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A circular trans-acting hepatitis delta virus ribozyme

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ABSTRACT

A circular *trans*-acting ribozyme designed to adopt the motif of the hepatitis delta virus (HDV) *trans*-acting ribozyme was produced. The circular form was generated *in vitro* by splicing a modified group I intron precursor RNA in which the relative order of the 5′ and 3′ splice sites, flanking the single HDV-like ribozyme sequence-containing exon, is reversed. Trans-cleavage activity of the circular HDV-like ribozyme was comparable to linear permutations of HDV ribozymes containing the same core sequence, and was shown not to be due to linear contaminants in the circular ribozyme preparation. In nuclear and cytoplasmic extracts from HeLa cells, the circular ribozyme had enhanced resistance to nuclease degradation relative to a linear form of the ribozyme, suggesting that circularization may be a viable alternative to chemical modification as a means of stabilizing ribozymes against nuclease degradation.

INTRODUCTION

The genomic and antigenomic RNAs of hepatitis delta virus contain RNA sequences that self-cleave *in vitro* in the presence of Mg^{2+} (1,2,3). The minimal sequences for rapid self-cleavage, 1 nucleotide 5′ to the cleavage site and 84 nucleotides 3′ of the cleavage site (4,5), may assume a four-stem pseudo-knotted structure that is unique to ribozymes from this virus (5–8). This secondary structure is consistent with sequence similarities and variations in the genomic and antigenomic ribozymes (5,6) as well as experimental data from *in vitro* mutagenesis (5,8–13), nuclease and chemical probing (6,12), and chemical modification interference (14). Alternative models have been considered by several groups for both the genomic and antigenomic ribozyme sequences (3,10,12,14–16) but these have not yet been shown to be predictive for ribozyme design, while the pseudoknot model has (8).

A short stem (stem I) containing 6 Watson–Crick basepairs and a possible GU basepair adjacent to the cleavage site defines a loop with a sequence that pairs with the 3′ end of the self-cleaving domain (stem II). A trans-acting form of the ribozyme is generated by separating the sequence between stem I and stem II (7,8); the 5′ side of stem I becomes the substrate and the ribozyme sequences are essentially within the loop defined by stem II (Fig 1A). Circularly permuted forms of trans-acting ribozymes can be generated in which stem II is closed and the transcription is initiated and terminated within stem IV (compare Fig 1A and Fig 1 B) (8). These results suggested that it may be possible to generate a covalently closed circular form of the delta ribozyme which retains trans-cleavage activity.

For *in vivo* uses, small RNAs in the form of antisense oligoribonucleotides (17), site-specific RNA-cleaving ribozymes (18–23), or decoy structures (24) may be more effective if stabilized against degradation by nucleases. A number of approaches are being explored to extend the half-life of small RNAs of these designs, including the use of chemically synthesized oligonucleotides containing modified sugars (25–28) or internucleotide linkages (29–32). For example, the hammerhead ribozyme can be extensively modified to protect it against degradation without loss of activity, providing key residues remain ribose (25–28). Obviously, chemical modification would not work with *in vivo* expressed RNA sequences and there also may be a greater need for toxicity evaluation with modified RNA than with natural RNA. Another approach involves the addition of stabilizing sequences such as stem and loop structures to the ends of the ribozyme (33) with the expectation that such structures will make ends more resistant to exonuclease attack. However, flanking sequences also can influence ribozyme activity (4,34,35), and therefore embedding the ribozyme within other sequences may prove counter-productive in those cases where it leads to less active structures. If the major route for small RNA degradation *in vivo* is exonucleolytic, it is predicted that elimination of ends by circularizing the RNA would increase the half-life of the RNA since the rate limiting step in degradation would become endonucleolytic cleavage of the RNA (36).

Using a group I permuted intron–exon (PIE) RNA sequence in which the order of the splice sites has been reversed, it is possible to catalyze the circularization of a variety of RNA exon sequences (37, unpublished results). As reported here, a PIE construct was used to generate a novel circular RNA sequence designed to adopt the HDV ribozyme structural motif (Fig 1C). We demonstrate that the circular ribozyme is active in trans, and is stable in HeLa cell nuclear and cytoplasmic extracts.

MATERIALS AND METHODS

Enzymes and reagents

T7 RNA polymerase was purified from an over-expressing clone provided by W.Studier (38). Sequenase (modified T7 DNA polymerase) was purchased from US Biochemicals (Cleveland).

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Other enzymes, nucleotides, ^{32}P-labeled nucleotides and chemicals were purchased from commercial sources. Nuclear and cytoplasmic extracts from HeLa cells (39) were provided by P.Modrich.

Construction of the ribozyme PIE plasmid

The fused-exon sequence of pPR100 (37) was modified by introducing two *Nhe*I sites (40) into the sequence forming the top of the anticodon stem of the tRNA derived exon, cutting this DNA with *Nhe*I to release most of the exon sequence and religating the vector to generate a plasmid (pPR120) with a shorter exon sequence, 5′ AAAATCGCTAGCGACTT, containing a single *Nhe*I site (underlined). Two synthetic oligodeoxyribonucleotides (5′-CTAGGCGAAT GGCTTCGGCC AGCCTC-CTCG CGGCCCGACC TGGG and 5′-CTAGCCCAGG TCGGGCCGCG AGGAGGCTGG CCGAAGCCAT TCGC) were annealed and ligated into *Nhe*I cut pPR120 DNA to introduce an HDV ribozyme motif into the fused exon, generating pRC1. Plasmids containing inserts of the proper orientation were identified by restriction digestion and dideoxy sequencing (41) of miniprep DNA. Plasmid DNA was purified from overnight cultures by equilibrium density centrifugation in CsCl containing ethidium bromide (42).

Ribozyme synthesis

Ribozymes CDC200 and PDC7 were prepared by *in vitro* transcription of *Bam*HI cut pCDC200 and pPDC7 DNA with purified T7 RNA polymerase as described (37), and the RNA was purified by electrophoresis on 8% polyacrylamide gels containing 8 M urea. For labeled RNA, [α^{32}P]CTP was included in the transcription mix at 1 μCi/μl. Ribozyme RC1 was prepared by transcription of *Bam*HI cut pRC1; after a two hour incubation at 37°C, in which about 50% of the transcript had spliced, the [Mg^{2+}] was raised to 50 mM, an additional 0.2 mM GTP was added and the reaction was incubated for an additional 30 min to increase the yield of spliced product to 60−80%. The RNA was purified by gel electrophoresis under denaturing conditions. Splicing conditions for the isolated PIE-ribozyme precursor were: 40 mM Hepes (pH 7.5), 200 mM NaCl, 0.05 mM EDTA, 20 mM $MgCl_2$, and 0.2 mM GTP at 37°C for 60

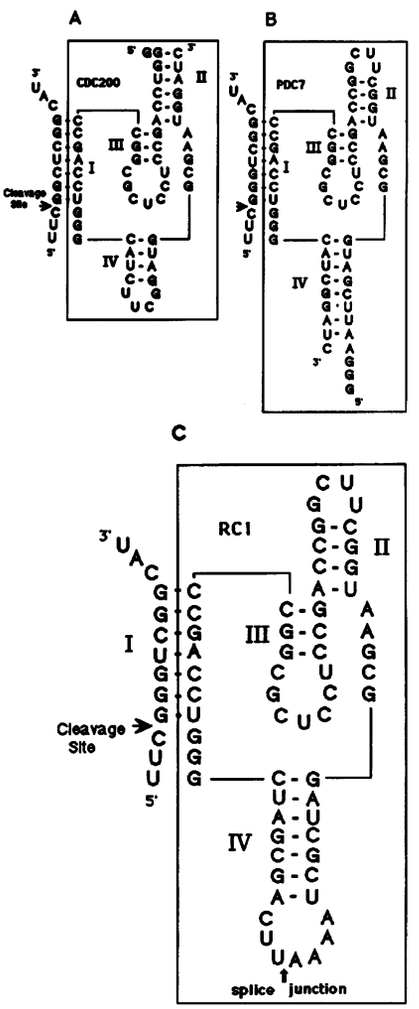

Figure 1. Linear and circular HDV ribozymes. (A and B) Sequences and proposed secondary structures of two linear HDV ribozymes (8), CDC200 and PDC7, showing the interaction with the substrate oligonucleotide through an intermolecular stem I interaction. The entire ribozyme sequence (boxed) is shown, and stems I through IV are numbered according to the convention used with the self-splicing form of the HDV ribozyme. (C) Sequence and proposed secondary structure of the circular ribozyme, RC1, binding the substrate oligonucleotide. 5′ to 3′ is counter clockwise for the circle.

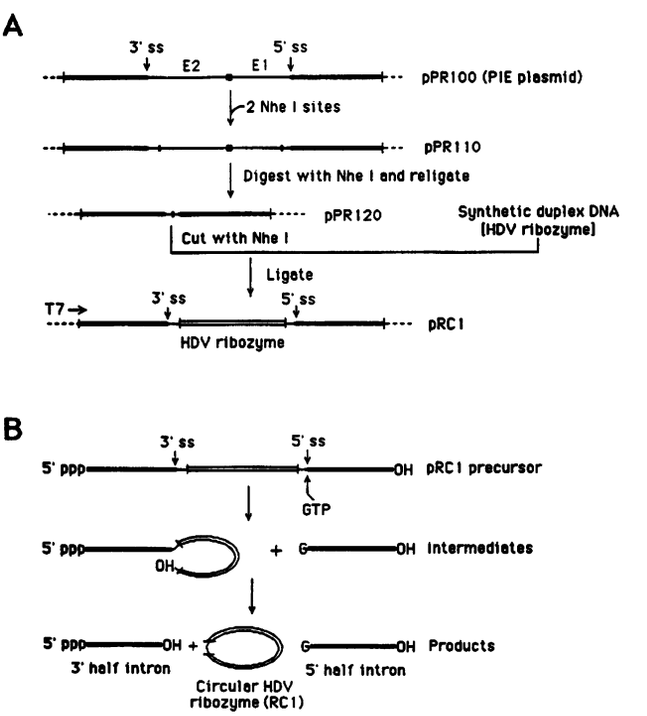

Figure 2. (A) Construction of HDV ribozyme-PIE plasmid (pRC1). Thick lines represent the divided and permuted intron sequences, thin lines the fused 5′ and 3′ exons (E1 and E2), and the double lines represent the HDV ribozyme. Plasmid pPR100 was described previously (37). pPR110 contains two *Nhe*I sites in the exon, pPR120 contains a shortened exon with one *Nhe*I site and pRC1 contains an HDV ribozyme sequence inserted as part of the exon (see text). (B) Splicing pathway of a permuted intron exon−ribozyme sequence to produce the circular ribozyme RC1. The splicing pathway is presumed to be the same group I mechanism for the pRC1 precursor RNA as was demonstrated for the pPR100 precursor (37). The 5′ and 3′ splice sites are indicated; guanosine nucleotide attack at the 5′ splice site initiates a reaction that results in production of the circular exon.

min. For the nicking experiment, RNA in 50 mM $NaHCO_3$ (pH 9.0) and 1 mM EDTA was heated to 95°C for 3 min. The products were separated by electrophoresis on an 8% and/or 12% polyacrylamide gel containing 8M urea. The identity of the circular HDV ribozyme RNA was confirmed by ribonuclease T1 digestion and fingerprinting (43) of RNA labeled during transcription with [α^{32}P]GTP (37).

Linear (randomly nicked) RC1 was prepared from circular RC1. Twenty μg of RC1 was heated to 95°C for 5 min in 100 mM $NaHCO_3$, 1 mM EDTA in 0.1 ml. The reaction was cooled, 0.1 ml of formamide and tracking dyes was added and the products fractionated on a 15% polyacrylamide gel containing 8 M urea. The linear (faster migrating band) and circular forms were located by UV shadowing and recovered.

Substrates for trans cleavage reactions

Substrates for trans cleavage were 5′ end-labeled 13-mer (Fig 1), DHS1 (7), and end-labeled 8 mer substrate, DHS4 (7). Both oligonucleotides were synthesized at US Biochemicals (Cleveland). Products of the reaction with 13 mer (^{32}P-UUC>p) were analyzed by electrophoresis in 20% polyacrylamide gels under denaturing conditions (7). Products of the reactions with the 8 mer (^{32}P-C>p) were analyzed by TLC on PEI plates in 1 M LiCl (7).

Nuclear and cytoplasmic extracts

HeLa cell extracts were a gift from P.Modrich. The nuclear extract (34 mg/ml) (39) was used as provided without further treatment. The cytoplasmic extract was obtained as a frozen ammonium sulfate precipitate which was resuspended in, and dialyzed against, 25 mM Tris−HCl (pH 7.5), 100 mM KCl, 0.1 mM EDTA, 10% glycerol, 2 mM DTT, 0.1% isopropanol saturated with PMSF (at room temperature). A precipitate that formed during dialysis was removed by centrifugation. The protein concentration of the cytoplasmic extract was estimated by the dye-binding method (44).

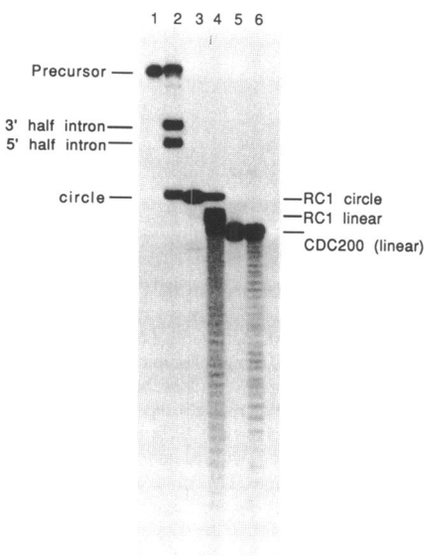

Figure 3. Splicing reaction of the PIE-RC1 precursor and identification of the circular product. Lane 1, gel-purified uniformly-labeled precursor; lane 2, precursor subjected to splicing conditions at 37°C for 60 min; lane 3, gel purified circular RNA; lane 4, purified circle subjected to partial alkaline hydrolysis; lane 5, gel purified linear ribozyme CDC200; lane 6, purified CDC200 subjected to partial hydrolysis. The products were fractionated by electrophoresis in a 15% polyacrylamide gel containing 8 M urea and an autoradiogram of the gel is shown.

RESULTS AND DISCUSSION

Splicing of PIE sequences to produce a circular ribozyme

We have previously shown that splicing of the PIE precursor, PR100, generates a circular exon; evidence for the circle consisted of: anomalous electrophoretic mobility in polyacrylamide gels under denaturing conditions, which is indicative of a non-linear structure; a ribonuclease T1 fragment consistent with the ligation product; and primer extension across the ligation junction also consistent with the ligation product (37). A circular form of the hepatitis delta virus (HDV) ribozyme was generated

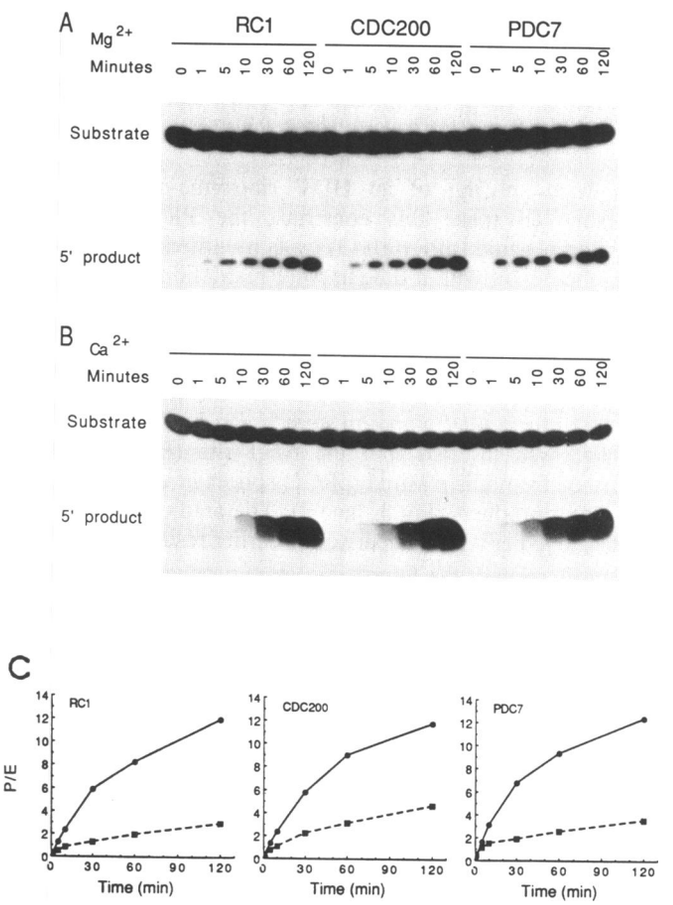

Figure 4. Trans cleavage by circular and linear HDV ribozymes. (**A** and **B**) Cleavage of 5 ′ end-labeled substrate 13-mer (see Fig. 1) by ribozymes RC1, CDC200, and PDC7 at 37°C in either Mg^{2+} (A) or Ca^{2+} (B). The reactions contained 10 nM ribozyme, 200 nM unlabeled substrate, a trace amount (<0.5 nM) of ^{32}P-labeled substrate, 40 mM Tris−HCl (pH 8.0), 1 mM EDTA, and either 11 mM $MgCl_2$ or 11 mM $CaCl_2$. The ribozyme and substrate were preincubated separately at 37°C for 5 min prior to mixing; aliquots were removed at the times indicated and mixed with an equal volume of formamide containing 50 mM EDTA and tracking dyes and fractionated on a 20% polyacrylamide gel containing 7 M urea. (**C**) Quantitation of the data from (A) and (B) comparing turnover in Mg^{2+} (squares) and Ca^{2+} (circles) for each of the ribozymes. The ratio of moles of product generated per mole of ribozyme (P/E, calculated as the fraction of substrate cleaved times the initial substrate concentration divided by the concentration of ribozyme) is plotted verses time.

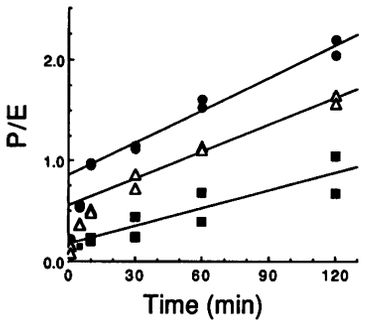

Figure 5. Cleavage activity of linear and circular forms of RC1. Closed circles; 40 nM RC1; closed squares, 40 nM nicked RC1; open triangles, 20 nM each circle and linear RC1. Substrate (200 nM unlabeled and trace [^{32}P]13-mer) and ribozyme were incubated separately in 40 mM Tris−HCl (pH 8.0), 1 mM EDTA, and 11 mM $MgCl_2$ at 37°C for 5 min and mixed to start the reaction. Aliquots were removed and mixed with an equal volume of formamide containing 50 mM ETDA to stop the reactions. Products were fractionated on 20% polyacrylamide gels, bands located by autoradiography, excised and counted for Cherenkov scintillation. P/E is as defined in the legend to Fig. 4. The lines shown are fit to the steady state region of each reaction and have equations of y = 0.16 + 0.0059x, y = 0.55 + 0.0089x, y = 0.86 + 0.011x.

by *in vitro* splicing of an HDV ribozyme-containing PIE precursor. To make this construct, the PR100 sequence-containing plasmid (pPR100) (37) was modified by introducing a shortened exon with a unique *Nhe*I restriction site (pPR120, Fig 2A), but the sequences involved in splice-site selection were not altered. Synthetic duplex DNA containing a ribozyme sequence, which was a composite of sequences from the genomic and antigenomic ribozymes together with novel sequences required to form a tetraloop on stem II and *Nhe*I sticky ends in stem IV, was inserted into the *Nhe*I site (Fig 2A). RNA transcribed from this plasmid (pRC1) splices *in vitro* to generate three major products (Fig 3, lane 2). The predicted sizes of the products together with comparison to earlier results (37) allowed the identification of the fastest migrating product as a candidate for the circular exon. RNA isolated from the candidate band was treated with alkali to partially nick it; with this treatment, some of the RNA displayed a discrete increase in mobility consistent with conversion to a linear species (Fig. 3, lanes 3 & 4), thus confirming a non-linear structure for the original form. A smear of fragments below the faster migrating species is expected for molecules which are nicked more than once. In comparison, a linear form of the same HDV ribozyme (CDC200) (8) generated a smear of fragments upon alkaline treatment (Fig 3, lanes 5 & 6). These data indicate that the fastest migrating band generated in the splicing reaction was circular.

The circular RNA is a trans-active ribozyme

At 37°C in 10 mM Mg^{2+}, the circular ribozyme, RC1, cleaved a 5′ end-labeled oligonucleotide to a similar extent as linear ribozymes CDC200 and PDC7 (8) that contained the same core structure (Fig. 4A). An initial burst and slow turnover at 37°C in Mg^{2+} was observed with both the circular and linear forms of the HDV ribozyme (Fig. 4C, dashed lines), and was consistent with earlier results interpreted to indicate that the initial round of cleavage was relatively fast, but there was a change in the rate limiting step so that turnover is slow (8). However, the HDV ribozyme is active with a variety of divalent cations, and in the trans reaction with this substrate, substitution of Ca^{2+} for Mg^{2+}

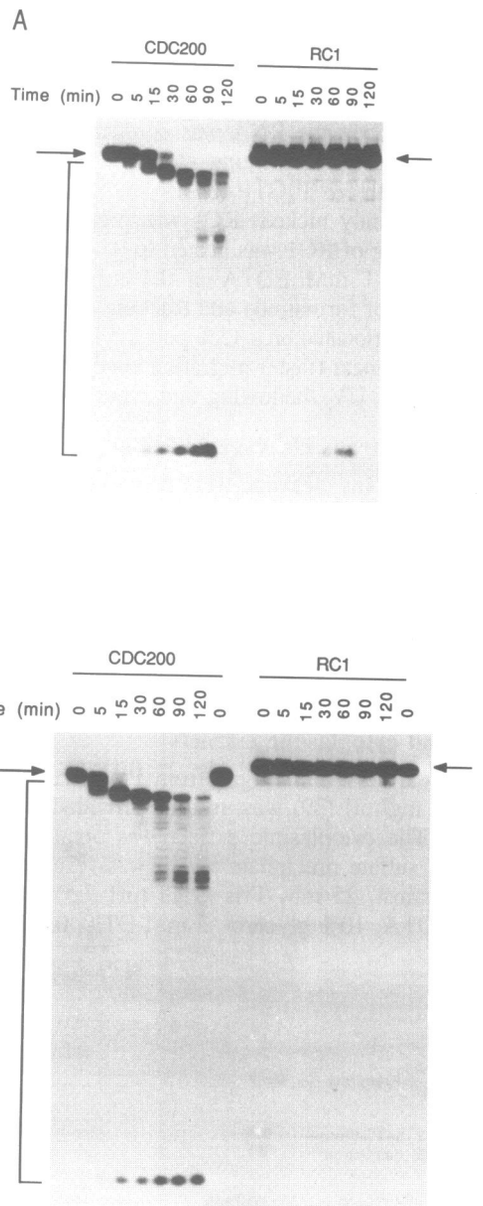

Figure 6. Stability of CDC200 and RC1 to degradation in HeLa cell extracts. (A) Cytoplasmic extract. (B) Nuclear extract. RC1 and CDC200, internally labeled with ^{32}P, were incubated at 37°C in 50 μl reactions containing 50 mM Tris−HCl (pH 7.5), 100 mM NaCl, 10 mM $MgCl_2$, 5 μg of carrier tRNA, and either 0.9 mg/ml of cytoplasmic extract or 0.7 mg/ml of nuclear extract. 5 μL aliquots were removed at the times indicated and were quenched with EDTA and formamide and fractionated on a 15% polyacrylamide gel under denaturing conditions. Arrows indicate the position of the intact ribozyme; the degradation products are bracketed. The data was quantified by cutting out precursor and products from the gel and measuring Cherenkov scintillation. The value for the half life ($t_{1/2}$) of each ribozyme, given in the text, was obtained from $t_{1/2} = 0.693/k$ (k is the first-order rate constant estimated by fitting the data to $F=c(1-e^{-kt})$, where F is the fraction of RNA degraded, t is time, and c is the end point).

enhances turnover approximately 10-fold (Perrotta & Been, unpublished results). In 10 mM Ca^{2+} at 37°C with 10 nM ribozyme and 200 nM substrate, turnover was approximately 0.2−0.3 min^{-1} for all three ribozymes (Fig. 4B & 4C). Thus in both Mg^{2+} and Ca^{2+}, RC1 displayed kinetics similar to those of the two linear ribozymes.

Activity of the circular ribozyme is not due to linear contaminants

It is expected that some of the circular form of RC1 would be randomly nicked during purification, and some of these linear forms would be active. However, the following observations demonstrate that the observed activity of RC1 is due primarily to the circular ribozyme and not exclusively to linear forms. First, RC1, CDC200, and PDC7 had similar specific activities, within a factor of 2 (Fig. 4 and data not shown), yet no linear form was detected when the circular RC1 RNA was re-analyzed on a polyacrylamide gel under denaturing conditions which separated circles and linears. In a control lane in which the same quantity of RC1 was partially nicked by alkaline hydrolysis, the linear form was clearly apparent when it represented less than 20% of the total RNA in the lane (data not shown). These results place a generous upper limit on the amount of contaminating linear form. Second, the specific activity of the gel-purified (randomly-nicked) linear form of RC1 was less than the circular RC1. In reactions containing 10 mM Ca^{2+}, 200 nM substrate (5′ end-labeled C*GGGUCGG, refs 7,8) and either 40 nM linear (nicked) RC1 or 40 nM circular RC1, product was formed at the rate of 0.057/min and 0.29/min (moles product/mole of ribozyme/min), respectively (data not shown). Thus, under turnover conditions in Ca^{2+}, the circle generated product 5 times faster than the nicked RC1. Similar rates were obtained at other ribozyme concentrations: 8 nM circle, 0.25/min; 20 nM linear, 0.064/min. In 10 mM Mg^{2+}, conditions showing less turnover, a difference in burst size for the two forms of RC1 was seen (Fig 5). The circular form generated a burst of 0.86 moles of product per mole of enzyme (P/E), while the linear form generated a burst of 0.17 P/E. Mixing equal amounts of linear and circular forms generated a burst of intermediate value (0.55 P/E) (Fig 5). By either analysis, the results can be interpreted to indicate that the linear (randomly nicked) RC1 contains approximately 20% active species. That value would be consistent with the prediction that nicking RC1 in nonessential sequences (eg. at or near stem-loops II and IV) would convert it to a linear form which is still active, while nicking in other regions may inactivate the ribozyme. This difference was seen with two independent preparations of ribozymes in which linears and circles received the same treatments. The size of the burst with RC1 in Mg^{2+} suggests that 80−90% of the ribozyme is active. These findings implicate the circle, rather than a very robust linear form, as the major active species of RC1.

The circular HDV ribozyme is more resistant to nuclease degradation

The stability of the circular ribozyme relative to a linear ribozyme was tested in partially fractionated HeLa cell extracts. In the presence of cytoplasmic extract where a linear form of the ribozyme (CDC200) was rapidly degraded ($t_{1/2}$ ~13 min), RC1 showed little evidence of degradation after 1.5 to 2 hrs (Fig. 6A). The half-life for the circular ribozyme was estimated to be at least 14 hrs. The presence of any linear contaminants in the circle preparation would mean that this difference is a lower estimate. In a nuclear extract the difference was also large; the half-life of the circular form was estimated to be greater than 24 hrs compared to about 5 min for the linear form under the same conditions (Fig. 6B). These data suggest that elimination of ends protects the RNA by making it resistant to exonucleases, although the possibility that the circular form is more compact and therefore may be more endonuclease resistant as well cannot be ruled out at this point. Nevertheless, the half-life of the circular form is increased by 65 to 300 fold, depending on the extract, relative to a linear ribozyme of similar size and sequence. Enhanced stabilities of RNA circles has also been observed when circular group I introns were injected into frog oocytes (36).

Previously, it has been shown that circular RNAs are often products of self-splicing and self-cleaving reactions, and are able to interconvert between linear and circular forms through self-catalyzed reactions (45,46,47). This study demonstrates that it is possible to generate small circular ribozymes by PIE splicing which are stable as circles and do not autocatalytically interconvert with linear forms. These circular ribozymes retain structures required for formation of a substrate binding site and a catalytic site, and therefore can act in trans. For certain structural studies of ribozymes, the circular forms of the RNA may prove useful since they would be expected to have fewer stable folding options available and therefore more likely to assume a unique structure. While we have not demonstrated such a structural effect, we have shown that this small circular RNA shows enhanced resistance to degradation in nuclease rich cellular extracts. Presumably this resistance is due to lack of ends and as such circular RNA may offer some advantages over non-modified linear forms for uses *in vivo*. Finally, circularization by splicing is a method by which to process an RNA to eliminate sequences at the ends of the primary transcript which could interfere with the desired function. Although circle production by splicing of group I PIE sequences *in vivo* has not been examined, messenger exon sequences with an inverted order of splice sites have been detected by PCR (48,49). A more recent report extends these findings and shows that pre-mRNA can splice to produce circles of exon sequences when the splice sites are utilized in reverse order (50). In this case, the RNA circles are stable in the cytoplasm despite the absence of modifications generally associated with the ends of messengers (50). These exciting findings suggest that, with the appropriate vectors, it may be possible to exploit various splicing systems, in place of or in addition to the self-splicing group I introns, to generate circular RNA sequences in specific cell types or subcellular locations.

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REFERENCES

1. Kuo,M.Y.-P., Sharmeen,L., Dinter-Gottlieb,G. and Taylor,J. (1988) *J. Virol.* **62,** 4439−4444.
2. Sharmeen,L., Kuo,M.Y.-P., Dinter-Gottlieb,G. and Taylor,J. (1988) *J. Virol.* **62,** 2674−2679.
3. Wu,H.-N., Lin,Y.-J., Lin,F.-P., Makino,S., Chang,M.-F. and Lai,M.M.C. (1989) *Proc. Natl. Acad. Sci. U.S.A.* **86,** 1831−1835.
4. Perrotta,A.T. and Been,M.D. (1990) *Nucleic Acids Res.* **18,** 6821−6827.
5. Perrotta,A.T. and Been,M.D. (1991) *Nature* **350,** 434−436.
6. Rosenstein,S.P. and Been,M.D. (1991) *Nucleic Acids Res.* **19,** 5409−5416.
7. Perrotta,A.T. and Been,M.D. (1992) *Biochemistry* **31,** 16−21.

8. Been,M.D., Perrotta,A.T. and Rosenstein,S.P. (1992) *Biochemistry* **31**, 11843–11852.
9. Thill,G., Blumenfeld,M., Lescure,F. and Vasseur,M. (1991) *Nucleic Acids Res.* **19**, 6519–6525.
10. Wu,H.-N. and Huang,Z.-S. (1992) *Nucleic Acids Res.* **20**, 5937–5941.
11. Suh,y.-A., Kumar,P.K.R., Nishikawa,F., Kayano,E., Nakai,S., Odai,O., Uesugi,S., Taira,K. and Nishikawa,S. (1992) *Nucleic Acids Res.* **20**, 747–753.
12. Wu,H.-N., Wang,Y.-J., Hung,C.-F., Lee,H.-J. and Lai,M.M.C. (1992) *J. Mol. Biol.* **223**, 233–245.
13. Thill,G., Vasseur,M. and Tanner,N.K. (1993) *Biochemistry* **32**, 4254–4262.
14. Belinsky,M., Britton,E. and Dinter-Gottlieb,G. (1993) *FASEB J* **7**, 130–136.
15. Branch,A. and Robertson,H.D. (1991) *Proc. Natl. Acad. Sci. U.S.A.* **88**, 10163–10167.
16. Smith,J.B., Gottlieb,P.A. and Dinter-Gottlieb,G. (1992) *Biochemistry* **31**, 9629–9635.
17. Weintraub,H., Izant,J.G. and Harland,R.M. (1985) *Trends in Gen.* **1**, 22–25.
18. Haseloff,J. and Gerlach,W.L. (1988) *Nature* **334**, 585–591.
19. Cech,T.R. (1988) *JAMA* **260**, 3030–3034.
20. Cameron,F.H. and Jennings,P.A. (1989) *Proc. Natl. Acad. Sci.USA* **86**, 9139–9143.
21. Cotten,M. and Birnstiel,M.L. (1989) *EMBO J.* **8**, 3861–3866.
22. Sarver,N., Cantin,E.M., Chang,P.S., Zaia,J.A., Ladne,P.A., Stephens,D.A. and Rossi,J.J. (1990) *Science* **247**, 1222 –1225.
23. Saxena,S.K. and Ackerman,E.J. (1990) *J. Biol. Chem.* **265**, 17106–17109.
24. Sullenger,B.A., Gallardo,H.F., Ungers,G.E. and Gilboa,E. (1990) *Cell* **63**, 601–608
25. Pieken,W.A., Olsen,D.B., Benseler F., Aurup,H. and Eckstein,F. (1991) *Science* **253**, 314–317.
26. Paollella,G., Sproat,B.S. and Lamond,A.I. (1992) *EMBO J.* **11**, 1913–1919.
27. Taylor,N.R., Kaplan,B.E., Swiderski,P., Li,H. and Rossi,J.J. (1992) *Nucleic Acids Res.* **20**, 4559–4565.
28. Goodchild,J. (1992) *Nucleic Acids Res.* **20**, 4607–4612.
29. Matsukura,M., Shinozuka,K., Zon,G., Mitsuya,H., Reitz,M., Cohen,J.S. and Broder,S. (1987) *Proc. Natl. Acad. Sci. USA* **84**, 7706–7710.
30. Mori,K., Boiziau,C., Cazenave,C., Matsukura,M., Subasinghe,C., Cohen,J.S., Broder,S., Toulme,J.J. and Stein,C.A. (1989) *Nucleic Acids Res.* **17**, 8207–8219.
31. Eckstein,F. and Gish,G. (1989) *Trends Biochem. Sci.* **14**, 97–100.
32. Chowrira,B.M. and Burke,J.M. (1992) *Nucleic Acids Res.* **20**, 2835–2840.
33. Sioud,M. and Drlica,K. (1991) *Proc. Natl. Acad. Sci., U.S.A.* **88**, 7303–7307.
34. Forster,A.C. and Symons,R.H. (1987) *Cell* **49**, 211–220.
35. Woodson,S.A. and Cech,T.R. (1989) *Cell* **57**, 335–345.
36. Harland,R. and Misher,L. (1988) *Developement* **102**, 837–852.
37. Puttaraju,M. and Been,M. D. (1992) *Nucleic Acids Res.* **20**, 5357–5364.
38. Davanloo,P., Rosenberg,A.H., Dunn,J.J. and Studier,F.W. (1984) *Proc. Natl. Acad. Sci. U.S.A.* **81**, 2035–2039.
39. Holmes,J., Clark,S. and Modrich,P. (1990) *Proc. Natl. Acad. Sci. U.S.A.* **87**, 5837–5841.
40. Kunkel,T.A., Roberts,J.D. and Zakour,R.A. (1987) *Meth. Enzym.* **154**, 367–382.
41. Sanger,F., Nicklen,S. and Coulson,A.R. (1977) *Proc. Natl. Acad. Sci. U.S.A.* **74**, 5463–5467.
42. Maniatis,T., Fritsch,E.F. and Sambrook,J. (1982) *Molecular Cloning: A Laboratory Manual* (Cold Spring Harbor University Press, Cold Spring Harbor).
43. Barrell,B.G. (1971) in Cantoni,G.L. and Davies,D.R. (eds) Procedures in Nucleic Acid Research. Harper and Row, New York. Vol. 2, pp. 751–779.
44. Bradford,M. (1976) *Anal. Biochem.* **72**, 248–254.
45. Zaug,A.J., Kent,J.R. and Cech,T.R. (1984) *Science* **224**, 574–578.
46. Sullivan,F.X. and Cech,T.R. (1985) *Cell* **42**, 639–648.
47. Buzayan,J.M., Gerlach,W.L. and Bruening (1986) *Nature* **323**, 349–353.
48. Nigro,J.M., Cho,K.R., Fearon,E.R., Kern,S.E., Ruppert,J.M., Oliner, J.D., Kinzler,K.W. and Vogelstein,B. (1991) *Cell* **64**, 607–613.
49. Cocquerelle,C., Daubersies,P., Maje'rus,M.-A., Kerckaert,J.-P. and Bailleul,B. (1992) *EMBO J.* **11**, 1095–1098.
50. Cocquerelle,C., Mascrez,B., He'tuin,D. and Bailleul,B. (1993) *FASEB* **7**, 155–160.