RNA (1998), *4*:340–345. Cambridge University Press. Printed in the USA.

METHODS REPORT

Enzymatic incorporation of 2′-thio-CTP into the HDV ribozyme

KRISTEN RAINES and PHILIP A. GOTTLIEB
Department of Biological Science, SUNY at Buffalo, Buffalo, New York 14260, USA

ABSTRACT

We have synthesized the analogue 2′-deoxy-2′-thio-CTP (CTP-SH) and tested its ability to support RNA transcription in place of CTP. The modified nucleotide in a transcription reaction and in the absence of CTP generated the appropriately sized fragment when a mutant T7 polymerase (Y639F) was used. Wild-type polymerase was unable to generate RNA under the same conditions. Transcription was optimal around pH 7.5 and was dependent upon CTP-SH concentration. Transcripts containing the analogue were efficiently isolated using a thiol-activated sepharose column. Insertion of CTP-SH into the HDV ribozyme, replacing all cytidine residues with 2′-thiocytidine, appears to inhibit self-cleaving activity, even in the presence of manganese. The ability to introduce the CTP-SH analogue enzymatically into RNA opens the way for new structure–function studies where the 2′-hydroxyl can be efficiently replaced by a thiol group.

Keywords: nucleotide analogue; 2′-thio-CTP; ribozyme; mutant polymerase

INTRODUCTION

A major focus of ribozyme studies is to determine which chemical groups are directly involved in the folding of the ribozyme and which participate directly in catalytic activity. A useful approach in this regard is to insert modified nucleotides, to generate precise modifications in the RNA, and to measure their effect on catalytic function. Substitution by analogue nucleotides is ideal for this purpose, because they minimally perturb the overall structure, but allow isosteric changes in the RNA.

We are interested in understanding the self-cleaving activity of the HDV ribozyme using a modified nucleotides approach. The HDV RNA enzyme catalyzes the site-specific cleavage of RNA through a 2′-hydroxyl located in the active site (Kuo et al., 1988; Wu et al., 1989). The resulting reaction liberates the 5′ hydroxyl with the formation of a 2′,3′ cyclic phosphate and is mediated by divalent ions such as magnesium. Alterations to the 2′ position at the cleavage site, such as a thiol group, would be a major step forward in our efforts to analyze the catalytic requirements of this ribozyme. This functional group could participate in self-cleaving activity via thiolate anion and may provide new insights into the role of metals in the catalytic mechanism (Dantzman & Kiessling, 1996).

Recent work by three groups has begun exploring the effect of 2′-thiol analogues on RNA. Reese et al. (1994) synthesized 2′-thiouridylyl (3′-5′) uridine and were able to fragment the dinucleotide at high pH to produce uracil and uridine 5′-phosphate, whereas Dantzman and Kiessling (1996) showed that the 2′-thiouridine-3′-(*p*-nitrophenyl) phosphate is subject to hydrolysis or transesterification reactions, depending on pH. The latter work is especially relevant to ribozyme studies because it demonstrates that the thiol group can carry out attack on the 3′ phosphodiester bond. Hamm and Piccirilli (1997) have synthetically introduced 2′-mercaptocytidine nucleoside analogue into a deoxyoligonucleotide, opening the way for new studies using phosphoramidite chemistry. In that work, the 2′-thiocytidine nucleoside, once incorporated, was shown to have a half-life of 3,000 h at pH 7.5. However, a convenient method for incorporation of 2′-thionucleoside analogues into large fragments of RNA has not been reported previously.

For this reason, we have begun investigating methods to insert 2′-thiol groups into RNA by transcription. In this work, we synthesized 2′-thiocytidine triphosphate, which has a thiol group at the 2′-position instead

Reprint requests to: Philip Gottlieb, 670 Hochstetter Hall, Department of Biological Science, SUNY at Buffalo, Buffalo, New York 14260, USA; e-mail: philgott@ubunix.acsu.buffalo.edu.

Abbreviations: 2′-thio-CTP or CTP-SH, 2′-deoxy-2′-thiocytidine triphosphate; NPS-Cl, 2-nitrophenylsulfenylchloride; NPS-S-cytidine, 2′-deoxy-2′-(2-nitrophenyldithio)cytidine; NPS-S-CTP, 2′-deoxy-2′-(2-nitrophenyldithio) cytidine triphosphate; TEAB, triethylammonium bicarbonate; DEAE, diethylaminoethyl.

of a hydroxyl. We demonstrate that this analogue supports transcription when a mutant T7 polymerase is used at an appropriate pH. Once incorporated, the thiol group allows the resulting transcript to be isolated by a thiol-specific affinity column. This provides a general approach to the enzymatic incorporation of 2′-mercapto analogues for structure and functional studies on self-cleaving and other RNA molecules. When the HDV ribozyme was completely substituted with the analogue, no self-cleaving activity was observed in the presence of either magnesium or magnesium supplemented with manganese.

RESULTS AND DISCUSSION

We synthesized the triphosphate 2′-thiocytidine, a cytidine analogue whose 2′ hydroxyl group is replaced with a thiol group. The starting material for this reaction was the 2′-mercaptocytidine nucleoside protected at the 2′ position with a nitrophenolsulfenyl group (Divakar & Reese, 1982; Matsuda & Miyasaka, 1983). The NMR data are consistent with this compound (Fig. 1A), including a coupling constant ($J_{1'\text{-}2'} = 9.2$) for the H_1' proton characteristic for these nucleoside derivatives (Divakar & Reese, 1982; Matsuda & Miyasaka, 1983), confirming the presence of the thiol group in the correct anomeric configuration.

The analogue, NPS-S-cytidine, was then converted to the NPS-S-CTP (Fig. 1B) by reaction with phosphoryloxychloride followed by the addition of pyrophosphate. The phosphorous NMR was consistent with a triphosphate after DEAE and reverse-phase HPLC column purification. The NPS protecting group was removed in the last step by the addition of DTT under slightly basic conditions (Fig. 1C), thereby maintaining the integrity of the 2′-thiol during the various chemical reaction steps. The UV spectrum demonstrated that the signature peak for the NPS group at 356 nm was no longer apparent, consistent with its removal in the purified triphosphate. The HPLC analysis showed a shift in retention time; the NPS-S-CTP was 27.1 min, whereas that of the analogue triphosphate was 17.3 min.

When considering the possibility of transcribing with CTP-SH, we were concerned about the ionization of the thiol group under conditions normally used to transcribe RNA (pH 8.0). The thiol group has a significantly lower pK_a than its hydroxyl counterpart and, at the higher pH value, a significant population of the CTP-SH analogue could be deprotonated (Imazawa et al., 1975). NMR measurements of protonation of 2′-thiol group of 2′-deoxy-2′-thiouridine 3′-(*p*-nitrophenyl phosphate) showed a pK_a of approximately 8.2 (Dantzman & Kiessling, 1996). In the ionic form, the analogue might not support transcription. Consequently, we began our initial investigations by transcribing at pH 6.5 to maintain the protonated form. Additionally, we wanted to minimize the number of cytidine residues in the tran-

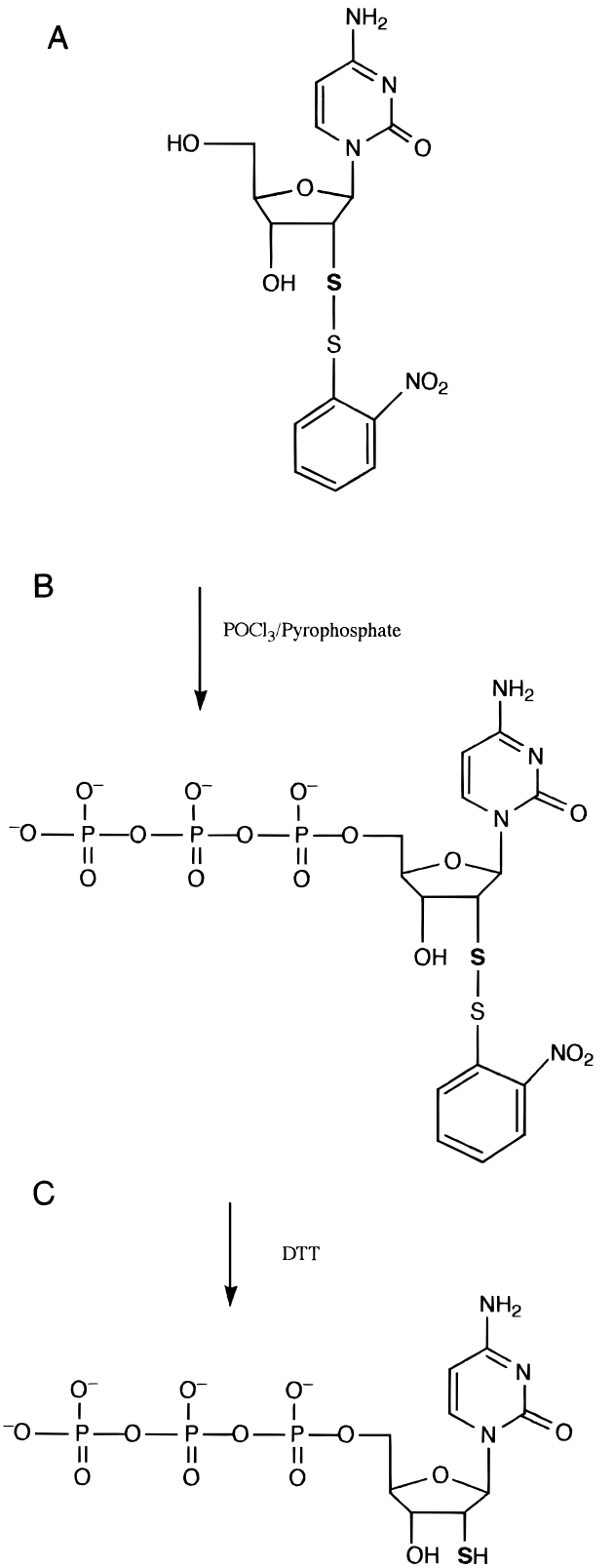

FIGURE 1. Synthetic scheme for the conversion of the NPS-cytidine (**A**) nucleoside to the triphosphate protected at the 2′-position (**B**). The last step is the deprotection was the addition of DTT (**C**) to liberate the triphosphate containing the 2′-thiol modification.

script and therefore used a plasmid that contained a segment of the tryptophan leader sequence from *Bacillus subtillis* that has only four cytidines in a 63-nt transcript (Gollnick, 1994). When CTP was substituted with CTP-SH in the reaction mixture and allowed to react in the presence of wild-type T7 polymerase and other NTPs, we were unable to observe any RNA transcription (Fig. 2, lane 3), similar to the reaction that contained no CTP (Fig. 2, lane 4). The control reaction (Fig. 2, lane 2), which had the CTP nucleotide, provided us with a measure of transcription activity under these conditions. Interestingly, an equimolar mixture of CTP and CTP-SH reduced the level of transcription, indicating that the analogue CTP-SH may inhibit normal T7 RNA polymerase activity (Fig. 2, lane 1).

We then attempted to incorporate the CTP-SH analogue into RNA by using a mutant polymerase with a relaxed specificity for nucleotides that bear modifications at the 2′ position (Sousa & Padilla, 1995). This polymerase, Y639F, has the same error rate of incorporation as the wild-type and therefore has the same specificity for insertion of the appropriate nucleotide. When the Y639F T7 polymerase was used with the CTP-SH analogue, a transcript was observed (Fig. 2, lane 7) that was identical in size to a transcript generated in the presence of CTP (Fig. 2, lane 6). No transcript was observed in the absence of both CTP or CTP-SH (Fig. 2, lane 8), and a mixture of the nucleotides supported transcription (Fig. 2, lane 5). For these conditions, the quantity of RNA achieved with CTP-SH was lower than that with CTP-OH.

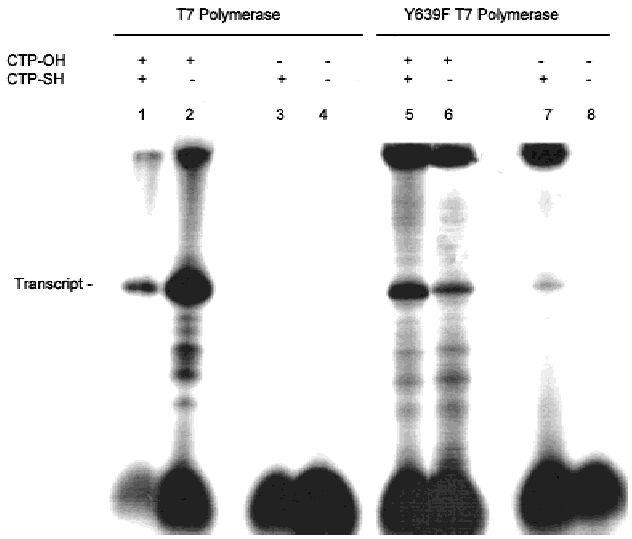

FIGURE 2. Analysis of transcription in the presence of CTP-SH or its natural counterpart. Linearized plasmid containing a partial sequence of the *trp* leader was transcribed in the presence of CTP, the CTP-SH analogue, or in combination. Lanes 1–4, transcriptions in the presence of T7 polymerase; lanes 5–8, utilization of the mutant polymerase Y639F T7 polymerase. Transcription was performed at pH 6.5, 37 °C, and 1 μg of plasmid for 1 h. The concentration of CTP or CTP-SH was 0.4 mM. Analysis was performed on a 12%–8 M urea polyacrylamide gel.

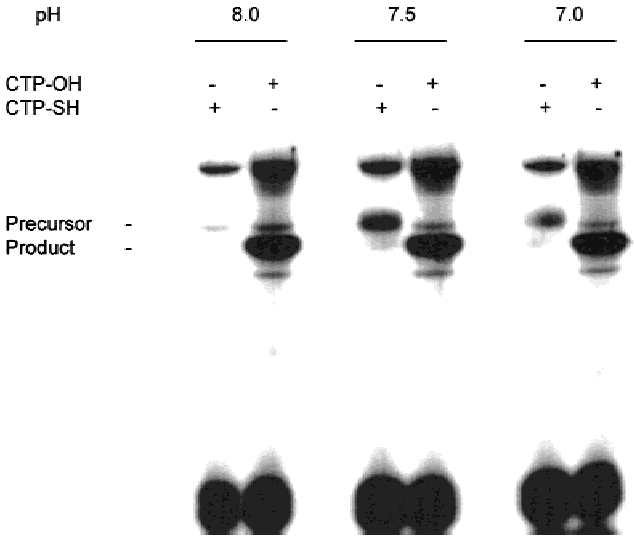

FIGURE 3. Transcription in the presence of CTP-SH as a function of pH. Linearized plasmid containing the template for the HDV ribozyme was transcribed at indicated pH values in the presence of either CTP or CTP-SH at 37 °C using 1 μg of plasmid and the mutant T7 polymerase for 1 h. Under these conditions, greater than 95% of transcripts that are made in the presence of CTP will self-cleave to product HDV.

In order to further define conditions for transcription using CTP-SH, buffered solutions at 7.0, 7.5, and 8.0 were used with the plasmid dSIV, which encodes the HDV ribozyme. As can be seen in Figure 3, optimal production of RNA in the presence of the analogue was pH 7.5; a significant increase in RNA yield was observed at pH 7.5 when compared to the yield at pH 8.0, 7.0, and 6.5. Similar results were observed for the *trp* leader sequence (data not shown). Transcription reactions in the presence of the CTP-SH nucleotide at pH 8.0 produced only minor levels of RNA in comparison to those observed at the lower pH values (Fig. 3). We postulate that CTP-SH is deprotonated at the higher pH and cannot support transcription, a supposition that is consistent with the measured pK_a of a dinucleotide with a 2′-thiol (Dantzman & Kiessling, 1996). Transcription was also dependent on the concentration of the analogue. Increasing concentrations of CTP-SH increased the yield of RNA in a range of concentrations consistent with the requirements of the polymerase (Fig. 4). We compared the efficiency of transcription with CTP (0.5 mM) versus RNA with CTP-SH (0.5 mM), when other nucleotides are saturating (1 mM) at pH 7.5. The yield of the CTP-SH-substituted RNA was 41% compared to that of normal transcription at pH 7.5 (data not shown; based on Phosphorimager densitometry).

To confirm the presence of the thiol in the RNA transcript, we used an affinity column that retains molecules based on the presence of thiol groups. Transcribed, labeled HDV RNA, after phenol extraction and ethanol precipitation, was applied to an activated isopropylthiol Sepharose column and washed extensively.

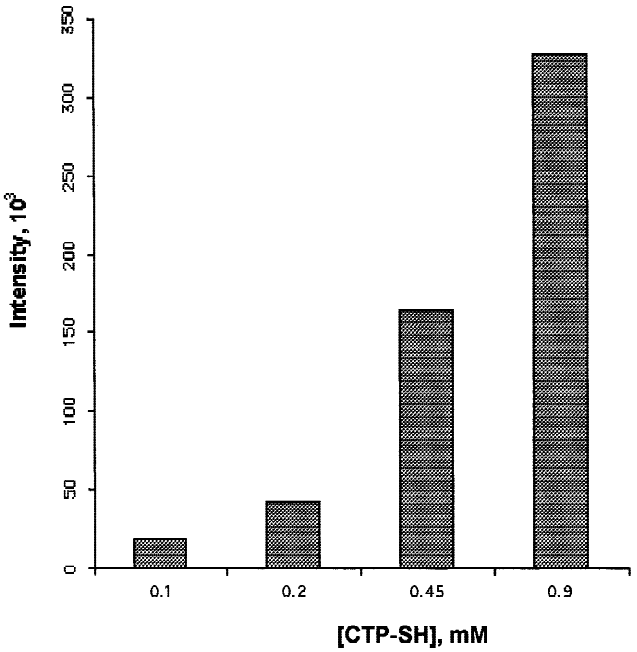

FIGURE 4. Dependence of RNA transcription on CTP-SH concentration. The plasmid containing the template for the HDV ribozyme was transcribed at pH 7.5 in the presence of varying concentrations of CTP-SH at 37 °C, 1 μg of plasmid, and 1 μL of T7 polymerase (1 mg/mL). Concentrations of the remaining NTPs were 0.5 mM. The amount of RNA produced was quantified by a Phosphorimager.

To release the RNA retained to the column, increasing concentrations of DTT were used (Fig. 5A). After the addition of 500 mM DTT, no additional label remained on the column. A gel analysis of the fractions showed that the correct-sized fragment was removed when 500 mM DTT was present (Fig. 5B). As a control, we applied RNA transcribed with natural CTP to the column and treated it identically to the analogue-substituted RNA (Fig. 5A), yielding approximately 1.2 pmol of RNA. A comparison of these two RNA transcripts clearly indicated that only the thiol-substituted RNA was efficiently retained on the column and eluted with high concentrations of DTT. However, at 20 mM DTT, both columns appear to have released some labeled material. We hypothesize that some RNAs are trapped in the gel matrix nonspecifically and the addition of DTT alters the resin sufficiently to cause their release.

In this work, we have demonstrated that CTP-SH can replace CTP in a transcription reaction to produce the correct-sized RNA fragment. The fact that the analogue can be efficiently introduced into RNA via transcription opens the way for a number of new studies. This initial work addressed the question whether total substitution of the HDV ribozyme with this analogue can inhibit cleavage activity. Figure 3 demonstrated that the substituted RNA remained as precursor, whereas virtually all of the "normal" unsubstituted RNA was converted to product when transcribed at 37 °C (Fig. 3). Substituted RNA isolated from the affinity column was also used to measure the effect of manganese on the catalytic activity of RNA, in an attempt to rescue the activity by allowing thiol groups to coordinate with this divalent cation. The CTP-SH RNA was incubated at 37 °C in the presence of 10 mM magnesium and in the absence or presence of 1 mM manganese buffered at pH 7.0. No self-cleaving activity was observed (data not shown).

The reasons why the self-cleaving activity is inhibited when the CTP-SH analogue is inserted will require further analysis. Because this preliminary work describes the complete incorporation of CTP-SH, it is difficult to assess how the analogue affects the active site, how it perturbs metal coordination in the ribozyme structure, and how intramolecular interactions are affected when the thiol group is present. However, modifications to this approach, for example, single-hit incorporation of the analogue, should provide a simple method to address some of these issues.

MATERIALS AND METHODS

General procedures

Proton NMR was performed on a Brucker 250 MHz in either deuterated DMSO or D_2O using TMS as a standard. Phosphorous NMR (100 MHz) was done in D_2O. Carbon NMR (125 MHz) was done in deuterated DMSO on a Varian 500 MHz spectrometer. TLC was performed on precoated aluminum sheets of silica gel 60/kieselguhr F254 (EM Industries) with indicated solvent systems. UV spectra were collected with a Beckman DU640 spectrophotometer. Reverse-phase HPLC chromatography was done with an ABI C18 semi-preparative or analytical column.

Synthesis of CTP-SH

The starting material for the synthesis of the triphosphate was NPS-S-cytidine (Fig. 1A). This was achieved based on procedures described previously (Divakar & Reese, 1982; Matsuda & Miyasaka, 1983). The characteristics for the NPS-S-cytidine compound that was first purified on a silica column and then crystallized from aqueous ethanol are listed below. Mass spectrum FAB (MNa^+) 435 ($C_{15}H_{16}N_4O_6S_2Na$); δ_H [$(CD_3)_2SO$] 3.55 (2H, m) 3.62 (1H, dd, J = 5.1, 9.2), 3.72 (1H, m), 4.03 (1H, m), 5.03 (1H, m), 5.52 (1H, d J = 7.4), 6.14 (1H, d), 6.26 (1H, d J = 9.2), 7.18 (2H, br s), 7.48 (1H, m), 7.6 (1H, d, J = 7.6), 7.74 (1H, m), 8.11 (1H, d, J = 8.2), 8.21 (1H, d, J = 8.3); δ_C [$(CD_3)_2SO$] 59.28, 61.75, 72.54, 86.57, 87.01, 95.01, 126.35, 127.26, 127.34, 135.20, 136.36, 141.35, 145.20, 155.63, 165.63.

Conversion to the triphosphate was accomplished according to Wieczorek et al. (1994). Briefly, NPS-S-cytidine (0.19 mmol, 78 mg) was dissolved in 1 mL of trimethylphosphate. To this was added phosphoryloxychloride (0.38 mmol, 35.4 μL). The reaction was followed by TLC using the solvent system of BuOH:NH_3OH:$H_2$0 (7:1:2). After the conversion of the starting material, the solution was cooled to 4 °C and 440 mg (1.4 mmol) of pyrophosphate tributylamine salt (Sigma) dissolved in 1.2 mL of freshly distilled DMF was added to the

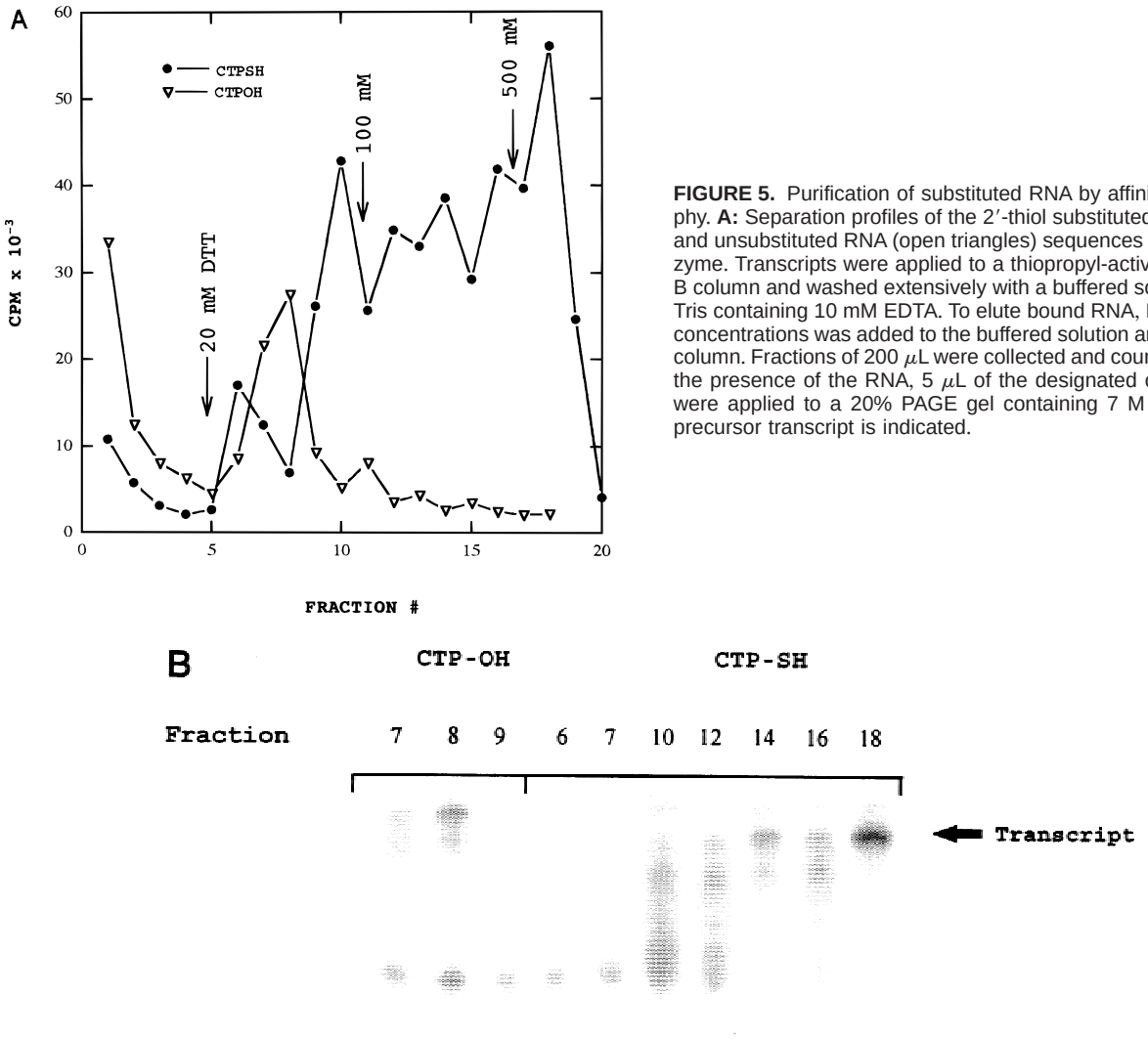

FIGURE 5. Purification of substituted RNA by affinity chromatography. **A:** Separation profiles of the 2′-thiol substituted (closed circles) and unsubstituted RNA (open triangles) sequences of the HDV ribozyme. Transcripts were applied to a thiopropyl-activated Sepharose B column and washed extensively with a buffered solution of 10 mM Tris containing 10 mM EDTA. To elute bound RNA, DTT at indicated concentrations was added to the buffered solution and applied to the column. Fractions of 200 μL were collected and counted. **B:** To verify the presence of the RNA, 5 μL of the designated column fractions were applied to a 20% PAGE gel containing 7 M urea. The HDV precursor transcript is indicated.

solution. The mixture was allowed to react for 15 min on ice and was quenched by the addition of 2 mL of cold triethylamine and 4 mL of H_2O. The solution was stored at $-20\,^{\circ}C$ overnight.

Purification of the triphosphate was accomplished by diluting the crude reaction mixture with 100 mL of 0.1 M TEAB, pH 7.5. The solution was applied to a DEAE cellulose (Whatman) column previously equilibrated with 0.1 M TEAB, pH 7.5. The column was developed with a gradient of 0.1–1.0 M TEAB. The desired material, NPS-S-CTP, was collected, dried, and repurified by reverse-phase HPLC (C-18) using a gradient of 2–25% acetonitrile buffered with 0.1 M triethylammonium acetate, pH 7.0. The overall yield for these steps was approximately 12% (14 mg). UV spectrum (H_2O, pH 4.5) λ_{max} 275, 359 nm; $\delta_H(D_2O)$ 4.05 (1H, m) 4.1 (1H, m), 5.62, (1H, d, $J = 7.6$), 6.21 (1H, d,$J = 9.8$), 7.34 (1H, m), 7.43 (1H, d, $J = 7.7$), 7.60 (1H, m), 8.02 (1H, d, $J = 8.2$), 8.06 (1H, d, $J = 8.2$) (Note: This represents a partial list of protons. Protons from the triethylamine counterion make it difficult to assess all upfield protons); δ_P (D_2O) -10.24 (d), -11.06 (d), -22.64 (t).

To remove the NPS group, the material was dissolved in 500 μL of Tris-HCl buffer, pH 8.5, and to this was added DTT to a final concentration of 100 mM. After 10 min at room temperature, the reaction was quenched by the addition of acetic acid to bring the solution to pH 5. Removal of the NPS group from the nucleotide was confirmed by the absence of 360 nm absorbency and by HPLC analysis using the above gradient. The latter method demonstrated the loss of starting material with the concomitant appearance of a faster migrating fraction. Final purification was achieved by passing the material through a Sephadex G-10 sizing column. The nucleotide was always stored in the presence of DTT at $-20\,^{\circ}C$. Based on UV absorbency at 260 nm, HPLC analysis of the final material indicated that the purity of the CTP-SH analogue was 94%.

Transcription

All transcriptions were performed using either a plasmid template containing the leader sequence of the *B. subtilis* trp operon (gift of Paul Gollnick, SUNY at Buffalo) (Gollnick, 1994) or the self-cleaving HDV ribozyme (gift of Mike Been, Duke University) (Perrotta & Been, 1992). The former sequence has four cytidine residues in the RNA, two of which are within

the first 10 nt required for initiation. Because of the limited number of C residues, it was used in the initial experiments. Transcription was performed in solutions buffered with either 40 mM Tris·HCl or 40 mM MOPS·NaOH, depending on the pH required, containing 10 mM DTT and 15 mM $MgCl_2$. The final concentrations of nucleotides were 0.5 or 1.0 mM for ATP, GTP, and 200 μM UTP, and α-^{32}P-UTP (10–80 μCi); the concentration of CTP or its analogue counterpart is indicated for each experiment. All reactions were done in a 20-μL volume at specified temperatures using either T7 polymerase or T7 mutant polymerase as indicated. A typical reaction was done with 1 μg of plasmid incubated for 1 h at 37 °C. The transcription reaction was terminated by the addition of 5 μL of 80% formamide, 0.1% bromophenol blue, 0.1% xylene cyanol, and 20 mM EDTA. The solution was applied to a 12% polyacrylamide–8 M urea gel and the transcripts were detected by a Phosphorimager (Molecular Dynamics).

Affinity column

Three-hundred milligrams of thiopropyl-activated sepharose 6B (Sigma) were allowed to swell in a solution of 10 mM Tris and 10 mM EDTA, pH 7.0 at 4 °C. After 1 h, the resin was centrifuged at 500 rpm for 20 s and excess solution was removed. One milliliter of buffer was added, gently mixed, and allowed to sit for 30 min. This was followed by a repeat of the centrifugation step and removal of buffer. To prevent nonspecific binding of RNA, the resin was treated with tRNA by adding 1 mL of buffer and 50 μL of a 10 mg/mL solution of tRNA. This mixture was incubated for 20 min and the unbound tRNA was then removed by two washes as described above.

Transcribed RNA with either CTP or CTP-SH was added to the activated sepharose and allowed to react for 40 min at 4 °C. Each resin was introduced into a 1-mL syringe. The columns were rinsed with 4 mL of buffer to elute unbound material and then various concentrations of DTT in buffer (see Fig. 5) were added to elute bound RNA. Fractions of 200 μL were collected and analyzed by gel electrophoresis.

Y639F mutant T7 polymerase

The plasmid containing the mutant T7 polymerase Y693F was a gift of Rui Sousa. The protein was purified according to the methods outlined previously (Bonner et al., 1992).

ACKNOWLEDGMENTS

This work was supported by NIH grant GM52033. We thank Margaret Hollingsworth, Paul Gollnick, and Gail Dinter-Gottlieb for critically reading this manuscript. We are grateful to Hiro Suga for suggesting the use of the thiol affinity column, to Janet Morrow for many helpful discussions, and to Danny Epstein for the carbon NMR data analysis.

Received August 1, 1997; returned for revision August 27, 1997; revised manuscript received December 22, 1997

REFERENCES

Bonner G, Patra D, Lafer EM, Sousa R. 1992. Mutations in T7 polymerase that support the proposal for a common polymerase active site structure. *EMBO J 11*:3767–3775.

Dantzman CL, Kiessling LL. 1996. Reactivity of a 2′-thio nucleotide analog. *J Am Chem Soc 118*:11715–11719.

Divakar KJ, Reese CB. 1982. Reaction between 2,2′-anhydro-1-β-D-arabinosyluracil and thiolate ions. *J Chem Soc Perkin Trans I*:1625–1628.

Gollnick P. 1994. Regulation of the *Bacillus subtilis* trp operon by an RNA-binding protein. *Mol Microbiol 11*:991–997.

Hamm ML, Piccirilli JA. 1997. Incorporation of 2′-deoxy-2′-mercaptocytidine into oligonucleotides via phosphoramidite chemistry. *J Org Chem 62*:3415–3420.

Imazawa M, Ueda T, Ukita T. 1975. Nucleosides and nucleotides. XII. Synthesis and properties of 2′-deoxy-2′-mercaptouridine and its derivatives. *Chem Pharm Bull 23*:604–610.

Kuo MYP, Sharmeen L, Dinter-Gottlieb G, Taylor J. 1988. Characterization of self-cleaving RNA sequences on the genome and antigenome of human hepatitis delta virus. *J Virology 62*:4439–4444.

Matsuda A, Miyasaka T. 1983. Facile synthesis of 2′-deoxy-2′-arylthiouridines. *Heterocycles 20*:55–58.

Perrotta AT, Been MD. 1992. Cleavage of oligoribonucleotides by a ribozyme derived from the Hepatitis Delta virus RNA sequence. *Biochemistry 31*:16–20.

Reese CB, Simons C, Zhang PZ. 1994. The synthesis of a 2′-thiouridylyl-(3′-5′)-uridine. *J Chem Soc Chem Commun 1994*: 1809–1810.

Sousa R, Padilla R. 1995. A mutant T7 polymerase as a DNA polymerase. *EMBO J 14*:4609–4621.

Wieczorek A, Dinter-Gottlieb G, Gottlieb PA. 1994. Evidence that total substitution of adenine with 7-deazaadenine in the HDV antigenomic ribozyme changes the kinetics of RNA folding. *Bioorg Med Chem Lett 4*:987–994.

Wu HN, Lin YJ, Lin FP, Makino S, Chang MF, Lai MC. 1989. Human hepatitis delta RNA subfragments contain an autocleavage activity. *Proc Natl Acad Sci USA 86*:1831–1835.