Satellite Cereal Yellow Dwarf Virus-RPV (satRPV) RNA Requires a DouXble Hammerhead for Self-cleavage and an Alternative Structure for Replication

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The 110 nt hammerhead ribozyme in the satellite RNA of cereal yellow dwarf virus-RPV (satRPV RNA) folds into an alternative conformation that inhibits self-cleavage. This alternative structure comprises a pseudoknot with base-pairing between loop (L1) and a single-stranded bulge (L2a), which are located in hammerhead stems I and II, respectively. Mutations that disrupt this base-pairing, or otherwise cause the ribozyme to more closely resemble a canonical hammerhead, greatly increase self-cleavage. In a more natural multimeric sequence context containing the full-length satRPV RNA and two copies of the hammerhead, wild-type RNA cleaves much more efficiently than in the 110 nt context. Mutations in the upstream hammerhead, including a knock-out in the catalytic core, affect cleavage at the downstream cleavage site, indicating that multimers of satRPV RNA cleave via a double hammerhead. The double hammerhead includes base-pairing between two copies of the L1 sequence which extends stem I. Disruption of L1-L1 base-pairing slows cleavage of the multimer. L1-L2a base-pairing is required for efficient replication of satRPV RNA in oat protoplasts. Mutations that affect self-cleavage of the multimer do not correlate with replication efficiency, indicating that the ability to self-cleave is not a primary determinant of replication. We present a replication model in which multimeric satRPV RNA folds into alternative conformations that cannot form in the monomer. One potential metastable intermediate conformation involves L1-L2a base-pairing that may facilitate formation of the double hammerhead. However, we conclude that L1-L2a also performs some other essential function in the satRPV RNA replication cycle, because the L1-L2a base-pairing is more important than efficient self-cleavage for replication.

Keywords: hammerhead ribozyme; pseudoknot; RNA folding; rolling circle RNA; viroid-like RNA

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Introduction

Circular (D type) satellite RNAs (Mayo et al., 1995) and viroids (Branch & Robertson, 1984) replicate via a rolling circle mechanism, in which the infectious circular (+) RNA is copied into linear, multimeric (−) strands (Bruening et al., 1991). The negative strand is used for the synthesis of multimeric (+) RNAs as either linear multimeric or circular monomeric forms. The multimeric (+) RNA cleaves and ligates, yielding circular (+) monomeric RNAs (reviewed by Symons, 1992, 1997). The infectious monomeric RNAs range from about 225 to 450 nt and do not encode any open reading frames. Satellite RNAs differ from viroids in that
they depend on their helper viruses for replication and encapsidation. They have no sequence similarity to their helper virus genome. In contrast, viroids are autonomously replicating subviral pathogens that do not require a helper virus. Thus, they must be replicated by a pre-existing host RNA polymerase(s) and they are not encapsidated (reviewed by Diener, 1987; Riesner & Gross, 1985; Semancik, 1987; Symons, 1990).

Under in vitro conditions, most viroids form a very stable rod-like structure, whereas satellite RNAs tend to form branched structures. Alternative, metastable foldings have been suggested to be involved in the viroid replication process (Hecker et al., 1988; Loss et al., 1991; Riesner et al., 1992). Multi-hairpin viroid structures are formed relatively quickly during the in vitro synthesis of oligomeric RNA (Hecker et al., 1988) and folding simulations of potato spindle tuber viroid (PSTVd) RNA suggest that the RNA folding pathway plays an important role in the viroid replication cycle (Gultyaev et al., 1998). The functional importance of RNA folding pathways is not likely confined to viroids. Here we propose such alternatives in a satellite RNA.

The subject of this study is satellite cereal yellow dwarf virus-RPV (satRPV) RNA which was previously called satellite barley yellow dwarf virus (sBYDV) RNA (Miller et al., 1991). Its helper virus, the former RPV serotype of BYDV (BYDV-RPV), has been reclassified as cereal yellow dwarf virus-RPV (CYDV-RPV) in the new genus Polerovirus (D’Arcy et al., 1999). The serotypes that remain classified as BYDV do not support any known satellite RNA. SatRPV RNA and nepoviral satellite RNAs differ from other D type (rolling circle replication mechanism) satellite RNAs in that the encapsidated form of the RNA is linear (Prody et al., 1986; Miller et al., 1991). The molecule is circularized only after entering the cell (Chay et al., 1997). The encapsidated forms of other D type satellite RNAs are predominately circular (Symons, 1997).

Multimeric forms of D type satellite RNAs and a few viroids undergo self-cleavage at the plus strand hammerhead ribozyme site to generate the monomeric unit (Forster & Symons, 1987; Symons, 1997). The hammerhead structure of (+) polarity satRPV RNA differs from canonical hammerheads (Vaish et al., 1998) in at least three features (Figure 1; Miller & Silver, 1991). Firstly, the trinucleotide at the 5' side of the cleavage site is AUa, instead of GUC, which is found in naturally occurring hammerheads except those of cherry small circular viroid-like RNA (AUa in (+) strand, GUA in (−) strand) (Di Serio et al., 1997), the minus strands of satellite lucerne transient streak virus RNA (satLTSV; Keese et al., 1983), and satellite velvet tobacco mottle virus RNA (satVTMoV; Haseloff & Symons, 1982) which cleave at GUA. Secondly, an unpaired cytosine base (C24) is present immediately 3' to the conserved CUGANGA sequence, and an unpaired adenine base (A73) occurs immediately 5' of the conserved GAAA sequence, opposite C24 (Figure 1). The bases at these positions are normally Watson-Crick base-paired to each other at the proximal end of stem II (Bruening, 1989), except in carnation stunt viroid-like (CarSV) RNA (−) strand which has unpaired C and A bases at the same positions (Hernandez Figure 1. Alternative structures in the hammerhead ribozyme in satRPV (+) strand RNA. Numbering of nucleotides is based on the full-length satellite RNA (Miller et al., 1991); the three major stem-loops are numbered as described by Hertel et al. (1992). Single-strand bulges and loops in the three main stems are prefixed with L (Miller & Silver, 1991). A cleavage site is indicated by arrowhead, the absolutely conserved nucleotides of the catalytic core are in bold. (a) Two-dimensional representation drawn as described by Miller & Silver (1991). The pseudoknot alternative to the hammerhead formed by base-pairing between bases in loops L1 and L2a is indicated by the double-headed arrow. Mutations used in the study are indicated. (b) The same structures drawn to resemble the three-dimensional crystal structure as described by Pley et al. (1994) and Scott et al. (1995).
et al., 1992), and satLTSV RNA which has an unpaired U base at the position homologous to C24 (Forster & Symons, 1987). The most striking and, so far, unique feature of the satRPV ribozyme is that the loop (L1) at the end of stem I can base-pair with a five base, single-stranded bulge (L2a) in the compound stem II (Figure 1). This L1-L2a base-pairing forms an alternative tertiary structure consisting of a kind of pseudoknot (or “kissing stem-loop”) that greatly inhibits self-cleavage of a 110 nt transcript comprising the (‡) strand satRPV RNA ribozyme (Miller & Silver, 1991). This led us to propose a molecular switching model in which this tertiary structure controls the rate of self-cleavage and possibly other functions of the satellite RNA.

We now find that self-cleavage occurs much faster in the larger than unit length satellite RNA than in the 110 nt hammerhead context. Our results show that the (‡) satRPV RNA cleaves via a double hammerhead. We also show that base-pairing between L1 and L2a is essential for satRPV replication in oat protoplasts even though it is not present in the double hammerhead and it inhibited self-cleavage of the 110 nt ribozyme. On the basis of these results, we propose a sliding model for the possible folding pathway of multimeric (‡) satRPV RNA.

Results

Self-cleavage of wild-type and mutated transcripts in the 110 nt hammerhead context

We reported previously that the alternative tertiary structure, which contains base-pairing between L1 and L2a, attenuated self-cleavage of the isolated 110 nt ribozyme of satRPV (+) strand (Miller & Silver, 1991). Transcripts containing each of the single mutations that disrupt L1-L2a base-pairing (G8U and C32A, Figure 1), self-cleave over 100-fold more rapidly than wild-type RNA (Table 1). The double mutant G8U/C32A that restored base-pairing, cleaved much more slowly than either of the single mutants. Therefore, alternative tertiary structure (L1-L2a base-pairing), attenuates self-cleavage of the ribozyme in the isolated 110 nt context (Miller & Silver, 1991).

We next examined the role of the unpaired nucleotides (C24 and A73) situated at the proximal end of stem II. Insertion of unpaired bases at these sites was shown to greatly reduce cleavage of an efficient, artificial hammerhead (Ruffner et al., 1990; Long & Uhlenbeck, 1994). Bases C24 or A73 were deleted in satRPV RNA hammerhead mutants C24Δ and A73Δ, respectively (Figure 2). The cleavage rates of both single deletion mutants did not differ significantly from that of wild-type (WT) (Figure 2). However, the hammerhead containing the double mutation, C24Δ/A73Δ, had at least a 50-fold shorter half-life (39 minutes) than either of the single mutants or the wild-type sequence (Figure 2). Thus deletion of both C24 and A73 together favored formation of hammerhead over the pseudoknot, or it increased cleavage chemistry once the hammerhead formed, or both.

Mutant C24Δ/A73Δ is interesting because it cleaves rapidly even though it can potentially form the L1-L2a helix. To test whether disrupting the L1-L2a helix could further increase cleavage of C24Δ/A73Δ, we added the mutation C32A which disrupts L1-L2a base-pairing. The half-life of C24Δ/A73Δ/C32A (six minutes), is indeed faster than that of C24Δ/A73Δ (Figure 2), but it is not significantly different from the five minute half-life A73 together favored formation of hammerhead over the pseudoknot, or it increased cleavage chemistry once the hammerhead formed, or both.

Table 1. Half-lives of wild-type and mutant satRPV RNA transcripts

<table>
<thead>
<tr>
<th>Mutant</th>
<th>110 nt hammerhead structure alone (minutes)</th>
<th>1.5-mer structure (minutes)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild-type</td>
<td>&gt;2000</td>
<td>45</td>
</tr>
<tr>
<td>C24Δ</td>
<td>&gt;2000</td>
<td>75</td>
</tr>
<tr>
<td>A73Δ</td>
<td>&gt;2000</td>
<td>55</td>
</tr>
<tr>
<td>C24Δ/A73Δ</td>
<td>39</td>
<td>90</td>
</tr>
<tr>
<td>C24Δ/A73Δ/C32A</td>
<td>6</td>
<td>35</td>
</tr>
<tr>
<td>C32A</td>
<td>5b</td>
<td>60</td>
</tr>
<tr>
<td>G8U</td>
<td>11b</td>
<td>&gt;350</td>
</tr>
<tr>
<td>G8U/C32A</td>
<td>180b</td>
<td>75</td>
</tr>
<tr>
<td>G8U/G19A</td>
<td>NT</td>
<td>∞</td>
</tr>
</tbody>
</table>

See Figure 1 for positions of altered bases. Δ, deletion. NT, not tested.

The half-lives (t_{1/2}) of uncleaved RNAs are calculated from cleavage assays as in Figures 3 and 4.

b Data from Miller & Silver (1991).

![Figure 2](image-url)
of the C32A mutant alone (Table 1). Either the effects of the C24A/A73A double deletion and the C32A mutation are not additive or it is quite possible that this cleavage rate represents the detection limit of our assay.

**Self-cleavage of wild-type and mutated transcripts in the context of greater than full-length satellite RNA**

All the above self-cleavage studies were conducted in the context of the isolated 110 nt ribozyme. In order to analyze cleavage and replication rates in a more natural context, 470 nt, “one-and-a-half-meric” transcripts (1.5-mer, designated by the prefix 1.5-) were constructed with the same mutations that were studied in the isolated 110 nt ribozyme. These transcripts contained two copies of the hammerhead structure (HH1 and HH2; Figure 3(a)) flanking the full-length monomeric unit. In all the mutants, only the first hammerhead (HH1) was mutated. The resulting full-length monomer obtained after cleavage at both hammerheads contains the desired mutation, because all mutations were 3’ of the cleavage site. The cleavage rates at HH1 and HH2 were observed by measuring accumulation of the 5’ and 3’ fragments (5EF and 3EF), respectively, that flank the full-length monomer. The overall result of cleavage at either site was also observed by monitoring the decay of the 1.5-mer.

The 45 minute half-life of the wild-type 1.5-mer (1.5-WT), calculated from three separate assays (Table 1), was at least 45-fold shorter than that of the 110 nt hammerhead alone (>2000 minutes, Table 1). Several mutants that greatly increased self-cleavage of the 110 nt hammerhead (C32A, G8U/C32A, C24A/A73A, C24A/A73A/C32A) had little or a slight negative effect on self-cleavage of the 1.5-mer (Table 1 and Figure 3). This is probably due to the much more efficient cleavage of wild-type 1.5-mer compared to the 110 nt transcript. The effects of mutations in L1 and L2a were nearly the opposite of those observed in the 110 nt context: mutant 1.5-G8U cleaved much more slowly than wild-type, cleavage of 1.5-C32A was slightly reduced, and the double mutant, 1.5-G8U/C32A cleaved at wild-type rates (Table 1 and Figure 3).

Another surprise was that the mutations in HH1 affected cleavage mainly at the HH2 cleavage site (CS2) as indicated by accumulation of fragments 5EF-M (348 nt) and 3EF (122 nt) (Figure 3). One possible explanation is that multimeric satRPV RNA self-cleaves via a double hammerhead that requires base-pairing between the two copies of L1, as shown in Figure 4. This prevents formation of the alternative pseudoknot structure (base-pairing between L1 and L2a) and explains why the 1.5-G8U mutation, which weakens the L1-L1 base-pairing, has such a strong negative effect on the cleavage rate of the 1.5-mer. It does not explain why restoration of L1-L2a base-pairing in the double mutant, 1.5-G8U/C32A, restores the cleavage rate (see Discussion).

Additional support for a double hammerhead structure comes from the behavior of a single base mutation that arose fortuitously in a 1.5-G8U clone. The essential CUGA sequence in HH1 was mutated to CUAA (1.5-G8U/G19A, Figures 1 and 5). Because G19 (G5 in standard hammerhead nomenclature; Hertel et al., 1992) is an essential base in the catalytic pocket, the G19A mutation completely destroys ribozyme function (Ruffner et al., 1990; Symons, 1992; Long & Uhlenbeck, 1994). The 1.5-G8U/G19A RNA cleaved normally at CS1 but even more slowly (if at all) than G8U at CS2 (Figure 5, see the accumulation of 5EF (26 nt) and 3EF (122 nt), respectively). The simplest explanation is that CS1 was cleaved by HH2 via a double hammerhead structure (Figure 4). The lack of accumulation of the 122 nt fragment (3EF) from CS2 also indicates that very little, if any, cleavage occurred via a single hammerhead.

**Biological activity of mutant satRPV RNAs**

Previously, we proposed that the L1-L2a base-pairing was required for a role in replication other than self-cleavage (Miller & Silver, 1991). To determine whether it has a role in the satRPV RNA replication cycle, plant cells were inoculated with satRPV RNAs containing mutations that affect L1-L2a base-pairing and with other hammerhead mutants. In separate inoculations, each monomeric mutant was electroporated along with CYDV-RPV helper virus genomic RNA into oat protoplasts. After 36 hours, Northern blot hybridization of total RNA revealed accumulation of oligomeric series of both strands of satRPV RNA, indicating rolling circle replication (Figure 6). Mutant G8U accumulated approximately 50-fold less plus strand than wild-type satRPV. The C32A mutant replicated even more poorly in both strands. The double mutant G8U/C32A, which restores L1-L2a base-pairing, replicated only threefold less efficiently than wild-type satRPV RNA. Mutant G8U/G19A, which abolished plus strand cleavage by hammerhead structure, replicated poorly, as expected. Interestingly, both G8U and G8U/G19A accumulated significant amounts of minus strand. Mutations C24A, A73A and C24A/A73A, had little or no effect on replication efficiencies, although A73A was enriched for high molecular mass plus strands (Figure 6). All the mutants except C32A accumulated some minus strand. It appears that the mutation in L2a was more harmful to replication than the mutation in L1, even though the double mutant restored nearly complete replication activity. Clearly, base-pairing between L1 and L2a is much more important than their primary sequences for replication of satRPV RNA.
Discussion

Effects of mutations on self-cleavage rate of 110 nt hammerhead context

The results presented here support our notion that satRNAs are so compact that any given sequence may participate in more than one function. These different functions, such as self-cleavage, ligation, origins of replication and assembly may be determined by different conformations of the same sequence. According to the rolling circle model, multimeric copies are formed from a monomeric, circular template. The multimer must then fold in a way to allow the hammerhead conformation to form, despite potential competing folding possibilities. Uhlenbeck and colleagues (Fedor & Uhlenbeck, 1992; Stage-Zimmermann & Uhlenbeck, 1998) described three major steps in the cleavage process: formation of an active ribozyme structure, phosphodiester bond cleavage, and dissociation of the cleaved fragments. Often the rate of formation of the active structure is slower than the actual cleavage rate and sometimes a portion of the molecules remain in an inactive conformation and never cleave (Stage-Zimmermann & Uhlenbeck, 1998). In the 110 nt context, we proposed that the structure exists in an equilibrium between the inactive pseudoknot (base-pairing between L1 and L2a) (Figure 1) and the hammerhead, with the majority of the molecules remaining folded in the inactive pseudoknot conformation (Miller & Silver, 1991). Alternatively, because the crystal structure indicates that L1 and L2a may be in proximity in the hammerhead conformation (Pley et al., 1994; Scott et al., 1995), base-pairing between them may not greatly distort the active site and rapid cleavage may occur in the pseudoknot context, but the reverse reaction (re-ligation) may be allowed, due to L1-L2a base-pairing holding the cleavage products in place long enough to allow re-ligation.

The unpaired nucleotides (C24 and A73) situated at the proximal end of stem II of the hammerhead may provide sufficient length and flexibility in stem II to allow L1-L2a base-pairing (Figure 1). Thus, deletion of both bases may move the folding equilibrium toward the hammerhead, in the 110 nt context. Alternatively, this double deletion may directly increase the activity of the catalytic core (Ruffner et al., 1990), and thus increase the actual chemical rate of cleavage. However, mutant 110 nt transcripts that disrupt base-pairing between L1 and L2a, G8U, C32A and C24Δ/A73Δ/C32A, cleave much faster than C24Δ/A73Δ. This is consistent with the first possibility, that C24Δ/A73Δ shifts structural equilibrium toward the hammerhead rather than increasing the cleavage rate per se, because G8U and C32A still have the C24-A73 mismatch, as does a very rapid cleaving mutant that has a minimal stem II with no L2a sequence at all (M5 by Miller & Silver, 1991). Furthermore, deletion of C24 and A73 had little effect on the rate of cleavage (or replication) in the 1.5-mer structures (1.5-C24Δ/A73Δ; Figure 3). Thus, C24 and A73 play a role in the formation of the alternative, non-cleaving structure in the 110 nt context, but not in the multimeric context.

Cleavage via double hammerhead

Wild-type satRPV RNA in the 1.5-meric context cleaved much faster than in the 110 nt hammerhead context (Table 1). Mutations in HH1, including one that inactivates the active site, affected cleavage at cleavage site 2 (CS2) much more than at CS1 (Figure 3). This strongly supports cleavage via a double hammerhead (Figure 4). Attenuation of self-cleavage by “intra-monomer” L1-L2a base-pairing seems to occur only in the 110 nt hammerhead context in which it is the strongest uninterrupted helix. In contrast, in the 1.5-mer, base-pairing between L1 and L1 (and adjacent bases), and/or between L2a and L2a are predicted by MFOLD (Zuker, 1989) to be more stable than L1-L2a. Thus, the double hammerhead which requires L1-L1 base-pairing forms readily (Figure 4). Consequently, the 1.5-G8U mutant, in which the L1-L1 helix is weakened, cleaved more slowly than the other mutants. The L2a-L2a helix is not necessary for the double hammerhead, thus altering it had little effect on the cleavage rate of 1.5-C32A (Figure 3).

The double hammerhead could not form in the circular monomer. This would be an advantage because it would ensure that the circular template for (−) strand synthesis would remain intact. This also implies that the linear forms that are encapsidated are derived only from cleaved (+) multimer and not from a circular template. Thus, we propose that the origin of assembly structure is not present in the circular monomer.

Most satellite RNAs undergo intramolecular self-cleavage via single hammerhead structures. However, those with a very short stem III are known (Forster et al., 1988; Hernandez et al., 1992), or predicted (Collins et al., 1998) to cleave via a double hammerhead that extends the length of stem III. Stability of the single hammerhead stem III was found to be an important factor in determining whether self-cleavage occurs by a single or double hammerhead structure (Sheldon & Symons, 1989). However, satRPV RNA cleaves via a double hammerhead despite a stable stem III (ten base-pairs with a one-base bulge) in the single and double hammerheads. In this case, the weak four-base stem I in the single hammerhead structure is strengthened by the adjacent L1-L1 base-pairing in the double hammerhead (Figure 4). The only other natural hammerhead with a stem I this short is in the (−) strand of CarSV RNA. However, it self-cleaves efficiently as a single hammerhead (Hernandez et al., 1992). This is also the only other hammerhead with the C-A mismatch at the proximal end of stem II. To our
Figure 3 (legend opposite)
knowledge satRPV RNA would be the first satRNA whose ribozyme requires a double hammerhead for which the double hammerhead stabilizes stem I rather than stem III.

**Replication in oat protoplasts**

The effect of covarying mutants G8U, C32A and G8U/C32A on replication in protoplasts showed that base-pairing between L1 and L2a is essential for replication of satRPV RNA, independent of their primary sequences. The slightly reduced accumulation of (+) strand on infection with the double mutant G8U/C32A compared to wild-type could reflect the slightly weaker base-pairing between L1 and L2a. The wild-type helix has a \( \Delta G = -12.0 \text{ kcal/mol} \) versus \(-10.6 \text{ kcal/mol}\) for G8U/C32A. The G8U mutation weakens L1-L1 base-pairing in the double hammerhead and greatly slows the cleavage rate of the 1.5-mer (Table 1), yet this mutant replicates significantly, albeit at a low level (Figure 6). The fact that it produces substantial (−) strand supports the notion that cleavage is not necessary for a satRNA to act as a replicase template (Sheldon & Symons, 1993).

In contrast, mutant 1.5-C32A is virtually dead, even though it has only a slightly reduced cleavage rate and is not involved in the double hammerhead. Thus, efficiency of multimer cleavage in vitro is not a determinant of replication efficiency.

The L1-L2a helix may play a positive role in some function other than self-cleavage, such as replicase recognition or ligation. The unpaired ACAAA sequence, or its complement, in stem II of the hammerhead could be a replication origin, as it is identical with the conserved 5′-terminal five bases of the genomic and subgenomic RNAs of one of its helper viruses, beet western yellow virus (Rasochova et al., 1997) and differs by one transition from the 5′ end of CYDV-RPV (ACGAA; R. Beckett, unpublished data). Because it is opposite L2a in stem II, this ACAAA sequence would likely have different conformations in the presence or absence of L1-L2a base-pairing.

L1-L2a base-pairing may be required for circularization. Alternative conformations for cleavage and ligation have been identified in other RNAs. In PSTVd, which requires host components for cleavage, the switch from cleavage to ligation is driven by a change from a tetraloop to a conformation that resembles the loop E structure found in 5S ribosomal RNA (Baumstark et al., 1997).

Chay et al. (1997) showed that linear satellite tobacco ringspot virus (satTRSV) RNA folds into a non-hammerhead conformation to facilitate efficient ligation to form a circular molecule. The ligation structure is the most stable predicted structure for monomeric satTRSV RNA. It juxtaposes the 5′ and 3′ ends of the linear form in close proximity to form a circle.
proximity via base-pairing. In satRPV RNA, L1-L2a base-pairing leads to a non-hammerhead structural arrangement in the most stable predicted structure of monomeric satRPV RNA (Figure 7(e)). However, several bases remain single stranded at the 5' end of satRPV RNA, unlike satTRSV, and considerable flexibility would be predicted between the 5' and 3' ends. Perhaps the pseudoknot structure is the optimal ligation substrate. The 5' and 3' ends would be held in close proximity with little flexibility, owing to the complex base-pairing (Figure 1(b)).

Alternative conformations of the very different, complex ribozyme in the antigenomic RNA of hepatitis delta virus (HDV) may serve similar biological roles as those in satRPV RNA. A short duplex, P2a, can inhibit and promote ribozyme activity depending on salt concentrations, in vitro (Perrotta & Been, 1998). In this ribozyme, a four-nucleotide sequence within the self-cleaving domain pairs with a sequence just outside the 3' boundary of the ribozyme. Thus, it also appears that the inhibitory effect is due to an alternative secondary structure or a distortion of the three-dimensional structure (Perrotta & Been, 1998).

Sliding model: possible folding pathway of the multimeric (+) satRPV RNA in the cleavage reaction

We propose that satRPV RNA undergoes a series of conformational changes, depending on the stage of replication. The predicted secondary structure of lowest free energy state for multimeric (+) satRPV contains L1-L1 base-pairing but does not form hammerheads owing to the presence of additional L2a-L2a base-pairing that prevent formation of stem II (stable-conformation; Figure 7(a)). A slight change in the stable conformation can generate either of two possible intermediate conformations (C-form and R-form; Figure 7(b) & (c)) which contain base-pairing between L1 and L2a. The C-form contains inter-monomeric base-pairing between L1 and L2a, whereas the L1 and L2a sequences that base-pair in the R-form are from the same monomeric satRPV unit. The R-form is a concatemer of the most stable monomeric satRPV RNA structure (Figure 7(e)). Because it doesn’t form a hammerhead, it may be the conformation of the stable, linear multimers that are present in significant quantities in virions (Miller et al., 1991). The C-form structures contain the distal end of stem II, which could serve as a nucleation point for the transition to the structure that contains the active double-hammerhead (active conformation; Figure 7(d)). This requires disruption of L1-L2a base-pairing and re-formation of L1-L1 helix. Thus, we call this a sliding model, in which the two strands of the stacked helices involving L1 and L2a (in the stable structure) slide relative to each other to form the L1-L2a helix in the intermediate structure (C-form), and then again to re-form the L1-L1 helix in the active double-hammerhead conformation. After cleavage, folding into the most stable monomeric structure (Figure 7(e)) would facilitate separation of the cleavage products, and prevent the reverse reaction, by disruption of stem I and the proximal end of stem II. Preliminary structure probing data support the most stable monomer structure (data not shown).
otherwise, cleavage at CS1 by HH2 would allow refolding into a single hammerhead for cleavage by the same catalytic domain at CS2. In other self-cleaving satellite and viroid RNAs, the hammerhead is also absent in the most stable, predicted conformation of the full-length monomer (Keese et al., 1988; Hernandez et al., 1992; Chay et al., 1997; Navarro & Flores, 1997; Collins et al., 1998).

This model also explains why restoration of L1-L2a base-pairing in the double mutant, 1.5-G8U/C32A, restores the cleavage rate even though the L1-L1 base-pairing is weakened. Without L1-L2a base-pairing, the multimer may remain trapped in the stable conformation (Figure 7(a)) even if L1-L1 base-pairing is weakened. L2a-L2a (with flanking base-pairs) is the strongest helix in the stable structure. Only in wild-type or G8U/C32A 1.5-mers, could L1-L2a form transiently to produce the C-form that we propose is the intermediate required for formation of the active double hammerhead conformation (Figure 7(d)).

All of the above models will be difficult to test because of the difficulty of capturing metastable conformations in vivo and assigning their biological functions. Progress has been made in identifying folding intermediates of the Tetrahymena group I intron ribozyme and PSTVd RNA. The folding pathway has been predicted to be important in the replication of PSTVd (Gast et al., 1996; Gultyaev et al., 1998), and alternative conformations have been demonstrated to have different functions (Baumstark et al., 1997). Both the kinetics of folding and the presence of intermediates of the Tetrahymena ribozyme were observed by a kinetic oligonucleotide hybridization assay (Zarrinkar & Williamson, 1994; Treiber et al., 1998) and synchrotron hydroxyl radical footprinting (Sclavi et al., 1998).

While conformational changes associated with functional changes have been demonstrated for other satellite and viroid monomers, here we provide evidence of alternative conformations that can form only in a multimeric context. The alternative structures of satRPV multimeric RNA provide a new example of the complex and subtle means by which a small, non-coding RNA can provide structural information needed to effect its replication by host and viral proteins. Any studies of RNA structure and function, or RNA-protein interactions should bear in mind the multifunctional, structurally flexible potential of any given RNA sequence.

**Materials and Methods**

**Synthesis of hammerhead context and 1.5-mer structures**

RNA molecules were synthesized by *in vitro* transcription of satRPV sequences in pGEM3Zf(−) (Promega, Madison, WI). Plasmid pSS1 contains the wild-type (+) strand satRPV hammerhead sequence: bases 310-322 and 1-89 of the satellite sequence (Miller et al., 1991). Cleavage occurs between bases 322 and 1. Plasmids and
RNAs derived from them are named using the formula xNy, where x is the wild-type base, N is its position in satRPV RNA, and y is the new base (Δ indicates deletion). Multiple mutations are separated by slashes. A plasmid and its transcript are named similarly with the deletion). Multiple mutations are separated by slashes. A

Figure 7 (legend shown opposite)
deletion (Δ) is underlined. To construct the 1.5-meric satRPV cDNA clones (transcripts preceded by 1.5-), the above wild-type or mutant derivatives of pSS1 were amplified with the following primers: MA418, 5′ CTCAAGCTTATTTCGTGG 3′; and MA419, 5′ CTCA-GATCTTATTCGGTG 3′. The PCR products were digested at their ends with HindIII and BglII (underlined) and cloned into the wild-type dimeric cDNA clone, pT7Sat (Raschova & Miller, 1996), which had been digested with the same enzymes. The presence of the mutations was verified by sequencing.

Self-cleavage assays

The [α-32P]UTP-labeled RNAs were transcribed in vitro as described (Miller & Silver, 1991) from EcoRI-linearized plasmids. The RNA transcript (SS1) from Xbal-linearized pSS1 and the mutant derivatives, contained only three vector-derived bases at the 5′ end and at most six at the 3′ end. Uncleaved transcripts were purified by elution in elution buffer (0.5 % NH4OAc; 1 mM EDTA; 0.1 % (w/v) SDS) after electrophoresis of transcripts on a 6 % polyacrylamide, 7 M urea gel. Conditions for self-cleavage of SS1 were 50 mM Tris-HCl (pH 7.5), 10 mM MgCl2 at 37 °C. Self-cleavage reactions contained approx. 30,000 cpm gel-purified, uncleaved transcription product in cleavage buffer (50 mM Tris-HCl (pH 7.5), 10 mM MgCl2). Reactions were started by the addition of MgCl2. To stop the reaction, 8 µl aliquots were removed at designated time points, added to an equal volume of stop buffer (95% formamide, 0.5 mM EDTA, 0.025 % SDS), and then immediately frozen at −80 °C. Samples were thawed and denatured by boiling for one minute and then quenching on ice immediately prior to electrophoresis on 6 % polyacrylamide, 7 M urea sequencing-type gels. Gels were exposed on a Phosphorimager and the bands quantified using ImageQuant (Molecular Dynamics, Sunnyvale, CA).

Isolation of satRPV RNA monomer

Unlabeled, 1.5-meric satRPV RNAs were synthesized by in vitro transcription of EcoRI-linearized templates using phage T7 RNA polymerase (RiboMax kit, Promega). To induce self-cleavage, the RNA transcripts were incubated at 37 °C in cleavage buffer for approximately three hours. The 322 nt monomer obtained by self-cleavage was eluted with elution buffer from a 6 % acrylamide, 7 M urea gel after electrophoresis and staining with ethidium bromide.

Electroporation of protoplasts and extraction of total RNA

Oat (Avena sativa cv. Stout) protoplasts were isolated from cell suspension culture (cell line S226 obtained from Howard Rines, USDA/ARS, University of Minnesota) as described by Dinesh-Kumar & Miller (1993). Protoplasts were electroporated with 50 ng of viral RNA purified from a mixture of CYDV-RPV and BYDV-PAV viruses, and 20 ng of gel-purified monomeric satRPV RNA transcript. At designated times, cells were collected by centrifugation, quick frozen in liquid nitrogen, and stored at −80 °C. Total RNA was isolated from protoplasts using the RNeasy Plant Mini kit (Qiagen, Valencia, CA). The RNA was ultimately eluted from the resin by resuspension in DEPC-treated water.

RNA analysis

Denaturing 1.5 % agarose gel electrophoresis and Northern blot hybridization was as performed as described by Raschova & Miller (1996). Each lane was loaded with equal amounts of total RNA as determined by spectrophotometry and confirmed by ethidium bromide staining of ribosomal RNA before Northern blot hybridization. The 32P-labeled RNA probes were synthesized by in vitro transcription according to the manufacturer’s instructions (Fromega, Madison, WI) using [α-32P]CTP label. Antisense satRPV RNA probe was synthesized by SP6 RNA polymerase transcription of HincII-cut plasmid pT7Sat (Raschova & Miller, 1996). Sense satRPV RNA probe was prepared by T7 RNA polymerase transcription of EcoRI-cut plasmid pT7Sat. Antisense CYDV-RPV probe, complementary to bases 809-1191 was prepared by T7 RNA polymerase transcription of Apal-cut plasmid pML5 (Raschova et al., 1997). Northern blot hybridization was as described by Passmore & Bruening (1993) using 5 × 109 to 10 × 109 cpm/ml of radioactive probe (32P-labeled transcript) per hybridization. Membranes hybridized with probes were exposed to PhosphorImager screens. Before re-probing with another probe, blots were stripped by boiling in 0.1 × SSC and 0.1 % SDS, and exposed to ensure that all counts had been removed. The MFOLD program is used to predict secondary structure of lowest free energy state for RNAs (Zuker, 1989).

Figure 7. Possible conformations of multimeric (+) satRPV RNA to facilitate the cleavage reaction via the sliding model. The conformations differ only in the hammerhead regions of multimeric satRPV RNAs; the secondary structure of the remainder of the satRPV RNA sequence (continuous lines) is predicted to be identical with that shown in the monomeric satRPV RNA structure (e). Cleavage sites are indicated by solid triangles. L1 and L2a sequences are boxed and shaded. Superscripts indicate the satRPV monomeric unit to which each copy of L1 and L2a belong. For clarity, each satellite monomeric unit is shown in a different color. Curved broken lines at right of each structure indicate indefinite number of monomeric repeats. (a) Stable conformation. The predicted secondary structure of lowest free energy state for multimeric (+) satRPV RNA (using MFOLD) containing n copies of the monomer. (b) The stable conformation may rearrange by sliding of the two main strands on the horizontal axis relative to each other to form the intermediate C-form Metastable conformation. This structure has L1-L2a base-pairing between monomeric units. (c) Alternatively, the stable structure may fold so that L1-L2a from the same monomeric unit base-pair to each other (R-form). This is a concatenation of the most stable monomeric structure (e). Both metastable structures include the distal end of hammerhead stem II (shaded gold). (d) The horizontal strands in C-form metastable structure may slide again, re-forming the L1-L1 base-pairing, but with L2a single stranded in stem II, to generate functional “inter-monomeric” hammerheads (double hammerheads). (e) After cleavage, the monomeric products could fold to the most stable monomeric conformation (predicted by MFOLD and confirmed by chemical and enzymatic probing (not shown)).
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