approximately 90 bases upstream (−90) and 6 bases downstream (+6) of the transcription start site, and the promoter for the 1.7-kb sgRNA encompassing approximately 90 bases upstream (−90) and 4 bases downstream (+4) of the start site. However, the minimal promoter for basal level transcription appears to be different for the two promoters. Deletion of the region between −66 and −90 upstream of the 1.45-kb sgRNA start site eliminated detectable sgRNA accumulation, while deletions within 10 bases upstream of the 1.7-kb sgRNA start site still allowed for the accumulation of low levels of the sgRNA (Fig. 6B, lane E-10).

Computer secondary structure analysis revealed that the TCV sgRNA promoters located on the minus-strand of the TCV genome have similar stem–loop structures (Fig. 8). Both promoters consist of a putative hairpin comprising most of the promoter sequence just 3′ of the transcription start site. The stems at the base of both hairpins are particularly stable, with many consecutive G:C base pairs. The promoter at the 3′ end of plus-strand sat-RNA C (a subviral satellite RNA associated with TCV) that is used for minus-strand synthesis is also a hairpin with a number of consecutive G:C base pairs at the base of the stem (Song and Simon, 1995a). Similar stable hairpins are also predicted at the 3′ ends of the plus-strands of the genomic RNAs for TCV and for the related carmoviruses carnation mottle virus (CarMV) and cardamine chlorotic fleck virus (CCFV; Song and Simon, 1995a). Interestingly, minus-strand synthesis of genomic and subviral RNAs begins from the 3′ end of the plus-strand template and passes through the stem–loop, while sgRNA transcription begins 5′ of the hairpin and does not include the hairpin. These different modes of transcription would allow the promoter to be included in the complementary strands of full-length genomic and subviral RNA while the promoter is absent from the sgRNA transcripts. Although there is some evidence to support replication of sgRNAs of coronaviruses (Sawicki and Sawicki, 1990; Schaad and Baric, 1994), the lack of sgRNA promoters in the sgRNA transcripts would likely preclude the replication of TCV sgRNAs.

The location of the TCV sgRNA promoters primarily upstream of the transcription start sites is similar to that

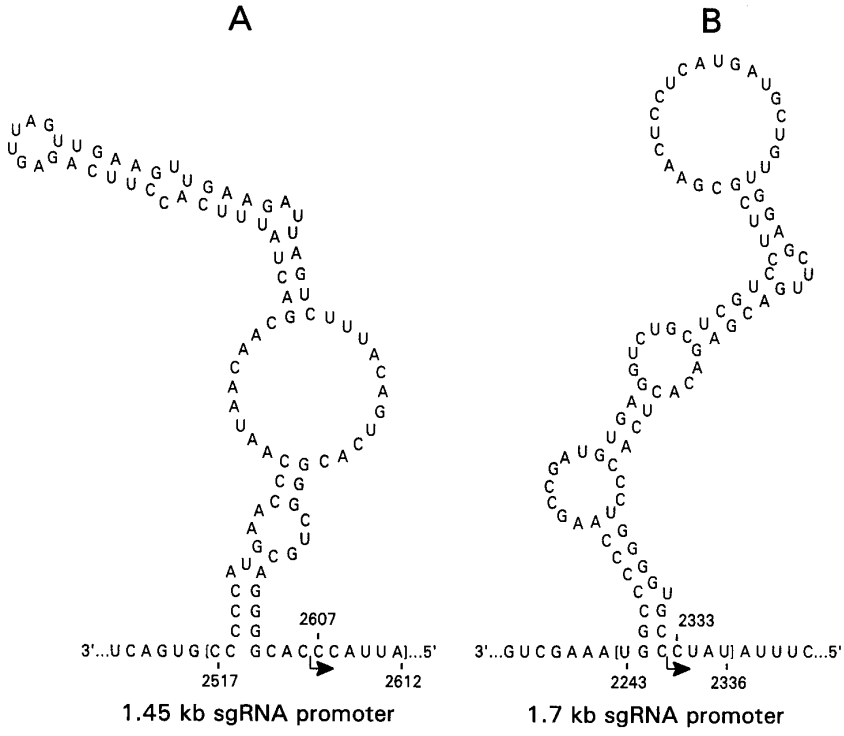

FIG. 8. Computer predicted secondary structures for the (A) 1.45- and (B) 1.7-kb sgRNA promoters. Minus-strand sequence is shown. Brackets enclose the promoter boundaries derived from the deletion analysis. Arrows denote the transcription start sites.

found for many mapped sgRNA promoters (e.g., AlMV, Van der Vossen *et al.,* 1995; BMV, French and Ahlquist, 1988; CMV, Boccard and Baulcombe, 1993). This is in contrast to the sgRNA promoter for BNYVV, which is located mainly downstream of the start site (Balmori *et al.,* 1993). CNV, a member of the tombusvirus group that is closely related to the carmovirus group, also has the promoter for the 0.9-kb sgRNA mainly upstream of the transcription start site (+6 to −20 bases; Johnston and Rochon, 1995). However, this promoter comprises a much shorter sequence than the TCV sgRNA promoters and shares no sequence similarity with the TCV sgRNA promoters.

The 96-base 1.45-kb sgRNA promoter and the 94-base 1.7-kb sgRNA promoter were active when translocated in the TCV genome, generating the synthesis of new sgRNAs in infected protoplasts. This result indicates that these promoter sequences contain all the elements required for activity *in vivo.* Virus containing promoters in both the natural and transplanted locations produced substantially higher levels of sgRNA from the transplanted promoter (the promoter closest to the 5′ end of the minus-strand) than the same promoter in the natural location (see Figs. 3 and 5). The same preferential use of sgRNA promoters that are proximal to the 5′ end of the minus-strand was also found for BMV (French and Ahlquist, 1988) and CMV (Boccard and Baulcombe, 1993), while preferential transcription for 3′ (minus-strand) proximal promoters was found for AlMV (van der Vossen *et al.,* 1995), BNYVV (Balmori *et al.,* 1993), and Sindbis virus (Raju and Huang, 1991). In addition, levels of the genomic RNA and natural sgRNAs decreased in protoplasts infected with TCV containing active and inactive sgRNA promoter inserts in the CP ORF (SG3+, 3−, 6, 7, and 8) when compared with cells infected with wt TCV (Figs. 3 and 5), possibly due to lack of encapsidation (protection) by the disrupted CP. However, levels of the viral genomic and natural sgRNA RNA levels increased when deletions inactivated the transplanted 1.7-kb sgRNA promoter (Fig. 6C, compare lanes H+67 to H+4 with lanes H+1 to H−19), suggesting that both promoter position and encapsidation (protection) contribute to the differential viral RNA accumulation of mutant and wt TCV.

Only the 1.45-kb sgRNA promoter could serve as an independent promoter *in vitro,* suggesting that additional sequences and/or factors not present in our *in vitro* RdRp mixture are required for *in vitro* activity of the 1.7-kb sgRNA promoter. While others have studied sgRNA promoters using *in vitro* RdRp assays (Marsh *et al.,* 1988; Van der Kuyl *et al.,* 1990), this is the first report of a defined sgRNA promoter able to function independent of additional genomic RNA sequence. In addition to producing a product the size of MDV RNA synthesized from the transcription start site (225 bases), a product of ~550 bases was synthesized by the 96-base 1.45-kb sgRNA promoter in amounts that varied depending on the sequence at the 3′ end of the template. Sizing of the 550-base product following oligonucleotide-directed RNase H digestion suggested a hairpin structure, composed of both template and radiolabeled product, similar to that

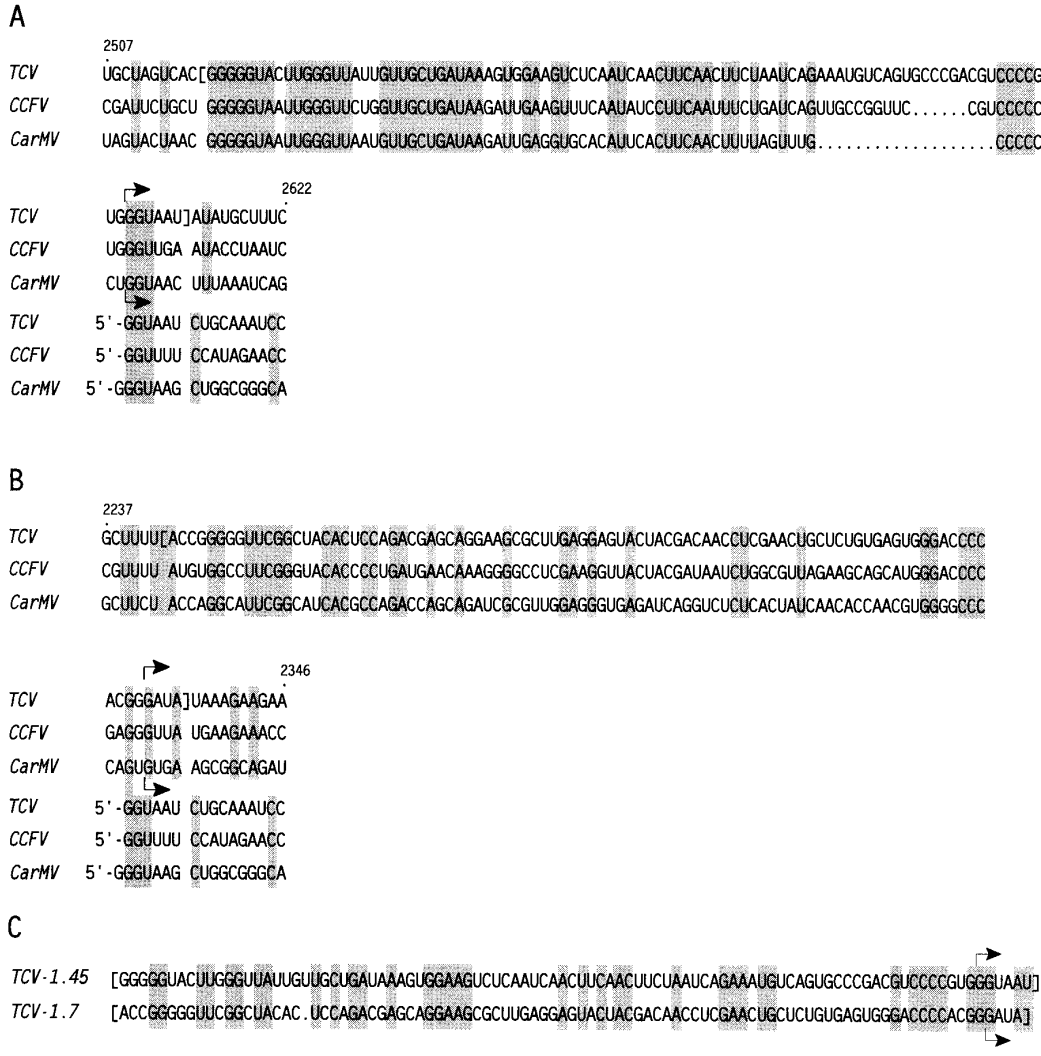

FIG. 9. Comparison of the TCV (A) 1.45- and (B) 1.7-kb sgRNA promoters with corresponding sequences in related carmoviruses. Identical nucleotides are shaded. Sequence from the plus-strands of the viral genomic RNAs is shown. Brackets enclose the TCV sgRNA promoters defined by deletion analysis. Arrows denote the TCV and CarMV (Carrington and Morris, 1986) transcription start sites in the complementary minus-strands. CCFV, cardamine chlorotic fleck virus (Oh *et al.,* 1995); CarMV, carnation mottle virus (Guilley *et al.,* 1985). Sequences surrounding the transcription start sites are also compared to the sequences at the 5′ ends of the respective genomic RNAs. (C) Sequence similarity between the 1.45- and 1.7-kb TCV sgRNA promoters.

previously described for L-RNA produced using sat-RNA C minus-strands as template (Song and Simon, 1995a, 1995b).

TCV shares 63% genome similarity with CCFV and 48% genome similarity with CarMV (Guilley *et al.,* 1985). Alignment of the 1.45- and 1.7-kb sgRNA promoter sequences with the corresponding regions in the CCFV genomic RNA revealed 69 and 60% sequence similarity, respectively, while alignment with CarMV genomic RNA indicated 58 and 51% sequence similarity, respectively (Fig. 9A). Since the sequence similarity in the region could be influenced by the presence of the sgRNA promoters within ORFs, alignments were also made for the 100 bases upstream and 100 bases downstream of the sgRNA promoters. For the 1.45-kb sgRNA promoter region, such alignments indicated 55% upstream similarity and 66% downstream similarity for CCFV and 42% upstream similarity and 38% downstream similarity for CarMV. For the 1.7-kb sgRNA promoter region, CCFV had 61% upstream and 50% downstream sequence similarity, while CarMV had 45 and 38% sequence similarity. These values suggest that at least for CarMV, sequence similarity in the sgRNA promoter regions is greater than in the surrounding region, indicating that carmoviruses may employ similar mechanisms for synthesis of their sgRNAs.

Alignment of the TCV 1.45- and 1.7-kb sgRNA promoters shows 40% sequence similarity with the highest level of similarity in the vicinity of the transcription start sites. Alignment of the 5′ ends of the TCV and CarMV sgRNAs (Carrington and Morris, 1986) and the putative 5′ ends of the CCFV sgRNAs with the 5′ ends of the carmovirus genomic RNAs revealed that the four 5′ bases were conserved ('GGUA/U'). Sequence similarity between the 5′

ends of the genomic RNAs and the 5′ ends of sgRNAs has also been found for many other viruses such as BMV (Marsh and Hall, 1987; Marsh *et al.,* 1989), CMV (Boccard and Baulcombe, 1993), AlMV (Van der Kuyl *et al.,* 1990), red clover necrotic mottle virus (Zavriev *et al.,* 1996), barley yellow dwarf virus (Kelly *et al.,* 1994), and maize chlorotic mottle virus (Lommel *et al.,* 1991). Such sequence similarity may assist the viral replicase in recognizing and interacting with specific minus-strand signals for plus-strand RNA synthesis (Pacha *et al.,* 1990; Pogue *et al.,* 1990).

Interestingly, the longest stretch of sequence identity in the region of the two TCV sgRNA promoters is the 12-base sequence "CUACAACUCUCU" located 16 bases downstream of the 1.45-kb sgRNA start site and 46 bases downstream of the 1.7-kb sgRNA start site. Deletion of this conserved sequence had no effect on the accumulation of either sgRNA in protoplasts. Since many studies of putative sgRNA promoter sequences have relied partially on sequence similarity (e.g., Dinesh-Kumar *et al.,* 1992; Meulewaeter *et al.,* 1992; Zavriev *et al.,* 1996), this observation suggests that putative promoters defined by sequence similarity should be confirmed by promoter mapping.

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