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Biochemistry

RNA-mediated ligation of self-cleavage products of a *Neurospora* mitochondrial plasmid transcript

(ribozyme/VSRNA/intron)

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ABSTRACT *Neurospora* VS RNA is a mitochondrial single-stranded RNA that combines certain features of catalytic RNAs and group I introns. We report here that monomeric VS RNA synthesized *in vitro* by self-cleavage of a multimeric transcript can perform an RNA-mediated self-ligation reaction producing circular RNAs indistinguishable from those isolated from mitochondria. We conclude that the active site for the ligation reaction is present in the RNA itself. Also, the mechanism for aligning the termini to be ligated may be different from mechanisms previously described. The lack of sequence similarity between VS RNA and previously characterized catalytic RNAs suggests that VS RNA is an independently evolved ribozyme capable of both cleavage and ligation.

We have recently described a self-cleaving RNA, called VS RNA, that is found as an abundant single-stranded molecule in the mitochondria of Varkud-1c and a few other natural isolates of *Neurospora* (1, 2). The majority of VS RNA isolated from mitochondria is an 881-nucleotide circle, with smaller amounts of linear and multimeric RNAs also present. VS RNA is complementary to one strand of a double-stranded circular DNA, termed VS DNA, which is organized as a population of head-to-tail multimers; this organization is typical of several other *Neurospora* mitochondrial DNA plasmids (3–5). The monomer unit length of VS DNA is 881 base pairs (bp), equivalent to the length of VS RNA.

VS RNA synthesized *in vitro* by T7 RNA polymerase transcription of a cloned VS DNA template self-cleaves at a specific position in a protein-independent reaction producing 2′,3′-(cyclic)phosphate and 5′-hydroxyl termini (1). These termini are also produced by hammerhead catalytic RNAs, which include several plant viral satellite RNAs (6–10), one viroid RNA (11), and the transcript of a nuclear satellite DNA of a newt (12). Some nonhammerhead RNAs, such as the RNA of human hepatitis δ virus (HDV) (13–15) and the negative-strand RNA [(−)RNA] of the satellite of tobacco ringspot virus (STobRV) (16), also produce these termini. VS RNA shows no substantial sequence similarity to any of the above RNAs, suggesting that the nucleotides and/or the RNA structure involved in VS RNA cleavage may be different from those in previously characterized RNAs. The biological role of the self-cleavage reaction is thought to be the production of monomer-length RNAs from multimeric precursors.

Monomer-length viroid, viral satellite, and HDV RNAs are present *in vivo* as circular molecules; however, it is not known whether the ligation is catalyzed by the RNA itself or by cellular proteins. A limited extent of RNA-mediated ligation of a hammerhead-containing RNA has been observed after prolonged incubation at low temperature in the presence of zinc (7). Fragments of the antigenomic strand of HDV RNA can also be ligated *in vitro* (17). An observation interpreted as rapid ligation of HDV genomic RNA fragments (18) has recently been shown be an artefact of the experimental procedure (19, 20). An RNA ligase capable of joining 2′,3′-(cyclic)phosphate and 5′-hydroxyl termini has been identified in several organisms (21–23), and it has been speculated that such a protein enzyme may be responsible for the production of circular forms of at least some of these RNAs *in vivo* (24, 25). In contrast, STobRV (−)RNA, and especially smaller derivatives of it, ligate quite efficiently in the absence of proteins (ref. 16; P. A. Feldstein and G. Bruening, personal communication).

Like these other RNAs, multimeric forms of VS RNA are also found in RNA isolated from mitochondria; however, VS RNA appears to be synthesized from a DNA template (VS DNA; ref. 1 and J. Kennell, A. Lambowitz, B.J.S., and R.A.C., unpublished results), rather than a complementary RNA as is the case for the plant viroids and viral satellite and HDV RNAs (6). The VS plasmid may actually be a satellite of an additional, larger mitochondrial plasmid, which is also present in all natural isolates in which VS RNA is found (2).

In this report we show that multimeric VS RNA synthesized *in vitro* can perform both self-cleavage and ligation reactions in the absence of proteins, indicating that the active site(s) for both reactions are contained within the RNA itself. Because VS RNA shows no substantial sequence similarity to previously characterized self-cleaving RNAs, it may be an independently evolved ribozyme.

MATERIALS AND METHODS

Procedures for growth of *Neurospora* and isolation of mitochondrial DNA and RNA have been described (1, 26, 27). Mitochondrial DNA from Varkud-1c was partially digested with *Sca* I and cloned in the *Sma* I site of the vector pTZ19R (Pharmacia), which contains a T7 promoter upstream of the cloning site, to produce plasmid pS6319. The organization of the insert in this clone was confirmed by restriction mapping and partial sequencing (data not shown).

In vitro synthesis, denaturation, electrophoresis, and sequencing of RNA were performed as described elsewhere (ref. 1 and references therein). RNA was eluted from polyacrylamide gels by crushing and soaking in 0.15 M sodium acetate, pH 6.0/0.5% sodium dodecyl sulfate/10 mM EDTA for 2 h at room temperature while rotating.

Polynucleotide kinase reactions were carried out as described previously (1, 28); 3′-end-labeling with [5′-^{32}P]pCp and RNA ligase was carried out essentially as described by Bruce and Uhlenbeck (29). Calf intestinal alkaline phosphatase reactions

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Abbreviations: HDV, hepatitis δ virus; STobRV, satellite of tobacco ringspot virus.

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were carried out in 100 μl of buffer (50 mM Tris·HCl, pH 8.0/1.0 mM EDTA) containing 2 units of enzyme at 37°C for 30 min.

To obtain monomer RNA for evaluation of protein-independent ligation (see Fig. 4), radioactively labeled precursor RNA (PMMD) was synthesized by T7 RNA polymerase transcription in the presence of [α-^{32}P]ATP and the self-cleavage products were separated by polyacrylamide gel electrophoresis as in Fig. 1C. Linear monomer RNA, M, was isolated from the gel and incubated at room temperature for the times indicated in the figure in the presence of 20 mM Tris·HCl at pH 8.0 and 50 mM $MgCl_2$. After incubation, sodium acetate was added (to 0.3 M), and RNAs were precipitated with ethanol at −20°C, washed with 70% (vol/vol) ethanol, resuspended in 95% (vol/vol) formamide/0.05% bromphenol blue/0.05% xylene cyanol, heated to 70°C, electrophoresed in an 8 M urea/4% polyacrylamide gel, and visualized by autoradiography.

RESULTS

Many self-cleaving RNAs, including VS RNA, are thought to be synthesized as multimers *in vivo*. Multimeric transcripts synthesized *in vitro* to mimic this organization have been used previously to demonstrate self-cleavage and ligation of a different RNA, (−)RNA of STobRV (16). To use this same experimental approach, we constructed a clone, pS6319, that contains a circularly permuted trimeric copy of a VS DNA template in the plasmid vector pTZ19R (Fig. 1A). When linearized downstream of the insert and transcribed *in vitro* from the T7 promoter upstream of the insert, an RNA is produced that contains two adjacent complete VS RNA monomer (M) units flanked by approximately one half of a monomer on the promoter-proximal side (P) and another half on the promoter-distal side (D). This transcript contains three copies of the previously characterized VS RNA self-cleavage site between nucleotides 620 and 621 indicated by the arrowheads at the top of Fig. 1A. Cleavage at this site would be expected to yield guanosine 2′,3′-(cyclic)phosphate and adenosine 5′-hydroxyl termini (1).

In addition to the expected full-length transcript (designated PMMD, using the nomenclature of Buzayan *et al.*, ref. 16), 12 smaller RNAs were also observed when the products of *in*

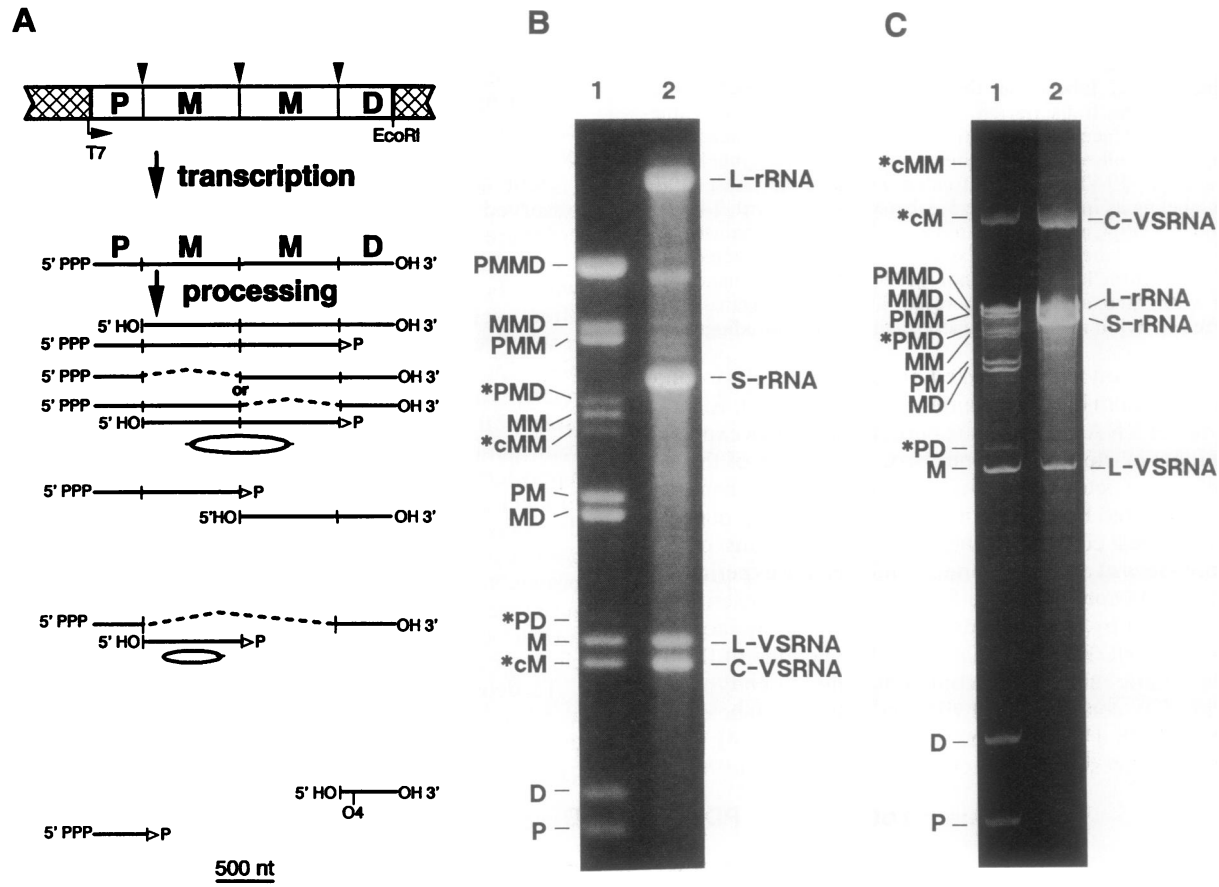

FIG. 1. Self-cleavage and ligation of multimeric VS RNA synthesized *in vitro*. (*A*) Organization of clone pS6319 and a summary of the RNAs produced during *in vitro* transcription (*B* and *C*). RNAs are designated by using the nomenclature of Buzayan *et al.* (16): P, promoter-proximal fragment; M, linear monomer; D, promoter-distal fragment. cM and cMM denote circular monomer and dimer RNAs, respectively. The transcript PMMD contains 41 nucleotides (nt) of vector sequence at the 5′ terminus, 2643 nt of VS RNA, and 19 nt of vector sequence at the 3′ terminus. The 3′-hydroxyl (OH), 5′-hydroxyl (HO), 2′,3′-(cyclic)phosphodiester (>P), and 5′-triphosphate (PPP) termini deduced from Fig. 2 (and data not shown) are indicated. O4 indicates the region of the RNAs complementary to the oligodeoxynucleotide primer used for sequencing (see Fig. 3). Broken lines indicate the positions from which monomer sequences have been excised and the flanking sequences ligated. Arrowheads mark the position of the previously characterized self-cleavage site at nucleotide 620 (1). (*B* and *C*) pS6319 was linearized at the *Eco*RI site and transcribed by T7 RNA polymerase (the arrow labeled T7 at the top of *A* marks the start site of transcription) for 60 min at 37°C. The products were treated with DNase I, extracted with phenol/chloroform/isoamyl alcohol, and precipitated with ethanol. RNAs were either denatured with glyoxal and separated by electrophoresis on a 1.45% agarose gel (*B*) or denatured by heating to 65°C in 64% (vol/vol) formamide and separated on a 4% polyacrylamide/8 M urea gel (*C*). RNAs were visualized by staining with ethidium bromide. Lanes 1 contain products of T7 runoff transcription; lanes 2 contain total RNA isolated from Varkud-1c mitochondria. Asterisks denote RNAs produced by ligation. Mitochondrial RNAs are L- and S-rRNA, large and small mitochondrial ribosomal RNAs; L- and C-VSRNA, linear and circular VS RNA. The other prominent mitochondrial RNA, migrating between the rRNAs in *B*, is a group II intron excised from the cytochrome oxidase subunit 1 pre-mRNA (30).

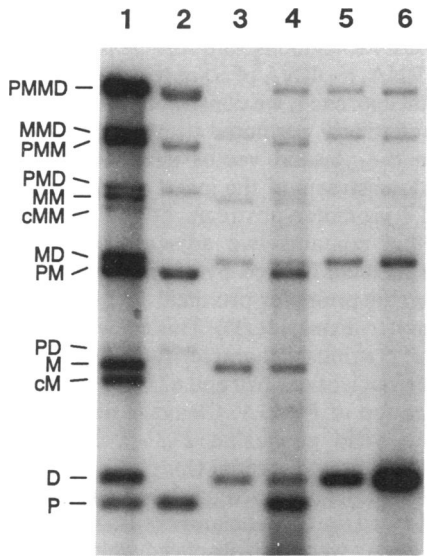

FIG. 2. End-labeling of RNAs produced during runoff transcription of pS6319. Nomenclature is as in Fig. 1. Lane 1, control (to visualize all RNAs) internally labeled with [α-^{32}P]ATP during transcription; lane 2, labeled at the 5′ end of the transcript with [γ-^{32}P]GTP during transcription. RNAs in lanes 3–6 were synthesized in the absence of radioactive nucleotides and labeled after transcription as follows: lane 3, labeled at 5′-OH with polynucleotide kinase and [γ-^{32}P]ATP; lane 4, treated with alkaline phosphatase prior to labeling as in lane 3; lane 5, labeled at 3′-OH with T4 RNA ligase and [5′-^{32}P]pCp; lane 6, treated with alkaline phosphatase prior to labeling as in lane 5. After labeling, RNAs were extracted with phenol/chloroform/isoamyl alcohol, precipitated with ethanol, denatured with glyoxal, electrophoresed in a 1.45% agarose gel, transferred to nylon membrane, and visualized by autoradiography.

vitro transcription were analyzed by agarose gel electrophoresis and ethidium bromide staining (Fig. 1*B*, lane 1). Eight of these smaller RNAs have electrophoretic mobilities expected for products of cleavage at one, two, or all three of the VS RNA cleavage sites. The identification of these bands is further supported by Northern hybridizations using oligonucleotide probes complementary to defined regions of VS RNA (not shown) and by 5′- and 3′-end-labeling experiments (Fig. 2). RNAs containing the 5′ end of the T7 transcript (P) were identified by labeling during transcription by incorporation of [γ-^{32}P]GTP, which is the first nucleotide of the T7 transcript (lane 2). RNAs containing the 3′ end of the transcript (D) contain a 3′-hydroxyl that was labeled by incubation with [^{32}P]pCp and RNA ligase (lane 5). Self-cleavage produces a 2′,3′-(cyclic)phosphate as indicated by (*i*) failure of band P to label with [^{32}P]pCp and RNA ligase even after prior treatment with alkaline phosphatase (lanes 5 and 6), and (*ii*) nuclease digestion and thin-layer chromatography of eluted band P, which identified guanosine 2′,3′-(cyclic)phosphate as the terminal nucleotide (experiment not shown, performed as described in ref. 1). The downstream cleavage product, band D, contains a 5′-hydroxyl terminus that labels with [γ-^{32}P]ATP and polynucleotide kinase (lane 3). Also, reverse transcriptase sequencing of D (Fig. 3) shows a strong termination band at A 621, confirming that cleavage occurred between G 620 and A 621—the same cleavage site as characterized previously with monomer VS RNA (1).

Reverse transcriptase sequencing showed that M is a mixture of monomer-length linear RNAs (Fig. 3). Some M RNAs have a 5′ end at A 621, suggesting that they are the expected cleavage products or possibly circles specifically reopened at this position. In some molecules the sequence continues well past 621; these RNAs may be derived by nicking of circles at other positions, or possibly by ligation (see below) of linear M RNAs during the sequencing reactions. Taken together, these observations indicate that the predominant cleavages in the trimeric VS RNA transcripts occur by the same mechanism and are at the same position (between G 620 and A 621) as in the monomeric VS RNA characterized previously. This site also corresponds to a prominent 5′ end of the linear VS RNA isolated from mitochondria, suggesting that the same cleavage reaction may occur *in vitro* and *in vivo* (1).

Four additional RNAs, indicative of ligation, were also observed (indicated by asterisks in Fig. 1 *B* and *C*). cM and cMM are circular monomeric and dimeric VS RNAs, respectively. PMD and PD are the linear ligation products that would be predicted from joining of the flanking sequences after excision of one or two of the internal monomers, respectively. The identification of these bands is based on (*i*) electrophoretic mobility on agarose and polyacrylamide gels relative to previously characterized circular and linear RNAs isolated from mitochondria (Fig. 1 *B* and *C*), (*ii*) electrophoretic mobilities on agarose gels of cM and cMM isolated from a polyacrylamide gel (not shown), (*iii*) failure of cM and cMM to label at either the 5′ or 3′ end by any of the procedures described in the legend of Fig. 2, (*iv*) further incubation of gel-purified PD, which produced the expected self-cleavage products, P and D (data not shown), and (*v*) reverse transcriptase sequencing (Fig. 3 and data not shown). The formation of these additional four RNAs indicates that both cleavage and ligation can occur *in vitro*.

To determine if ligation could be mediated by the RNA alone (i.e., free of T7 polymerase and other components of the transcription reaction), the linear monomer (M) produced by cleavage was isolated from a denaturing polyacrylamide

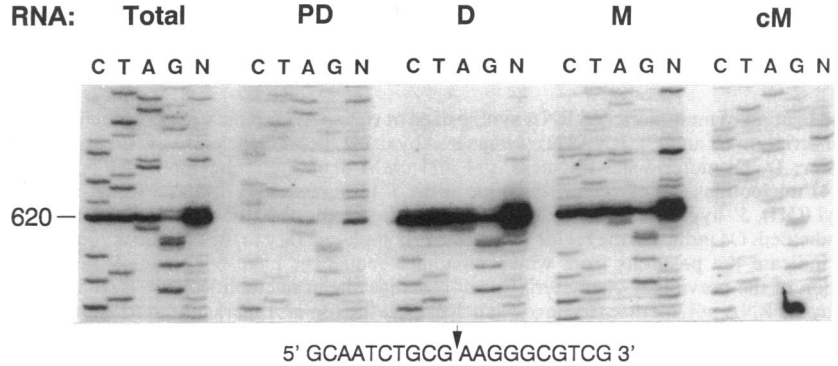

FIG. 3. Nucleotide sequence surrounding the cleavage and/or ligation sites. Total RNA isolated from Varkud-1c mitochondria and RNAs PD, D, M, and cM, which had been individually eluted from a gel similar to lane 1 in Fig. 1*C*, were used as templates for dideoxy sequencing using avian myeloblastosis virus reverse transcriptase and ^{32}P-end-labeled oligonucleotide O4 (see Fig. 1*A*). The arrow marks the location of the self-cleavage site at position 620.

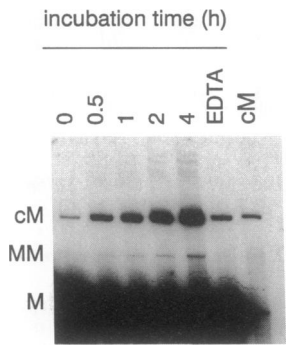

FIG. 4. Protein-independent ligation of linear monomer RNA. Radioactively labeled PMMD RNA was synthesized by T7 transcription in the presence of [α-^{32}P]ATP, and the self-cleavage products were separated by polyacrylamide gel electrophoresis as in Fig. 1*C*. Linear monomer RNA, M, was isolated from the gel and incubated at room temperature for the times indicated in the figure in the presence of 20 mM Tris·HCl at pH 8.0 and 50 mM $MgCl_2$. In the lane labeled EDTA, 5 mM EDTA was substituted for the $MgCl_2$, and the sample was incubated for 4 h. As a mobility marker, radioactively labeled circular monomer RNA was also isolated from a gel containing the products of T7 transcription and was electrophoresed in the lane labeled cM.

gel similar to that shown in Fig. 1*C*. Incubation of this RNA in the presence of Tris buffer and 50 mM magnesium, but in the absence of any proteins, resulted in the time-dependent production of at least two ligated products. RNAs comigrating with circular monomer (cM) and linear dimer (MM) are indicated in Fig. 4. Some slower-migrating RNAs that may be larger multimers are also detectable. Ligation also occurred when incubations were carried out with lower magnesium concentrations [10 or 20 mM; however, the yield of ligated products was lower than at 50 mM (data not shown)]. The yield of ligated products was greatly reduced when EDTA was substituted for magnesium during the incubation (Fig. 4); however, incubation in the presence of EDTA did result in some increase in the amount of cM. Also, a small amount of cM was visible even at the start of the incubation, suggesting that some of the M RNA ligated during isolation from the gel. We have not determined whether this apparently "magnesium-independent" ligation is due to tightly bound magnesium or to a reaction that does not require magnesium specifically. Nonetheless, the production of some cM and MM in the absence of proteins indicates that the sequences and structures required for ligation are contained within the RNA.

Since the major self-cleavage site is located after nucleotide 620 (Fig. 3), it is likely that the resulting 2′,3′-(cyclic)-phosphate and 5′-hydroxyl termini are involved in formation of the ligated RNAs. However, we cannot strictly rule out the possibility of cleavage and ligation at a different site, which would leave only a small amount of linear monomer intermediates that would be difficult to identify against the background of nicked circles. We think this less likely, because cleavage at such a hypothetical site should also leave linear terminal products with sizes different from P and D, and we have not observed such RNAs (Fig. 2). Taken together, the analyses of the major cleavage and ligation products suggest that self-ligation represents reversal of the self-cleavage reaction.

DISCUSSION

We have previously shown that *Neurospora* mitochondrial VS RNA synthesized *in vitro* performs a site-specific self-cleavage (1). The reaction produces 2′,3′-(cyclic)phosphate and 5′-hydroxyl termini (1), typical of reactions performed by several other self-cleaving RNAs (6). In this report we show that a longer transcript whose structure mimics that of a natural multimeric VS RNA is also capable of RNA-mediated cleavage at the same site, producing a monomer-length linear VS RNA. Subsequent incubation of these linear monomers yields circular monomers and smaller amounts of other ligated products, indicating that the active site(s) for both cleavage and ligation are contained within the RNA. Because VS RNA does not show substantial sequence similarity to previously characterized RNAs, it may be an RNA that has independently evolved the ability to perform cleavage and ligation reactions.

RNA-mediated ligation of 2′,3′-(cyclic)phosphate and 5′-hydroxyl termini has been observed *in vitro* with fragments of some other catalytic RNAs (7, 17). In one mechanism, which may not depend on a specific nucleotide sequence, two substrate RNAs can be aligned on a complementary template and joined by either 2′-5′ or 3′-5′ phosphodiester bonds (31). Fragments of HDV RNA can be ligated *in vitro*, apparently by this mechanism, and a region of HDV RNA complementary to the termini is thought to be appropriately base paired in the native RNA (17).

If a complementary template were available, VS RNA fragments might ligate similarly. However, several observations suggest that simply aligning the termini on a complementary template is not the mechanism used for VS RNA ligation. Although continuous regions of VS RNA that could span the 620/621 junction and thereby provide the complementary alignment template are present, the longest is only six nucleotides, and two of the resulting base pairs would be G·U pairs (5′-UUUCGU-3′; bases 105–110, numbered as in ref. 1). More importantly, even this short sequence is absent from a deletion derivative of VS RNA that nonetheless makes circular RNA *in vivo* (unpublished data); however, we cannot rule out the possibility that an alternative shorter template, perhaps assisted by a trans-acting cellular factor, could substitute *in vivo*.

An RNA secondary structure in which the terminal bases of the substrate RNAs are not paired to a template, although some of the flanking bases are, may be involved in cleavage and ligation of STobRV (−)RNA (32–35). This RNA, and especially smaller derivatives of it, ligates efficiently in the absence of proteins (ref. 16; P. A. Feldstein and G. Bruening, personal communication). A similar secondary structure, composed of short helices surrounding a symmetrical internal bulge that contains the self-cleavage site, can be drawn for VS RNA; however, deletion and substitution mutants that would remove the hypothetical upstream helix are still capable of self-cleavage, suggesting that the functional secondary structures of VS RNA and STobRV (−)RNA are very different (J. Olive and R.A.C., unpublished data).

VS RNA may represent a molecular fossil of the RNA world that has retained both RNA-mediated cleavage and ligation abilities. Alternatively, it may be a more sophisticated RNA that, later in evolution, acquired one or more of its activities. RNA-mediated cleavage and ligation are also characteristic of self-splicing group I and group II introns (reviewed in refs. 36 and 37), and the excision of monomer RNAs followed by ligation of the adjacent regions is reminiscent of the excision of introns and ligation of flanking exons seen in RNA splicing (24, 38). The RNAs of VS and some other mitochondrial plasmids (1, 39), as well as some nonmitochondrial circular RNAs (40, 41), contain many of the conserved sequences and secondary structures characteristic of group I introns. These similarities are intriguing from an evolutionary perspective, and it has been speculated that some of these elements may have evolved from or into group I introns (38, 42); however, the group I structure is apparently not involved in reactions mediated by these RNAs. For example, VS RNA is not spliced or even cleaved at the positions predicted from the group I structure; instead,

it self-cleaves at a site outside of the group I core structure in a reaction that produces a 2',3'-(cyclic)phosphate, rather than the 3'-hydroxyl characteristic of group I introns (1). The peripheral location of the cleavage site and the chemistry of the reaction are consistent with the idea that VS RNA might be a chimera formed by insertion of a novel catalytic RNA into a group I intron.

The VS plasmid is capable of horizontal transfer between strains of *Neurospora* (2). The discovery that both the self-cleavage and ligation of VS RNA are RNA-mediated suggests that the potential host range of this element could be even wider, since no specific preexisting endonuclease and ligase would be required in the recipient organism. RNAs possessing both of these activities, such as self-splicing introns and VS RNA, have the potential to function as mobile elements, since they may be able to insert themselves into a suitable sequence context in a gene or its transcript without necessarily disrupting genetic functions in the recipient (43–45). These abilities, coupled with the similarities between VS RNA and group I introns, make VS RNA a good candidate for an evolutionary intermediate between a self-splicing intron and a mitochondrial plasmid or other type of mobile element.

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