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A hammerhead ribozyme allows synthesis of a new form of the *Tetrahymena* ribozyme homogeneous in length with a 3′ end blocked for transesterification

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ABSTRACT

The L-21 *Sca*I form of the *Tetrahymena* ribozyme acts as a sequence-specific endonuclease. This ribozyme has a homogeneous 5′ end but a somewhat heterogeneous 3′ end, as is typical of RNA synthesized by transcription *in vitro*. To produce a more homogeneous ribozyme for both structural and enzymological studies, a hammerhead ribozyme was inserted at the 3′ end of the *Tetrahymena* ribozyme. During transcription the hammerhead moiety self-cleaves to produce the L-21 A *Tetrahymena* ribozyme, which ends at A410 with a 2′,3′-cyclic phosphate terminus. The new ribozyme has endoribonuclease activity equivalent to that of L-21 *Sca*I under conditions where binding of substrate is rate-limiting, as well as under conditions where chemical cleavage by guanosine is rate-limiting. However, the L-21 A has lost activity in oligo(C) disproportionation (e.g., 2 $pC_5 \rightarrow pC_4 + pC_6$), consistent with the previous proposal that this reaction occurs predominantly through a covalent ribozyme-substrate intermediate involving the 3′-terminal hydroxyl group of the ribozyme. Formation of such an intermediate would be prevented by the 2′,3′-cyclic phosphate terminus. Thus the L-21 A ribozyme has simplified enzymatic activity, being fully active as an endonuclease but blocked for disproportionation.

INTRODUCTION

Self-splicing of the *Tetrahymena* pre-rRNA exemplifies intramolecular catalysis: the intervening sequence (IVS) folds to form a catalytic center that mediates its excision from the precursor RNA and the ligation of the flanking exons (1). The catalytic core of the IVS can be separated from the splice sites in such a way that it retains activity, being able to cleave and join other substrate RNA molecules in *trans* (2–6). One particularly useful form of this ribozyme is the L-21 *Sca*I RNA, which is missing 21 nt from the 5′ end of the IVS and has a 3′ end formed by run-off transcription at a *Sca*I site in the plasmid DNA template (7). This ribozyme acts as a sequence-specific endonuclease, binding guanosine (or GTP) and using it as a nucleophile. It has been the subject of mechanistic investigations (8–12), and its substrate-binding properties and folding pathway have been analyzed (13–15).

One concern regarding the L-21 *Sca*I ribozyme is the heterogeneity of its 3′ end (7). The RNA is prepared by *in vitro* transcription with T7 RNA polymerase, which frequently adds one or more non-encoded nucleotides to the 3′ end of a run-off transcript (16). Such 3′-terminal heterogeneity can be problematic for NMR spectroscopy and could potentially interfere with crystallization (17). Furthermore, the 3′ end of the ribozyme can participate in catalysis. If the ribozyme ends at the natural 3′ splice site (nucleotide G414) and has a 3′-OH group, it can use this G-OH to catalyze disproportionation of oligoribonucleotides to form both longer and shorter chains (ref. 2; Fig. 1A). Alternatively, the ribozyme can bind exogenous GTP or G (guanosine) and use it as a nucleophile to catalyze direct cleavage of RNA substrates, without formation of a covalent intermediate (3,5; Fig. 1B). Ribozymes containing a 3′ terminal G-OH can use either this endogenous G or exogenous G as a nucleophile, potentially complicating mechanistic studies.

To provide a *Tetrahymena* ribozyme with a uniform 3′-terminus for both structural and mechanistic studies, we designed the double ribozyme shown in Fig. 2. The L-21 ribozyme sequence is followed by a hammerhead catalytic domain (24). Thus, self-cleavage of the hammerhead within the primary transcript should produce a *Tetrahymena* ribozyme with a discrete 3′ end at A410. Similar uses of a hammerhead ribozyme in *cis* to specify the 3′ end of an RNA transcript have been reported (25,26). Because hammerhead cleavage leaves a 2′,3′-cyclic phosphate terminus (27–29), the resulting 'L-21 A' form of the *Tetrahymena* ribozyme is expected to have its 3′ end blocked for transesterification. We find that L-21 A has the same activity as the L-21 *Sca*I ribozyme in the endoribonuclease reaction, where the ribozyme catalyzes transesterification with exogenous guanosine and therefore does not utilize its own 3′ terminus as a nucleophile. However, the L-21 A ribozyme has very little oligo(C) disproportionation activity relative to ribozymes with

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primarily G-OH 3'-termini, and its residual activity can be explained by the small amount of G-OH termini remaining in the L-21 A preparation.

MATERIALS AND METHODS

pTZL-21H2 Template. The *ScaI* endonuclease restriction fragment from pTZ18U (30) containing the f1 origin of replication was inserted into plasmid pT7L-21 (7) to make pTZL-21. Supercoiled pTZL-21, which has two *ScaI* sites, was digested with *ScaI* in the presence of 5 μg/ml ethidium bromide, which inhibits endonuclease digestion of linear DNA, to generate a linear molecule with a singie *ScaI* cut. Linear pTZL-21 was further digested with *Hin*dIII to remove a 30 bp fragment at the 3' end of the DNA, which was then replaced with a 48 bp synthetic oligonucleotide duplex containing a hammerhead ribozyme sequence. We used phagemid mutagenesis (31) to make subsequent changes in the hammerhead which yielded pTZL-21H2. The expected nucleotide sequence of RNA transcribed from this plasmid is given in Fig. 2. All cloned plasmids were characterized by dideoxy sequencing (32).

L-21 H2 RNA Transcription. In vitro transcription proceeded for 0.5–4 hr at 30°C under the following conditions: 5 μg/ml pTZL-21H2 DNA, CsCl banded and linearized with *Hin*dIII endonuclease; 40 mM Tris-HCl, pH 7.5; 15 mM $MgCl_2$; 1 mM of each nucleoside triphosphate; 2 mM spermidine; 5 mM dithiothreitol; and 500 units T7 RNA polymerase per mg of DNA. The polymerase was isolated from *Escherichia coli* strain BL21 containing plasmid pAR1219 (33). A working stock of L-21 A ribozyme was prepared from a 10 ml *in vitro* transcription reaction. It was separated from unprocessed L-21 H2 precursor and a minor product called MB (middle band) RNA by electrophoresis in a 4% polyacrylamide gel containing 7 M urea. The RNA was electroeluted from the gel slice, phenol/chloroform extracted, and precipitated with ethanol. The pellet was resuspended in 10 mM Tris-HCl, pH 7.5, 1 mM EDTA (TE) and stored at −20°C.

Removal of 2',3'-cyclic phosphate. Approximately 90 picomoles of gel-purified L-21 A RNA was incubated in 750 μl of filter-sterilized 1 M sodium formate, pH 3.5 at room temperature for 5 hr to open the 2',3'-cyclic phosphate resulting from hammerhead ribozyme cleavage (27). The pH was adjusted by addition of 75 μl 1 N NaOH and 13.5 μl 1 M Tris-HCl, pH 8. The RNA was subsequently treated with calf intestinal phosphatase (Dupont NEN) at 37°C for 30 min to remove the terminal phosphate from either the 2' or 3' position. After phenol/chloroform extraction, the RNA was precipitated with ethanol and resuspended in 50 μl TE.

Analysis of 3'-terminal nucleotide. RNA labeled at the 3' end with [^{32}]-pCp and RNA ligase (34) was completely digested in 4–14 μl reactions with 1 μl of 200 units/ml ribonuclease T1, 250 units/ml ribonuclease T2, and 50 μg/ml RNase A (37°C, 1–2 hr). Approximately 20,000 cpm in 2 μl of each sample was spotted on separate 10 cm^2 Kodak 13254 cellulose TLC plates with fluorescent indicator. A 2 μl sample of 10 mM unlabeled nucleoside 3'-monophosphates, Np, was also applied to the origin of each plate. The plates were developed in the first dimension

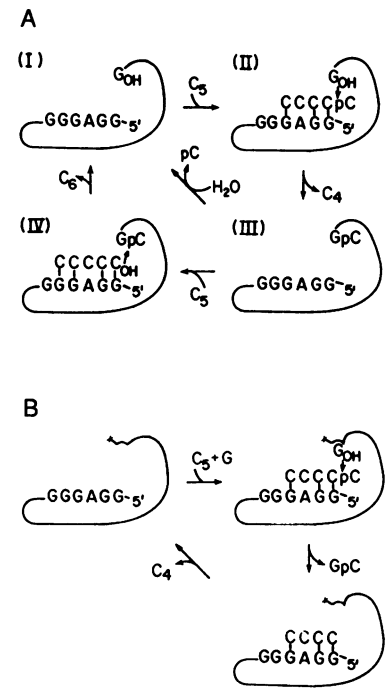

Fig. 1. The *Tetrahymena* ribozyme can catalyze reactions either involving or not involving its 3'-terminal G-OH. (A) Model for the mechanism of oligo(C) disproportionation (2,18). The free ribozyme (I) binds the C_5 substrate by Watson-Crick base-pairing and tertiary interactions (not shown; see ref. 9–11,13,14) such that the ribozyme's 3'-terminal guanosine (G414) can perform nucleophilic attack (II). The resulting transesterification reaction produced C_4 and the covalent intermediate (III), which has pC joined to the 3' end of the ribozyme. A second C_5 molecule can then bind and undergo reverse transesterification (IV), producing C_6 and regenerating the free ribozyme (I). The net reaction (outside pathway) is $2\ C_5 - C_4 + C_6$. Subsequent cycles produce longer and shorter oligomers of C. (B) Model for the mechanism of endonucleolytic cleavage of an oligopyrimidine by guanosine (3). The X at the 3'-end of the ribozyme indicates that this terminus does not participate in the reaction. The ribozyme binds C_5 in its 5'-exon-binding site and guanosine in its G-site (19,20). The resulting transesterification reaction produces C_4+GpC. The net reaction is $C_5 + G - C_4 + GC$.

(50:30:0.8, isobutyric acid:0.5 M NH_4OH:0.1 M EDTA; ref. 35) for 50 min and left to dry in a hood overnight. They were then developed in the second dimension (70:15:15:1, isopropanol:conc. HCl:H_2O:0.1 M EDTA) for 1.5 hr, dried, autoradiographed and scanned on a Molecular Dynamics Phosphorimager.

Endonuclease activity. The L-21 A and L-21 *ScaI* ribozymes were incubated with substrate, 5'-^{32}P-$G_2CCCUCUA_5$, in 10 or 20 mM $MgCl_2$ and 50 or 100 mM Tris-HCl, pH 7.5 at 50°C. Ribozyme (E), RNA substrate (S), and guanosine (G) concentrations were varied to measure different kinetic parameters. Substrate and ribozyme were pre-incubated separately at 50°C for 10 min. Reactions were initiated by mixing and time points were taken at 0, 0.25, 0.5, 0.75, 1, 2, and 7 min. Reactions were stopped by the addition of an equal volume of 50 mM EDTA, 10 M urea, 0.04% bromophenol blue, 0.04% xylene cyanol, 0.1×Tris-Borate buffer, pH 8.4 and subjected to electrophoresis in a 20% polyacrylamide 7 M urea gel. The dried gel was scanned on a Phosphorimager. A semilogarithmic plot of the fraction of substrate remaining (S_t/S_o, where S_t=the amount of substrate at time t) *vs* t was used to obtain k_{obs} ($k_{obs} = -$slope). Plots were linear for at least 2 half-lives.

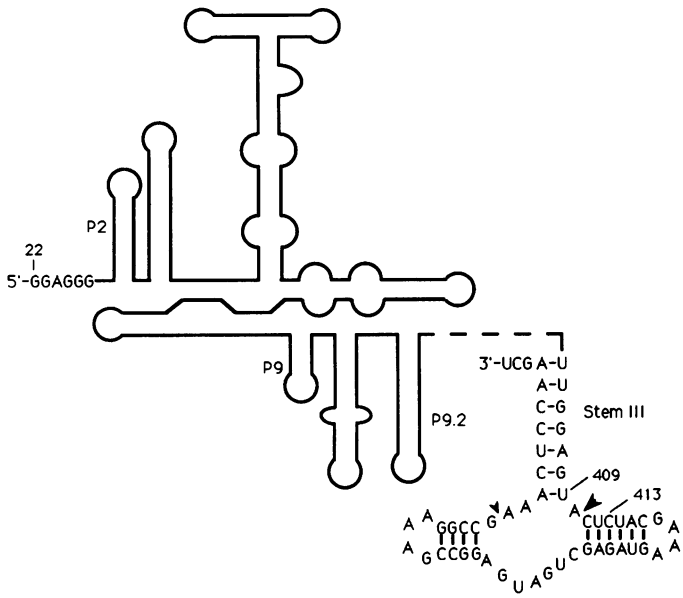

Fig. 2. L-21 H2 RNA, portrayed in its expected secondary structure. The L-21 ribozyme extends from G22 through C413, and includes the solid line where the sequence is not explicitly shown. The nucleotides at the 3′ end were designed to form a hammerhead ribozyme domain to cleave the IVS after A410 (large arrow). The resulting L-21 A RNA is expected to be one nt longer than an L-21 *ScaI* RNA produced by precise transcription termination. The small arrow indicates the approximate site of IVS ribozyme-catalyzed hydrolysis that is thought to produce MB RNA during transcription. This longer RNA is also an active ribozyme (see text). Secondary structure of IVS RNA from (21–23); secondary structure of hammerhead ribozyme from (24).

Oligo(C) disproportionation reaction. Reactions with pentacytidylic acid and four different ribozymes were performed in 50 mM Tris-HCl, pH 7.5, 20 mM $MgCl_2$ at 42°C, the same ionic conditions and temperature used in previous studies (2,36). The concentration of ribozyme was 0.25 μM, and the concentration of C_5 was either 1.25 μM or 5 μM. Control reactions to monitor endoribonuclease activity contained 0.5 mM guanosine. Substrate and ribozyme were pre-incubated separately at 42°C for 10 min prior to mixing at t=0. Reactions were processed and data collected as described above.

RESULTS AND DISCUSSION

Synthesis and characterization of the L-21 A ribozyme

Plasmid pTZL-21H2 (constructed as described in Methods) was transcribed with phage T7 RNA polymerase at 30°C. The RNA transcript (Fig. 2) underwent efficient self-cleavage during transcription. As shown in Fig. 3, the major product (L-21 A) co-migrated with L-21 *ScaI* RNA during gel electrophoresis, while a minor product (MB, middle band) had an electrophoretic mobility intermediate between those of the primary transcript and the major L-21 A product. Changing the transcription conditions influenced the ratio of L-21 A RNA to MB RNA. Raising the $MgCl_2$ concentration from 15 mM to 50 mM increased the ratio, while raising the temperature from 30°C to 37°C reduced it.

The efficiency of L-21 A production was higher with pTZL-21H2 than with several other hammerhead-containing constructs (data not shown). For example, an earlier construct with a stem III of 4 base pairs connecting the *Tetrahymena* IVS

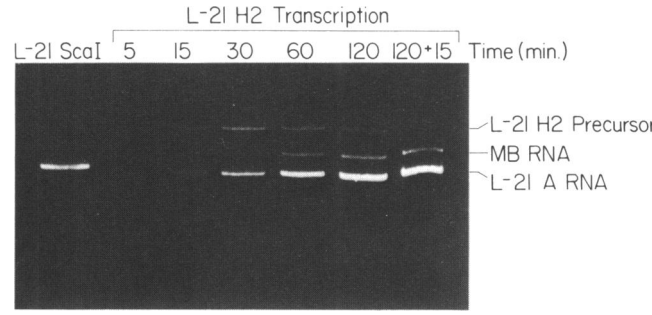

Fig. 3. Transcription and processing of L-21 H2 RNA *in vitro* to produce the L-21 A ribozyme. Transcription using pTZL-21H2 DNA and phage T7 RNA polymerase proceeded for 5–120 min at 30°C. Samples were analyzed by 4% polyacrylamide/urea gel electrophoresis. Ethidium-bromide stained gel is shown. Lane (120+15), 120 min transcription followed by 15 min incubation at 50°C. This treatment leads to additional processing of the L-21 H2 precursor, but appears to favor production of the undesired MB RNA.

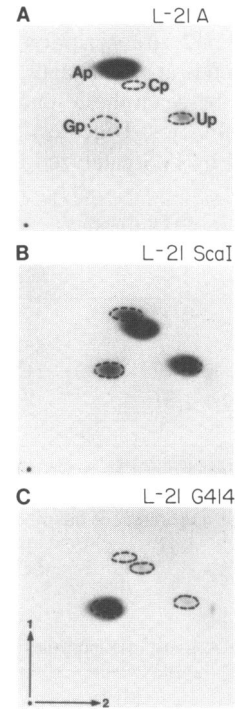

Fig. 4. 3′-Terminal nucleotide analysis of L-21 A, L-21 *ScaI* and L-21 G414 ribozymes. Outlining indicates position of the four unlabeled nucleoside 3′-monophosphate markers as visualized by UV shadowing.

and the hammerhead was found to cleave much less efficiently at both 30°C and 50°C than the RNA with the 7 base pair stem III shown in Fig. 2. Stabilizing stem III by increasing the number of base pairs has been previously shown to enhance hammerhead cleavage (37).

When the transcription products (Fig. 3) were treated with RNA ligase and [^{32}P]-pCp, only the L-21H2 precursor and the MB RNA were labeled, indicating 3′-hydroxyl termini. The L-21 A RNA was not labeled, consistent with it containing a 2′,3′-cyclic phosphate terminus produced by hammerhead self-cleavage. This RNA was treated sequentially with mild acid and calf intestinal phosphatase to open the putative 2′,3′-cyclic

phosphate and remove the phosphate monoester. It was then 3′-end-labeled with RNA ligase and [^{32}P]-pCp. Sequencing by the enzymatic method (38) gave the expected sequence (Fig. 2), beginning with U409; the terminal residue cannot be determined by this method.

The identity of the 3′-terminal nucleotide of L-21 A and MB RNAs was determined by complete RNase digestion and two-dimensional thin-layer chromatography (Fig. 4 and data not shown). For comparison, the same analysis was performed on two other ribozymes: L-21 *Sca*I RNA, prepared by run-off transcription (7); and L-21 G414 RNA, the 3′ end of which is produced by IVS-catalyzed hydrolysis at the natural 3′ splice site (G414) (3,39,40). The L-21 *Sca*I RNA had the most heterogeneous 3′ end, with a distribution of nucleotides quite similar to that determined earlier for a different preparation of that ribozyme (Table 1). The L-21 G414 RNA ended primarily in G, as expected. L-21 A ended predominantly in A; combined with the sequence analysis, this confirmed the site of hammerhead self-cleavage at A410 as designed (Fig. 2).

MB RNA ended primarily in G (Table 1). Although the enzymatic sequence of MB RNA could not be obtained, 5′ end-labeling of pTZL-21H2 transcription/processing products revealed an oligonucleotide approximately 12 nt long which could represent the distance between the 3′ end of MB RNA and the 3′ end of the initial transcript. Together, these data suggest that MB RNA is produced by IVS-catalyzed hydrolysis following a G residue within the hammerhead catalytic domain (small arrowhead in Fig. 2). Such a reaction would be analogous to 3′-splice site hydrolysis following G414 in *Tetrahymena* pre-rRNA (39).

Table 1. 3′ Terminal Nucleotide Distribution

Ribozyme	A	C	G	U
L-21 A	93	–	2	5
L-21 G414	1	6	85	8
L-21 *Sca*I	9	49	11	31
L-21 *Sca*I*	12	47	9	32
MB	5	–	87	7

Percent of each nucleotide determined by analysis of chromatographs such as those shown in Fig. 4.
*Analysis of an independent preparation of this ribozyme as reported by Zaug *et al.* (7).

Endonuclease activity of L-21 A

The endoribonuclease activities of the L-21 A and L-21 *Sca*I ribozymes were compared side-by-side using an oligoribonucleotide substrate (S) 5′-^{32}P-GGCCCUCUAAAAA. (This oligoribonucleotide forms a matched duplex with the substrate-binding site of the ribozyme). The two ribozymes had the same activity under a variety of conditions (Table 2). The correspondence of the observed reaction rate (k_{obs}) to rate constants for individual steps in the cleavage of the matched RNA substrate has been described by Herschlag et al. (10,12) for the L-21 *Sca*I ribozyme (50°C, 10 mM $MgCl_2$). For the L-21 *Sca* I ribozyme, $(k_{cat}/K_m)^S$, the second-order rate constant for reaction of E·G and free S, represents binding of S. The second order rate constant for reaction of E·S and free G, $(k_{cat}/K_m)^G$, represents the rate of the chemical step. Finally, $k_c(-G)$ is the rate of the chemical step of the guanosine-independent hydrolysis reaction. For $(k_{cat}/K_m)^G$ and $k_c(-G)$, a high concentration of E was used to promote rapid and quantitative binding of S; evidence that the concentration was high enough is provided by the observation that a 2-fold increase in ribozyme concentration had no effect on the reaction rate (lines 3–4 and 7–8 of Table 2). The finding that each of these kinetic parameters is very similar for the L-21 A and L-21 *Sca*I ribozymes is expected, because none of these reactions is thought to depend on a ribozyme 3′-terminal hydroxyl group.

The L-21 A ribozyme, with its 2′,3′-cyclic phosphate 3′-terminus, remains capable of catalyzing sequence-specific cleavage of the RNA substrate in the absence of G (Table 2). This observation provides strong additional support for our previous conclusion (10) that G-independent cleavage can be largely explained as hydrolysis, rather than transesterification by those L-21 *Sca*I ribozyme molecules that end in G-OH. However, guanosine-independent cleavage by the L-21 *Sca*I ribozyme may be slightly faster than that by the L-21 A RNA (0.4 min^{-1} *vs*

Table 2. Endoribonuclease Activity of Ribozymes at 50°C

Ribozyme	[E] nM	[S] nM	[G] μM	k_{obs}[a] min^{-1}	$(k_{cat}/K_m)^S$ 10^8 $M^{-1}min^{-1}$	$(k_{cat}/K_m)^G$ 10^5 $M^{-1}min^{-1}$	$k_c(-G)$ min^{-1}
L-21 A	10	0.04	800	1.2	1.2[b]		
	50	0.5	2	1.2		6[c]	
	50	0.5	0	0.3			0.3[d]
	100	0.5	0	0.3			
L-21 *Sca*I	10	0.04	800	1.0	1.0[b]		
	50	0.5	2	1.2		6[c]	
	50	0.5	0	0.4			0.4[d]
	100	0.5	0	0.4			

First row of determinations for each ribozyme ($(k_{cat}/K_m)^S$) was done with 20 mM $MgCl_2$; all other measurements with 10 mM $MgCl_2$. [More detailed experiments with the L-21 *Sca*I RNA have shown only modest differences between 10 and 20 mM $MgCl_2$. The $(k_{cat}/K_m)^S$ rate constant increases by ~50% when $MgCl_2$ is increased from 10 and 20 mM. Because the rate of the chemical step also increases slightly with higher Mg^{2+}, it is likely that binding of S continues to be rate-limiting for $(k_{cat}/K_m)^S$ at 20 mM $MgCl_2$ (T. McConnell, D. Herschlag, and T.R.C., unpublished).]
[a]In any given experiment, the determination of k_{obs} is quite precise: the linear least-squares fit to each data set gave a correlation coefficient r in the range $r = -0.94$ to -0.98 ($r_{ave} = -0.97$), where an exact fit to a straight line with negative slope would give $r = -1$. However, possible inaccuracies in [E] and possible preparation-to-preparation variations in ribozyme activity limit the accuracy of each value to approximately a factor of 2 (for a more complete discussion see ref. 10).
[b]Calculated as $(k_{cat}/K_m)^S = k_{obs}/[E]$ (ref. 10).
[c]k_{obs} was first corrected to account for the contribution of binding of S as described (12); the correction amounted to about 20% for k_{obs}(2 μM G) and was insignificant for $k_{obs}(-G)$. The corrected values of k_{obs} were then used to calculate $(k_{cat}/K_m)^G = (k_{obs}(2\ \mu M\ G) - k_{obs}(-G))/[G]$ (ref. 11).
[d]$k_c(-G) = k_{obs}$ (ref. 10).

0.3 min^{-1} in side-by-side comparisons, Table 2). It is therefore possible that some of the reaction of the former ribozyme might proceed by transesterification using a 3′-terminal G. Note that 9−11% of the L-21 *Sca*I RNA has a 3′-terminal G-OH (Table 1).

Oligo(C) disproportionation activity of ribozymes with different 3′-termini

The activity of the L-21 A ribozyme in disproportionation of pC_5 was compared to that of the L-21 *Sca*I and L-21 G414 ribozymes (Fig. 5). The L-21 A ribozyme had only 1−2% the disproportionation activity of L-21 G414 (Table 3, and data not shown for an experiment at 5 μM S, 0.25 μM E). A control experiment in the presence of 0.5 mM G showed that the L-21 A ribozyme had normal activity as an endonuclease (Table 3). Thus, the low disproportionation activity of L-21 A RNA does not reflect a low inherent activity of the ribozyme under these conditions.

The data of Table 3 lead to the hypothesis that all three preparations of ribozyme might be catalyzing disproportionation by the mechanism of Fig. 1A, which requires a free 3′ G-OH. That is, the 15% residual disproportionation activity of the L-21 *Sca*I RNA equals the percent of molecules with a 3′-terminal G-OH within a factor of 2 (Table 3). [Note that ribozymes with 3′-terminal G-OH groups on 'tethers' of different length and sequence could have different reaction rates.] Similarly, the L-21 A ribozyme might have ~2% residual disproportionation activity because ~2% of the molecules in the population have G-OH 3′-termini.

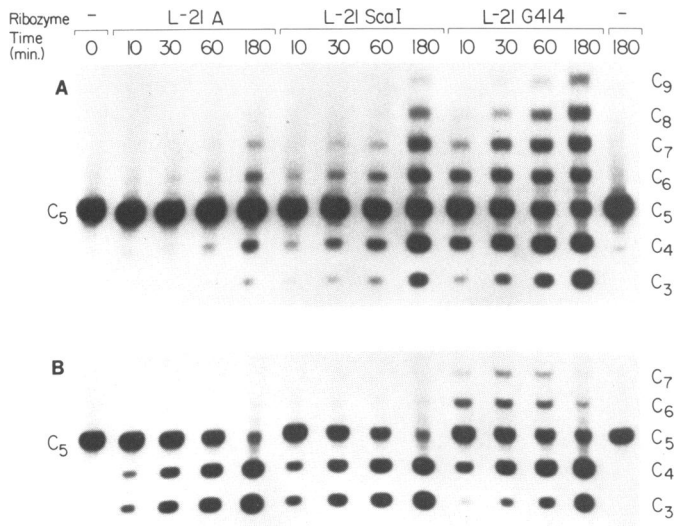

Fig. 5. Comparison of activity of ribozymes in reactions with pentacytidylic acid. (A) Disproportionation of 1.25 μM [5′-^{32}P]-pC_5; reaction described in METHODS. (B) Reactions as in A with the addition of 0.5 mM guanosine, which cleaves the oligo(C) by transesterification.

Table 3. C_5 Disproportionation Activity of Ribozymes at 42°C

Ribozyme	Fraction 3′-terminal G*	k_{obs} (min^{-1}) $[C_5]$ = 1.25 μM	$[C_5]$ = 1.25 μM, [G] = 0.5 mM
L-21 A	0.02	0.0003	0.02
L-21 *Sca*I	0.11	0.003	0.03
L-21 G414	0.85	0.02	0.03
MB	0.87	0.02	0.03

[E]=0.25 μM. [S] was subsaturating (data not shown), as expected based on K_m=42 μM measured for pC_5 under these conditions with a different form (L-19) of this ribozyme (2).
*from Table 1.

This hypothesis requires that ribozymes with 3′-terminal G-OH groups at certain positions other than 414 be active in using their 3′-terminal G as a nucleophile. One likely source of the 2% residual 3′-terminal G in the L-21 A RNA preparation is MB RNA, which migrates near L-21 A on a preparative electrophoresis gel. Tests of the enzymatic activity of MB RNA revealed it to be fully active in both oligo(C) disproportionation and endoribonucleolytic cleavage of RNA (Table 3). Thus, the residual disproportionation activity of the L-21 A ribozyme preparation could be explained by 2% contamination with MB RNA. Alternatively, some or all of the residual activity could be due to a different mis-cleaved form of the IVS that co-migrates with L-21 A RNA.

Earlier attempts to synthesize the ribozyme without its 3′-terminal G-OH have involved runoff transcription at a restriction site that precedes G414 (e.g., *Sca*I), or removal of the 3′ end by periodate oxidation and β-elimination (which should leave a 3′-phosphate). The removal of G414 was found to greatly reduce disproportionation activity, but surprisingly did not eliminate it (36,41). An interesting explanation for this residual activity was suggested by Kay & Inoue (36): the ribozyme might catalyze 'pyrimidine exchange' (the direct attack of one pC_5-OH on an internal phosphorus atom of another pC_5, without formation of the covalent nucleotidyl ribozyme intermediate of Fig. 1A). These same authors (36) stated an alternative possibility, that a 3′-heterogeneous population of ribozyme might contain some molecules with a G-OH 3′ end and therefore catalyze oligonucleotide disproportionation through a covalent intermediate. Our data clearly support the latter hypothesis. How, then, could the β-eliminated ribozyme retain disproportionation activity? A reasonable hypothesis is that the β-eliminated ribozyme first transfers its 3′-terminal phosphate to the 3′ end of pC_5 using its known transphosphorylation activity (42). If ribozyme molecules without 3′-hydroxyl groups catalyze direct pyrimidine exchange, the rate is <1% that of the reaction that utilizes a terminal G-OH.

CONCLUSIONS

A self-cleaving hammerhead RNA domain has been inserted at the 3′ end of the L-21 form of the *Tetrahymena* ribozyme. The resulting L-21 A ribozyme has a 3′ end at a specific nucleotide (A410) and terminates with a 2′,3′-cyclic phosphate. The homogeneity of this ribozyme relative to previous constructs should make it a preferred form for structural studies, including attempts at crystallization. The blocked 3′-terminus should simplify certain mechanistic studies: unlike preparations of the L-21 *Sca*I ribozyme, which contain some molecules with a 3′ terminal G-OH that can be used as a nucleophile, the L-21 A ribozyme appears to be restricted to catalyzing reactions using an external nucleophile (such as guanosine).

The use of hammerhead domains for specifying precise termini on long RNA transcripts has general utility. In addition to this and other examples where 3′ termini are specified on *in vitro* RNA transcripts (25,26), unique ribozyme 5′-termini can be formed by hammerhead cleavage (C. Rusconi, A. Zaug, J. Piccirilli and T.R.C., unpublished data).

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