Catalytically active geometry in the reversible circularization of 'mini-monomer' RNAs derived from the complementary strand of tobacco ringspot virus satellite RNA

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ABSTRACT

The less abundant polarity of the satellite RNA of tobacco ringspot virus, designated sTobRV(–)RNA, contains a ribozyme and its substrate. We demonstrate that the ribozyme can catalyze the ligation of substrate cleavage products and that oligoribonucleotides, termed 'mini-monomers' and containing little more than covalently attached ribozyme and substrate cleavage products, circularized spontaneously, efficiently and reversibly. The kinetics of ligation and cleavage of one such mini-monomer was consistent with a simple unimolecular reaction at some temperatures. Evidence suggests that the circular ligation product includes a 5 bp stem that is connected to a 4 bp stem by a bulge loop. Reduction of the bulge loop to one nt is expected to place the 4 and 5 bp helices in a nearly coaxial, rather than an angled or parallel, orientation. Such molecules did not circularize in a unimolecular reaction but did when incubated with second, *trans*-acting oligoribonucleotides that had either the original or a substituted 4 bp helix. These results suggest that a bulge loop that is too small prevents formation of geometry essential for unimolecular ligation. We suggest the term 'paperclip' to represent the arrangement of RNA strands in the region of sTobRV(–)RNA that participates in the cleavage and ligation reactions.

INTRODUCTION

RNA self-cleavage and ligation reactions are steps in replication models for the satellite RNA of tobacco ringspot virus (1, 2) (sTobRV RNA) and of certain other small RNA molecules (3–5). Dimeric and trimeric forms of the most abundant polarity of sTobRV RNA, arbitrarily designated as (+), self-cleave (6) to release the unit length, 359 nucleotide residue (nt), monomeric sTobRV(+)RNA (7). This phosphotransfer reaction generates a 5'-hydroxyl and a 2',3'-cyclophosphate as the new terminal groups. The nucleotide sequences required for the reaction of

sTobRV(+)RNA are confined to a contiguous region that includes the junction phosphodiester at which cleavage occurs. The self-cleaving sequence conforms to a hammerhead secondary structure model (8, 9), and the reaction is only weakly reversible to form dimeric (6) or circular sTobRV(+)RNA (Possmore and Bruening, submitted). In contrast, the complementary polarity, monomeric sTobRV(–)RNA not only cleaves but also readily ligates to linear dimers and circles (10, 11) and has a likely secondary structure (12–15) that does not correspond to a hammerhead. One other known self-cleaving RNA exhibits self-cleavage to generate a 2',3'-cyclophosphate end group and forms circles readily (16, 17).

In order to identify nucleotide sequences and other structural requirements for ligation, we studied non-enzymic ligations with 1, 2 or 3 oligoribonucleotides, derived from sTobRV(–)RNA sequences, as reactants. The single oligoribonucleotide reactions employ 'mini-monomer' (miniM) constructions in which sequences of sTobRV(–)RNA necessary for the cleavage and ligation reactions have been retained and have been connected together into a single molecule by non-sTobRV(–)RNA sequences. Analysis of miniM reactions revealed an aspect of the geometry of active molecules as well as time courses and temperature dependences of the equilibration of linear and circular oligoribonucleotides.

MATERIALS AND METHODS

Transcript nomenclature

We prepared *in vitro* transcripts of the form P_i-D_k and E_j (Figure 1A, B), derived from separate regions of the sTobRV(–)RNA molecule. E_j acts as a ribozyme, mediating the cleavage of P_i-D_k to generate P_i and D_k (13, 14) that were used here in ligation reactions. In P_i-D_k the promoter proximal portion, P_i , and promoter distal portion, D_k , of the transcript are joined by the junction phosphodiester, represented by a hyphen. Subscripts i , k , and j designate numbers of nt that correspond to sTobRV(–)RNA sequences, except that C > p replaces A > p

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as the 3' nt of P₆ and P₁₂. It is known that this substitution, of cytidylate for adenylate, has little effect on the cleavage reaction (15).

Each of the oligoribonucleotides miniM_n, where n equals the number of nt in the connecting loop, L_n, also can be designated in the form D₈L_cE₄₈L_nP₅. In these molecules, two loops, the fixed 5 nt L_c and the varied L_n, connect sequences that correspond to the P, D and E oligoribonucleotides of Figure 1A. Figure 2 defines the loops of the miniM_n series for n = 37, 36, 9, 7, 5, 3 and 1. As was the case for P₆ and P₁₂, above, the 3' terminal A>p was replaced by C>p. Results from our unpublished experiments show that, when A>p was replaced by each of the other N>p in miniM_n = 9 oligoribonucleotides and when A>p was replaced by C>p in miniM_n = 36 oligoribonucleotides, there was no change in the extent of circularization. Oligoribonucleotides designated 'miniM_n(GCGC)', where n = 8, 5 and 1, have the indicated L_n loops (Figure 2), but the four base pair stem contributed by P₅ and E₄₈ has been altered to rGCGC/GCGC from the usual rUGAC/ACUG.

Plasmids

All oligodeoxyribonucleotides were obtained from Operon Technologies, Inc., Alameda, CA, except for GB11, GB12, and GB31, which were synthesized in this laboratory on a Pharmacia Gene Machine. The nucleotide sequence of the insert of each plasmid used as template was confirmed by dideoxynucleoside triphosphate-terminated chain extension (18). **pT7(-)60*40:** the ApaI site-containing precursor of pT7(-)54*40 (14) was linearized with ApaI, made blunt ended by treatment with bacteriophage T4 DNA polymerase and 4 dNTPs (19), and ligated to non-phosphorylated linker dGTGGCCAC, which possesses a restriction endonuclease Ball site. The ligation reaction mixture was heated to 70°C, quickly chilled on ice, and purified using a Qiagen column as described by the supplier (20) to remove unligated linkers. The plasmid was 5'-phosphorylated, ligated, and used to transform *E. coli* strain D5Hα. pT7(-)60*40 was selected on the basis of its sensitivity to Ball, sequence, and ability to serve as template for synthesis of the cleavable oligoribonucleotide P₁₂-D₉.

pT7(-)miniM_n=37: the SpeI-HindIII polylinker fragment of plasmid pT7(-)222*163 (14) was replaced by a SpeI-HindIII fragment of plasmid p20SD(-)3 (21), connecting the E₄₈ sequence of pT7(-)222*163 to the P₅-D₉ sequence from p20SD(-)3 and forming loop L_n. A segment of the insert of the resulting plasmid pMP1 was amplified by use of the polymerase chain reaction (PCR), with a program of 25 cycles of 1 min each at 94°C, 42°C, and 72°C and using universal M13 sequencing primer and oligodeoxyribonucleotide MMC1: dGGGAATTCTG-ACAGTCCTGTCCGTATGACAGAGAAGTCAACC (providing the P₅-D₈L_c sequence and priming on E₄₈). The product was electrophoretically purified, cut with EcoRI and HindIII, and inserted into pTZ18R (U.S. Biochemicals, Inc., Cleveland, OH) cut with the same enzymes. **pT7(-)miniM_n=36:** the ApaI-HindIII polylinker fragment of pT7(-)222*163 (14) was replaced with a ApaI-HindIII fragment of the ApaI-site-containing precursor of plasmid pT7(-)54*40 (14), giving plasmid pEPD15. The insert for plasmid pT7(-)224*175 was prepared by the PCR using dGT-CAAGGGTACCAG and dATCGAATTAATACGACTCAC-TATAGAAACAGAGAAGTC as primers and pT7(-)222*163 as template. The product was digested with Asp718I and was inserted into a modified version of plasmid pIBI20 (International

Biotechnologies, Inc.), cut with EcoRV and Asp718I, giving pT7(-)224*175. Inserted sequences of pT7(-)224*175 and pEPD15 each were PCR-amplified using the M13 universal primer and MMC1. The pT7(-)224*175-derived product was cut with EcoRI and PstI to give a DNA fragment encoding the P₅-D₈L_cE₄₈ sequence and a portion of the loop L_n polylinker. The pEPD15-derived PCR product was cut with PstI and HindIII to give a DNA fragment encoding a portion of the loop L_n polylinker and P₅-D₉ sequence of miniM_n=36. The two DNA fragments were ligated into pTZ18R cut with EcoRI and HindIII.

pT7(-)miniM_n=9: pT7(-)miniM_n=36 was cut with SmaI, and the SmaI linker dCCCCGGGG was inserted. The resulting plasmid pT7(-)miniM_n=36Sma was cut with ApaI and SmaI, the ends were blunted by the action of bacteriophage T4 DNA polymerase and four dNTPs, and the product was ligated to generate the oligo-dC tract of pT7(-)miniM_n=9. **pT7(-)miniM_n=7** was prepared from pT7(-)M_n=36Sma, cut with ApaI and AvaI and treated with mung bean nuclease (22). **pT7(-)miniM_n=5** was prepared similarly to pT7(-)miniM_n=9, except that the construction was initiated from pT7(-)miniM_n=36. **pT7(-)miniM_n=3:** pT7(-)M_n=36Sma was prepared from *Escherichia coli* strain GM₄₈ (*dam*⁻, *dcm*⁻). The plasmid was cut by treatment with ApaI and Asp718I, incubated with bacteriophage T4 DNA polymerase and four dNTPs and ligated. **pT7(-)miniM_n=1** was obtained by cleaving pT7(-)miniM_n=36 to completion with ApaI and partially with RsaI and blunting and ligating as for pT7(-)miniM_n=3.

Construction of plasmids **pT7(-)miniM_n=8(GCGC)** and **pT7(-)miniM_n=5(GCGC)** was initiated by PCR amplification of the insert of pT7(-)miniM_n=9 using universal primer and reverse(-5) primer (dCAGGAAACAGCTATGA). A 0.001 dilution of this product was subjected to PCR (5 cycles of 1 min each at 94°C, 37°C, and 72°C and 25 cycles of 1 min each at 94°C, 55°C, and 72°C) using oligodeoxynucleotides GB11 (pdGCGCCGTCTGTCCGTATGACAGAGAAGCGCACCA) and GB12 (pdGGATGAACAGGACGGCGCGGG). These primers are capable of priming at several positions in the oligo-dC tract of loop L_n of pT7(-)miniM_n=9. Advantage was taken of the single, non-complementary, 3'-terminal dA present on the bulk of the PCR product to introduce a HindIII site at both ends of the PCR product by ligation of a 'PCR linker' (Feldstein and Bruening, to be submitted). The purified ligation product was digested with HindIII and cloned into HindIII digested pTZ19R. Inserts were screened for size and orientation by PCR and for activity by transcription of purified PCR product.

Transcriptions

Oligoribonucleotides were prepared by *in vitro* transcription as described (14) in reaction mixtures that contained 2 U/μl bacteriophage T7 RNA polymerase, 500 μM of each rNTP, and, where needed, [α-³²P]rCTP (50 nCi/ml). High specific activity transcripts were prepared as described (23).

pT7(-)222*163 (14), linearized with either the restriction endonuclease RsaI or SpeI, was the template, respectively, for *in vitro* synthesis of 5'-triphosphate oligoribonucleotides E₄₇ and E₆₀. pT7(-)54*40 and pT7(-)60*40, linearized with the restriction endonuclease DraI, were the templates, respectively, for *in vitro* synthesis of 5'-triphosphate oligoribonucleotides P₆-D₉ and P₁₂-D₉, (Figure 1A, B).

Plasmids of the series pT7(-)miniM_n=37 through pT7(-)miniM_n=1, linearized with the restriction endonuclease HindIII, were templates for the synthesis of the corresponding

miniM_n. The template for the *in vitro* synthesis of miniM_{n=8}(GCGC) was a purified PCR product using as template pT7(-)miniM_{n=8}(GCGC) and as primers reverse(-5) and GB12. The template for the *in vitro* synthesis of miniM_{n=1}(GCGC)-D₈ was a purified PCR product using as template pT7(-)miniM_{n=5}(GCGC) and as primers reverse(-5) and GB31 (dGGATGAACAGGACGGCGCGACCAGGTAAT-ATAC), the latter introducing a single dC in place of the 5 nt of the L_n loop of pT7(-)miniM_{n=5}(GCGC).

Self-cleavage reactions

Reactants were incubated, under the conditions indicated, usually in 1×Tris-processing buffer (10 mM Tris, 6 mM MgCl₂, 2 mM spermidine-HCl, pH 7.5). In later experiments several buffers were compared, and 1×MES-processing buffer (10 mM morpholino-*N*-ethanesulfonic acid, 6 mM NOOH, 6 mM MgCl₂, 2 mM spermidine-HCl, pH 6.2) supported the most rapid ligation rates and was adopted for some later experiments. As necessary, transcription reaction mixtures or electrophoretically purified transcripts were incubated further to generate desired cleavage products. In the extreme case, transcripts of pT7(-)miniM_{n=1} were incubated with E₄₈ for 1 hr at 37°C in 1×MES processing buffer to release the desired miniM_{n=1}

oligoribonucleotides. Electrophoretically purified P₆-D₉ and P₁₂-D₉ oligoribonucleotides, which are not self-cleaving, were incubated with E₄₇ or E₆₀, respectively, for 5 hr at 37°C in 1×Tris-processing buffer to generate P₆, D₉ and P₁₂.

Circular and linear oligoribonucleotides were resolved by electrophoresis through 8% or 12% polyacrylamide gel in 7 M urea and were recovered from gel slices by soaking (24). Oligoribonucleotide samples at approximately 0.5 μM in 0.01 mM Na₂EDTA, 0.09 mM Na₃EDTA, pH ~7.8, were brought to temperature. At zero time, 0.25 volume of 5×Tris-processing buffer was added. Samples were taken at intervals and were added to 2 volumes of 90°C stop solution (15 mM sodium EDTA in 97% formamide). Samples were incubated at 90°C for 1 min and cooled on ice. Products were analyzed by electrophoresis through polyacrylamide gel in 7 M urea. After electrophoresis, the gel was dried under vacuum and subjected to autoradiography. Radioactivity of bands was determined by two-dimensional β-particle counting with an AMBIS Radioanalytic Detector (Automated Microbiological Systems, Inc., San Diego, CA). The fraction of circles was taken as the ratio of the background-corrected radioactivity in bands, for circular molecules divided by the sum for circular and linear molecules. A simple equilibrium between circular and linear molecules is expected to conform to the time course predicted by

$$\ln \frac{(F_{\infty} - F)}{(F_{\infty} - F_0)} = -(k_1 + k_2)t$$

(adapted from (25)), where F, F₀ and F_∞ are the fractions of circles at time t, zero time, and at equilibrium, respectively. The rate constants are, for conversion of linear molecules to circular molecules, k₁, and for the reverse reaction, k₂. Data (F,t) were fit to the exponential form of the above equation by linear regression, giving F_∞, (k₁ + k₂), and the correlation coefficient r.

RESULTS AND DISCUSSION

Ligation of substrate cleavage products by the action of a sTobRV(-)RNA-derived ribozyme

The oligoribonucleotide set P₆, D₉ and E₄₇ (Figure 1A) corresponds closely to the minimum sequence required for the sTobRV(-)RNA self-cleavage reaction (13), whereas the set P₁₂, D₉ and E₆₀ has opportunity for additional association between the E and P molecules (Figure 1B). In a 12 hr incubation, ligation of P₆ to D₉ by the action of E₄₇ was observed at 4°C but was not detected at 23°C or 37°C (Figure 1C, lanes 1–3), even after long autoradiographic exposure. For the set P₁₂, D₉ and E₆₀, ligation products were detected at all three temperatures (Figure 1C, lanes 4–6).

Circularization of miniM oligoribonucleotides that contain ribozyme and substrate sequences

MiniM_n oligoribonucleotides were released from two-junction transcripts of the form P₆-miniM_n-D₉ (i.e., P₆-D₈L_nE₄₈ L_nP₅-D₉) by self-cleavage reactions (Figure 2) and were active in both unimolecular and bimolecular ligation reactions (Figure 3). In fact, multimers (up to linear hexamers and circular trimers for miniM_{n=9}) were detected in the transcription reaction mixture (data not shown). Appearance of a band of lesser mobility reveals formation of circular miniM (C-miniM_n) from electrophoretically purified linear miniM_n molecules (L-

Figure 1. Ligation of two oligoribonucleotides by the action of a third oligoribonucleotide, all with sequences derived from the negative polarity satellite RNA of tobacco ringspot virus, sTobRV(-)RNA. **A.** A secondary structure, supported by various results (12–15), of a complex in which oligoribonucleotides P₆ and D₉ are joined during the attack of the guanosine 5'-hydroxyl group of D₉ on the cytidylate cyclophosphate of P₆, as mediated by E₄₇. Dots indicate base pairing in the complex required for cleavage of P₆-D₉ by E₄₇ (12–14). **B.** A related secondary structure model for P₁₂, D₉, and E₆₀. Numbering of nucleotide residues is according to the positive polarity sTobRV(+)RNA sequence (7). Vector-derived nt are represented as lower case letters. **C.** 2.5 μM each of P₆, D₉, and E₄₇ (lanes 1–3) or P₁₂, D₉, and E₆₀ (lanes 4–6), labeled from [α-³²P]rCTP, were incubated together in 1× processing buffer for 12 hr at 4°C, 23°C, or 37°C (lanes 1 and 4, 2 and 5, and 3 and 6, respectively). Electrophoresis through 20% polyacrylamide gel revealed starting materials and the ligation products P₆-D₉ and P₁₂-D₉ at positions indicated at the sides of the autoradiogram.

Figure 2. Representation of transcripts, self-cleavage reactions and spontaneous circularization of miniM_n oligoribonucleotides with various sizes of loop L_n. Linear L-miniM_n molecules are also defined as connected sequence segments D₈L_cE₄₈L_nP₅, the segments being located by rectangles or shaded boxes as indicated. P₅, D₈, and E₄₈ are defined as in the Figure 1 legend and Materials and Methods, and the constant terminal loop L_c and the varied bulge loop L_n are defined in the figure such that, for example, L-miniM_{n=3} has an L₃ of sequence rACC. L-miniM_n molecules are derived from P₆-miniM_n-D₉ (P₆-D₈L_cE₄₈L_nP₅-D₉) by cleavage at the phosphodiester junctions J(-) in transcripts as indicated. L-miniM_n molecules spontaneously form circular oligoribonucleotides C-miniM_n in an equilibrium represented by the double-headed arrow. The top line of the C-miniM_n structure is oriented 5' to 3', and the 5' and 3' extensions of the transcript have 7 and 6 vector-derived nt, respectively.

miniM_n). The circles were identified as such by their anomalous mobility shifts with changes in gel concentration, as compared to the lesser shifts observed for the linear molecules. Additionally, 'nicking' (10) of electrophoretically-purified, presumed circles with ribonuclease T₁, caused them to comigrate with the corresponding linear oligoribonucleotide, as expected (data not shown).

To investigate the contribution to the ligation reaction of the nt found in the set P₁₂, D₉ and E₆₀ but not the set P₆, D₉ and E₄₇, we made a miniM construction, miniM_{n=37}. Loop L₃₇ (Figure 2) consists of sTobRV(-)RNA nt 175-163 and nt 77-54, part of which appear in Figure 1B. In contrast, loop L₃₆ of miniM_{n=36} has a distinct sequence, being a set of restriction endonuclease sites. The lesser circularization of miniM_{n=37} than of miniM_{n=36} (Figure 3, lanes 1, 2) suggests that the specific sTobRV(-)RNA sequences of loop L₃₇ are not required for efficient circularization and that the additional sequence of the set P₁₂, D₉ and E₆₀, compared to P₆, D₉, and E₄₇ (Figure 1), enhanced ligation by stabilizing the interaction of P and E. Lack of a specific nt sequence requirement in L_n is further supported by results presented below.

To investigate the effects on the ligation reaction of changes in the relative geometry of the two stems connected by loop L_n, we prepared miniM_n molecules in the series n = 9, 7, 5, 3 and 1, corresponding to oligoribonucleotide lengths of 75, 73, 71, 69, and 67 nt, respectively. We selected oligo-rC for the L_n loops as a nt sequence least likely to interact with other parts of the miniM_n molecule. When incubated for 1 hr at 400 nM,

Figure 3. Spontaneous circularization of L-miniM_n molecules and a comparison of the effects of seven bulge loops on the ligation reaction. Each electrophoretically purified L-miniM_n oligoribonucleotide, as indicated by the numbers above the lanes, was incubated at 400 nM (lanes 1-7) and at 0.4 nM (lanes 1'-7') for 1 hr at 37°C. Analysis was by electrophoresis through 8% polyacrylamide gel and autoradiography. The linear molecules (L) migrated more rapidly than the circular molecules (C) or the linear dimers (2).

miniM_{n=9} and miniM_{n=7} circularized to extents similar to extents observed for miniM_{n=36} (Figure 3, lanes 2, 3 and 4), providing additional evidence that the nt sequence of L_n is not critical to the circularization reaction. Yields declined substantially for miniM_{n=5} and miniM_{n=3} (Figure 3, lanes 5, 6). Two dimensional β -particle counting gave a yield for C-miniM_{n=1} of less than 0.008 [calculated as C-miniM_{n=1}/(C-miniM_{n=1} + L-miniM_{n=1})].

Reactions at 100 nM were assessed for the period 30 sec through 20 hours for L-miniM_n, n = 9, 7, 5, 3, and 1 (data not shown). Initial rates of reaction were similar for n = 9 through 5. No further reaction was observed after 8 min, and the apparent equilibrium composition of C-miniM_n declined for the series n = 9, 7, and 5. For miniM_{n=3} and miniM_{n=1} a slow increase in C-miniM_n was observed through 20 hr. However, the yield of C-miniM_{n=3} and C-miniM_{n=1} were 0.25 to 0.3, as was observed for C-miniM_{n=5}. Thus for the series n = 5, 3 and n = 1, the yield of C-miniM was little changed but the initial rates declined dramatically.

L-miniM_n molecules also were incubated at 0.4 nM (Figure 3, lanes 1'-7'). The initial concentration of L-miniM_n had little effect on the yield of circles for molecules with loops L_n of n = 9 and n = 7 (Figure 3, lanes 3, 3', 4, 4'). However, dilution of reactants to 0.4 nM markedly decreased the yield of circles for molecules with loops L_n of n = 5 and n = 3. Circles were not detected for 0.4 nM miniM_{n=1} (Figure 3, lane 7'), even at long autoradiographic exposures. The observed concentration dependence of the limited circularization of L-miniM_{n=1} suggests that the reaction may be a bimolecular or higher order reaction. Incubation of L-miniM_{n=9} at 100 nM and 1 nM gave indistinguishable initial rates and yields of C-miniM_{n=9} (data not shown). Although it is possible that some molecules of the miniM_{n=9} preparation undergo a reaction that is bimolecular, the very similar reaction rates at 100 nM and 1 nM would require a K_m for this reaction that would be smaller than the K_m for any

Figure 4. Influence of transacting oligoribonucleotides on the circularization of L-miniM_{n=1}. **A.** [³²P]miniM_{n=1} was incubated at 2.0 nM for 1 hr at 37°C in either 10 mM MES, 6 mM NaOH, pH 6.2, (lane 1) or in 1×MES-processing buffer (lanes 2–4), which contains magnesium ions and spermidine. Additions were unlabeled 1 μM miniM_{n=1}-D₉ (lane 3) or 1 μM miniM_{n=1}(GCGC)-D₈ (lane 4). Samples were analyzed by electrophoresis through 12% polyacrylamide gel. Locations of L-miniM_{n=1} and C-miniM_{n=1} are indicated beside lane 4. Neither long autoradiographic exposures nor adjustment of the contrast or range of β-particle scanning gave any indication of a discrete band at the position of C-miniM_{n=1} in lanes 1 and 2. Nevertheless, β-particles were counted in the region of lanes 1 and 2 to which C-miniM_{n=1} migrated in lanes 3 and 4. The background-corrected radioactivities for C-miniM_{n=1}, expressed as a percentage of the radioactivities for the circular and linear molecules combined, are presented above each lane. **B.** Nucleotide sequence and experimentally-supported secondary structure of miniM_{n=1}-D₉, specified also by marked sequence regions as D₈L_cE₄₈L₁P₅-D₉. **C.** Nucleotide sequence and presumed secondary structure of miniM_{n=1}(GCGC)-D₈, which has a stem, indicated by bold font, that is different from the corresponding stem of miniM_{n=1}-D₉. For both molecules, L₁ consists of a single cytidylate residue. 3'-terminal, vector-derived sequences are indicated by lower case letters.

known ribozyme reaction (26, 27). In a postulated bimolecular reaction, miniM_{n=9} molecules would be required to act as both enzyme and substrate, causing the initial rate to be strongly sensitive to the initial miniM_{n=9} concentration. Therefore, the lack of concentration dependence for the circularization of L-miniM_{n=9} is consistent with a unimolecular reaction.

Circularization of miniM_{n=1} by miniM_{n=1} derivatives acting in *trans*

A bimolecular reaction was arranged (Figure 4A) using an analogue of miniM_{n=1}, miniM_{n=1}-D₉ (D₈L_cE₄₈L₁P₅-D₉; Figure 4B), that in preliminary experiments indicated activity as a facilitator of C-miniM_{n=1} formation. The phosphodiester junction-containing miniM_{n=1}-D₉ at concentrations of 200 nM to 1600 nM released miniM_{n=1} with increasing yield at increasing initial concentrations. In these reaction mixtures, C-miniM_{n=1} and L-miniM_{n=1} were generated at a 1:3 ratio, i.e. in a greater yield of C-miniM_{n=1} than was achieved with L-

Figure 5. Comparison of the inter-conversion of linear and circular forms of oligoribonucleotides miniM_{n=9} (panels A and B) and miniM_{n=8}(GCGC) (panel C). The change in one stem, for miniM_{n=9} compared to miniM_{n=8}(GCGC), is indicated by the partial structures in panels B and C. Incubations were in 1×Tris-processing buffer. Open and closed symbols present data from reactions initiated with electrophoretically-purified preparations enriched in linear and circular molecules, respectively. Aliquots taken at times indicated were analyzed by electrophoresis through 8% polyacrylamide. For panels A and C, the temperature was a constant 37°C (○, ●) or a constant 55°C (□, ■). In panel B, the temperature was shifted at time zero to 45°C after 30 min at 23°C (▲, △). The curves in panel C are least square fits to an equation for approach to equilibrium of a single-reactant, single-product reaction (25).

miniM_{n=1} as the reactant, regardless of the initial concentration of oligoribonucleotide (data not shown). The folding of miniM_{n=1}-D₉ may predispose the released L-miniM_{n=1} to internally ligate. Alternatively, or additionally, miniM_{n=1}-D₉ may be more effective than miniM_{n=1} in acting in *trans* to catalyze circularization of miniM_{n=1}. To test the latter hypothesis, we supplied unlabeled 1 μM miniM_{n=1}-D₉ to a reaction mixture containing 2 nM [³²P]L-miniM_{n=1} in 1×MES processing buffer. At 37°C, the yield of [³²P]C-miniM_{n=1} was greater than 0.05 in 1 hr (Figure 4A, lane 3). No significant accumulation of [³²P]C-miniM_{n=1} was detected when [³²P]L-miniM_{n=1} was incubated without miniM_{n=1}-D₉ in MES buffer, without or with magnesium ions and spermidine-HCl (Figure 4A, lanes 1, 2). These results are consistent with miniM_{n=1}-D₉ acting on L-miniM_{n=1} to facilitate its circularization. Consequently, circularization of L-miniM_{n=1}, when incubated alone, very likely is a bimolecular (or higher order) reaction.

Figure 6. Near-equilibrium compositions and rates, as a function of temperature, for reactions initiated from preparations of electrophoretically purified linear (open symbols) and circular (closed symbols) forms of $\text{miniM}_{n=9}$ (panel A) and of $\text{miniM}_{n=8}(\text{GCGC})$ (panels B and C). The distinct stem of the two oligoribonucleotides is indicated in panels A and B. Results were derived from analyses of reactions like those presented in Figure 5A and C. The near-equilibrium fraction of circles was calculated in two ways. In one, the fraction was the average of the 8 min and 16 min time points ($\square, \blacksquare$), these paired time points having a maximum difference of 0.025. In the other, the fraction of circles at infinite time was derived by curve fitting (e.g., Figure 5C), with symbols consisting of circle with error bars as indicated to the right of the panels. Symbols define two aspects of the fit to the data. The length of the vertical line of each symbol is proportional to the logarithm of the deviation of $1/r^2$ from 1, where r is the correlation coefficient. The horizontal line of each symbol indicates by its length and direction the time, greater or less than zero time, that the fitted curve predicts for a fraction of circles corresponding to the fraction present in the starting material. In panel C, rate constants for the formation of circles from linear molecules (k_1) and for the back reaction (k_2) are presented for points selected by criteria described in the text.

We were able to obtain the $\text{miniM}_{n=1}-\text{D}_9$ used in the experiment presented in Figure 4A because $\text{P}_6-\text{miniM}_{n=1}-\text{D}_9$ cleaved efficiently only at the first junction (corresponding to the first reaction in Figure 2). Presumably catalytically active folding for cleavage at the 5'-most junction of the transcript $\text{P}_6-\text{D}_8\text{L}_c\text{E}_{48}\text{L}_n\text{P}_5-\text{D}_9$ is independent of the $\text{L}_n\text{P}_5-\text{D}_9$ sequence that would not be present in nascent chains corresponding of nt sequence $\text{P}_6-\text{D}_8\text{L}_c\text{E}_{48}$. Cleavage at the 5'-most junction releases $\text{D}_8\text{L}_c\text{E}_{48}\text{L}_n\text{P}_5-\text{D}_9$ (i.e., $\text{miniM}_{n=1}-\text{D}_9$; Figure 3B), which has

two D segments. We postulate that the D_9 portion of the molecule must compete with the D_8 portion in helix formation, and if D_9 does displace D_8 , the resulting small loop L_n of $\text{miniM}_{n=1}-\text{D}_9$ will not allow a unimolecular reaction. The observed concentration dependence of the self-cleavage of $\text{miniM}_{n=1}-\text{D}_9$, implying a bimolecular (or higher order) reaction, and the action of $\text{miniM}_{n=1}-\text{D}_9$ in *trans* to circularize $\text{L-miniM}_{n=1}$, certify the presence of sequences in $\text{miniM}_{n=1}$ that are functional in the self-cleavage and ligation reactions, even though $\text{L-miniM}_{n=1}$ itself did not detectably circularize at low concentrations.

Evidence for the importance of tertiary interactions in the intermolecular circularization reaction

The $\text{miniM}_{n=1}/\text{miniM}_{n=1}(\text{GCGC})-\text{D}_8$ pair (Figures 2, 4C) of molecules differ from the $\text{miniM}_{n=1}/\text{miniM}_{n=1}-\text{D}_9$ pair (Figures 2, 4B) because the 4 bp stems adjacent to bulge loop L_n are distinct for the two members of the former pair and the same for the members of the latter pair. Nevertheless, $\text{miniM}_{n=1}(\text{GCGC})-\text{D}_8$ was able to facilitate the circularization of $\text{miniM}_{n=1}$ (Figure 4A; lane 4). This result suggests that the circularization-facilitating association between two $\text{miniM}_{n=1}$ molecules does not involve the disruption of the secondary structure of either reactant, but more likely the tertiary structure. Importance of a tertiary interaction is consistent with another observation. Even at 4°C, the circularization of $[^{32}\text{P}]\text{L-miniM}_{n=1}$ facilitated by $\text{miniM}_{n=1}-\text{D}_9$ proceeded to the extent of 0.04 in 1 hr (results not shown). Comparisons with results presented in Figure 3, lane 3 suggest a smaller temperature coefficient for this circularization reaction than might be expected if the melting of a short helix (28) were rate limiting. We interpret results described in this paragraph and the observed poor circularization of L-miniM_n molecules for $n = 1$ and $n = 3$ as evidence favoring specific tertiary interactions, both intramolecular and intermolecular, in the RNA-facilitated ligation and cleavage reactions of $\text{sTobRV}(-)\text{RNA}$ and its oligoribonucleotide derivatives. Intermolecular associations appear also to be catalytically relevant for deletion mutants of a *Tetrahymena* ribozyme (29) and for 'double-hammerhead' ribozymes (30).

Non-coaxial orientation of stems in the 'paperclip' model structure

The single-cytidylate of loop L_1 in $\text{miniM}_{n=1}$ (Figure 2) should force the 5 base pair stem and the 4 base pair stem, that loop L_n connects, to be coaxial or nearly so. $\text{L-miniM}_{n=1}$ apparently is unable to circularize in a unimolecular reaction (Figure 3). Thus the unimolecular cleavage and ligation reactions of $\text{sTobRV}(-)\text{RNA}$ are not allowed if the two stems in question are nearly coaxial. Our results suggest (i) that catalytically significant geometry is allowed when the two loop L_n -connected stems of Figure 2 assume an orientation that is much closer to a parallel than to a coaxial arrangement and (ii) that such geometry is necessary for the cleavage and ligation reactions exhibited by the minim molecules and $\text{sTobRV}(-)\text{RNA}$. The non-coaxial orientation of hences in the active conformation may be considered to resemble a paperclip. We proposed the term 'paperclip structure' to designate the conformation of the self-cleaving and ligating sequence of $\text{sTobRV}(-)\text{RNA}$ (as cited by Symons, 1992 (5)) and the similar sequences of satellite RNAs associated with arabis mosaic virus (31) and and chicory yellow mottle virus (32).

Time course of unimolecular ligation and cleavage reactions

Because L-miniM_{n=9} circularized more readily than its smaller homologues circularized, especially in dilute solution, we presume that L-miniM_{n=9} circularizes predominantly in a unimolecular reaction in which the binding step of the formal trimolecular reactions of Figure 1 would not occur. We examined at several temperatures the time course for conversion of either purified L-miniM_{n=9} or purified C-miniM_{n=9} to a mixture of circular and linear molecules (Figures 5A and 6A). Because of spontaneous opening of electrophoretically purified C-miniM_{n=9}, even in the absence of added magnesium ions, we rarely obtained a C-miniM_{n=9} preparation that contained less than about 10% L-miniM_{n=9}. The reactions initiated with preparations of circular and linear molecules did not converge to the same composition, suggesting the presence of more than one conformational or sequence species in one or both of the preparations of L-miniM_{n=9} and C-miniM_{n=9}. Incubations of purified linear and purified circular molecules for periods of up to 16 hr, at temperatures in the range 4°C to 37°C, did not result in convergence to the same composition. Although, the convergence was greater at the higher temperatures (results not shown), no reaction course followed a path predicted by a simple approach to equilibrium (25) between L-miniM_{n=9} and C-miniM_{n=9}. The existence of forms of C-miniM_{n=9} with different propensities to linearize also is indicated by the results from a temperature shift experiment (Figure 5B). A 30 min incubation at 23°C of preparations enriched in L-miniM_{n=9} and in C-miniM_{n=9} brought the preparations to compositions (Figure 5B, zero time point) more similar to each other than was achieved in 16 min (Figure 6A). Subsequent incubation at 45°C for 8 min caused the fractions of circles in the two preparations to decrease and to diverge approximately to the values expected from Figure 6A, implying the presence of slowly-reacting or non-reacting C-miniM_{n=9} molecules in electrophoretically-purified preparations. As is consistent with these observations, the time courses of the reaction did not fit closely to the form expected for a simple inter-conversion between circular and linear molecules (Figure 6A).

Several heating and cooling regimes were tested in attempts to generate a single reactive form in preparations of the C-miniM_{n=9} and L-miniM_{n=9}. These treatments did not alter the distribution of active and less active molecules as assessed by subsequent incubation in buffers containing magnesium ions and spermidine-HCl (data not shown). One potential application of the miniM_n oligoribonucleotides is as subjects for nuclear magnetic resonance or X-ray diffraction studies. The existence of more than one form of C-miniM_{n=9} in electrophoretically purified preparations, whether because of conformation or because of distinct sequences resulting from infidelity during transcription, is likely to be detrimental to possible physical studies of these molecules. The presence of non- or slowly-reacting species after electrophoretic purification also was seen for the self-splicing intron of *Tetrahymena* (33).

The time course of inter-conversion of circular and linear oligoribonucleotides was repeated with miniM_{n=8}(GCGC) (Figures 5C and 6B). For reactions initiated with C-miniM_{n=8}(GCGC) and with L-miniM_{n=8}(GCGC), at least at temperatures of 35°C to 55°C, a closer convergence in composition was observed (Figures 5C and 6B) than was achieved for the miniM_{n=9} system (Figures 5A and 6A). The closeness of fit of the miniM_{n=8}(GCGC) reactions to a time course expected for a single-reactant, single-product reaction allows both k_1 and k_2 to be calculated from data for reactions initiated with

either L-miniM_{n=8}(GCGC) or C-miniM_{n=8}(GCGC), providing there is a significant change (e.g., 0.2 or greater fraction circles produced from linear molecules) in composition during the course of the reaction. Only for miniM_{n=8}(GCGC) at temperatures 37°C and 45°C for the forward reaction and 37°C, 45°C and 55°C for the reverse reaction were these conditions closely approached or satisfied (Figure 6B and 6C). Analysis of the forward and reverse reactions gave similar values at 37°C and 45°C for k_1 and for k_2 , as is consistent with a single-reactant, single-product reaction. Our results suggest that the rates are greatest close to 45°C and that they are less than 1 min⁻¹ for miniM_{n=8}(GCGC). Rough analyses for miniM_{n=9} suggest constants closer to 1 min⁻¹ at 45°C, although the result is of course less reliable (Figures 5A, 6A). Unlike the trimolecular ligation reactions of Figure 1 and certain bimolecular cleavage reactions (34), L-miniM_{n=9} ligated spontaneously over the wide range of 4°C to 75°C (not shown), suggesting that as expected there is additional stability, beyond what is available in the bi- and tri-molecular reactions, afforded by incorporating ribozyme and substrate sequences into one molecule.

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REFERENCES

1. Bruening, G. (1990) *Semin. Virol.* **1**, 127–134.
2. Bruening, G., Passmore, B.K., van Tol, H., Buzayan, J.M. and Feldstein, P.A. (1991) *Molec. Plant-Microbe Interact.* **4**, 219–225.
3. Symons, R.H. (1989) *Trends Biochem. Sci.* **14**, 445–450.
4. Symons, R.H. (1991) *CRC Crit. Rev. Plant Sci.* **10**, 189–234.
5. Symons, R.H. (1992) *Annu. Rev. Biochem.* **61**, 641–671.
6. Prody, G.A., Buzayan, J.L., Schneider, I.R. and Bruening, G. (1986) *Science* **231**, 1577–1580.
7. Buzayan, J., Gerlach, W.L., Bruening, G., Keese, P. and Gould, A.R. (1986) *Virology* **151**, 186–199.
8. Forster, A.C. and Symons, R.H. (1987) *Cell* **49**, 211–220.
9. Haseloff, J. and Gerlach, W.L. (1988) *Nature* **334**, 585–591.
10. Buzayan, J.M., Gerlach, W.L. and Bruening, G. (1986) *Nature* **323**, 349–353.
11. van Tol, H., Buzayan, J.M. and Bruening, G. (1991) *Virology* **180**, 23–30.
12. Haseloff, J. and Gerlach, W.L. (1989) *Gene* **82**, 43–52.
13. Feldstein, P.A., Buzayan, J.M. and Bruening, G. (1989) *Gene* **82**, 53–61.
14. Feldstein, P.A., Buzayan, J.M., van Tol, H., deBear, J., Gough, G.R., Gilham, P.T. and Bruening, G. (1990) *Proc. Nat. Acad. Sci. USA* **87**, 2623–2627.
15. Hampel, A., Tritz, R., Hicks, M. and Cruz, P. (1990) *Nucleic Acids Res.* **18**, 299–304.
16. Seville, B.J. and Collins, R.A. (1990) *Cell* **61**, 685–696.
17. Seville, B.J. and Collins, R.A. (1991) *Proc. Nat. Acad. Sci. USA* **88**, 8826–8830.
18. Sanger, F., Niklen, S. and Coulson, A.R. (1977) *Proc. Nat. Acad. Sci. USA* **74**, 5463–5467.
19. Tabor, S. and Struhl, K. (1987) In Ausubel, F.M., Roger, B., Kingston, R.E., Moore, D.D., Smith, J.A., Seidman, J.G. and Struhl, K. (eds), *Current Protocols in Molecular Biology*. Greene Publishing Associates and Wiley Interscience, New York, pp. 3.5.10–3.5.12.
20. Anonymous, (1988) in *Qiagenology Applications Protocols* (Qiagen, Inc., Chatsworth, CA, pp. 20.
21. Ponz, F., Rowhani, A., Mircetich, S.M. and Bruening, G. (1987) *Virology* **160**, 183–190.
22. Hammond, A.W. and D'Alessio, J.M. (1986) *Focus* **8**, 4–6.
23. Anonymous, (1989) *Promega Biotechnology*, pp. 43–43.
24. Gish, G. and Eckstein, F. (1988) *Science* **240**, 1520–1522.

25. Benson, S.W. (1960) *The Foundations of Chemical Kinetics Series*, (McGraw-Hill Book Co., Inc., New York.
26. Fedor, M.J. and Uhlenbeck, O.C. (1990) *Proc. Natl. Acad. Sci. USA* **87**, 1668–1672.
27. Herschlag, D. and Cech, T.R. (1990) *Biochemistry* **29**, 10159–10171.
28. Cantor, C.R. and Schimmel, P.R. (1980) *Biophysical Chemistry, Part III, The Behavior of Biological Macromolecules*. W.H. Freeman and Co., San Francisco, pp. 1215–1217.
29. Doudna, J.A. and Szostak, J.W. (1989) *Nature* **339**, 519–522.
30. Forster, A.C., Davies, C., Sheldon, C., Jeffries, A.C. and Symons, R.H. (1988) *Nature* **334**, 265–267.
31. Kaper, J.M., Tousignant, M.E. and Steger, G. (1988) *Biochem. Biophys. Res. Commun.* **154**, 318–325.
32. Rubino, L., Tousignant, M.E., Steger, G. and Kaper, J.M. (1990) *J. Gen. Virol.* **71**, 1897–1903.
33. Walstrum, S.A. and Uhlenbeck, O.C. (1990) *Biochemistry* **29**, 10695–10702.
34. Hampel, A. and Tritz, R. (1989) *Biochemistry* **28**, 4929–4933.