Satellite RNA in Particles of Strawberry Latent Ringspot Virus

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(Accepted 7 July 1982)

SUMMARY

RNA from particles of isolate H of strawberry latent ringspot virus (SLRV) comprises species of mol. wt. $2.9 \times 10^6$ (RNA-1), $1.4 \times 10^6$ (RNA-2) and $0.4 \times 10^6$ (RNA-3). Isolates obtained by inoculating plants with RNA-1 plus RNA-2 lacked RNA-3, even after repeated subculturing. RNA-3 was produced when it was inoculated in mixtures with the RNA of such isolates, but not when it was inoculated alone. Most of the nucleotide sequence of RNA-3 was absent from either RNA-1 or RNA-2. RNA-3 is therefore a satellite RNA. SLRV isolates containing or lacking satellite RNA induced indistinguishable symptoms in test plants. Like the genome RNA of SLRV, satellite RNA was found to be polyadenylated and to be bound covalently to protein. Satellite RNA induced the synthesis of a 38000 mol. wt. polypeptide in rabbit reticulocyte lysates. Several properties of SLRV satellite RNA therefore resemble those of tomato black ring virus satellite RNA.

INTRODUCTION

Strawberry latent ringspot virus (SLRV) has been classified as a putative nepovirus (Murant, 1981) because it possesses many features characteristic of nepoviruses. In particular, its genome RNA consists of two species of mol. wt. $2.9 \times 10^6$ and $1.4 \times 10^6$ (Murant et al., 1981), it includes polyadenylate sequences (Mayo et al., 1979a) and is linked to a small protein (Mayo et al., 1982). However, SLRV differs from other nepoviruses in that the coat protein of SLRV particles comprises two different polypeptides of mol. wt. 44000 and 29000 (Mayo et al., 1974; Gallitelli et al., 1982) instead of one polypeptide of mol. wt. 52000 to 58000 (Mayo et al., 1971).

Particles of some isolates of several nepoviruses contain RNA species additional to the two comprising the virus genome. Those found in particles of tobacco ringspot virus and tomato black ring virus have been shown to be satellite RNA because they depend on the helper virus for multiplication but are not an essential part of the helper virus genome (Schneider, 1969, 1971; Murant et al., 1973) and neither has extensive sequence homology with the genome RNA of their respective helper viruses (Rezaian & Jackson, 1981; Robinson, 1982). The additional RNA species found in particles of myrobalan latent ringspot, chicory yellow mottle and arabis mosaic viruses are also likely to be satellite molecules (Murant & Mayo, 1982). A minor RNA (RNA-3, mol. wt. $0.4 \times 10^6$) was found in particles of the H isolate of SLRV (Mayo et al., 1974; Gallitelli et al., 1982) but not in particles of the J (Mayo et al., 1974), peach or olive (Gallitelli et al., 1982) isolates, and it was suggested that this too is a satellite RNA. In this paper we present evidence that this is so, and describe some properties of the satellite RNA.

METHODS

Virus propagation and purification. The H (Hampshire or T39) isolate from strawberry (Lister, 1964), and isolates experimentally generated from it were propagated in Nicotiana clevelandii and purified from systemically infected and inoculated leaves about 14 days after inoculation. Leaves were triturated in 0.07 M-sodium phosphate buffer, pH 7, containing 0.1% mercaptoacetate and 0.01 M-EDTA, using a mechanical blender. The extract was filtered through muslin and clarified by adding 8.5% (v/v) n-butanol and centrifuging for 10 min at 10000 g. The virus particles were then concentrated and purified by precipitation with 10% (w/v) polyethylene glycol 6000 and 0.17 M-NaCl, followed by two or three cycles of high- and low-speed centrifugation. Sedimented virus particles were

0022-1317/82/0000-5274 $02.00 © 1982 SGM
region of the gel containing RNA-1 and RNA-2 was cut out. RNA was then eluted and prepared using avian myeloblastosis virus reverse transcriptase with random DNA fragments as primers as [3SS]methionine (600 Ci/mmol; Amersham International) and then incubated for 60 min at 30 °C. Following described by Robinson et al., RNA was recovered from the fractions by precipitation from 70% ethanol at −18 °C by adding 2.5 vol. ethanol. RNA was extracted from leaf tissue using the same reagents and procedure.

**Sucrose density gradient centrifugation of RNA.** To purify RNA-3 from RNA preparations, samples of about 100 μg RNA were centrifuged in gradients of 10 to 40% sucrose in 0.1 M-Tris–HCl, 0.1 M-NaCl, 1 mM-EDTA, 0.1% SDS, pH 7-5, for 16 h at 20 °C in a SW27.1 rotor (Beckman). Gradients were fractionated by upward displacement through a u.v. absorptiometer and RNA was recovered from the fractions by precipitation from 70% ethanol at −18 °C. The RNA-3 preparations used in the experiments failed to induce lesions when inoculated at about 1 μg/ml on leaves of *C. amaranticolor*, showing that they were essentially free from contamination by SLRV genome RNA.

Radioactive RNA was centrifuged in similar gradients for 15 h at 25 °C in a SW50.1 rotor (Beckman). Fractions (0.1 ml) were collected after absorptiometry, and radioactivity was determined, following the addition of 0.1 ml H₂O and 3 ml Biofluor (New England Nuclear), by scintillation counting.

**Electrophoresis of RNA in polyacrylamide gels.** Samples of about 25 μg RNA were analysed by electrophoresis in gels (100 × 6 mm diam.) of 2-6% acrylamide, 0.13% bisacrylamide in 36 mM-Tris–HCl, 30 mM-Na₂HPO₄, pH 7.8. Infective RNA was eluted by washing gels in 0.01 M-sodium acetate, 1 mM-magnesium acetate, pH 5.5, and homogenizing the relevant portion of the gel with 0.5% bentonite in the same buffer, as described by Harrison et al. (1972).

**Hybridization of RNA with complementary DNA.** [3H]-labelled cDNA copies of SLRV RNA-3 (cDNA-3) were prepared using avian myeloblastosis virus reverse transcriptase with random DNA fragments as primers as described by Robinson et al. (1980). Reaction mixtures (50 μl) contained either 50 μCi [Me-3H]TTP (sp. act. 42 Ci/mmoll or 50 μCi [5-3H]dCTP (sp. act. 21 Ci/mmoll; both from Amersham International) together with 0.67 mM each of the other three deoxynucleoside 5'-triphosphates. Conditions for the formation of hybrids between cDNA and RNA, and for their assay using S1 nuclease were as described by Robinson et al. (1980). When cDNA-3 was prepared using [3H]dCTP, about 30% of the labelled product became resistant to digestion with S1 nuclease after incubation without added RNA for 2.5 h at 60 °C or 4 °C. This suggested that the preparation contained complementary homopolymeric sequences, with the labelled species being 3H-oligothymylate transcribed from the polyadenylate of the template. The blank value decreased to about 10% when [3H]dCTP was used as the label, and all the results refer to this cDNA.

**Chromatography on oligo(dT)-cellulose.** RNA (50 to 100 μg) was dissolved in 0.4 M-NaCl, 0.5% SDS, 0.01 M-Tris–HCl, pH 7-4 (‘high-salt’) and applied slowly to columns (about 9 mm × 6 mm diam.) of oligo(dT)-cellulose (Type 7; P-L. Biochemicals). Columns were washed with about 5 ml ‘high salt’ buffer to remove RNA lacking poly(A), followed by about 5 ml 0.05% SDS, 0.01 M-Tris–HCl, pH 7-4 (‘low-salt’) to elute polyadenylated RNA (Mayo et al., 1979a).

**Iodination of RNA.** Samples of 100 to 300 μg RNA were iodinated using Na¹²⁵I and chloramine T as described by Mayo et al. (1979b). Iodinated RNA was separated from non-covalently bound ¹²⁵I by centrifugation to near-equilibrium in caesium trichloroacetate solution followed by sucrose gradient centrifugation in buffer containing SDS, as described by Mayo et al. (1979b).

**Translation of RNA in reticulocyte lysates.** Samples of 10 μl rabbit reticulocyte lysate (nuclease-treated, messenger-dependent; Amersham International) were mixed with 2 to 4 μl RNA-3 (0-6 to 2.4 μg) and 1 μl (45 μCi) [³⁵S]methionine (600 Ci/mmoll; Amersham International) and then incubated for 60 min at 30 °C. Following treatment with ribonuclease A and EDTA (Shih et al., 1978), SDS (2% w/v) and 2-mercaptoethanol (1% v/v) were added, and after heating at 100 °C for 2 min the radioactive polypeptides were analysed by electrophoresis in 8% polyacrylamide gels using a discontinuous buffer system (Laemmli, 1970).

**RESULTS**

**Permanent removal of RNA-3 from isolates**

Following electrophoresis of RNA in an acrylamide gel similar to that shown in Fig. 1(b), the region of the gel containing RNA-1 and RNA-2 was cut out. RNA was then eluted and
Satellite RNA of SLRV

Fig. 1. Ultraviolet densitometer scans of 2.6% acrylamide gels following electrophoresis of SLRV RNA. (a) RNA from particles of isolate HA; (b) RNA from particles of an isolate obtained by adding RNA-3 to inocula of isolate HA.

Fig. 2. Kinetics of hybridization of cDNA-3 with RNA-3 (○) and with RNA from particles of isolate HA of SLRV that comprised approximately equal weights of RNA-1 and RNA-2 (▲).

inoculated on to leaves of Chenopodium murale. After 10 days, individual lesions were cut from the leaves and extracts of each were inoculated singly on to C. quinoa. After the infections had become systemic, sap from the apical tissue of these C. quinoa plants was used to inoculate Nicotiana clevelandii. After three successive subcultures in N. clevelandii, the RNA from purified particles of the isolates was examined by gel electrophoresis. No RNA-3 was detected in any of the six isolates tested (Fig. 1a). When purified RNA-3 was added to inocula containing RNA extracted from particles of each of three isolates lacking RNA-3, the virus recovered from the infected plants contained RNA-3 (Fig. 1b). RNA-3 therefore multiplied in leaves infected with these isolates and was incorporated into virus-like particles. One RNA-3 free isolate, designated HA, was selected for further tests.

Test for sequence homology between RNA-3 and RNA from SLRV(HA)

The upper curve in Fig. 2 shows the kinetics of hybridization of cDNA-3 with an excess of RNA-3. At the highest concentrations of RNA about 70% of the cDNA became resistant to S1 nuclease, and this value is taken to represent 100% hybridization. When RNA was omitted from hybridization mixtures about 10 to 12% of the cDNA had become resistant to S1 nuclease 2.5 h after the start of the reaction; nuclease resistance developed slowly and is therefore unlikely to
Table 1. Assay for RNA-3 nucleotide sequences in leaf samples

<table>
<thead>
<tr>
<th>Inoculum</th>
<th>Interval between inoculation and sampling</th>
<th>% ct/min hybridized*</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>-</td>
<td>2</td>
</tr>
<tr>
<td>RNA-3†</td>
<td>2 h</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>7 days</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>14 days‡</td>
<td>3</td>
</tr>
<tr>
<td>Isolate H†</td>
<td>2 h</td>
<td>8</td>
</tr>
<tr>
<td></td>
<td>7 days</td>
<td>53</td>
</tr>
</tbody>
</table>

* Values were corrected for 10% self-annealing of the cDNA. In parallel tests, 65% hybridized with excess RNA-3 and 20% hybridized with excess of a mixture of RNA-1 and RNA-2.
† Inoculum RNA concentrations were 0.2 μg/ml (RNA-3) and 4 μg/ml (isolate H).
‡ Three leaves immediately above those inoculated.

represent a 'snap-back' reaction. This value was taken as 0% hybridization. The curve shows a single sharp transition with a $R_{0.5}$ (initial RNA concentration $\times$ time, for 50% hybridization) of $1.8 \times 10^{-3}$ mol.s/l. The relatively large correction necessary for self-annealing of cDNA-3 makes this value of $R_{0.5}$ somewhat unreliable and therefore, although it can be concluded that the reaction involves a major component (or components) of the RNA-3 preparation, no inferences about the nucleotide sequence complexity of RNA-3 are justified.

When cDNA-3 was hybridized with RNA from particles of isolate HA (RNA-1 + RNA-2), about 30% (corrected as above) of the cDNA reacted (Fig. 2; lower curve). However, because the RNA-3 preparation used as template to synthesize cDNA-3 almost certainly contained some fragments of RNA-1 and RNA-2, some reaction between cDNA-3 and RNA-1 + RNA-2 was expected. Nevertheless, 70% of the reactive cDNA-3 did not react with either RNA-1 or RNA-2, implying that at least 70% of the sequence of RNA-3 does not occur in either RNA-1 or RNA-2.

Tests for independent replication of RNA-3

Purified preparations of RNA-3 induced no symptoms in inoculated plants, but as a further test for possible multiplication, leaves of *N. clevelandii* were inoculated with purified RNA-3 and samples (1 to 2 g) were taken from these leaves after either 2 h or 7 days. Samples were also taken from leaves above the inoculated leaves 14 days after inoculation, from healthy leaves, and from similar plants inoculated with isolate H. RNA was extracted from these samples and the fraction that was insoluble in 2 M-LiCl was allowed to react with cDNA-3. The results (Table 1) show that no RNA-3 was detectable in plants inoculated with RNA-3, but that it was readily detected 7 days after inoculating leaves with isolate H.

As a more sensitive test for multiplication of RNA-3 in inoculated leaves, RNA was extracted from leaves of *N. clevelandii* following inoculation with RNA-3 and added to RNA extracted from particles of isolate HA. *N. clevelandii* plants were inoculated with these mixtures and, following a further passage in *N. clevelandii*, the RNA composition of the resulting virus particles was analysed by polyacrylamide gel electrophoresis. RNA-3 was detected when RNA was extracted from leaves sampled about 2 h after inoculation but not when the leaves were sampled 7 days after inoculation. Presumably, the test detected a small amount of biologically active RNA-3 from the inoculum that was retained by the inoculated leaf, and this RNA was inactivated during the 7 days following inoculation.

Biological effects of RNA-3

Isolate HA was compared with an isolate obtained when RNA-3 was added to inocula of RNA from isolate HA. RNA-3 did not alter the symptoms induced in infected plants of *C. amaranticolor*, *C. quinoa*, *C. murale* or *Cucumis sativus*, nor did it induce symptoms in plants of *N. clevelandii*, *N. tabacum* cv. Samsun NN, *N. benthamiana* and *N. debneyi* which were infected without symptom by both isolates.
Satellite RNA of SLRV

Fig. 3. Centrifugation of $^{125}$I-labelled RNA of SLRV(H). The upper curve (dotted) is the trace from the u.v. absorptiometer (A$_{254}$) and the lower curve (•) shows the radioactivity. The numbered arrows indicate the positions of the three RNA species of SLRV(H), confirmed by a parallel separation of unlabelled RNA.

Fig. 4. Translation of SLRV RNA-3 in messenger-dependent rabbit reticulocyte lysate. [35S]methionine-labelled polypeptides were separated by electrophoresis in an 8% polyacrylamide gel. The autoradiogram shows products synthesized in mixtures containing (a) no added RNA, or (b) 0.24 mg/ml SLRV RNA-3. The numbered arrows indicate the positions of marker proteins: 1, bovine serum albumin (67000); 2, ovalbumin (45000); 3, α-chymotrypsinogen (25700). The bar indicates the top of the gel.

Polyadenylation of RNA-3

Most RNA in preparations from SLRV particles binds to oligo(dT)-cellulose at high ionic strengths (Mayo et al., 1979a). Analysis of RNA fractions from a similar experiment by electrophoresis in polyacrylamide gels showed that all three species of SLRV RNA were bound. To confirm that RNA-3 was bound, and therefore presumably polyadenylated, 52 μg of RNA-3 was applied to a column containing about 100 mg oligo(dT)-cellulose. About 8 μg of RNA was eluted by ‘high-salt’ buffer, and the remainder (85%) remained bound until eluted in ‘low-salt’ buffer. In other experiments the proportion bound was 60 to 70%, and gel electrophoresis showed that some of the RNA eluted by ‘high-salt’ buffer was of the same size as RNA-3; however, the results show that most RNA-3 molecules contained sufficient poly(A) to bind to oligo(dT)-cellulose.

Protein linked to RNA-3

Evidence was obtained previously that SLRV RNA is linked to a protein; as in all nepoviruses examined, the infectivity of SLRV RNA was decreased by protease treatment and the RNA could be made highly radioactive by iodination (Mayo et al., 1982). To ascertain whether RNA-3 is linked to protein, RNA was iodinated, purified and separated into species by centrifugation in a sucrose gradient (Fig. 3). The results showed that in this preparation, which
was unusually rich in RNA-3 (compare Fig. 1), the fractions of greatest radioactivity coincided with the peak of RNA-3. The radioactivity corresponding with the peak of RNA-2 was difficult to resolve from polydisperse radioactivity and RNA-1 was close to the bottom of the tube. Thus, RNA-3 was more radioactive than RNA-2 on a weight (absorbance) basis, but the species were more nearly equally radioactive on a molar basis. The results are therefore consistent with a protein molecule being linked to RNA-3, as it is to genome RNA (Mayo et al., 1982).

**Messenger activity of RNA-3**

When RNA-3 was added to reticulocyte lysates the synthesis of a polypeptide with a mol. wt. of about 38000 was stimulated (Fig. 4). Neither SLRV RNA-1, SLRV RNA-2 nor tobacco rattle virus RNA-2 induced the synthesis of this polypeptide. It therefore seems likely that this polypeptide is the translation product of RNA-3; indeed its mol. wt. corresponds to a nearly complete translation of the molecule. No bands were detected in positions expected for polypeptides of the mol. wt. of SLRV coat proteins (44000 and 29000).

**DISCUSSION**

The results show that RNA-3, the minor RNA of mol. wt. 0.4 × 10^6 found in particles of the H isolate of SLRV, is a satellite RNA. Little if any of its nucleotide sequence occurs in either RNA-1 or RNA-2; it is not essential for the replication of RNA-1 and RNA-2, and it does not replicate independently of RNA-1 and RNA-2. It is thus neither a subgenomic fragment, nor a part of the genome of SLRV, nor genomic RNA of a different virus. However, as for several other satellite RNA species, the evidence that the helper virus is essential for replication is not unequivocal. When RNA-3 is inoculated alone it might replicate, but only in the inoculated cell: perhaps only by examining inoculated protoplasts will it be possible to test this idea. A possible parallel is suggested by the finding that although inocula must contain both species of genome RNA for plants to become infected with cowpea mosaic or tomato black ring viruses, protoplasts can be infected by the larger RNA species alone (Goldbach et al., 1980; Robinson et al., 1980).

SLRV satellite RNA resembles that of tomato black ring virus in several ways: both satellites resemble the genome RNA of their helper viruses in being polyadenylated molecules covalently bound to a small protein, both are mRNA species coding for relatively large proteins that account for virtually all the coding capacity of the RNA molecules and neither causes any obvious modification of the symptoms induced in host plants by their helper viruses. This type of satellite RNA therefore seems distinct from that exemplified by the satellite RNA of tobacco ringspot virus, which has a much smaller mol. wt., of about 10^5, is not polyadenylated, lacks messenger activity, and which causes a pronounced change in the symptoms shown by infected plants and indeed interferes with the replication of the helper virus (Murant & Mayo, 1982). Further work is needed both to assess the biological significance of this distinction between types of satellite, and to understand the function(s) of the proteins for which satellite RNA of tomato black ring and strawberry latent ringspot viruses are messengers.

We gratefully acknowledge a gift of reverse transcriptase from Dr J. Beard, Life Sciences Inc., St Petersburg, Florida, U.S.A., and excellent technical assistance from Susan Bradley and Ann Jenkins.

**REFERENCES**


Satellite RNA of SLRV


(Received 1 June 1982)