The multiplication in plants of arabis mosaic virus satellite RNA requires the encoded protein

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Oligonucleotide-directed mutagenesis was used to create two mutations at each of three positions within the open reading frame (ORF) of a cDNA clone representing a satellite RNA from a lilac isolate of arabis mosaic nepovirus (ArMV). Three of the six mutants, in which stop codons were introduced at three different sites, did not direct synthesis of a translation product. The other three mutants, in which stop codons were not introduced, directed synthesis of a translation product (39K) although, in two of these, the mutation led to a single amino acid substitution. When Chenopodium quinoa plants were inoculated with in vitro transcripts from each of the six mutants together with the genomic RNA molecules (RNA-1 and RNA-2) of ArMV, progeny RNA was detected only with two of the three mutants in which the nucleotide changes did not introduce a stop codon to the coding region. To look for complementation, two deletion mutants were made. In these, 113 or 117 nucleotides were removed from two consecutive regions within the ORF. Two insertion mutants (in which the deleted sequences were replaced with a 130 nucleotide sequence from RNA-2 of cherry leaf roll nepovirus) were also made. Transcripts from none of these mutants retained messenger activity and none was detected either in C. quinoa plants or in virions, even in the presence of wild-type satellite RNA.

Arabis mosaic virus (ArMV) is a nepovirus (Harrison & Murant, 1977), with a bipartite RNA genome of $M_r$ of $2.4 \times 10^6$ for RNA-1 and $1.4 \times 10^6$ for RNA-2. An ArMV isolate from lilac (ArMV-L) contains a satellite RNA that depends on ArMV both for replication and encapsidation, and that has no extensive sequence homology with either of the two genomic RNAs (Liu et al., 1991b). This satellite RNA is a single-stranded linear molecule of 1104 nucleotides with structural and biological properties in common with other protein-encoding satellite RNAs of nepoviruses (for review, see Fritsch & Mayo, 1989) such as tomato black ring virus (TBRV; Meyer et al., 1984) or grapevine fanleaf virus (GFLV; Pinck et al., 1987; Fuchs et al., 1989). The protein encoded by the satellite RNA of TBRV has been synthesized in infected tobacco protoplasts (Fritsch et al., 1978) and that of the satellite RNA of GFLV has been detected in infected plants (Moser et al., 1992). The satellite RNA in ArMV-L contains a single open reading frame (ORF) encoding 393 amino acids with a theoretical $M_r$ of 38849 (Liu et al., 1990). In rabbit reticulocyte lysate, the virion-derived satellite RNA directs the synthesis of a protein with an $M_r$ of 39K. A cDNA clone (M1A3/3) of the satellite RNA yielded biologically active transcripts (Liu et al., 1991a) and this paper records the preparation and bioassay of mutants derived from this cDNA clone.

ArMV-L and a satellite-free derivative of it (ArMV-SF; Liu et al., 1991b) were the sources of the satellite and viral genomic RNA in these experiments. Virus was propagated in Chenopodium quinoa and, using methods described by Liu et al. (1991b), RNA was extracted from virions purified from systemically invaded leaves.

The cDNA clone M1A3/3, which was the template for site-specific mutagenesis, was constructed by inserting the cDNA sequence representing the satellite RNA into EcoRI/BamHI-cut pT7T3 18U (Pharmacia) under the control of the T7 promoter. Mutations (mutants 1 to 6, Fig. 1) were introduced by using each of the following six oligonucleotide primers, respectively:

- primer 1: 5' GGATCCGAATTCTGAAAAATTTCTATGGA-TAGCCATATTAACGCG 3';
- primer 2: 5' GGATCCGAATTCTGAAAAATTTCTATGGA-TAGCCATATTAACGCG 3';
- primer 3: 5' ATACAAGCTTGCGAGTG 3';
- primer 4: 5' ATACAAGCTTGCGAGTG 3';
- primer 5: 5' TCGACACTCGCTAGCCCACAG 3';
- primer 6: 5' TCGACACTCGCTAGCCCACAG 3'.

Underlined sequences in primers 1 and 2 are recognition sites for EcoRI. All oligonucleotide primers were synthesized in an Applied Biosystems Model 380B automatic DNA synthesizer.

Oligonucleotide-directed mutagenesis was carried out in two ways. Mutants 1 and 2 were made using PCR and either primer 1 or primer 2 plus primer 7 (5' AGC-
Mutant clones of ArMV-L satellite RNA. The two or three 5′-most ATG codons are shown in bold. Underlined sequences are recognition sites for the restriction enzymes indicated. Letters in lower case represent base substitutions.

TAGGATCTTTTTTTTTGAGTTGAC 3′, which is complementary to the 3′ end sequence of M1.A3/3. The underlined sequence indicates a BamHI cleavage site. Protocols for PCR and cloning of the products into EcoRI/BamHI-cut pT7T3 18U were as described by Liu et al. (1991a). Mutations were confirmed by direct sequencing of the plasmid DNA (Murphy & Kavanagh, 1988). Mutants 3, 4, 5 and 6 (Fig. 1) were made by using a uracil-containing DNA template, substantially as described by Kunkel et al. (1987). However, when preparing uracil-containing DNA templates, double-stranded M1.A3/3 plasmid DNA was used to transform Escherichia coli (mut ung F' strain RZ1032). Following infection of the RZ1032 culture with the M13K07 helper phage (Pharmacia), single-stranded M1.A3/3 containing uracil residues was isolated using the manufacturer's protocol. Clones of mutants 3, 4, 5 and 6 were selected by restriction enzyme mapping since a new restriction enzyme site was created at each mutation site.

Mutations made as described above at positions 21 to 23, 126 to 128 and 243 to 245 gave rise to mutants 1 and 2, mutants 3 and 4, and mutants 5 and 6 respectively (Fig. 1). Mutants 1, 3 and 5 each had two nucleotides changed, resulting in a TAG stop in the ORF, whereas 2, 4 and 6 each also had one to three nucleotides changed but in such a way that they did not introduce a stop codon in the coding region. The nucleotide changes resulted in a substitution of lysine for methionine in mutant 4 and of aspartic acid for proline in mutant 6. In mutant 2, the change of two nucleotides did not alter the serine codon, so there was no change in the amino acid sequence.

Using methods described by Liu et al. (1991a) the plasmid DNA was transcribed in vitro and the RNA was either translated or bioassayed. In rabbit reticulocyte lysate, transcripts from mutants 2, 4 and 6 directed the synthesis of a 39K protein, whereas transcripts from mutants 1, 3 and 5 did not yield detectable amounts of 35S-labelled polypeptides (Fig. 2). Although the base sequence surrounding the third in-frame methionine codon (AAUAUGGG) most closely fits with the consensus sequence proposed by Kozak (1984) or Lütcke et al. (1987), data from our experiments suggested that the translation of the satellite RNA initiated at the first AUG codon of the ORF, which has a U at position −3 and a G at +4.

To determine whether the mutant satellite RNAs replicated in plants, in vitro transcripts from 2 μg of template DNA (from two separate bacterial colonies) of all six mutants were mixed with 2 μg of virion RNA (RNA-1 and -2) from ArMV-SF, and inoculated onto C. quinoa. The amount of RNA transcripts in inocula was monitored by co-electrophoresis against standards. Subsequently, total nucleic acid extracted from systemically invaded foliage (sampled 7 to 10 days after inoculation) was tested for the presence of the satellite RNA by Northern transfer hybridization with a 32P-labelled probe prepared by nick-translating plasmid DNA (M1.A3/3). Satellite RNA was detected only in extracts from plants that had been inoculated with mutant 2 (lane 3, Fig. 3) or mutant 6 transcripts plus...
As shown in Fig. 4, two deletion mutants and two insertion mutants were constructed from mutant 5 or mutant 3–5 (which had itself been made from mutant 3 with primer 5). Deletion mutant D-1 was made by digestion of mutant 3–5 with NheI (cleaving at positions 124 and 241), removal of the 117-nucleotide fragment and re-ligation. Deletion mutant D-2 was constructed by digesting mutant 5 with NheI and Styl (cutting at positions 241 and 358 respectively). After removal of the 113-nucleotide fragment, the larger product of mutant 5 was treated with the Klenow fragment of DNA polymerase I and then religated. D-1 and D-2 each retained a unique NheI recognition site at positions 124 and 241 respectively. To construct the insertion mutants, a 130-nucleotide XbaI/SpeI fragment from a clone (pT7T3 18U 22; Scott et al., 1992) representing RNA-2 of cherry leaf roll nepovirus (CLRV) was inserted into NheI-cut D-1 and D-2 to produce CD-1 and CD-2 respectively. As a consequence of the deletion or insertion, none of these deletion and insertion mutants retained the ORF encoding the 39K protein. In vitro transcription of D-1 or D-2 yielded RNA that migrated faster during agarose gel electrophoresis than the transcripts of M1A3/3 or virion-derived satellite RNA, whereas the transcripts from CD-1 or CD-2 were indistinguishable from these markers.

When in vitro transcripts of D-1, D-2, CD-1 or CD-2 were inoculated, together with virion RNA of ArMV-SF, onto C. quinoa, tests performed as described above did not reveal satellite RNA sequences either in plants or in virions. To determine whether the wild-type satellite RNA would complement the changes in the deletion mutants (D-1, D-2) or insertion mutants (CD-1, CD-2), plants were inoculated with in vitro transcripts from these clones mixed with virion-derived RNA from ArMV-L (i.e. the satellite and the genomic RNAs). RNA was then extracted from inoculated leaves or from virions purified from systemically invaded tissue and was analysed by agarose gel electrophoresis and Northern hybridization. When 32P-labelled M1A3/3 plasmid DNA was used as a probe for the progeny RNA of D-1 and D-2, only the wild-type satellite RNA was detected; no M1A3/3 labelled bands migrating faster than, and additional to, the normal satellite RNA were identified. In seeking the progeny RNA of CD-1 and CD-2, the 130-nucleotide sequence of CLRV was labelled with 32P and used as a probe in Northern hybridization. This did not hybridize with any of the RNA species (RNA-1, -2 or the satellite RNA) that were detected by ethidium bromide staining.

Our ability to detect progeny of mutants 2 and 6 but not of 1 and 5 strongly suggests that the protein encoded in the ArMV-L satellite RNA is important for its accumulation in plants. Because mutant 3 resembled mutants 1 and 5 in lacking messenger activity in vitro, it was not surprising that it did not appear to replicate in plants. It was unexpected, however, that mutant 4, which had retained its ability to direct the synthesis of satellite-encoded protein, also failed to replicate to detectable levels. A plausible reason is that the single amino acid
substitution, introduced by site-specific mutation, altered the three-dimensional structure of the protein thereby destroying its functions(s). The possibility cannot be excluded, however, that the single nucleotide substitution at this site is itself directly responsible for eliminating biological activity. There are several possible explanations of why the in vitro transcripts from clones D-1, D-2, CD-1 and CD-2 did not replicate in plants even in the presence of wild-type satellite RNA. The satellite-encoded protein may not act in trans, the deletions and insertions may have changed the secondary structure of the satellite RNA and made it unrecognizable by viral replicase(s) or the natural satellite RNA may replicate more efficiently than the in vitro transcripts of these mutants, so that the multiplication of the natural satellite RNA inhibits (rather than complements) the multiplication of the transcripts.

Since satellites depend on helper viruses for replication, it is generally believed that they use helper-encoded polymerases for their replication. This assumption has been confirmed for a distinctly different satellite RNA of cucumber mosaic virus (Wu et al., 1991). The role of the protein encoded by the ArMV-L satellite is unknown, but it might act by modifying the helper-encoded replicase, as suggested by Fritsch et al. (1978) for the satellite RNA of TBRV. Another possibility is that the satellite protein is involved in the encapsidation of the satellite RNA within helper virus particles. Thus, our inability to detect progeny of mutants that did not synthesize a protein may reflect the fact that the transcripts replicated in inoculated cells but were not packaged and did not move to other cells in the infected plant. This possibility can be investigated by challenging protoplasts with the same transcripts.

References


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