**In vitro synthesis of biologically active transcripts of tomato black ring virus satellite RNA**

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Synthetic transcripts of tomato black ring virus satellite RNA (TBRV satRNA), isolate L, were prepared from cDNA cloned in the Bluescribe transcription vector. Transcripts with 49 (T49L) or two (T2GL) extra nucleotides at their 5' ends and 42 extra nucleotides at their 3' ends were able to induce, but to different extents, the synthesis *in vitro* of the satRNA-encoded 48K protein. However, when inoculated into *Chenopodium quinoa* together with TBRV L genomic RNAs, only T2GL was biologically active, in the presence or absence of a 5' cap analogue in the transcription reactions. Analysis of the 5' and 3' termini of the satRNA isolated from plants showed that non-viral extensions were not maintained in the transcript progeny.

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**Introduction**

Tomato black ring virus satellite RNA (TBRV satRNA) belongs to the group of large nepovirus satellite RNAs which, in spite of their size and their messenger RNA activity, require a helper virus both for their replication and encapsidation (Fritsch & Mayo, 1989).

The multiplication of satRNA interferes only slightly, if at all, with the multiplication of TBRV genomic RNAs (Murant et al., 1973; Doz et al., 1980), but the association between the satRNA and the helper virus appears to be highly specific (Doz et al., 1980; Murant & Raschké, 1982). Nevertheless, analysis of the nucleotide sequence of TBRV satRNA (Meyer et al., 1984) confirmed earlier observations made by Robinson (1982) that it shares only limited sequence homology with genomic RNAs. SatRNA resembles TBRV genomic RNA in having a 5'-linked genome-linked protein (VPg) (Koenig & Fritsch, 1982; Mayo et al., 1982) and a 3'-poly(A) tail (Mayo et al., 1979).

Analysis of different satRNAs associated with helper TBRV of two different serotypes showed that they share 60% sequence homology and allowed the localization of areas of sequence differences in non-coding and coding regions (Hemmer et al., 1987). All TBRV satRNAs encode a 48K protein, and a 48K protein is made by their translation *in vitro* (Fritsch et al., 1984), and, for TBRV isolate S, in infected protoplasts (Fritsch et al., 1978). This suggests that the 48K protein has an essential role in the multiplication of satRNA (perhaps in the replication process; Hemmer et al., 1987); however, the regions of the RNA implicated in the dependence on the helper and in the specificity of the satRNA/helper virus association are yet to be determined.

To investigate further the role of the coding and non-coding regions of TBRV satRNA in its helper virus-dependent replication and encapsidation, we have undertaken the construction of full-length cDNA clones from which biologically active transcripts can be generated. Infectious RNA transcripts from cloned cDNAs have been obtained successfully for several positive-stranded plant virus RNAs including satellite RNAs (Ahlquist et al., 1984; Dawson et al., 1986; Collmer & Kaper, 1986; Gerlach et al., 1986; Simon & Howell, 1987). Here we report the preparation of biologically active TBRV satRNA transcripts and the characteristics of the progeny RNA.

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**Methods**

*Virus isolate.* TBRV L is an isolate from Lanarkshire (U.K.) belonging to the Scottish serotype (Fritsch et al., 1984). The satellite RNA was originally present in this isolate. TBRV L(−) which lacks satellite RNA was a gift from M. A. Mayo (Dundee, U.K.).

*Preparation of virus RNAs.* Virus particles were extracted from systemically infected *Nicotiana clevelandii* leaves and total viral RNA was purified as described by Fritsch et al. (1978). SatRNA was isolated by the method described by Fritsch et al. (1984).

*Recombinant plasmids.* A full-length DNA copy of satRNA from TBRV L was obtained by joining together inserts from two recombinant plasmids, p335L and p3361L, which contained overlapping cDNAs of satRNA L that had been cloned in the PstI site of pBR322 by adding dG/dC homopolymer tracts to cDNA (Hemmer et al., 1987). p335L contains a copy of residues 1 to 1313 of satRNA L and p3661L contains a copy of residues 850 to 1376 followed by 13 adenosine
Fig. 1. Construction of full-length DNA copies of TBRV satRNA L. (a) Schematic representation of satRNA L [1376 nucleotides excluding poly(A)]. The open reading frame (residues 15 to 1277) is indicated by a large open bar. (b) Plasmids used to construct the full-length satRNA L clone p343L. The large open boxes indicate satRNA-specific sequences; numbers represent the cDNA coordinates. Heavy lines at the box ends indicate dG–dC tails. Ap\(^R\), ampicillin resistance gene; Tc\(^R\), tetracyclin resistance gene. (c) Plasmids containing a full-length DNA copy of TBRV satRNA. Double lines in p343L represent pBR322-specific sequences. Stippled thin bars in the other plasmids correspond to pBS(+) sequences. Black boxes represent T3 and T7 promoter sequences.

residues. p343L was constructed by a three-point ligation of the 0.9 kbp PstI–SphI fragment of p335L, the 1.2 kbp SphI–EcoRI fragment of p3361L and the 3.6 kbp EcoRI–PstI fragment of pBR322 (Fig. 1). The 1.4 kbp PstI fragment of p343L was then inserted into PstI-cut pBS(+) (Bluescribe M13+, Stratagene) to give the plasmid pB35L which contains a complete copy of satRNA L under the control of the T3 promoter (Fig. 1). The plasmid sequences between the T3 promoter and the 5’ terminal of the sat-cDNA were removed using the oligodeoxiribonucleotide-directed mutagenesis kit from Amersham (Nakayama & Eckstein, 1986). The oligodeoxiribonucleotide 5’ CATCTG-TATTCTTTCAACCTTATA GGAGG used is complementary to nucleotides 1 to 12 of the satRNA L and to the 3’ terminal part of the T3
promoter (underlined). Mutation resulted in the loss of the PstI site upstream of the insert. Recombinant cDNA clones with the modified sequence were characterized by a single PstI restriction site (Fig. 1) and the structure of the mutated region was checked by sequencing. The plasmid pB389L/S (Fig. 1) was obtained by replacing the 254 bp SmaI fragment of pB317L by the 269 bp SmaI fragment of p394S which contains the 3' terminal part of satRNA S including a 48 residue tail. The plasmid p394S was obtained by cDNA synthesis and cloning by the method of Heidecker & Messing (1983).

Cloning procedures were as described by Maniatis et al. (1982) and Hemmer et al. (1987). The JM101 and JM103 strains of Escherichia coli were used for transformation by recombinant pBS(+)..

In vitro transcription. BamHI-linearized plasmid DNA (1 μg) was incubated in 25 μl of 40 mM-Tris–HCl pH 8, 8 mM-MgCl₂, 2 mM-sterimidine, 10 mM-DTT, 500 μM each of ATP, GTP, UTP and CTP, 80 μg/ml of bovine serum albumin (Bethesda Research Laboratories), 10 units of RNasin (Stratagene) and 10 units of T3 RNA polymerase (Stratagene). Incubation was at 37 °C for 45 min. The yield was generally 5 to 10 μg of transcript per μg of template DNA. For the preparation of capped transcripts the initial concentration of GTP was reduced to 50 μM, and 500 μM-m7GpppG (Pharmacia) was added to the medium. Incubation was at 37 °C for 60 min with the addition of GTP to a final concentration of 500 μM at 30 min. The yield of capped transcripts was generally about three to four times lower than that of uncapped transcripts. The amount and integrity of the synthesized transcripts was evaluated by formaldehyde–agarose gel electrophoresis (Gustafson et al., 1982) of a 10-fold dilution of the transcription mixture. 32P-labelled antisense RNA probes were prepared in a 15 μl incubation medium containing 40 mM-Tris–HCl pH 8, 8 mM-MgCl₂, 2 mM-sterimidine, 10 mM-DTT, 1 mM each of ATP, CTP, GTP and TTT (ATP) at 800 Ci/mmol, 80 μg/ml of bovine serum albumin, 1 μg of linearized DNA, 10 units RNasin and 10 units of T7 RNA polymerase. Incubation was at 37 °C for 30 min. DNA was then eliminated by addition of 10 units of RNase-free DNase (Stratagene) and incubation at 37 °C for 20 min.

Labelled and unlabelled transcripts were purified by phenol extraction and ethanol precipitation in the presence of 600 mM-ammonium acetate. Transcripts were suspended in diethylpyrocarbonate-treated water (0.1%). The concentration of unlabelled transcripts was adjusted to approximately 1 mg/ml.

Cell-free translation. Transcripts were translated in wheatgerm extracts and in rabbit reticulocyte lysate, without previous elimination of the template DNA from the medium, and [35S]methionine-labelled translation products were analysed by SDS–PAGE as described by Fritsch et al. (1980).

Infectivity assay. For tests of transcript infectivity the DNA template was not eliminated from the reaction mixture before inoculation. Transcripts synthesized from 5 μg of the linearized DNA template were diluted in 250 μl of a solution containing 15 μg/ml of TBRV L(–) RNAs and 0.05% bentonite; celite-dusted leaves of Chenopodium quinoa were inoculated with 30 μl of this solution. When capped transcripts were to be inoculated, the smaller yield of synthesis was obtained by addition of 10 units of RNase-free DNAse (Stratagene) and incubation at 37 °C for 20 min.

Labelled and unlabelled transcripts were purified by phenol extraction and ethanol precipitation in the presence of 600 mM-ammonium acetate. Transcripts were suspended in diethylpyrocarbonate-treated water (0.1%). The concentration of unlabelled transcripts was adjusted to approximately 1 mg/ml.

Detection of progeny RNAs by hybridization experiments. Infected leaves (0.5 g) were ground in a mixture of 1 ml of a buffer containing 200 mM-Tris–HCl pH 9, 400 mM-KCl, 35 mM-MgCl₂, 200 mM-sucrose and 25 mM-EDTA and of 1 ml phenol/M-cresol (9/1, v/v). After centrifugation, the total RNA contained in the aqueous phase was precipitated from 70% ethanol. The different RNA species were separated by formaldehyde–agarose gel electrophoresis (Gustafson et al., 1982) transferred to nitrocellulose and hybridized with 32P-labelled antisense RNA probes (Zinn et al., 1983) specific to TBRV RNA-1,-2 or satRNA. Probes were complementary to nucleotides 4239 to 5636 for RNA-1 (Greif et al., 1988), to nucleotides 3345 to 4155 for RNA-2 (Meyer et al., 1986) and to nucleotides 594 to 1180 for satRNA (Hemmer et al., 1987).

Analysis of progeny RNA. Approximately 100 g of infected leaves were ground in 67 mM-sodium phosphate pH 6.8. RNA was extracted from purified virus as described by Fritsch et al. (1978) and analysed by formaldehyde–agarose gel electrophoresis. The 3' end of the progeny RNA was analysed after transfer to nitrocellulose by hybridization with a 32P-labelled antisense RNA probe, obtained by in vitro transcription of Parl-linearized pBS(+) by T7 RNA polymerase, which is complementary to the 18 3' terminal residues of the pB317L transcript.

Primer extension. The 5' terminus of the progeny satRNA was characterized by primer extension of the 5' 32P-labelled oligodeoxynucleotide 5' CAGAAAGGCTGGAGCGTG, which is complementary to nucleotides 121 to 138 of satRNA L, by reverse transcriptase (Life Sciences) as described by Meyer et al. (1986). Dideoxynucleotide sequencing reactions using the same oligonucleotide primer and single-stranded DNA template containing the satRNA L-specific sequence were performed as described by Sanger et al. (1977). The extension products were co-electrophoresed with the sequence reaction products in a 6% polyacrylamide sequencing gel.

The 5' 32P-labelled oligodeoxynucleotide 5' TATTGTGCTCTG-GAG, which is complementary to nucleotides 1357 to 1370 of TBRV satRNA S (Meyer et al., 1984), was used as a primer to determine the sequence of the 3' end of progeny satRNA. Progeny satRNA was isolated from the virus extracted from plants infected with transcripts of pB389L/S. The extension products were sequenced by the chemical degradation method (Maxam & Gilbert, 1980).

Results

Structure of the synthetic TBRV satRNA L transcripts

In vitro transcription of BamHI-linearized pB35L and pB317L (Fig. 1) yielded satRNA L transcripts T49L and T2GL (Fig. 2a) with 3'-terminal non-viral extensions of 42 nucleotides arising from the cloning procedure used (Hemmer et al., 1987), and from the vector polylinker. T49L contains at its 5' end 49 non-viral nucleotides from the polylinker and a stretch of 14 G residues (Hemmer et al., 1987), whereas T2GL contains only two extra G residues at its 5' end (Fig. 2a). Analysis of the transcripts by formaldehyde–agarose gel electrophoresis (Fig. 2b) showed that both transcripts comigrated with genuine satRNA and that elimination of the 3' terminal residue of the T3 promoter of pB317L did not reduce the transcription efficiency of the DNA.
Cell-free translation of synthetic transcripts

As shown in Fig. 3 (lanes 1, 3 and 4) transcripts T2GL, T49L and satRNA incubated in wheatgerm extract induced the synthesis of a 48K protein. Although similar amounts of each RNA were translated, T49L induced synthesis of much smaller quantities of this protein than that induced by satRNA or T2GL, suggesting that the 49 non-viral nucleotides at the 5' of T49L hinder the translation of this transcript. Similar results were obtained when transcripts and satRNA were incubated in a rabbit reticulocyte lysate (Fig. 3, lanes 5, 7 and 8) where much more 48K protein was translated from T2GL than from T49L, but surprisingly T2GL was also much more efficient than satRNA which was translated very poorly [in contrast to genomic RNAs (Fig. 3, lane 9)]. To account for this result, we suggest that the absence of VPg and/or the presence of two extra G residues may enhance the translation of the RNA considerably in the reticulocyte system. However, we cannot rule out the possibility that by cloning we have selected a minor variant RNA which is more efficiently translated in vitro.

In the wheatgerm system the difference between the amounts of the 48K protein synthesized by T2GL and by satRNA is not very large and may not be significant because a certain variability of translation efficiency may also be observed with different preparations of the same RNA.

Biological activity of synthetic transcripts

Synthetic capped or uncapped transcripts T49L and T2G were inoculated into C. quinoa together with TBRV L(−)
Transcripts of TBRV satRNA

Fig. 4. Northern blot hybridization of RNA extracted from leaves inoculated with TBRV satRNA transcripts. RNAs were analysed after the first passage in C. quinoa leaves (lanes 2 and 3), the second (lanes 4, 5, 8, 9, 12, 13, 15 and 18 to 21) and/or the third passage (lanes 6, 7, 10 and 11). TBRV L(−) RNAs were coinoculated with uncapped T49L (lane 1), uncapped T2GL (lanes 2, 4, 6, 15, 19 and 21), capped T2GL (lanes 3, 5 and 7), uncapped T2GL/S (lanes 8 and 10), capped T2GL/S (lanes 9 and 11), BamHI-linearized pB317L (lane 13) or inoculated alone (lane 12). Leaves were also inoculated with TBRV L RNAs (lanes 14, 18 and 20). RNA extracted from healthy plants (lane 16), purified satRNA L (lane 17). The Northern blots were hybridized with 32P-labelled antisense RNA probes specific for RNA-1 (lanes 18 and 19), RNA-2 (lanes 20 and 21) or satRNA (lanes 1 to 17). The position of the TBRV RNA species is indicated on the right.

RNAs. After 10 days, total RNA was extracted from infected leaves and analysed by Northern blot hybridization experiments using 32P-labelled antisense RNA probes complementary to RNA-1, RNA-2 and satRNA. Also, two or three leaves were ground in a small volume of phosphate buffer and the crude extract was used to reinoculate C. quinoa plants. Two or three successive passages were performed in this manner and Northern blot analysis of RNA was done after each passage.

As shown in Fig. 4, satRNA-specific sequences could not be detected in infected leaves of plants inoculated with T2GL after the first passage (lanes 2 and 3) but after one or two further passages the presence of satRNA became evident (lanes 4 to 7). In contrast no satRNA multiplied when T49L was inoculated, even after two passages (lane 1). Capping of RNA transcripts did not result in a significant increase of satRNA multiplication (lanes 3, 5 and 7). Controls showed that satRNA could be detected easily in plants inoculated with TBRV L RNAs (lane 14) but not in those infected with TBRV L(−) RNAs (lane 12), or with a mixture of TBRV L(−) RNAs and BamHI-linearized pB317L (lane 13) even after two passages.

Hybridization of RNA with probes corresponding to RNA-1 or RNA-2 showed that multiplication of virus had occurred and that approximately the same amounts of RNA-1 and -2 had been produced when either natural satRNA or transcript RNA was present in the inoculum (Fig. 4, lanes 18 to 21). The yield of progeny satRNA was always lower when T2GL rather than satRNA was coinoculated with the genomic RNAs (lanes 14 and 15).

Nevertheless analysis of the RNA extracted from purified virus showed that T2GL progeny RNA was encapsidated (Fig. 5).

To show unambiguously that the appearance of progeny RNA resulted from the multiplication of the transcript T2GL template and not from a fortuitous contamination, a recombinant of TBRV satRNA was made by replacing the 209 nucleotides (including 13 A
(a) Comparison of the nucleotide sequence of the 3' regions of transcripts T2GL and T2GL/S (from residue 1183 to the 3' end of the transcripts). Upstream of these regions the sequences of both transcripts are identical. The sequence complementary to the oligodeoxynucleotide used in primer extension experiment is underlined. (b) Determination of the nucleotide sequence of the 3' region of T2GL/S progeny RNA. Chemical degradation sequencing of the primer extension products of T2GL/S progeny RNA. (i) Dideoxynucleotide sequencing reactions were made on single-stranded pB389L/S (ii) and pB317L (iii) using the universal M13 primer. The detected RNA sequences are shown from 5' (top) to 3' (bottom) and correspond to the segments indicated between arrows in 6(a).
Analysis of the 5’ and 3’ ends of progeny RNA

Primer extension experiments were done to determine whether the two extra 5’ nucleotides of the T2GL transcript were maintained in the progeny RNA. The faster migrating run-off cDNA of the progeny RNA has the same length as the run-off cDNA of satRNA and both are shorter than the run-off cDNA of T2GL (Fig. 7a). Parallel migration of products of dideoxynucleotide sequencing reactions performed with the same primer on single-stranded pB317L shows that the more rapidly migrating run-off copies of satRNA and of T2GL progeny RNA were exactly two nucleotides shorter than the run-off cDNA of T2GL. The upper run-off product, obtained with wild-type satRNA, with T2GL and, in relatively smaller amounts, with T2GL progeny, may have resulted from a further addition of one nucleotide to the cDNA by the reverse transcriptase. Surprisingly, an additional run-off cDNA was obtained with the T2GL progeny RNA. The origin of this product is unclear, but as it was larger than T2GL run-off cDNA, it is unlikely to have been a result of extension of the primer on unmodified progeny RNA.

To determine whether progeny RNA had also lost the 3’ extra nucleotides present in T2GL, a 32P-labelled antisense RNA probe complementary to the 18 last residues of T2GL was hybridized with the RNA transferred to a nitrocellulose sheet. Fig. 7(b) shows that T2GL (lanes 1 and 4) hybridized with this probe whereas similar quantities of satRNA (lane 2) or T2GL progeny RNA (lane 3) did not.

Discussion

The results obtained showed that synthetic transcripts of TBRV L satRNA, coinoculated with the genomic RNAs, were biologically active although their rate of multiplication appeared to be lower than that of their wild-type RNA. Use of a synthetic hybrid transcript provided evidence that the progeny RNA did not result from the amplification of traces of a contaminant satRNA, but from the multiplication of the inoculated transcript. As a consequence of these results it appears that the presence of VPg on the transcript is not a prerequisite for the multiplication of satRNA, in contrast to earlier observations showing that genomic RNAs were non-infectious when their VPg was degraded by protease treatment (Harrison & Barker, 1978). The role of VPg is still unknown, but if the VPg protects the RNA from RNase degradation in the plant cell, the high concentration of the inoculated transcripts could have prevented or compensated for partial degradation. Also the high stability of satRNA (Murant & Raschké, 1982) could have a similar effect. Unlike the results obtained with transcripts of other naturally non-capped virus RNAs (Vos et al., 1988; Domier et al., 1989) capping of the 5’ terminus of the synthetic satRNA transcripts had
no detectable effect on their multiplication. It remains to be demonstrated whether the addition of VPg occurred, and favoured further replication of the progeny RNA.

As with transcripts of other cloned cDNA of polyadenylated virus RNAs (van der Werf et al., 1986; Vos et al., 1988; Ziegler-Graff et al., 1988), multiplication of TBRV satRNA transcripts occurred with different lengths of extra viral nucleotides at the 3' end, and these extra nucleotides were not maintained in the progeny RNA. This suggests that the poly(A) stretch might be the signal (or a part of it) for the recognition of plus-strand biological activity of most studied transcripts (Dawson et al., 1989). The generation of such Y-extended molecules could correspond to an artefact generated by the primer extension reaction rather than a defective replication from cloned cDNA of the TBRV virus and production of infectious transcripts. Proceedings of the National Academy of Sciences, U.S.A. 83, 1982–1986.


We thank Annie Hoeft for the synthesis of oligonucleotides, and K. Richards for critically reading the manuscript.
Transcripts of TBRV satRNA


(Received 11 October 1989; Accepted 11 December 1989)