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Current Review

Replication of a Plant Virus Satellite RNA: Evidence Favors Transcription of Circular Templates of Both Polarities

George Bruening, Boni K. Passmore, Hans van Tol, Jamal M. Buzayan, and Paul A. Feldstein

Department of Plant Pathology, College of Agricultural and Environmental Sciences, University of California, Davis 95616 U.S.A.
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Small satellite RNAs. Two groups of satellite entities of plant viruses are satellite RNAs and satellite viruses. For either type of satellite, *dependence* and *lack of nucleotide sequence similarity* are the critical characteristics of the relationship between the satellite and its associated supporting virus. Members of several plant virus groups are known to have satellites, and where supporting virus and satellite have been resolved, the virus, as required by its definition, exhibits an independent capability of replication. In contrast, a satellite RNA or a satellite virus increases only in a host plant that is also infected by any one of a few specific viruses or virus strains. The satellite entity exhibits a degree of dependence that implies an intimate interaction during replication, and the satellite may be regarded as a molecular parasite of the virus. It is therefore surprising that neither a satellite RNA nor a satellite virus genomic RNA has extensive nucleotide sequence similarity with the genomic RNA(s) of the supporting virus. The two groups of satellite entities are distinguished according to the origin of the capsid: the genomic RNA of a satellite virus encodes its own coat protein, whereas a satellite RNA has no coat protein gene and accumulates in capsids composed of the coat protein of its supporting virus.

The intimate association of satellite and virus in the infected cell and the sequestration of virus coat protein by satellite RNA suggest that the supporting virus titer may be reduced in the presence of the satellite, as compared to infections by the virus alone. This is usually, but not always, the case. A satellite may alter the symptoms that the supporting virus alone would induce. In many systems a satellite attenuates symptoms, but examples of enhanced symptoms are also common. The sequences of two well-studied satellite RNAs have been introduced into plant genomes. When the satellite RNA-expressing, transgenic plants were inoculated with the corresponding supporting virus, the plants became infected but were strongly protected against the most severe effects of the virus (Baulcombe *et al.* 1986; Gerlach *et al.* 1987; Wu *et al.* 1988). The transgenic plants thus may be considered to have acquired a gene for resistance against a specific virus.

For all known satellite RNAs and satellite viruses of plants, replication appears to require the synthesis in the infected cells of RNA that is complementary in polarity to that of the most abundantly encapsidated form of the satellite. Among the many known satellite RNAs, several share the properties of similar size, lack of detected messenger RNA activity *in vitro*, and the presence in extracts of infected tissue of circular and multimeric forms of at least one polarity. In addition, linear forms have a 5′-hydroxyl and a 2′,3′-cyclophosphate as the terminal groups (e.g., Kiberstis *et al.* 1985; Buzayan *et al.* 1986a). The unit length of the known satellite RNAs with these characteristics is fewer than 500 nucleotide residues, and they may be designated as *small satellite RNAs*. Although messenger RNA activity has not been attributed, by analysis of sequences or *in vitro* translation, to either polarity of these RNAs, the polarity that accumulates in greatest amount is arbitrarily designated as plus, (+).

The characteristics listed in the previous paragraph are those of known satellite RNAs of supporting viruses from three groups: nepovirus, luteovirus, and sobemovirus. The encapsidated form of each of three nepovirus small satellite RNAs is linear. The satellite RNA of tobacco ringspot virus (sTobRV RNA) was the first of these to be discovered (Schneider 1969, 1971, 1977) and is the most studied. The satellite RNA of arabis mosaic virus (sArMV RNA) and satellite RNA of chicory yellow mosaic virus (sCYMoV RNA) show extensive nucleotide sequence similarity with sTobRV RNA (Kaper *et al.* 1988; Rubino *et al.* 1990). Recently, a small satellite RNA of barley yellow dwarf virus (sBYDV RNA), with an encapsidated linear form, was discovered in association with BYDV (W. A. Miller, T. Hercus, P. M. Waterhouse, and W. L. Gerlach; cited by Bruening 1989).

Small satellite RNAs are associated with four viruses of the sobemovirus group (Randles *et al.* 1981; Tien *et al.* 1981; Gould and Hatta 1981; Francki *et al.* 1983). The most abundantly encapsidated form is circular and has the potential for extensive internal base pairing. Thus, in this limited way, the sobemovirus satellite RNAs resemble the independently replicating, circular viroid RNAs and commonly have been referred to as "virusoids." Satellite RNAs of Solanum nodiflorum mottle virus and of velvet tobacco

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mottle virus (sSNMoV RNA and sVTMoV RNA, respectively) are very similar in sequence (Haseloff and Symons 1982). Two satellite RNAs of subterranean clover mottle virus (sSCMoV RNAs) have similar sequences in half of the molecule but diverge significantly in the other half. The sSCMoV RNAs have only limited sequence similarity with the sSNMoV RNA-sVTMoV RNA pair (Davies *et al.* 1990). Two very similar isolates of satellite RNA of lucerne transient streak virus (sLTSV RNA; Keese *et al.* 1983) are distinct from sobemovirus satellite RNAs of either the sSCMoV RNA group or the sSNMoV RNA group (Davies *et al.* 1990), whereas a third satellite RNA associated with LTSV is similar to the larger sSCMoV RNA in electrophoretic mobility and sequence as tested by hybridization (Dall *et al.* 1990). Finally, although it is not a satellite RNA, an unusual viroid, avocado sunblotch viroid (ASBVd), also has the characteristics of this broad group of small satellite RNAs (Symons 1981; Bruening *et al.* 1982; Hutchins *et al.* 1985).

Self-cleavage reactions of small satellite RNAs. sTobRV RNA (Prody *et al.* 1986; Buzayan *et al.* 1986b), sBYDV RNA (W. A. Miller, T. Hercus, P. M. Waterhouse, and W. L. Gerlach, unpublished), the small satellite RNAs of sobemoviruses (Forster and Symons 1987a, 1987b; Davies *et al.* 1990), and ASBVd (Hutchins *et al.* 1986) all exhibit, for at least one polarity of RNA, a specific, not enzymically catalyzed, self-cleavage reaction that is capable of releasing the unit-length RNA from a multimeric precursor. The new ends created by self-cleavage are a 5′-hydroxyl and a 2′,3′-cyclophosphate. The site at which cleavage occurs, the junction phosphodiester, connects the "monomeric" unit sequences of the repetitive sequence, multimeric RNA. The released, (+) polarity, monomeric RNA has been shown to be biologically active. Thus, a self-cleavage reaction of the type exhibited by these satellite and viroid RNAs may be termed "autolytic processing" because of its specificity and ability to generate functional RNA.

Rolling circle transcription. Although the sobemovirus satellite RNAs and ASBVd are predominantly circular and the encapsidated forms of the nepovirus and luteovirus satellite RNAs are linear, all of these RNAs are present both in circular and in linear forms in extracts of infected tissue. Thus, replication of these RNAs very likely has fundamental aspects in common, with the greater accumulation or encapsidation either of the linear form or of the circular form as a variation on an underlying theme. In "rolling circle transcription," a polymerase proceeds around a circular template to synthesize a multimeric transcript. Rolling circle transcription is suggested by the presence of both circles and multimers in infected tissue. Rolling circle transcription has long been considered to be a strong candidate as a step in the replication of small satellite RNAs and viroids (e.g., Kiefer *et al.* 1982; Branch and Robertson 1984; Hutchins *et al.* 1985; Symons *et al.* 1985; Bruening *et al.* 1988; Bruening 1990; Symons 1990).

In this current review we consider the evidence in favor of rolling circle transcription as two steps in a specific model for the replication of one small satellite RNA, sTobRV RNA. Figure 1 defines two general classes of a rolling circle model, with the junction dinucleoside phosphate sequences, and their complements, corresponding to those of sTobRV RNA. In both classes, linear, unit-length sTobRV (+) RNA circularizes in step 1, and in step 2 the sTobRV (+) RNA circle serves as template for rolling circle transcription, generating multimeric sTobRV (−) RNA. In a symmetrical model, Figure 1A, self-cleavage of multimeric sTobRV (−) RNA in step 3 gives unit-length, monomeric sTobRV (−) RNA. Steps 4, 5, and 6 correspond to steps 1, 2, and 3, respectively, but with the complementary orientation of the two polarities of RNA. In contrast, in the asymmetrical model of Figure 1B, multimeric sTobRV (−) RNA serves directly as the template for synthesis of multimeric sTobRV (+) RNA in step 3. In step 4, the cycle is completed by the autolytic processing of multimeric sTobRV (+) RNA.

The first evidence in support of rolling circle transcription in the replication of sTobRV RNA came from the analysis of melted, double-stranded RNA isolated from tissue infected with TobRV and sTobRV RNA (Kiefer *et al.* 1982). Hybridization probes revealed that both polarities of sTobRV RNA occurred as a series of multimers of the unit sequence. RNA from virion-like particles (sTobRV RNA in TobRV capsids) also exhibited a "ladder" of (+) polarity RNA multimers. As is indicated by the results of Kiefer *et al.* (1982) and in Figure 2, analyses of tissue-derived sTobRV RNA have revealed that the sTobRV (−) RNA series is more heavily skewed toward the high-order multimers than is the sTobRV (+) RNA series. This observation slightly favors the asymmetric model (Fig. 1B) for sTobRV (−) RNA replication in which multimeric sTobRV (−) RNA is the template for the synthesis of multimeric sTobRV (+) RNA.

sTobRV RNA circles *in vitro* and *in vivo*. Sogo and Schneider (1982) obtained evidence from electron microscopy for circular forms in double-stranded RNA preparations from plants infected with TobRV and sTobRV RNA. The inocula in these experiments had a ratio of satellite RNA to supporting virus genomic RNAs great enough to cause sTobRV RNA to predominate in the progeny TobRV capsids. Presumably, most of the recovered double-stranded RNA was also of sTobRV RNA origin. Linthorst and Kaper (1984) showed by hybridization the presence of circles of the (+) polarity in RNA extracted from infected tissues. The first observation of sTobRV (−) RNA circles was the result of the spontaneous ligation reaction exhibited by linear sTobRV (−) RNA derived from *in vitro* transcripts of cloned sequences (Buzayan *et al.* 1986b). When electrophoretically purified, linear, monomeric sTobRV (−) RNA was incubated in a buffered solution of magnesium ions and spermidine for 1 hr at 37° C (Buzayan *et al.* 1986b; Van Tol *et al.* 1991), "riboligation" produced circles in a yield of 50% or more. The reaction is the reverse of the self-cleavage reaction, and like the self-cleavage reaction, it has no requirement for added protein and almost certainly is a property of the RNA per se. However, neither sTobRV (+) RNA (Prody *et al.* 1986), nor any of the other satellite RNAs that have been tested, nor ASBVd showed significant reversibility of the self-cleavage reaction. Possibly linear sArMV (−) RNA and sCYMoV (−) RNA, which have sequences (Kaper

et al. 1988; Rubino *et al.* 1990) that are very similar to the self-cleaving sequences of sTobRV (−) RNA (Fig. 3; Haseloff and Gerlach 1989; Feldstein *et al.* 1989, 1990), will also circularize, but this has not been tested.

Undoubtedly a specific conformation of the sTobRV (−) RNA self-cleaving sequence renders it capable of efficient ligation. The secondary structure of the self-cleaving sequence of sTobRV (−) RNA is unlike the "hammerhead"

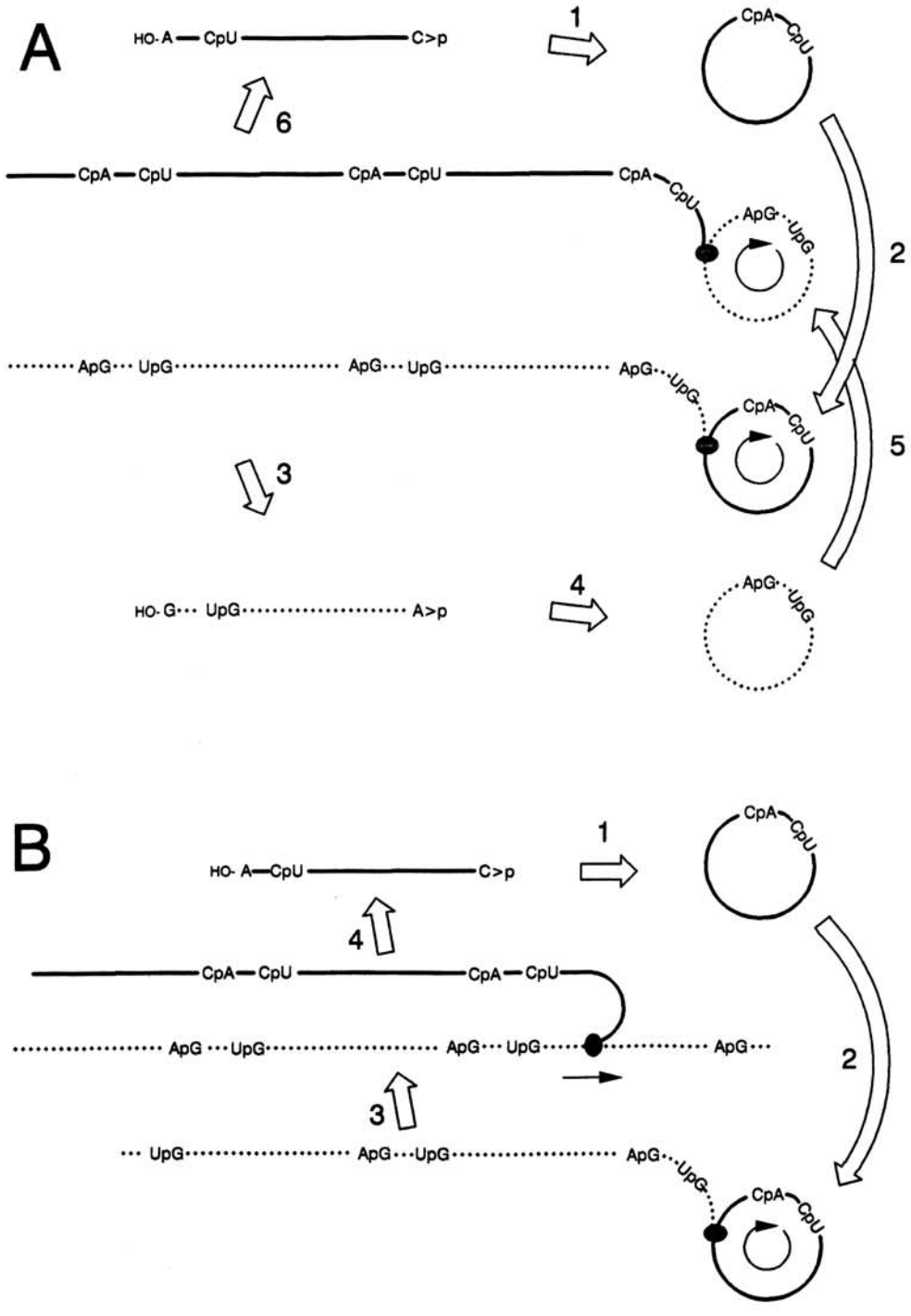

Fig. 1. Comparison of models for replication of satellite tobacco ringspot virus RNA (sTobRV RNA) that incorporate rolling circle transcription, **A**, symmetrically for the synthesis of both strands and, **B**, asymmetrically for the synthesis of one strand only. Heavy solid lines with inserted dinucleoside phosphate sequences represent what is arbitrarily designated as the positive polarity of the polyribonucleotide chain, sTobRV (+) RNA. Dotted lines represent the negative polarity chain, sTobRV (−) RNA. The (+) and (−) polarity sequences of the cleaved junction phosphodiester are given as dinucleoside phosphates CpA and ApG, respectively; UpG and CpU are the dinucleoside phosphate complements of the junction sequences. The straight portion of each chain is shown 5′ to 3′, left to right. The 5′-hydroxyl (HO-A and HO-G) and 2′,3′-cyclophosphate termini (C>p and A>p) of the unit-length, linear satellite RNA are indicated. Each solid ellipse represents an RNA-dependent RNA polymerase, which, for the purposes of these diagrams, is considered to be fixed in place as the template strand moves in the direction indicated by the small arrows. The major steps in each model, indicated by the large arrows, are discussed in the text.

structure that is consistent with the other self-cleaving sequences of small satellite RNAs and ASBVd (Hutchins *et al.* 1986; Forster and Symons 1987a, 1987b; Bruening 1989). The self-cleavage of sTobRV (−) RNA requires not only sequences in the immediate region of the junction, as represented by the top strand in Figure 3A, but also sequences distant in the polyribonucleotide chain, as represented by the bottom portion of Figure 3A (Haseloff and Gerlach 1989; Feldstein *et al.* 1989). Thus, the sTobRV (−) RNA structure encompasses a type of "ribozyme" in which one part of the sequence acts catalytically in *trans* on another, junction-containing part that has very limited sequence requirements (Feldstein *et al.* 1990). Although ribozymes with simple substrate requirements have also been derived from hammerhead sequences (Haseloff and Gerlach 1988), this required dividing the original, contiguous sequence into junction-containing and *trans*-acting portions.

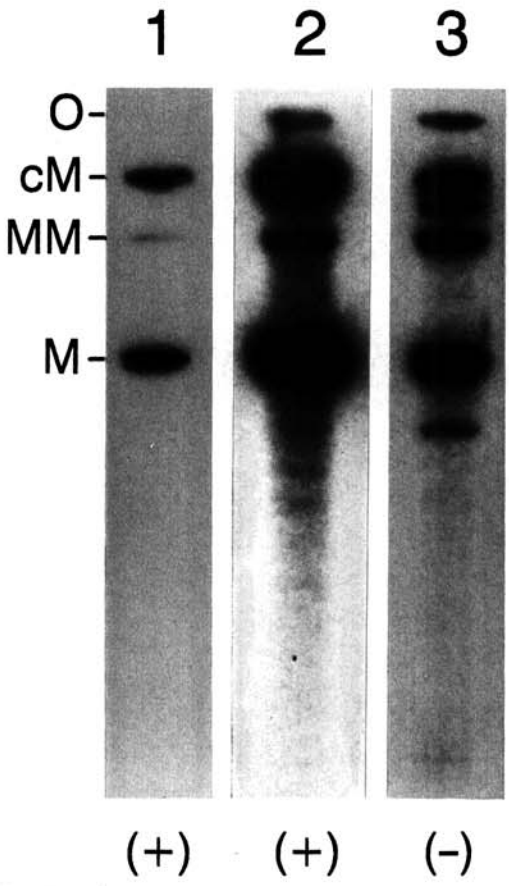

Fig. 2. Abundant circular forms of both polarities of satellite tobacco ringspot virus RNA (sTobRV RNA) in an extract from bean plants inoculated with TobRV and sTobRV RNA. For hybrid selection, 50 μg of denatured plasmid DNA, bearing a cDNA insert of a permuted, dimeric sTobRV RNA sequence, was attached to diazophenylthioether cellulose paper that was subsequently inactivated by treatment with glycine and thoroughly washed. One hundred micrograms of total RNA from infected bean plants was heated to 85° C for 10 min in buffered 65% formamide and incubated with the derivatized paper for 2 hr at 50° C. RNA was eluted from the thoroughly washed paper by heating it in 0.3 ml of sterile water in a boiling water bath for 60 sec and was concentrated, all by procedures similar to those of Valen (1987). Extract was analyzed by electrophoresis through a 6.5% polyacrylamide gel in 7 M urea, electroblotting to a charged nylon membrane, and hybridization to sTobRV RNA transcripts synthesized from solutions of the same composition and containing [α-^{32}P]rCTP. Probes were [^{32}P]sTobRV (−) RNA for lanes 1 and 2 and [^{32}P]sTobRV (+) RNA for lane 3; this is indicated below each lane by the polarity of the detected RNA. Autoradiographic exposures at −80° C were less than 1 hr (lane 1) and 36 hr (lanes 2 and 3). The position of the bottom of the sample well is indicated by O. Linear and circular forms of sTobRV RNA are indicated by M and cM; MM is dimeric sTobRV RNA. Components with mobilities between those of MM and cM are presumably the trimeric (lanes 2 and 3) and tetrameric forms (lane 3) of sTobRV RNA.

That circular sTobRV (−) RNA is present *in vivo*, as well as after spontaneous circularization of linear sTobRV (−) RNA *in vitro*, is shown in Figure 2, which displays RNA rapidly extracted under conditions that allow only very limited equilibration between the circular and linear forms of sTobRV (−) RNA. Experiments in which amounts of the two polarities of sTobRV RNA were compared, with or without any prior fractionation such as hybrid selection, revealed that sTobRV (+) RNA is present in about a 100-fold excess of sTobRV (−) RNA. Hybrid selection of sTobRV (−) RNA to remove excess sTobRV (+) RNA did not alter the observed distribution of sTobRV (−) RNA species (B. K. Passmore and G. Bruening, unpublished). For both sTobRV (+) RNA and sTobRV (−) RNA, linear and circular forms were about equally prevalent (Fig. 2). That both polarities of sTobRV RNA apparently are well represented by circles *in vivo* supports replication models that employ both polarities of circles as templates (Fig. 1A). Because the bulk of the encapsidated sTobRV (+) RNA is linear (Buzayan *et al.* 1986a), most or all of the surprisingly large amount of circular sTobRV (+) RNA observed in extracts of infected tissue must not be encapsidated.

Evidence for participation of sTobRV (−) RNA circles in replication. The presence of circular and multimeric satellite RNAs does not, of course, constitute proof of participation of rolling circle transcription in replication. Circular or multimeric RNA may be a dead-end product rather than a replication intermediate. Conceivably, input unit-length sTobRV (+) RNA could be enzymatically ligated *in vivo* to form a template for the synthesis of multimeric sTobRV (−) RNA. We believe this to be highly unlikely, however, because such a scheme will require a greater concentration of sTobRV (+) RNA inoculum than would be required if the template is unit-length. In addition, electrophoretically purified monomeric sTobRV (+) RNA was found to be at least as active in initiating replication as dimeric sTobRV (+) RNA (Prody *et al.* 1986). We assume that some of the observed sTobRV (+) RNA circles must serve as template for rolling circle transcription to account for the observed multimeric sTobRV (−) RNA. If circular sTobRV (+) RNA is the template for sTobRV (−) RNA synthesis, then symmetrical replication models will be supported strongly if sTobRV (−) RNA circles are found to be essential for sTobRV RNA replication.

A system in which circularization of sTobRV (−) RNA *in vitro* could be correlated with biological activity was derived from a study in which the cleavage reaction was inactivated by mutagenesis and, then, restored by introduction of a compensatory mutation. Two mutants, each with two adjacent base changes at a different site (Fig. 3), were prepared. These were used to determine whether

the postulated 4-base pair (bp) helix, in which the changes were made (Fig. 3B), actually participates in the self-cleavage reaction of sTobRV (−) RNA (Feldstein *et al.* 1990). The two base changes were introduced into a 15-nucleotide junction-containing oligoribonucleotide (corresponding to part of the top strand in Fig. 3A) or into a *trans*-acting oligoribonucleotide (part of the bottom strand, Fig. 3A). Each mutated oligoribonucleotide, when incubated with the appropriate wild-type oligoribonucleotide, did not engender a cleavage reaction. However, when the junction-containing and the *trans*-acting oligoribonucleotides, each with mutations at one site, were mixed, the former was cleaved at an efficiency corresponding to that observed when the two wild-type oligoribonucleotides were combined. Presumably, the restoration of the potential base pairs (Fig. 3B) also restored the 4-bp helix and hence the cleavage reaction.

The same two one-site mutations were introduced singly and together into an unpermuted monomeric sTobRV RNA sequence embedded in a circularly permuted dimeric sTobRV (−) RNA transcript (Van Tol *et al.* 1991). In contrast to the results with oligoribonucleotides, dimeric sTobRV (−) RNA bearing either one-site mutation cleaved. Cleavage occurred at both junctions and was nearly as efficient as the self-cleavage of the wild-type or two-site-mutated sTobRV (−) RNA. Thus, it was possible to recover monomeric, linear sTobRV (−) RNA of all four sequences (Fig. 3) in good yield. Each of the four sTobRV RNAs was incubated in a buffered solution of magnesium ions and spermidine, and each was converted, partially, to the circular form. However, the yield of circles from the one-site mutants was 6% or less, whereas 60% or more of the wild-type and two-site-mutated sTobRV (−) RNA appeared as circles after 1 hr at 37° C. Thus, the circularization reaction was more sensitive to interruption of the 4-bp helix (Fig. 3) than the self-cleavage reaction.

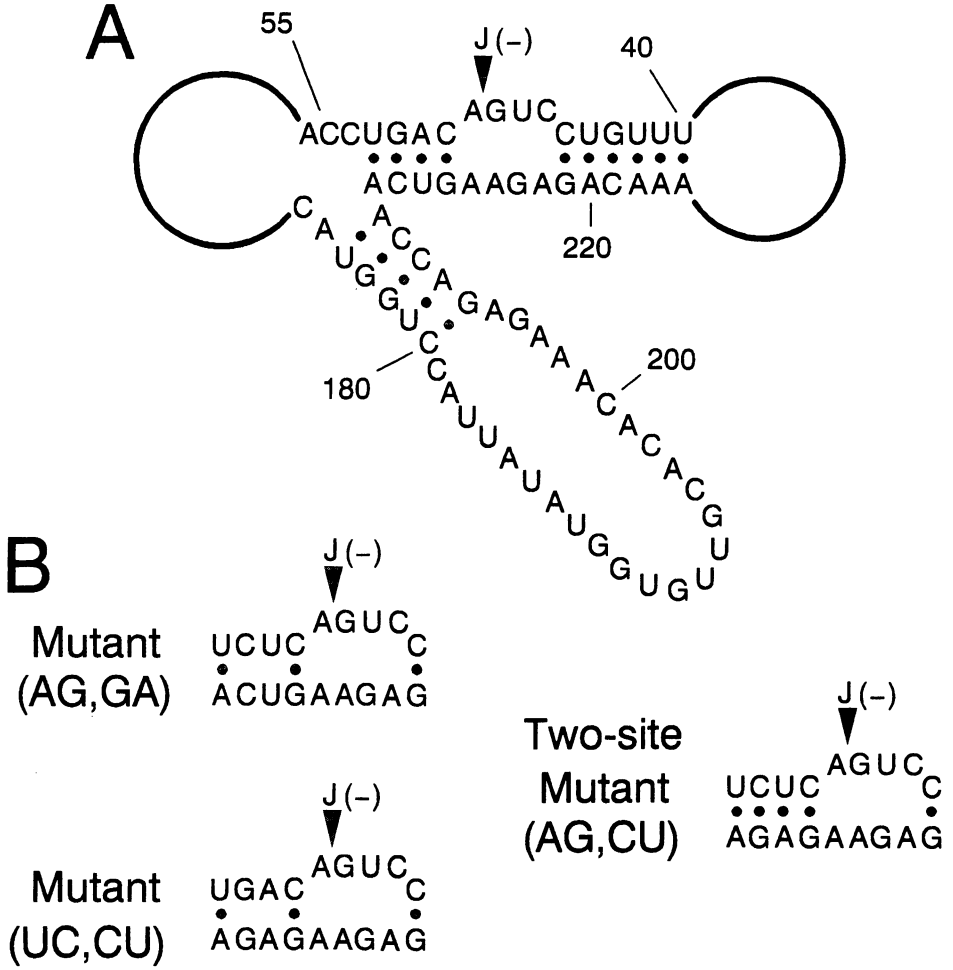

Fig. 3. Mutations that influence circle formation from the less abundant polarity of the satellite RNA of tobacco ringspot virus (sTobRV (−) RNA) and the ability of the corresponding sTobRV (+) RNA to increase when coinoculated with TobRV. **A**, A representation of the circular form of the wild-type sequence of sTobRV (−) RNA showing a central set of three helices that is essential to the sTobRV (−) RNA self-cleavage reaction. Base pairing is indicated by dots, and J(−) locates the junction phosphodiester that is formed in the circularization of linear sTobRV (−) RNA. **B**, The left two structures define two one-site mutants, sTobRV RNA (AG, GA) and sTobRV RNA (UC, CU), each of which has two adjacent base changes that interrupt the 4-base pair helix shown in **A**. The two-site mutant on the right, sTobRV RNA(AG, CU), incorporates both of the one-site mutations to restore the helix. Both base numbering and the bases identified in the designation of each mutant refer to the sTobRV (+) RNA sequence (Buzayan *et al.* 1986a).

When the cloned sequences described in the previous paragraph were transcribed into sTobRV (+) RNA, self-cleavage of the circularly permuted dimer was equally efficient for all four sequences. This is the expected result, because neither of the mutated sites falls within the sequence that forms the hammerhead structure. Virions and virion-like particles were recovered from bean (*Phaseolus vulgaris* L.) plants coinoculated with TobRV and electrophoretically purified sTobRV (+) RNA. Analyses of the RNA of these particles showed that neither of the one-site-mutated sTobRV (+) RNAs replicated and accumulated to a detectable level. Both the wild-type and the two-site-mutated sTobRV (+) RNAs accumulated to similar levels. Indeed, in mixed inoculations of TobRV with three proportions of wild-type sTobRV (+) RNA and the two-site-mutated sTobRV (+) RNA, the two sequences of the satellite RNA appeared to be equally competitive.

Thus, three *in vitro* reactions of sTobRV RNA were assessed: 1) self-cleavage of sTobRV (+) RNA, 2) self-cleavage of sTobRV (−) RNA, and 3) circularization of sTobRV (−) RNA. Only the last reaction was seriously affected by either of the two one-site mutations, and only the one-site-mutated sTobRV (+) RNAs failed to increase in bean tissue infected with TobRV. The observed correlation of sTobRV (−) RNA circularization and sTobRV (+) RNA-initiated replication strongly suggests that circularization of sTobRV (−) RNA, as it was observed *in vitro*, is necessary for sTobRV RNA replication. The results are entirely consistent with a replication model that has circular templates of both polarities (Fig. 1A). Such a replication model presumably applies also to sArMV RNA and sCYMoV RNA because of their structural similarities to sTobRV RNA. Recently, an *in vitro* transcript was prepared that has those sequence elements of sArMV (−) RNA that are similar to the self-cleaving sTobRV (−) RNA sequences. As expected, the sArMV (−) RNA-derived oligoribonucleotide exhibited self-cleavage (P. A. Feldstein, unpublished observation).

Generality of the symmetrical replication model. A symmetrical replication model is likely to apply to the nepovirus small satellite RNAs, to sBYDV RNA, to sLTSV RNA, and to ASBVd. Both polarities of these RNAs exhibit self-cleavage *in vitro*. However, three of the satellite RNAs of sobemoviruses, sSCMoV RNA, sSNMoV RNA and sVTMoV RNA, have a hammerhead structure only in the (+) polarity sequence and show no indication of a self-cleaving sequence of any kind in the (−) polarity sequence. Analyses of the (+) and (−) polarity satellite RNAs in extracts of leaves in which these small satellite RNAs were increased show a series of multimers for the (+) polarity RNA but only high molecular weight (−) polarity RNA (Chu *et al.* 1983; Hutchins *et al.* 1985; Davies *et al.* 1990). Results for sSCMoV RNA, sSNMoV RNA, and sVTMoV RNA are entirely consistent with replication models in which the only circular template is that of the (+) polarity, as illustrated in Figure 1B.

Summary. The sequences of sTobRV RNA and other small satellite RNAs appear to be neither transcribed into DNA nor translated into protein. Replication and the other activities, such as influence over supporting virus-induced symptom development, seem to require satellite sequences only in the form of RNA of both polarities. A model for the replication of sTobRV RNA employs circular RNAs both of the abundant polarity and of the less abundant polarity (sTobRV [−] RNA) as templates for the synthesis of complementary, multimeric RNA. In this model, processing of the multimeric RNA generates the respective unit-length, monomeric sTobRV RNA, completing the replication cycle. Evidence in favor of this model, which is qualitatively symmetric with regard to the participation of each polarity of RNA, includes the discovery of 1) circular and multimeric forms of both polarities of sTobRV RNA, 2) autolytic processing of both polarities of multimeric sTobRV RNA *in vitro*, and 3) an association, in a set of sTobRV RNA mutants, of the ability to initiate replication and the ability, of sTobRV (−) RNA, to circularize spontaneously *in vitro*.

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