The Embedded Ribonucleotide Assay: A Chimeric Substrate for Studying Cleavage of RNA by Transesterification

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Received January 16, 1996[⊗]

Abstract: The cleavage (transesterification) of polyribonucleotides is a process of considerable interest. The use of dinucleotide RNA fragments as substrates for the screening of RNA catalysis agents and mechanistic studies is widespread. This practice may not accurately predict the relative abilities of metal complexes to cleave polyribonucleotide substrates. We report the use of chimeric DNA/RNA molecules, containing RNA nucleotides embedded in DNA sequences, as substrates for studying the transesterification of RNA. The substrates, termed embRNA, display the simplicity of dinucleotide substrates while possessing the multiple phosphate and nucleobase metal-binding sites found in polyribonucleotides. In addition, the DNA residues provide an internal check for oxidative cleavage. The synthesis, purification, and activity of our first-generation embRNA, $T_{11}UT_7A$, is described. $T_{11}UT_7A$ is a substrate for the ribonuclease RNase 1, and RNase 1 cleavage provides an excellent measure of the extent of 2′-deprotection in the synthetic embRNA. Cleavage of $T_{11}UT_7A$ by hydroxide and a variety of metal ions and complexes is also reported, and the use of embRNA in kinetic assays is demonstrated. Competitive cleavage of RNA and DNA is built into the embRNA assay. With Pb(II), Ce(III), and Cu(II) reagents, we observed efficient RNA cleavage and no DNA cleavage. Kinetic comparison is made between embRNA $T_{11}UT_7$ and the analogous, all-RNA substrate U_{19}.

Many groups are actively developing RNA cleavage agents with possible medical applications, including the gene-specific, catalytic destruction of viral mRNA.[1−19] Generally, catalysts are chosen or rejected based on their ability to cleave dinucleotide substrates,[20−23] although we and others have reported studies based on metal-promoted cleavage of RNA oligomers and polymers.[9,24−26] The dinucleotide assays may provide misleading information about polynucleotide cleavage, due to the length-dependence of the RNA transesterification reaction (*vide infra*). Polymeric substrates themselves present a multiplicity of reaction sites that may not be kinetically equivalent. Furthermore, the products of each polymer cleavage reaction are substrates for further reaction, which can complicate kinetic studies beyond the initial rate regime. To overcome these limitations and to allow unprecedented control over the sequence and electrostatic context of RNA cleavage, we report a new assay for RNA transesterification that allows (1) a simplified study of high MW polynucleotide substrates, (2) an internal check for oxidative cleavage processes, (3) a systematic exploration of the sequence context effects on RNA cleavage, and (4) the study of competition between RNA and DNA cleavage. We have named this assay the Embedded Ribonucleotide Assay. It employs chimeric oligonucleotides that contain one or more RNA nucleotides inserted at controlled positions into DNA sequences. We use enzymatic cleavage of the embedded RNA (embRNA) to demonstrate biological activity and complete deprotection of the 2′-OH groups in our chemically-synthesized substrate. Our first embRNA substrate consists of RNA embedded into unmodified DNA, but the method is general and allows incorporation of DNA modifications at specific sites, including methylphosphonate linkages, deaza- or methylated nucleobases, or phosphorothioates. We show the first examples of competitive cleavage of RNA and DNA by

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⊗ Abstract published in *Advance ACS Abstracts,* July 1, 1996.

(1) Bashkin, J. K.; Frolova, E. I.; Sampath, U. *J. Am. Chem. Soc.* **1994**, *116*, 5981−5982.
(2) Bashkin, J. K.; Jenkins, L. A. *Comments Inorg. Chem.* **1994**, *16*, 77−93.
(3) Bashkin, J. K.; Sondhi, S. M.; Modak, A. S.; Sampath, U.; d'Avignon, D. A. *New J. Chem.* **1994**, *18*, 305−316.
(4) Bashkin, J. K.; Sampath, U. S.; Frolova, E. I. *Appl. Biochem. Biotechnol.* **1995**, *54*, 43−56.
(5) Bashkin, J. K.; Jenkins, L. A. *J. Chem. Soc., Dalton Trans.* **1993**, 3631.
(6) Bashkin, J. K. *Bioinorganic Chemistry of Copper*; Karlin, K. D., Tyeklar, Z., Eds.; Chapman and Hall: New York, 1993; pp 132−139.
(7) Bashkin, J. K.; McBeath, R. J.; Modak, A. S.; Sample, K. R.; Wise, W. B. *J. Org. Chem.* **1991**, *56*, 3168.
(8) Modak, A. S.; Gard, J. K.; Merriman, M. C.; Winkeler, K. A.; Bashkin, J. K.; Stern, M. K. *J. Am. Chem. Soc.* **1991**, *113*, 283.
(9) Stern, M. K.; Bashkin, J. K.; Sall, E. D. *J. Am. Chem. Soc.* **1990**, *112*, 5357.
(10) Bashkin, J. K.; Gard, J. K.; Modak, A. S. *J. Org. Chem* **1990**, *55*, 5125.
(11) Bashkin, J. K.; Xie, J.; Daniher, A. T.; Jenkins, L. A.; Yeh, G. C. *Ribozyme Mimics for Catalytic Antisense Strategies*; Meunier, B., Ed.; Kluwer: Netherlands, 1996; pp 355−366.
(12) Bashkin, J. K.; Xie, J.; Daniher, A. T.; Sampath, U.; Kao, J. L.-F. *J. Org. Chem.* **1996**, *61*, 2314−2321.
(13) Bashkin, J. K.; Sampath, U. S.; Frolova, E. I. *Appl. Biochem. Biotechnol.* **1995**, *54*, 43−56.
(14) Komiyama, M.; Inokawa, T.; Yoshinari, K. *J. Chem. Soc., Chem. Commun.* **1995**, 77−78.
(15) Morrow, J. R.; Shelton, V. M. *New J. Chem.* **1994**, *18*, 371−375.
(16) Magda, D.; Miller, R. A.; Sessler, J. L.; Iverson, B. L. *J. Am. Chem. Soc.* **1994**, *116*, 7439−7440.
(17) Polushin, N. P.; Chen, B.-C.; Anderson, L. W.; Cohen, J. S. *J. Org. Chem.* **1993**, *58*, 4606.
(18) Cech, T. R. *JAMA* **1988**, *260*, 3030−3034.
(19) Hovinen, J.; Guzaev, A.; Azhayeva, E.; Azhayev, A.; Lonnberg, H. *J. Org. Chem.* **1995**, *60*, 2205−2209.
(20) Linkletter, B.; Chin, J. *Angew. Chem., Int. Ed. Engl.* **1995**, *34*, 472−474.
(21) Kuusela, S.; Lönnberg, H. *J. Phys. Org. Chem.* **1993**, *5*, 347−356.
(22) Shelton, V. M.; Morrow, J. R. *Inorg. Chem.* **1991**, *30*, 4295−9.
(23) Breslow, R. *J. Am. Chem. Soc.* **1989**, *111*, 4473.
(24) Smith, J.; Anslyn, E. V. *Anal. Biochem.* **1994**, *220*, 53−57.
(25) Black, C. B.; Cowan, J. A. *Inorg. Chem.* **1994**, *33*, 5805−5808.
(26) Corcoran, R.; Labelle, M.; Czarnik, A. W.; Breslow, R. *Anal. Biochem.* **1985**, *144*, 563−568.

S0002-7863(96)00191-6 CCC: $12.00

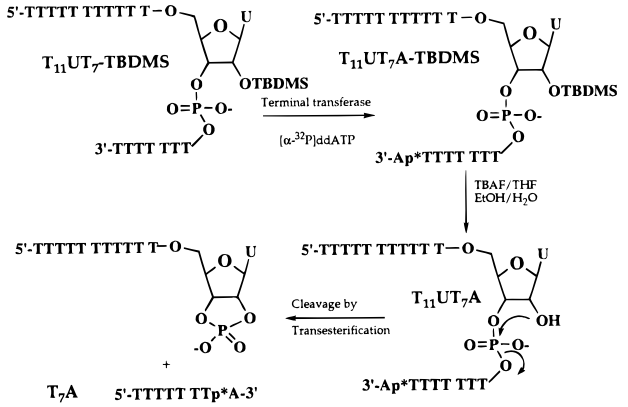

Figure 1. Synthesis and transesterification of the embedded RNA substrate $T_{11}UT_7A$.

Ce(III), Pb(II), and Cu(II) reagents. Both Ce[27,28] and Cu[29] reagents have previously been shown to cleave DNA *via* nucleophilic peroxide- and redox-mediated reactions, respectively. In addition, we have utilized the assay to successfully generate kinetic data which can be used to learn more about the mechanism by which metals promote RNA transesterification.

Chimeric substrates[30] have been previously used to monitor ribozyme reactions[31−33] and for the *in vitro* evolution of a "deoxyribozyme".[34] In contrast both to combinatorial synthesis of random sequence pools (and subsequent selection procedures) and to the systematic replacement of RNA residues by DNA in a complex ribozyme structure, our chimeric approach is designed to be relatively free of tertiary structural effects. We focus on fundamental aspects of ligation and electrostatic interactions between a catalytic metal (or metals), functional groups at the site of cleavage, and the nucleotides that flank the RNA site. DNA is *generally* inert[27,28,35] to hydrolysis by small molecule reagents at pH 7, and the DNA residues provide an internal check for any oxidative cleavage processes (or novel hydrolytic chemistry). Figure 1 shows the first embRNA substrate that we have studied, $T_{11}UT_7A$, and the cleavage products resulting from transesterification. In addition to the previously stated advantages, many reactions can be monitored simultaneously by polyacrylamide gel electrophoresis. The embRNA substrate provides a simple, convenient, efficient, and rapid system for screening and direct comparison of transesterification catalysts and for conducting kinetic studies on RNA transesterification.

Experimental Section

Synthesis and Labeling of the embRNA, $T_{11}UT_7$. The 2′-*tert*-butyldimethylsilyl-protected oligomer, $T_{11}UT_7$-TBDMS, was prepared by solid phase synthesis on an ABI 380B and purified by successive anion exchange and reverse phase HPLC. A Supelco LC-SAX HPLC column (25 cm × 4.6 mm) was used for ion exchange purification. Solvent A (0.1 M KCl, 10% acetonitrile) was run for 5 min following injection of the sample, followed by a 40-min gradient to solvent B (1.0 M KCl, 10% acetonitrile). The retention time for $T_{11}UT_7$-TBDMS was 27 min. Reverse phase desalting was done on a Supelco LC-18 column by eluting the sample for 15 min with water, followed by a 5-min gradient to a 60% acetonitrile solution. The retention time was 23 min.

The concentration of protected oligo was determined using UV spectroscopy (ϵ_{260} = 159 570 cm^{-1} M^{-1}).

$T_{11}UT_7$-TBDMS (10−30 pmol) was then 3′-end labeled using [α-^{32}P]-ddATP (Amersham) and terminal deoxynucleotidyl transferase (Promega). 3′-End labeling was accomplished by mixing the $T_{11}UT_7$-TBDMS, 5 μL 5 × reaction buffer provided by Promega (500 mM potassium cacodylate, 5 mM $CoCl_2$, 0.5 mM DTT, 5 mg/mL BSA), 10−20 units of terminal transferase, and 4 μL of [α-^{32}P]ddATP, and diluting to 25 μL. The reaction mixture was heated at 37 °C for 45-60 min. The reaction was stopped by heating at 70 °C for 10 min. The mixture was then extracted once with an equal volume of phenol/$CHCl_3$/isoamyl alcohol (25:24:1), and twice with $CHCl_3$. The extracted reaction mixture was dried and then reconstituted in 10 μL of 50% ethanol and 100 μL of 1.0 M tetra-*n*-butyl ammonium fluoride (TBAF).[36] This was allowed to react in the dark at room temperature for 12−24 h. When the same deprotection conditions were tried in the absence of ethanol, the reaction was slow and incomplete. RNase 1 cleavage is a good indication of the extent of deprotection, since the TBDMS-protected oligonucleotide is not cleaved by the enzyme. The product was purified by excision from a 20% denaturing polyacrylamide gel and desalted with Sephadex G-25. The authentic end-labeled cleavage product, T_7A, was also prepared to use as a standard.

Similarly, the $T_{11}UT_7$-TBDMS was 5′-end labeled using [γ-^{32}P] ATP and T4 polynucleotide kinase, deprotected with TBAF, and purified from a 20% denaturing polyacrylamide gel. The authentic 5′-end labeled product standards, 5′-p*T_{11}Up-3′ and 5′-p*$T_{11}U_{OH}$-3′, were also prepared.

The all-RNA substrate, U_{19}, was purchased from Oligos, Etc. The Cutrpy was synthesized by dissolving 1 equiv each of $CuCl_2$ and 2,2′,6′,6″-terpyridine in boiling ethanol and combining the two solutions. The Cutrpy precipitated immediately as a light green powder and was characterized by visible spectroscopy,[37] mass spectrometry, and elemental analysis. Buffers (HEPES, CHES, EPPS) and diethylpyrocarbonate (DEPC) were purchased from Sigma and used as received. The pH of the buffer solutions was measured using a Corning PS30 Check-mite pH sensor (accuracy ± 0.2 pH units). Reactions were run in 0.5 mL of polypropylene microcentrifuge tubes and incubated at 37 °C in a circulating water bath. All solutions were prepared using DEPC-treated water (0.1% DEPC, autoclaved for 40 min at 121 °C).

Cleavage of embRNA. The embRNA substrate $T_{11}UT_7A$ was treated with several cleavage agents, including NaOH, the ribonuclease RNase 1, and 1 mM concentration of a variety of metal ions and metal complexes. Hydroxide cleavage reactions contained ∼10^{-9} M embRNA, 0.07 M NaOH, and were run at 40 °C. The base-catalyzed reaction was quenched by neutralizing with 0.1 M HCl. The mixture was dried and resuspended in loading buffer prior to electrophoresis. RNase 1 cleavage reactions contained ∼10^{-9} M embRNA, enzyme reaction buffer from Promega, and 20 units of enzyme. The mixture was incubated at 37 °C for 1 h, quenched by heating to 70 °C for 10 min, and extracted once with phenol/chloroform/isoamyl alcohol (25:24:1) and twice with chloroform. The product was then dried and resuspended in loading buffer prior to electrophoresis. Reactions using metal ions and complexes contained ∼10^{-9} M embRNA substrate, 1 mM metals/complexes, 0.1 M $NaClO_4$, and 5 mM buffer. The Pb^{2+} and Cu^{2+} reactions (lanes 6−9 of Figure 2) contained MES buffer at pH 6.6. Reactions shown in lanes 12−19 of Figure 2 contained EPPS buffer at pH 8.1. Reactions were quenched with EDTA, dried, and resuspended in loading buffer prior to electrophoresis.

Rate constants were determined for the Ce^{3+}- and Cutrpy-promoted reactions using the 5′-end labeled substrate $T_{11}UT_7$, 0.5 mM metal concentrations, 0.1 M $NaClO_4$, and pH 7.0 (5.0 mM HEPES). For comparison, the rate constant for the cleavage of the all-RNA substrate, U_{19}, was also determined.[38] Kinetics were followed by quantifying the disappearance of starting material (as a percentage of total radioactivity in each lane) with time. In all cases, initial rate methods

(27) Komiyama, M.; Kodama, T.; Takeada, N.; Sumaoka, J.; Shiiba, T.; Matsumoto, Y.; Yashiro, M. *J. Biochem.* **1994**, *115*, 809−810.
(28) Takasaki, B. K.; Chin, J. *J. Am. Chem. Soc.* **1994**, *116*, 1121−1122.
(29) Sigman, D. S.; Mazumder, A.; Perrin, D. M. *Chem. Rev.* **1993**, *93*, 2295−2316.
(30) Duff, R. J.; de Vroom, E.; Geluk, A.; Hecht, S. M. *J. Am. Chem. Soc.* **1993**, *115*, 3350.
(31) Kuimelis, R. G.; McLaughlin, L. W. *J. Am. Chem. Soc.* **1995**, *117*, 11019−11020.
(32) Strobel, S. A.; Cech, T. R. *Biochemistry* **1993**, *32*, 13593.
(33) Dahm, S. C.; Uhlenbeck, O. C. *Biochimie* **1990**, *72*, 819.
(34) Breaker, R. R.; Joyce, G. F. *Chem. Biol.* **1994**, *1*, 223.
(35) Schnaith, L. M. T.; Hanson, R. S.; Que L., J. *Proc. Natl. Acad. Sci. U.S.A.* **1994**, *91*, 569−573.
(36) Scaringe, S. A.; Francklyn, C.; Usman, N. *Nucl. Acids Res.* **1990**, *18*, 5433−5441.
(37) Harris, C. M.; Lockyer, T. N. *Aust. J. Chem.* **1970**, *23*, 673−682.

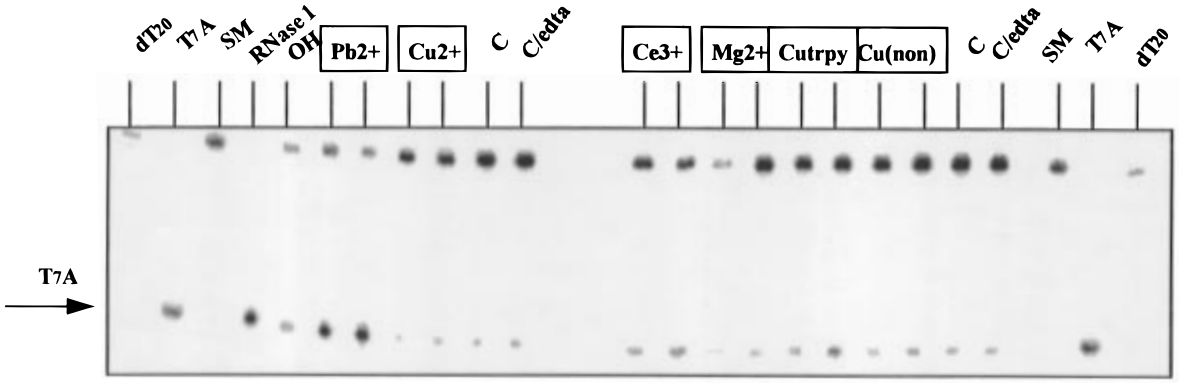

Figure 2. Image of a 20% polyacrylamide gel demonstrating the transesterification of the 3′-end labeled embRNA by various reagents. Time points were taken at 5 and 24 h for the reactions with metal ions and metal complexes (lanes 6−9 and 12−19). Lane 1: dT_{20} (size marker). Lane 2: T_7A (product standard). Lane 3: starting material, $T_{11}UT_7A$. Lane 4: RNase 1 cleavage. Lane 5: 0.07 M NaOH cleavage. Lanes 6 and 7: Pb^{2+} cleavage. Lanes 8 and 9: Cu^{2+} cleavage. Lane 10: MES buffer at 24 h (control, C). Lane 11: MES buffer + EDTA at 24 h (control, C/edta). Lanes 12 and 13: Ce^{3+} cleavage. Lanes 14 and 15: Mg^{2+} cleavage. Lanes 16 and 17: Cu(II)terpyridine cleavage. Lanes 18 and 19: Cu([9]aneN$_3$)$^{2+}$ cleavage. Lane 20: EPPS buffer at 24 h (control). Lane 21: EPPS buffer + EDTA at 24 h (control).

Table 1. Cleavage of $T_{11}UT_7A$ by Metal Ions and Metal Complexes[a]

metal ion/complex	buffer	pH	% cleavage (5 h)	% cleavage (24 h)
Pb^{2+}	MES	6.6	57	69
Cu^{2+}	MES	6.6	1	5
none (control)	MES	6.6	nd	0
none (control)	MES/EDTA	6.6	nd	1
Ce^{3+}	EPPS	8.1	9	20
Mg^{2+}	EPPS	8.1	nd	0
Cu(terpy)	EPPS	8.1	4	11
Cu([9]aneN$_3$)$^{2+}$	EPPS	8.1	1	2
none (control)	EPPS	8.1	nd	1
none (control)	EPPS/EDTA	8.1	nd	0

[a] Reaction conditions: ~10^{-9} M $T_{11}UT_7A$, 1 mM metals, 0.1 M $NaClO_4$, 5 mM buffer pH 6.6 and 8.1, 40 °C (not determined: nd).

were used. For this kinetic study, both the U_{19} and embRNA substrates were 5′-end labeled, because our 3′-labeling method uses terminal deoxynucleotidyl transferase, and this enzyme will not label all-RNA substrates.

Results/Discussion

Typical cleavage results are depicted in Figure 2, an electrophoresis gel image generated with a Molecular Dynamics phosphorimager. The reactions were analyzed on 20% denaturing polyacrylamide gels and quantified with the phosphorimager. The percent cleavage by metal ions/complexes after 5 and 24 h is shown in Table 1.

The embRNA is cleaved by RNase 1 only at the internal RNA site (U), and complete cleavage was observed in 1 h at 37 °C.[39] After 5 h at 40 °C, 0.07 M hydroxide cleaved 41% of the starting material. The ion Pb^{2+} was the most efficient cleavage agent of the metals studied. Similar results were obtained using 2 mM metals (data not shown). Under the conditions studied, none of the metal ions or complexes caused any significant DNA cleavage. To our knowledge, this represents the first examination of competitive RNA and DNA cleavage by Ce[27,28] and Cu[29] reagents, both of which can cleave DNA under special conditions. We conclude that RNA cleavage will remain a major pathway for Ce and Cu reactivity under most conditions, which may hamper efforts to develop selective Ce- and Cu-based DNA hydrolysis agents that function *in vivo*.

Using the 3′-end labeled substrate, the same cleavage product was observed for reactions with hydroxide, RNase 1, and all metal ions or complexes investigated, including those capable of redox chemistry. This was expected, since no external sacrificial redox equivalents (or nucleophilic hydrogen peroxide) were added. The observed 3′-end labeled product is 5′-T_7A-3′, which contains an intact 5′-OH, as determined by comparison to an authentic standard.

To further characterize the reaction, the $T_{11}UT_7$ substrate was 5′-end labeled and reacted with RNase 1, hydroxide, Pb^{2+}, and Cu(II)terpyridine (Cutrpy). These results are depicted in Figure 3. Again, all cleavage reactions gave the same initial product.[40] This initial product comigrates with an authentic sample of T_{11}Up-3′, the 3′-terminal phosphate standard that was prepared by chemical synthesis (Figure 3). RNase 1 is known to cleave RNA to the 2′,3′-cyclic phosphate and 5′-hydroxyl products. Hydroxide produces the cyclic phosphate initially but eventually produces a mixture of 2′- and 3′-monophosphates. Oligonucleotides with 2′,3′-cyclic phosphates or 3′-terminal phosphates are usually poorly resolved by electrophoresis,[31,41] so these two products should be indistinguishable under our electrophoresis conditions.

In the 5′-end labeled experiments, Pb^{2+} reacts with the initial cleavage product to produce a second product over time. 3′-Dephosphorylation by metals is a known reaction,[21,42] and we used authentic standards to determine that Pb^{2+} promotes the dephosphorylation of the embRNA product. The right-hand side of Figure 3 compares authentic, chemically-synthesized standards of the 3′-phosphate (T_{11}Up) and 3′-OH ($T_{11}U_{OH}$) products with the two products from the Pb^{2+}-cleavage reaction. The initial product formed by Pb^{2+} cleavage comigrates with the 3′-phosphorylated product. Pb^{2+} cleaves this to a product which comigrates with $T_{11}U_{OH}$. Thus, Pb^{2+} promotes the 3′-dephosphorylation reaction rather efficiently.

Rate constants were determined for the Ce^{3+}- and Cutrpy-promoted reactions (Table 2). Ce^{3+} promotes the transesterification of the embRNA 5.5 times faster than Cutrpy. In comparison with an all-RNA substrate (U_{19}), the embRNA is cleaved 15−17 times more slowly, which is consistent with the

(38) The k_{obs} reported for cleavage of the U_{19} is an approximate k_{obs}. The kinetics on the all-RNA substrate are complicated, because the initial cleavage products are substrates for further cleavage reactions, and some initial degradation products are present. Results such as these using all-RNA substrates further demonstrate the utility of the embRNA assay.

(39) For the 5′-end labeled substrate, a second product appears in the RNase 1 reaction lane, above the major product. We have not yet identified this minor species; it constitutes 4% of the product in Figure 3.

(40) The electrophoretic mobility of the products in the KOH lanes is slightly affected by the higher ionic strength in these lanes.

(41) Pan, T.; Uhlenbeck, O. C. *Nature* **1992**, *358*, 560−563.

(42) Jenkins, L. A.; Bashkin, J. K.; Pennock, J. D. to be submitted.

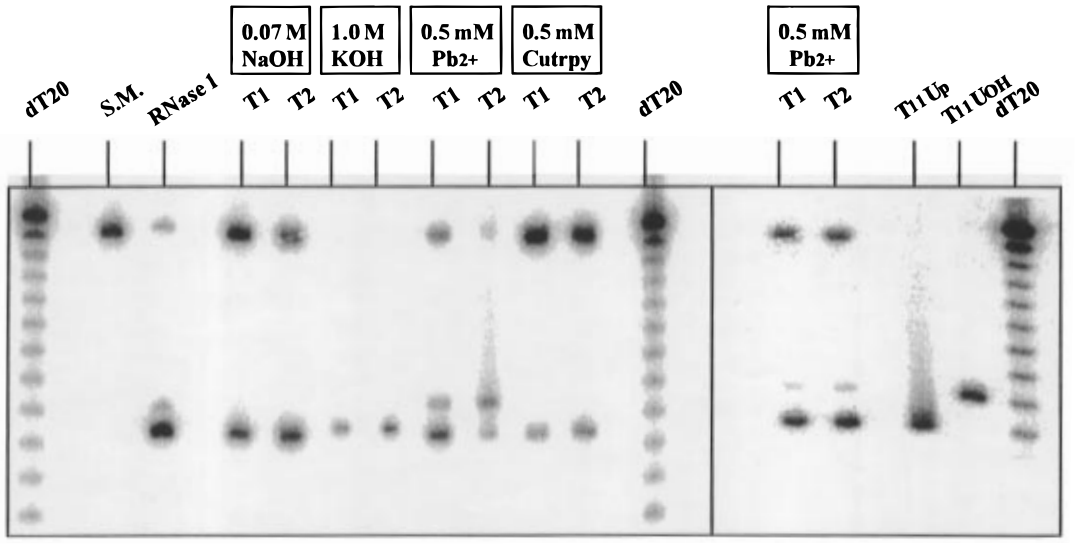

Figure 3. Image of a 20% polyacrylamide gel demonstrating the transesterification of the 5′-end labeled embRNA substrate. Two time points were taken for the hydroxide- and metal-promoted reactions as indicated. Lanes 1, 12, and 15: dT_{20} (size marker). Lane 2: Starting material (S. M.), $T_{11}UT_7$. Lane 3: RNase 1 cleavage. Lanes 4 and 5: 0.07 M NaOH and 2 h and 10 h. Lanes 6 and 7: 2 M KOH cleavage at 2 h and 7 h. Lanes 8 and 9: 0.5 mM Pb^{2+} cleavage and 2 h and 10 h. Lanes 10 and 11: 0.5 mM Cu(II)terpyridine cleavage 20 h and 41 h. Lanes 8b and 9b: Pb^{2+} cleavage at 2 h and 6 h. Lane 12: dT_{20} (size marker). Lane 13: $T_{11}U_P$ (3′-phosphate-containing product standard). Lane 14: $T_{11}U_{OH}$ (3′-OH-containing product standard). Lane 15: dT_{20} (size marker).

Table 2. Observed and Relative Rate Constants for the Transesterification of $T_{11}UT_7$ and U_{19} by Ce^{3+} and Cutrpy[a]

metal:substrate	k_{obs} (h^{-1})	relative rate
Cutrpy:embRNA	0.000149 ± 0.00007	1.0
Ce^{3+}:embRNA	0.0082 ± 0.0006	5.5
Cutrpy:U_{19}	0.025 ± 0.005	17
Ce^{3+}:U_{19}	0.12 ± 0.04	80

[a] Reactions were done in duplicate, and rate constants are reported the average (±1 standard deviation).

increased number of cleavage sites in the all-RNA substrate. However, the DNA sites of an embRNA substrate do not inhibit the RNA cleavage reaction.

The k_{obs} for the Cutrpy-promoted reaction can be divided by the [Cutrpy] to give a second-order rate constant, k_2, of 2.98 M^{-1} h^{-1} for transesterification. This k_2 can be compared to the analogous k_2 determined for the hydrolysis of 2′,3′-cAMP by Cutrpy of 40 ± 2 M^{-1} h^{-1}.[42] Cutrpy hydrolyzes the cyclic phosphate faster than it cleaves RNA by transesterification, so the product of transesterification should rapidly convert to 2′- and 3′-terminal phosphates in the presence of Cutrpy.

Length Dependence of RNA Transesterification. A length dependence has been observed previously,[43−45] but recent RNA cleavage studies using chemical reagents have almost always ignored these ideas. The importance of substrate length for medically-relevant reactions of ribozymes has recently been reported.[46] Reactions between DNA and Pt also depend on the length of the DNA.[47] The lack of correspondence between dinucleotide and oligonucleotide transesterification reactions is emphasized by the results with copper(II) terpyridine. Cutrpy cleaved the nanomolar embRNA substrate significantly faster than background (buffer) cleavage. Under the same conditions, Cutrpy failed to cleave ApUp above background levels, even with the ApUp concentration increased to micromolar levels (data not shown). Clearly, the relative reactivity of metal ions and metal complexes can be dramatically altered upon switching from dinucleotide to polynucleotide substrates. We suggest that, unlike ApA or other dinucleotides, polynucleotides provide the necessary electrostatic attraction and/or phosphate and nucleobase binding sites for the metal-promoted cleavage mechanism to operate. We are using the embRNA assay to investigate these possibilities.

Concluding Remarks

The embRNA assay provides a simple and rapid system for the screening of hydrolytic RNA cleavage agents. Since multiple metal binding sites are present, the embedded RNA assay is useful for studying polyribonucleotide cleavage, and it may present a different picture from dinucleotide assays. It has been used to screen new reagents, to rule out oxidative cleavage by an internal check for DNA cleavage, and to compare directly the relative efficiencies of the different reagents. It can be used to study sequence context and positional effects on RNA cleavage. In addition, the system is amenable to kinetic studies and can be used to learn more about the chemical mechanism of RNA cleavage, e.g., through the site-specific introduction of groups that block or enhance metal coordination near the RNA residue.

Acknowledgment. We thank A. Daniher for oligonucleotide synthesis. L.A.J. is a Dean's Fellow for 1994−1995. This work was supported in part by NSF Grant CHE-9318581. Acknowledgment is made to the donors of The Petroleum Research Fund, administered by the ACS, for partial support of this research.

JA960191Z

(43) Kierzek, R. *Nucl. Acids Res.* **1992**, *20*, 5073−5077.
(44) Ikenaga, H.; Inoue, Y. *Biochemistry* **1974**, *13*, 577−582.
(45) Butzow, J. J.; Eichhorn, G. L. *Biochemistry* **1971**, *10*, 2019−2027.
(46) Campbell, T. B.; Cech, T. R. *RNA* **1995**, *1*, 598−609.
(47) Elmroth, S. K. C.; Lippard, S. J. *J. Am. Chem. Soc.* **1994**, *116*, 3633−3634.