Complete replication of a satellite RNA *in vitro* by a purified RNA-dependent RNA polymerase

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The 334 nucleotide R satellite RNA was used as a template for purified RNA-dependent RNA polymerase (RdRp) from cucumber mosaic virus-infected tobacco plants. The products of the reaction were dsRNA and positive-strand RNA of the same size as the R satellite RNA. Similar products were obtained when T7 RNA polymerase positive-strand transcripts of a cDNA clone of the satellite RNA, designed to have the same 5' and 3' ends as the satellite RNA, were used as templates. The formation of the positive strands demonstrates complete replication of the satellite RNA. A positive-strand transcript with 65 and 255 additional nucleotides at the 5' and 3' ends of the satellite RNA respectively was also utilized as a template by the RdRp, but only dsRNA was formed. However, no products could be detected when the RdRp was programmed with transcripts corresponding to the negative-strand satellite RNA, either with no additional terminal nucleotides or with 24 and 310 additional nucleotides at the 5' and 3' ends respectively.

The genome of cucumber mosaic virus (CMV) is divided among three positive-strand RNAs, designated RNA 1 (3.4 kb), RNA 2 (3.0 kb) and RNA 3 (2.1 kb) which serve as mRNA for non-structural proteins 1a, 2a and 3a respectively. RNA 3 also encodes the virus coat protein which is translated from a subgenomic RNA, designated RNA 4. Proteins 1a and 2a are components of the RNA-dependent RNA polymerase (RdRp) (Hayes & Buck, 1990a), the enzyme complex responsible for replication of the virus genome, whereas protein 3a is thought to potentiate cell-to-cell movement of the virus.

Several isolates of CMV, but not all, also contain a satellite RNA (reviewed by Francki, 1985). Over 25 different CMV satellite RNAs have been characterized. They are all linear ssRNA molecules of approximately 334 to 386 nucleotides and are highly homologous. The satellite RNAs provide no functions necessary for the virus itself, contain sequences unrelated to CMV RNAs and are entirely dependent on CMV for replication and encapsidation. Satellite RNAs can alter the symptoms induced by the helper virus, the effect often depending on the host plant. For example, satellite I17N attenuates the symptoms on tobacco but induces necrotic death of tomato, whereas R satellite RNA attenuates symptoms on both hosts (Jacquemond & Lot, 1981; Jacquemond & Lauquin, 1988).

We have recently isolated the CMV RdRp and shown it to be capable of catalysing *in vitro* the complete replication process of genomic RNA, i.e. the synthesis of negative-stranded template RNA and the subsequent synthesis of positive-strand genomic RNA (Hayes & Buck, 1990a). We now show that the CMV RdRp is also capable of replicating CMV satellite RNA.

The 334 nucleotide R satellite RNA (Jacquemond & Lauquin, 1988) was purified by centrifugation on a sucrose gradient of total viral RNA produced by CMV-infected transgenic plants expressing an R satellite RNA (D. Tousch, M. Jacquemond & M. Tepfer, unpublished results). To determine whether the CMV RdRp could use satellite RNA as a template, 1 µg of RNA was added to 25 µl RdRp reaction mixture containing 50 mM-Tris–HCl pH 8.2, 4% glycerol, 10 mM-MgCl₂, 10 mM-DTT, 1 mM-ATP, 1 mM-CTP, 1 mM-GTP, 50 µM-UTP, 10 µCi [³²P]UTP (10 mCi/ml), and micrococcal nuclease-treated RdRp fraction 6, prepared essentially as described by Hayes & Buck (1990a) but with minor modifications (Hayes & Buck, 1992). After 1 h at 30 °C, a 5 µl aliquot of the reaction was spotted onto DE81 discs to determine the incorporation of [³²P]UMP into RNA (Hayes & Buck, 1990a). The remainder of the RdRp

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Fig. 1. Non-denaturing 8 M-urea-4% PAGE of products of RdRp reactions. Lane 1, 5' end-labelled R satellite RNA. Lanes 2, 5 and 8, products of RdRp fraction 6, no added RNA. Lane 3, product of RdRp fraction 6 programmed with R satellite RNA. Lane 4, 5' end-labelled pBLR T7 RNA polymerase positive-strand transcript. Lane 6, products of RdRp fraction 6 programmed with pBLR T7 RNA polymerase positive-strand transcript. Lane 7, end-labelled pBLR/PCR T7 RNA positive-sense polymerase transcript. Lane 9, products of RdRp fraction 6 programmed with pBLR/PCR T7 RNA polymerase positive-strand transcript. Equal c.p.m. were loaded in each lane.

Table 1. RNA synthesis catalysed by CMV RdRp using various templates

<table>
<thead>
<tr>
<th>Added RNA</th>
<th>C.p.m.</th>
</tr>
</thead>
<tbody>
<tr>
<td>No RNA</td>
<td>450</td>
</tr>
<tr>
<td>Viral RNA (CMV)</td>
<td>12700</td>
</tr>
<tr>
<td>Satellite RNA</td>
<td>8140</td>
</tr>
<tr>
<td>pBLR (+ transcript)</td>
<td>880</td>
</tr>
<tr>
<td>pBLR (- transcript)</td>
<td>480</td>
</tr>
<tr>
<td>pBLR/PCR (+ transcript)</td>
<td>6290</td>
</tr>
<tr>
<td>pBLR/PCR (- transcript)</td>
<td>520</td>
</tr>
</tbody>
</table>

* Approximately 1 μg of RNA was added in each case.  
† Incorporation of [32P]UMP as described by Hayes & Buck (1990a).

that the upper band in lane 3 represents newly synthesized single-stranded satellite RNA. Digestion of the gel-purified band with RNase H in the presence of oligonucleotides complementary to internal sequences of the satellite RNA (Hayes & Buck, 1990a) confirmed that the upper band was positive-sense satellite RNA and internally labelled (data not shown). This upper band was also sensitive to digestion by S1 nuclelease and RNase A in 2 × SSC confirming its identity as ssRNA (data not shown). In contrast, the faster migrating band was resistant to S1 nuclease and RNase A in 2 × SSC, but sensitive to RNase A in 0.1 × SSC (data not shown), showing that it is double-stranded (Ratti & Buck, 1979). Its electrophoretic mobility is consistent with it being the double-stranded form of the satellite RNA.

Having shown that the RdRp was capable of replicating the satellite RNA, we investigated the ability of the RdRp to process and replicate T7 RNA polymerase transcripts of the R satellite RNA cDNA clones. Cleavage of pBLR with HindIII and transcription with T7 RNA polymerase produces a 654 nucleotide RNA composed of positive-sense R satellite RNA with 65 extra nucleotides at the 5’ end and 255 nucleotides at the 3’ end (Fig. 1, lane 4) (D. Tousch, M. Jacquemond & M. Tepfer, unpublished results). When 1 μg of the transcript was added to the RdRp, the amount of [32P]UMP incorporated into RNA was much reduced compared to when satellite RNA was used as a template (Table 1). Analysis of the products on an 8 m-urea-polyacrylamide gel detected only a single band (Fig. 1, lane 6). After digestion with nuclease S1, or RNase A in 2 × SSC, a band with slightly greater mobility was detected (result not shown). This suggests that the reaction product was dsRNA with a single-stranded tail, which would be expected if internal initiation of negative-strand synthesis had occurred. No band corresponding to ssRNA was detected, even after prolonged exposure.

Cleavage of pBLR with XbaI and transcription with T3 RNA polymerase produced a 671 nucleotide RNA composed of negative-sense R satellite RNA with 24 extra nucleotides at the 5’ end and 310 nucleotides at the 3’ end (D. Tousch, M. Jacquemond & M. Tepfer, unpublished results). When 1 μg of the transcript was added to the RdRp, the amount of [32P]UMP incorporated into RNA was very low (Table 1). No bands corresponding to ss- or dsRNA were detected on an 8 m-urea-polyacrylamide gel (data not shown).

To investigate further whether the lack of ssRNA production with the T7 RNA transcript was a feature of the extra 5’ and 3’ nucleotides, the cDNA insert was amplified using the polymerase chain reaction (PCR) as previously described (Hayes & Buck 1990b), but using the 5’ oligonucleotide TAATACGACTCACTA-
TAGTTTTGTATGGGAGAATT and a 3' oligonucleotide GGGTCTGTAGAGGAATGTG with an extension step (72 °C) of 15 s. The 352 bp PCR product was then purified using a Stratagene PrimeErase Quik Push column as described by the manufacturer. T7 RNA polymerase transcription, using the promoter site within the 5' sequence of the product, resulted in a 334 nucleotide transcript (Fig. 1, lane 7) with the same 5' and 3' ends as the native satellite RNA. As expected, when used as a template for the RdRp, both ss- and dsRNA were produced (lane 9). Incorporation of [32P]UMP with this template was similar to that obtained using the purified satellite RNA.

The PCR reaction was then repeated using the 5' primer GTTTTTGTATGGGAGAATT and the 3' primer TAATACGACTCACTATAGGGTCCTGTA-GAGGAATGTG. T7 RNA polymerase transcription using the promoter site within the 3' sequence resulted in a 334 nucleotide transcript of negative-strand polarity corresponding to the complementary sequence of the satellite RNA with no terminal additions. However, when this transcript was used as a template for the RdRp reaction, there was no significant increase in [32P]UMP incorporation compared to the no template control (Table 1), and no labelled RNA species were detected by autoradiography (data not shown).

The observation that the CMV RdRp produces both negative- and positive-stranded RNA when provided with a satellite RNA template is consistent with the observation that the RNA is dependent upon the helper virus for replication. Although in vitro the CMV RdRp is able to recognize and replicate a positive-sense T7 RNA transcript identical to the satellite RNA, additional 5' and 3' flanking sequences result in only dsRNA synthesis. This, together with the lack of RNA synthesis with the pBLR negative transcript, indicates that the RdRp can recognize the promoter for negative- but not positive-strand synthesis internally. Internal recognition of a promoter for negative-strand synthesis by a partially purified brome mosaic virus RdRp was reported by Miller et al. (1986). Interestingly, high levels of single-stranded satellite RNA are detected in plants after inoculation with the pBLR positive transcript together with CMV RNAs 1, 2 and 3 (D. Tousch, M. Jacquemond & M. Tepfer, unpublished results). They proposed that positive-strand synthesis is initiated on the adjacent penultimate C residue of the negative strand. The unpaired G residue could be important for RdRp binding to the negative strand. (v) Another possibility is that synthesis of negative and positive strands could be coupled, with positive-strand synthesis requiring either the RdRp to recognize simultaneously sequences at the 5' end of the positive strand and at the 3' end of the negative strand, or the RdRp to remain bound to the 3' end of the negative strand as it is synthesized. Hence recognition and copying of a free negative strand may not occur in vivo. Experiments to distinguish these possibilities are in progress.

**Note in proof.** After this paper had been submitted for publication, Wu et al. (1991; *FEBS Letters* 292, 213–216) reported the replication of two different CMV satellite RNAs (1 and 7) by a partially purified RdRp.

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References


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