Comparison of the Nucleotide Sequences of Viroid-like Satellite RNA of the Canadian and Australasian Strains of Lucerne Transient Streak Virus

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SUMMARY

A complete cDNA copy of the viroid-like satellite RNA of the Canadian strain of lucerne transient streak virus (LTSV) has been cloned and the nucleotide sequence of the RNA has been determined using this and other clones. The sequence comprises 322 residues and shares 80% homology with satellite RNAs (stRNAs) of the Australasian isolates of LTSV. A proposed secondary structure for the RNA is highly base-paired and thermodynamically stable, and consists of a rod-like structure interspersed with single-stranded loops, an arrangement similar to that proposed for circular stRNA of other strains of LTSV and other sobemoviruses as well as for viroids. Whereas certain regions of the sequence are very similar or identical to those of stRNA from Australasian LTSV, including the putative self-cleavage sites on both the positive and negative sense forms of the RNA, other regions of the sequence are quite dissimilar between the Canadian and Australasian strains.

INTRODUCTION

Lucerne transient streak sobemovirus (LTSV) occurs widely in alfalfa in Canada. A Canadian strain (LTSV-Ca) of the virus that is distinct from the Australian (LTSV-Au) and New Zealand (LTSV-Nz) isolates both in host range and symptomatology has been described (Paliwal, 1983, 1984a). Particles of LTSV are isometric and contain at least two major RNA species: RNA 1 is a linear, single-stranded molecule with an approximate Mr of 1.4 x 10^6 (about 4.5 kb); RNA 2 is viroid-like in that it is a circular covalently closed molecule of 322 to 324 residues with a high degree of internal base pairing. The RNA 2 has been demonstrated to be a satellite RNA (stRNA), dependent on RNA 1 for its replication (Jones & Mayo, 1983; Paliwal, 1984a). The nucleotide sequences of the stRNA of LTSV-Au and LTSV-Nz isolates differ in only 10 of 324 nucleotides (Keese et al., 1983).

We report here the cloning of a complete cDNA copy of the Canadian stRNA and a comparison of the nucleotide sequences of stRNA of LTSV-Ca with those reported for LTSV-Au and LTSV-Nz.

METHODS

Virus and RNA. LTSV-Ca was purified from Trigonella foenum-graecum L. plants and viral RNAs were extracted as described by Paliwal (1984a).

Nucleic acids were fractionated in denaturing 4% polyacrylamide gels containing 8 M-urea at pH 8.3, and the bands corresponding to satellite circular LTSV RNA (RNA 2) were excised from the gels, eluted electrophoretically and precipitated with 2 volumes of ethanol. Circular RNA was dissolved in TE buffer (10 mM-Tris–HCl, 1 mM-EDTA, pH 7.5) and reprecipitated with ethanol several times.

Synthesis and cloning of double-stranded cDNA. LTSV-Ca stRNA (10 μg) was randomly linearized in 10 mM-Tris–HCl buffer pH 9.0 and heated at 100 °C for 2 min (Keese et al., 1983), treated with calf intestinal phosphatase (Boehringer Mannheim) (Maniatis et al., 1982), polyadenylated with 1.5 units of poly(A) polymerase (Bethesda Research Laboratories) in 50 mM-Tris–HCl pH 7.9 containing 50 μCi [32P]ATP, 1 mM-dithiothreitol, 10 mM-MgCl₂, 40 μg/ml bovine serum albumin for 30 min at 37 °C, phenol-extracted and precipitated with 2 volumes of
ethanol. First-strand cDNA was synthesized using \[^{32}P\]dATP (ICN Pharmaceuticals, Chemical and Radioisotope Division), reverse transcriptase (Life Sciences, St Petersburg, Fla., U.S.A.) and oligo(dT)\textsubscript{12-18} as primer. Poly(A)-tailed RNA was mixed with the primer and heated at 100 °C for 2 min before cDNA synthesis. Second-strand synthesis was essentially by the method of Gubler & Hoffman (1983) as described by AbouHaidar (1988). The double-stranded cDNA was then tailed with dC (10 to 15 nucleotides), annealed to dG-tailed pUC18, linearized with \textit{PstI} and added to competent \textit{Escherichia coli} cells, strain JM101 or HB101, as described by AbouHaidar (1988). 

In other cDNA preparations, random oligodeoxynucleotides (Pharmacia) or a synthetic oligonucleotide d(GTATGAGAGACTGACTGAA) were used as primers. Circular RNA 2 (stRNA) was mixed with these primers and heated at 100 °C for 2 min then snap-cooled on ice prior to cDNA synthesis. The double-stranded cDNAs obtained were ligated to \textit{BamHI} linkers, digested with \textit{BamHI} and purified by centrifugation through a 'spun-down' column of Sephadex G-50 (Maniatis et al., 1982). cDNAs were then ligated to pUC18 that had been linearized with \textit{BamHI} and dephosphorylated, and used to transform competent \textit{E. coli} cells. Useful recombinant plasmids were selected by Southern hybridization between DNA inserts and \[^{32}P\]-labelled cDNA probes made by reverse transcription from randomly primed stRNA (Taylor et al., 1976). 

**RESULTS AND DISCUSSION**

**Strategies for cloning and sequencing of LTSV-Ca stRNA**

Since stRNA of LTSV-Ca exists as single-stranded circular RNA, several approaches for cloning and sequencing were used. The linearization of RNA followed by poly(A) tailing resulted in the production of several short clones which were difficult to sequence because of homopolymer tails. The random primer method using circular stRNA as the template in combination with the addition of \textit{BamHI} linkers resulted in the production of several clones which contained sequences from one region (positions 75 to 150) (Fig. 1). In addition, the partial sequence of these clones allowed us to synthesize an oligonucleotide which was used as a primer for cDNA synthesis. Several complete clones of stRNA were then obtained and the nucleotide sequence was determined in two directions (Figs 1 and 2). The complete sequence was obtained from the sequence of two complete and several overlapping partial clones.

**Sequence and proposed secondary structure of LTSV-Ca stRNA**

The complete nucleotide sequence (presented in linear form) of LTSV-Ca stRNA is given in Fig. 2. The RNA comprises 322 nucleotides which can be arranged in an extensively base-paired, rod-like structure (Fig. 3). In this structure, 68.5% of the residues are base-paired (Fig. 3), 38% are G·C pairs, 28% are A·U pairs and only 2.5% are G·U pairs. These values are similar to those described for other viroid-like RNAs and viroids (Keese & Symons, 1987). The free energy calculated according to Zucker & Stiegler (1981) is about −635 kJ/mol. The structure of stRNA of LTSV-Ca resembles that of stRNAs of other strains of LTSV, viroid-like stRNAs from other sobemoviruses and viroids.

**Sequence homology between LTSV-Ca stRNA and stRNAs of other LTSV isolates**

The sequence homology between the stRNA of LTSV-Ca and those of LTSV-Au and LTSV-Nz is about 80% (Fig. 2). Certain regions of the nucleotide sequence are very similar between the three isolates (e.g. nucleotides 3 to 32, 40 to 49, 108 to 145, 148 to 161, 162 to 199, 206 to 217, 231 to 245 and 261 to 274) but the sequence of LTSV-Ca stRNA is distinct in other regions, such as that between nucleotides 60 and 102 (Fig. 2). The stRNA of LTSV-Ca is shorter than those of LTSV-Au and LTSV-Nz by only two nucleotides.

The LTSV-Ca stRNA can also be folded to form a hammerhead structure like that reported for both LTSV-Au and LTSV-Nz as well as for several other viroid-like stRNAs, viroids and satellite RNAs (Forster & Symons, 1987). This structure can be made in both the positive sense and negative sense strands of the LTSV-Ca stRNA (Fig. 4).
**Nucleotide sequence of LTSV satellite RNA**

Fig. 1. Partial restriction map and strategy for cloning and sequencing of LTSV-Ca RNA 2. Horizontal bars represent the cDNA clones obtained by either random (V1, V23, lt20 and lt24) or synthetic (V109) primers. Arrows indicate the direction and extent of the nucleotide sequence determined. P indicates the region complementary to the synthetic primer. Due to the circular nature of stRNA, V109 is presented in linear form with dotted ends. H, HhaI; N, NcoI; A, AvaI; S, SmaI; B, BglII.

Fig. 2. Alignment of the nucleotide sequences of LTSV-Ca RNA 2 (stRNA) and LTSV-Au strains (shown in linear form). The nucleotide sequence is that of the native (positive) sense RNA 2 (LTSV-Au, upper sequence; LTSV-Ca, lower sequence). In each case residue 1 is arbitrarily shown to correspond to the top end of the secondary structure shown in Fig. 3. The nucleotide sequence of RNA 2 of LTSV-Au is taken from Keese et al. (1983). Identical residues in LTSV-Ca and LTSV-Au strains (258 matches) are indicated by colons. The alignment was generated using the algorithm of Wilbur & Lipman (1983). K-tuple size, 1; window size, 20; gap penalty, 1.

![Fig. 1](image1)

![Fig. 2](image2)

The presence of self-cleavage domains on both the positive (native) sense and the negative sense RNA suggests the likely role of the self-cleavage in the replication process as reported by Forster & Symons (1987).

The sequence homology between the stRNA of LTSV-Ca and those of the two Australasian isolates revealed in this study is not unexpected. The highly conserved regions of the sequence are probably important for the RNA's biological functions; the only ones known thus far being the modification of LTSV-Ca lesion type in Chenopodium amaranticolor (Jones et al., 1983) and increasing the severity of symptoms in *T. foenum-graecum* caused by one of the helper viruses, southern bean mosaic virus (SBMV) (Paliwal, 1984b). However, the variable regions of the sequence of LTSV-Ca stRNA may be responsible for allowing this RNA to interact with the genomic RNAs of its helper viruses for its replication, packaging and transport. Although the homologous helper virus, LTSV-Ca, is closely related serologically to LTSV-Au and LTSV-Nz (Paliwal, 1984a), it is substantially different from them both in host range and symptomatology, suggesting differences between the nucleotide sequences of genomic RNA. LTSV-Ca stRNA must determine such differences. Furthermore, LTSV-Ca stRNA is not only supported for its replication by LTSV-Ca and other sobemoviruses (i.e. SBMV and turnip rosette virus), but also is encapsidated in their capsid proteins (Paliwal, 1984b; Y. C. Paliwal, unpublished results). Certainly, stRNA must contain specific recognition signals to enable its encapsidation by the coat proteins of these helper viruses.

Possible translation products of stRNA of LTSV-Ca

One open reading frame (ORF) found on the positive sense RNA capable of giving rise to a peptide of 25 amino acids, and two ORFs found in the negative sense RNA could give rise to two...
Fig. 3. Predicted secondary structure of stRNA of LTSV-Ca, determined using a computer program reported by Zucker & Stiegler (1981). Solid and dashed bars indicate the domains that are required for the hammerhead structures in Fig. 4. The solid and dashed bars demarcate the putative domains for self-cleavage of positive and negative sense RNA respectively. Arrows indicate the putative cleavage sites.

Fig. 4. Secondary structure models for the proposed self-cleavage domains of positive sense (a) or negative sense (b) RNA. Arrows indicate the predicted self-cleavage sites. The nucleotide residue numbers in the negative sense sequence are those of the complementary nucleotides in the positive sense sequence.
possible peptides of 32 and 29 amino acid residues. Other much smaller ORFs were also present in each strand.

Several possible translation products of the Australasian strains of LTSV were reported to be peptides shorter than 75 amino acids each (Keese et al., 1983). The peptide (25 amino acids) produced from the possible translation of LTSV-Ca was found to be similar to those produced from the translation of LTSV-Au and -Nz. The nucleotide sequences corresponding to these peptides are conserved in the three strains of LTSV (positions 125 to 198, Fig. 2). The other two peptides of LTSV-Ca do not share significant homology with any of those of the Australasian strains (data not shown). The small size and the differences in the amino acid sequences of these different peptides suggest that LTSV stRNA is not translatable. No in vitro translation products have been attributed to stRNA of LTSV (Morris-Krsinich & Forster, 1983).

From the data presented in this report, we can conclude that the nucleotide sequence and structure of the LTSV-Ca are quite different from those of the Australasian ones. The sequence differences between stRNAs of the three known strains of LTSV in conjunction with site-directed mutagenesis experiments may help in the elucidation of the relationships between structure and biological function of these viroid-like RNAs.

Furthermore, it will be of considerable interest to determine the molecular mechanisms of replication of LTSV-Ca RNA 1 and stRNA, in particular to find the recognition sites on each RNA for the RNA polymerase, the capsid protein and the viral transport protein(s).

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REFERENCES


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