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LETTER TO THE EDITOR

Effects of 3′-terminal phosphates in RNA produced by ribozyme cleavage

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In vitro runoff transcription using T7 RNA polymerase has been the method of choice to produce milligram quantities of RNA for structural studies. Unfortunately, the T7 enzyme often adds one or more extra nucleotides at the 3′ end, which results in a heterogeneous RNA product (Milligan et al., 1987). This heterogeneity can be observed at the 5′ end as well, depending on the transcription template (Ferre-D'Amare & Doudna, 1996). The lack of homogeneity, which potentially is deleterious for structural studies, can be overcome with the use of *cis*-and/or *trans*-acting ribozymes, which produce clean RNA ends after their catalytic reaction (Ferre-D'Amare & Doudna, 1996). This procedure has been scaled up for large quantities of RNA for NMR and X-ray crystallographic studies, and is useful for purification of larger RNAs (greater than 50 nt) where single-nucleotide resolution by gel electrophoresis is difficult. Ribozymes that have been used in this manner include the hairpin ribozyme, the hammerhead ribozyme (HH), the hepatitis delta ribozyme (δ), and the *Neurospora varkud* satellite RNA ribozyme (VS) (Guo & Collins, 1995; Price et al., 1995; Ferre-D'Amare & Doudna, 1996). All of these ribozymes leave a 5′-hydroxyl and a 3′-cyclic phosphate as products of cleavage.

During the course of a structural investigation of a selenocysteine insertion element from rat Type 1 iodothyronine 5′-deiodinase (D1 SECIS), we wished to prepare milligram quantities of an RNA that comprised the upper stem/loop of this RNA structure (41 nt; Fig. 1A). This stem/loop is a stimulator required for the insertion of selenocysteins at a specific internal UGA stop codon (Berry et al., 1991). Construction of the upper stem/loop RNA required that the base of the stem end with four noncanonical base pairs (Walczak et al., 1996), with no additional base pairing below this terminal quartet, as determined from in vitro assays (Martin et al., 1998). This RNA has been produced synthetically for crystallization trials, so we sought a thorough comparison between this molecule and the RNA produced by run-off transcription. Because the RNA to be studied begins with an adenosine and ends in a uridine, a ribozyme was used at each end to produce homogeneous RNA. The system used to accomplish this has been described previously (Ferre-D'Amare & Doudna, 1996) and the resulting T7-produced RNA would differ from the synthetic RNA only by the 3′-cyclic phosphate.

In making preliminary imino proton NMR assignments for both of these RNAs, we concluded that the base pairing for both RNAs is nearly identical, as evident from the one-dimensional NMR spectra collected in 90% H_2O (Fig. 2). Close inspection shows that there are subtle differences near the RNA helix end (bracketed region). The terminal U-U iminos of the ribozyme-cleaved RNA stem/loop are not observed at 25 °C, or these imino protons have altered chemical shifts and become degenerate with other iminos of the G-U base pairs (assignments to be presented elsewhere). In addition, the two preceding G-A base pairs change in chemical shift and are broadened, as evident in spectra collected at several temperatures (25 °C and 5 °C; Fig. 2 and insets). The resonance broadening results in a loss of NMR detection at temperatures above 35 °C. At the same temperature, these signals are detected with the synthetic RNA. These NMR spectral differences were not due to either 3′- or 5′-heterogeneities as determined by electrospray mass spectrometry of each RNA (Fig. 2). The observed spectral differences can be interpreted in at least two manners. The broadening could arise from flattening of the ribose pucker (O4′-endo or O4′-exo) imposed by the cyclic phosphate at the 3′ terminus (as seen with cyclic nucleo-

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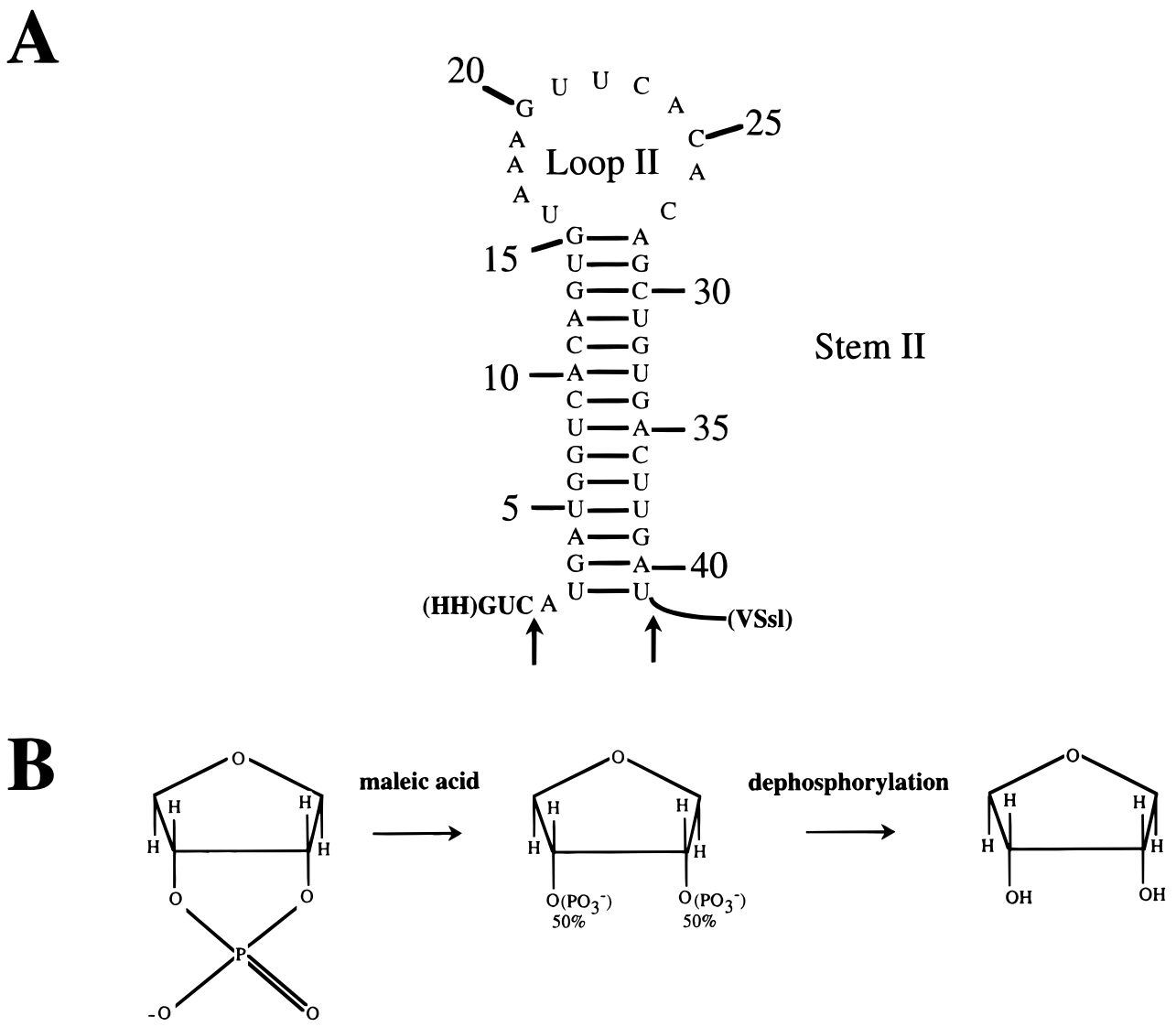

FIGURE 1. A: Secondary structure of the upper stem/loop from rat D1′ SECIS. Arrows represent the cleavage sites for the *cis*-hammerhead and the VS *trans*-ribozymes. VSsl represents the position of the VS stem/loop sequence required for trans VS cleavage and (HH)GUC represents the position of the required nucleotides for *cis*-hammerhead cleavage. **B:** Flow chart of the steps required for complete decyclization and dephosphorylation of 3′-cyclic phosphate termini. The 2′–3′-phosphate mixture is represented by $O(PO_3^-)$ in the second step.

tides; Saenger, 1984). This in effect could destabilize the last base pair by placing the sugar in a conformation away from a sugar pucker (3′-endo) that favors base pairing. In this case, the broadening (and imino exchange rates) could result from an increase in base pair opening equilibrium. Here the exchange rate is limited by the rate of opening/closing of the dynamic base pair. The broadening can alternatively be interpreted as a direct effect in the intrinsic catalytic exchange rate at these sites as a result of the cyclic phosphate in the ribozyme-cleaved molecule.

To test the hypothesis that suggests a destabilization of the terminal U-U base pair, the terminal cyclic phosphate was opened with maleic acid treatment (Abrash et al., 1967; Buzayan et al., 1986), and melting curves and ^{1}H NMR spectra were re-collected for this RNA. The decyclization can be scaled up to milligram quantities and is complete within 5 h as determined by mass spectrometry (not shown). This results in a 3′ terminus that has a mixed population of 2′- and 3′-phosphates (Fig. 1B), which have identical masses (Fig. 2). The melting transition of the decyclized T7 SECIS RNA did not change appreciably from the form with the cyclic phosphate (63.0 ± 1.0 °C versus 61.8 ± 1.0 °C) and is not significantly different from the chemically synthesized RNA (62.2 ± 1.5 °C). Inspection of the 1D NMR spectrum of the T7-produced RNA after maleic acid treatment (Fig. 2) shows that it closely resembles that of the synthetic RNA, with the G-A imino at 9.44 disappearing and two new iminos at 9.66 and 9.74 ppm

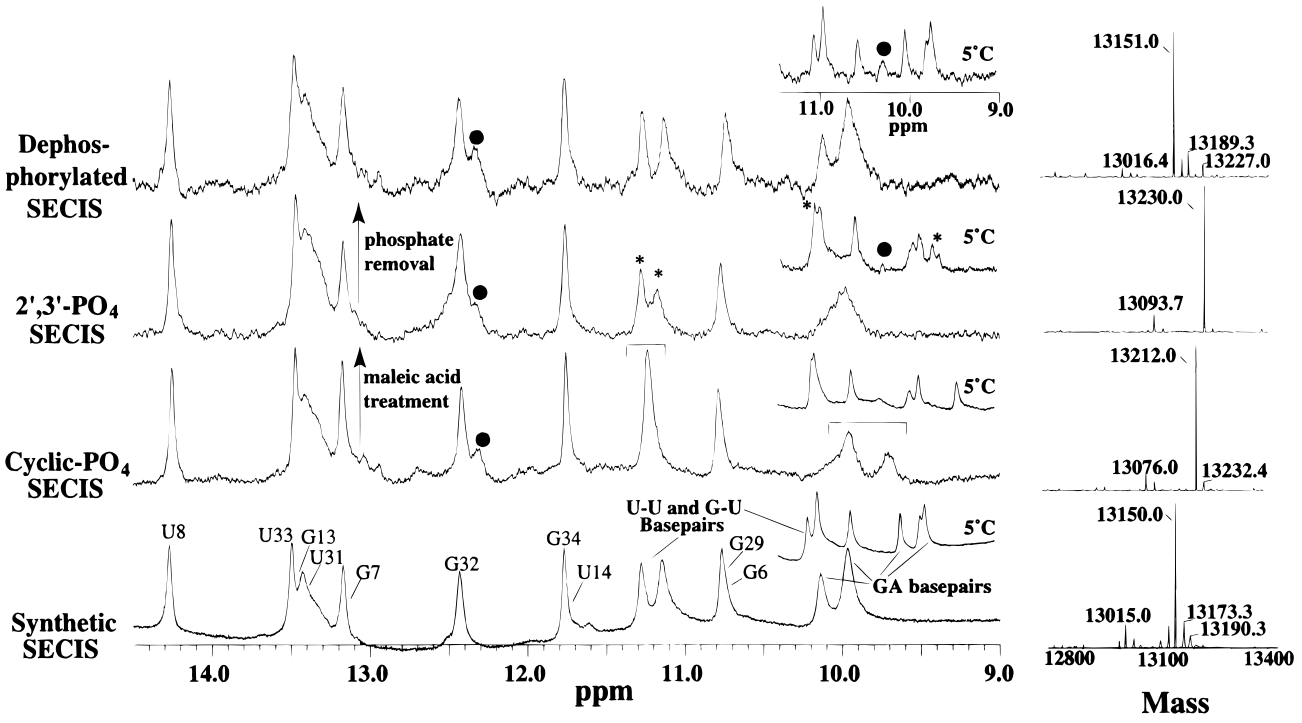

FIGURE 2. One-dimensional proton NMR spectra recorded in 90% H_2O (10 mM Na-phosphate, 50 mM NaCl, 100 μM EDTA, pH 6.80) at 500 MHz and 25 °C showing the imino regions for the synthetic SECIS RNA; the T7-produced SECIS with the cyclic-phosphate terminus; the T7-produced SECIS RNA after the decyclization reaction with maleic acid (details below); and the T7-produced RNA after removal of terminal phosphates (3′- and 2′-phosphates). Inset regions represent the upfield imino NMR spectral regions (11.5–9.0 ppm) at 5 °C for each. Processed electrospray mass spectra showing molecular weights for each RNA are included to the right of each NMR spectrum. Bracketed regions on the cyclic-phosphate RNA NMR spectrum point out the differences in the G-A base pair imino protons between the synthetic and T7-produced RNAs. Asterisks over the RNA with the opened terminal phosphates illustrate the regions of change after the decyclization reaction. Filled circles highlight an alternate form of this RNA that has not been assigned. Transcription reactions (20 mL) contained: 750 μL of linearized plasmid or 1 μM linear DNA templates, 25 mM $MgCl_2$, 8% PEG 8000, 50 μg/mL T7 RNA polymerase, 4 mM each NTP, 40 mM Tris-Cl, pH 8.0, 1 mM spermidine, 5 mM DTT, and 0.01% Triton X-100. The reactions were incubated 4 h at 37 °C prior to phenol/chloroform treatment (pH 4.5) and RNA precipitation from 0.3 M sodium-acetate and ethanol. The VS *trans*-ribozyme cleavage was performed in the transcription buffer without PEG 8000 after the RNA T7 product was dialyzed against distilled H_2O using Amicon concentrators. The efficiency of this *trans*-cleavage was increased by including several 3-min 95 °C denaturing steps over the 12-h cleavage period at 37 °C in a PCR machine. RNA was prepared for mass spectrometry by five consecutive precipitations from 3.5 M ammonium acetate and ethanol. The maleic acid treatment was completed as described previously at an RNA concentration of 1 mg/mL (Abrash et al., 1967; Buzayan et al., 1986), whereas dephosphorylation was performed as described earlier (Morse & Bass, 1997) using T4 polynucleotide kinase and shrimp alkaline phosphatase. The cyclic phosphate can also be opened with a mild HCl treatment (Forster et al., 1990). RNAs were gel-purified using 16% PAGE, electroeluted into 0.5× TBE prior to ethanol precipitation and dialysis into the NMR buffer. All one-dimensional NMR spectra were recorded using simple jump–return read pulses for water suppression. Mass spectra were obtained on a Micromass Quattro II mass spectrometer equipped with negative-ion electrospray ionization. RNA products were reconstituted in a solution consisting of 50% water, 47% acetonitrile, and 3% triethylamine, and were introduced into the mass spectrometer by direct infusion at a rate of 3 mL/min. Mass spectra were acquired over the mass range of 650–1050 *m/z*, with a core voltage of 32 eV. Multiply charged molecular ions were deconvoluted using MaxEnt software (Micromass, ver. 2.22) to generate molecular weight peaks in the spectra shown.

(5 °C insets). This most likely is two forms of the G-A pair closest to the helical 3′ end. The terminal U-U base pair imino also changes chemical shift (~11.25 ppm), nearly resembling that of the chemically synthesized RNA. Effects of the maleic acid treatment are restricted to the last three base pairs. Although the chemical shifts of the acid-treated RNA are near those of the chemically synthesized RNA, the same resonances seem to be broadened in comparison to the chemically synthesized RNA (Fig. 2 at 25 °C). This broadening mechanism must include a terminal phosphate in some form.

To test if it was a terminal phosphate(s) that was inducing this broadening, we removed both terminal phosphates using a combination of T4 kinase and shrimp alkaline phosphatase treatments (Morse & Bass, 1997). The removal of both phosphates was complete as monitored by the mass of the RNA (Fig. 2). The imino spectrum of the dephosphorylated RNA shows that it adopts an identical conformation to that of the synthetically produced RNA, whereas the broadened resonances of the ribozyme-produced RNA return to linewidths and temperature dependencies of the chemically synthesized RNA (Fig. 2). Thermal melting stud-

ies showed no significant differences in T_m between the three RNAs (synthetic, 62.2 ± 1.5 °C; cylic phosphate terminus, 61.8 ± 1.0 °C; and mixed 2′- and 3′-phosphate terminus, 63.0 ± 1.0 °C). This implies that either the broadening is a manifestation of a local unfolding phenomenon, with any effect on the global T_m being too subtle to detect in this manner, or that the broadening was the result of a terminal phosphate catalyzing exchange of nearby imino protons in the absence of enhanced open/closed ratio of these base pairs (the terminal three are this case).

It is well known that imino proton exchange is both base and acid catalyzed (Gueron & Leroy, 1995). In addition, phosphate, when used as a buffer can act as a effective extrinsic catalyst for imino exchange preferentially at helical termini and at A-U base pairs (Fritzsche et al., 1983). To test if the broadening seen in the T7-produced RNA was a result of phosphate-catalyzed imino exchange, we investigated the change in longitudinal relaxation (T_1) as a function of sodium phosphate buffer concentration using the synthetically produced RNA. This RNA has no terminal phosphates. All resolvable imino resonance had T_1 relaxation times measured over a phosphate concentration range from 10 to 250 mM at 5 °C (pH 6.80). As seen in Figure 3, four of the nine measured imino resonances have T_1s that are strongly dependent on phosphate concentration, with marked reduction in relaxation times with an increase in phosphate buffer concentration. Two of these are G-A iminos, one is a U degenerate with several U iminos in U-U pairs, and the fourth is a G-U imino above the quartet (U-U, A-G, G-A, U-U). The enhancements in relaxation rates arise from the catalyzed imino exchange contribution to the longitudinal relaxation. Enhancement of longitudinal relaxation rates at two G-A positions as well as the U-U pairs most likely arises from the intrinsic phosphate catalyst at the 3′ end of the T_7 RNA. The cyclic phosphate, in effect, raises the local concentration of the phosphate catalyst at the helix terminus. This exchange enhancement is not lim-

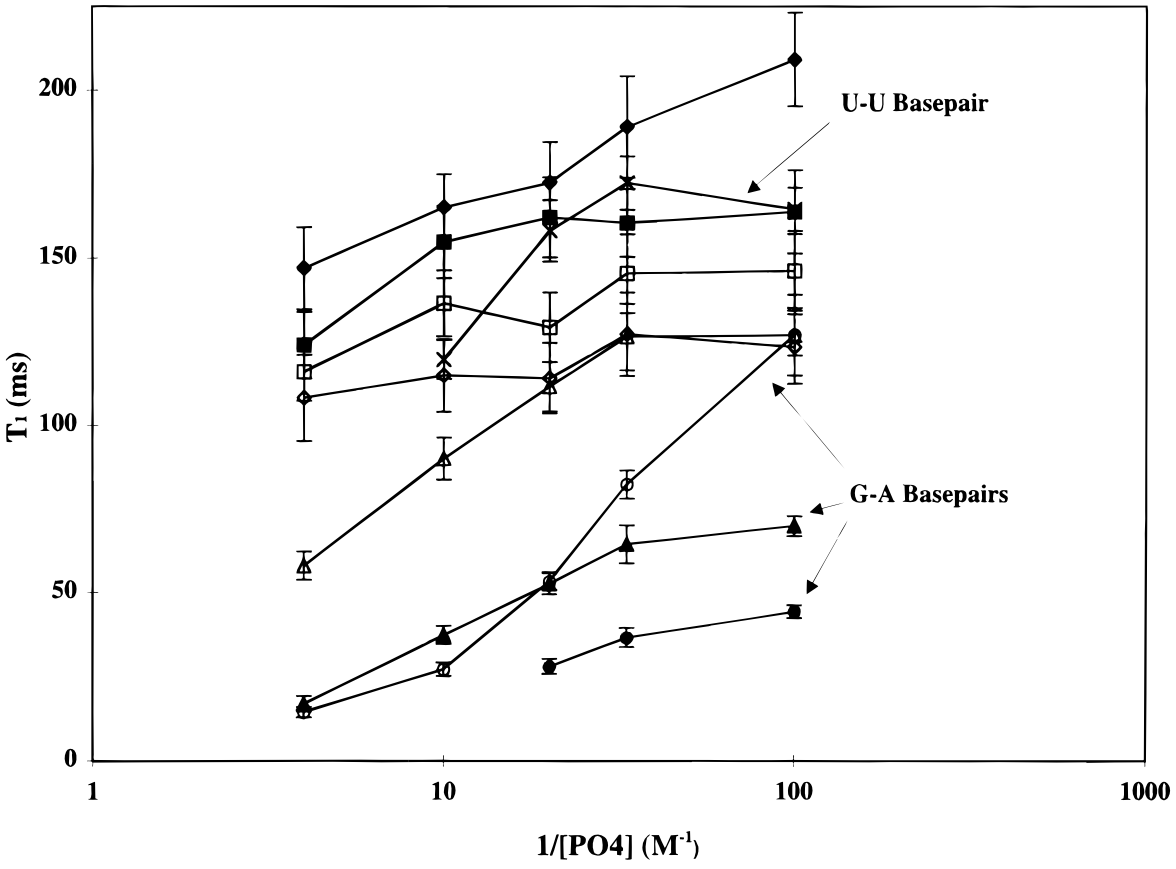

FIGURE 3. Longitudinal relaxation times (T_1) of iminos of the synthetic SECIS RNA as a function of inverse phosphate concentration. T_1 relaxation times were calculated using the inversion recovery method (Fritzsche et al., 1983) at pH 6.80, 50 mM NaCl, 100 μM EDTA, and fit to the theoretical exponential using a nonlinear fitting routine. Error bars represent R^2 values for each nonlinear fitting to the theoretical T1 function. The phosphate concentration of the RNA solution was adjusted by adding lyophilized solid sodium phosphate (pH 6.80) to make the final concentrations 10 mM, 30 mM, 50 mM, 100 mM, and 250 mM. All NMR experiments were collected at 5 °C in 90% H_2O. Longitudinal relaxation times for imino protons in the chemically synthesized RNA at 5 °C are represented as follows: 14.12 ppm (♦), 11.20 ppm (×), 12.19 ppm (□), 10.009 ppm (▲), 9.80 (●), 9.665 (○), 10.663 (△), 11.59 (◇), 12.94 ppm (■).

ited to the cyclic phosphate form because the T_1s of these same imino protons are enhanced in the RNA form that has both 3′- and 2′-phosphates at the 3′ terminus (Table 1).

The chemical shift differences between the three forms of the RNA are not completely understood. The shift differences (the last U-U pair and the last two G-A pairs) of the ribozyme-produced RNA that has the cyclic phosphate may arise from slight conformation alteration at the helical end brought about by the restrained sugar conformation. This could be explained by the flattening of the ribose pucker brought about by the cyclic phosphate. This subtle difference may change the stacking geometry of the last three pairs and result in shifts of the terminal G-A iminos. The cyclic phosphate does not appear to abolish the last base pair as suggested by the similar T_ms in all forms of the RNA, but does appear to produce a structural perturbation in the G-A tandem of the core region. Another structural alteration takes place as a result of the mixture of 2′- and 3′-phosphates at this helical end. Effects of 5′-phosphates in RNA and DNA can have differing trends depending on the secondary structure. DNA triplexes seem to be destabilized by a 5′-phosphates (Yoon et al., 1993), whereas RNA duplexes are stabilized by this phosphate (Freier et al., 1985). DNA duplexes do not have their melting temperatures changed appreciably (Yoon et al., 1993). Phosphates at the 3′ end in RNA have only small destabilization effects (Freier et al., 1985). Here, all forms of the 3′-terminal phosphate act similarly to the 3′-phosphate in RNA duplexes, with little thermodynamic alteration in the global melting temperature. Therefore, the initial resonance broadening observed in the ribozyme-produced SECIS RNA is the result of imino exchange catalysis by a terminal phosphate, and can happen in the absence of observable increased instability at the helix end. This can be explained by the nature of the GA tandem base pairs. Both of the G-A pairs in the core region of this RNA are expected to have a "sheared" geometry (Walter et al., 1994; Walczak et al., 1996, 1998; Wu et al., 1997). In this geometry, the iminos of these two Gs are not involved in hydrogen bonding of the pair, and are protected from exchange with solvent only by the two bases above and below. These iminos are placed in the major groove of the helix, and may be available for catalysis of exchange without opening of the base pair.

The use of *trans*-acting ribozymes to produce large quantities of homogeneous RNAs has been well documented. We present reasons that merit careful construction of RNAs, with special consideration of how to end helices that need to end in noncanonical base pairs. The presence of a 3′-phosphate, in the case of the SECIS RNA, increases the solvent exchange of this U-U pair and the iminos of the two preceding G-A base pairs. This effect is most severe with cyclic phosphate termini, followed by the mixed-isomer terminal phosphates. Although these observations may be unique to this RNA, the above terminal effects should be considered when designing RNA molecules for structural studies. A terminal phosphate in the context of the RNA at the 3′ end seems to contribute to the longitudinal relaxation rates of these iminos to the same extent as the RNA with mixed 2′–3′-phosphate termini. Both of these phosphate termini have their T_1s shortened to a smaller extent than the cyclic phosphate-ending RNA. Therefore, exchange broadening may be reduced for iminos at helical termini by extending the RNA one nucleotide past the last base pair, even when produced by the ribozyme method. If longer imino T_1s are sought, ending helices with a nucleotide lacking a phosphate could be considered. This will be most applicable to groups studying RNA structure by NMR methods, where observations of these protons may be critical. The simplest way to achieve this is by simple runoff transcription. In addition, we have presented a protocol where 3′-cyclic phosphates can be removed from RNA in milligram quantities, continuing to make the ribozyme-aided method available to produce large amounts of homogeneously sized RNAs.

TABLE 1. Longitudinal relaxation times (ms) of SECIS RNA imino protons at 5 °C.

Peak (ppm)	SECIS CP[a]	SECIS decyclic[b]	CS [10 mM][c]	CS [100 mM][d]
14.12	201.1	195.1	209.2	147
12.94	175.1	151.1	163.9	124
12.19	167.8	152.6	146.2	116.1
11.59	135.6	151.9	123.4	108.2
11.20	N/A	N/A	144.6	119.7
10.66	185.3	161.9	127	57.9
10.009			69.8	16.8
9.80			44.2	N/A
9.74		84.4	132.4	14.3
9.44	143.2			
9.865	87.3	63.4		
9.974	31.62	29.0		
9.90				
9.66		102.3		

[a]T7 produced SECIS RNA with cyclic phosphate.
[b]T7 produced SECIS RNA decyclized by maleic acid treatment.
[c]Chemically synthesized SECIS RNA in 10 mM sodium phosphate.
[d]Chemically synthesized SECIS RNA in 100 mM sodium phosphate.

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