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Isolation and Properties of a Single-Strand 5′→3′ Exoribonuclease from Ehrlich Ascites Tumor Cell Nucleoli†

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ABSTRACT: A single-strand-specific, nucleolar exoribonuclease from Ehrlich ascites tumor cells has been isolated and purified free from other nucleases. The exonuclease degraded single-stranded RNA processively from either a 5′-hydroxyl or a 5′-phosphorylated end and released 5′-mononucleotides. The enzyme digested single-strand poly(C), poly(U), and poly(A) equally well but did not degrade duplex poly(C)·poly(I) or poly(A)·poly(U). Less than 0.2% of duplex DNA or 1.5% of heat-denatured DNA was degraded under the conditions which resulted in greater than 26% degradation of RNA. The ribonuclease required Mg^{2+} (0.2 mM) for optimum activity and was inhibited by ethylenediaminetetraacetic acid but not by human placental RNase inhibitor. The native enzyme had a Stokes radius of 42 Å and a sedimentation coefficient ($s_{20,w}$) of 4.3 S. From these values, an apparent molecular weight of 76 000 was derived by using the Svedberg equation. The localization and unique mode of degradation suggest a role for the 5′→3′ exoribonuclease in ribosomal RNA processing.

In mammalian cells, the precursor of ribosomal RNA has a molecular weight of about 4.5×10^6 and a sedimentation coefficient of 45 S. The 45S precursor RNA is sequentially cleaved to yield mature 18S, 5.8S, and 28S rRNAs in addition to spacer fragments (Perry, 1976; Abelson, 1979). The arrangement of the primary transcript is thought to have the following structure: (5′)-spacer-18S-spacer-5.8S-spacer-28S-(3′) (Perry, 1976; Nazar, 1982; Dawid & Wellauer, 1976). The transcribed spacer sequences are removed stepwise at each maturation step (Perry, 1976; Abelson, 1979; Busch et al., 1982). The precursor 45S RNA molecule is enzymatically cleaved into intermediate 20S and 32S molecules which are further cleaved and trimmed to the final 18S, 5.8S, and 28S mature ribosomal RNA molecules (Nazar, 1982; Busch et al., 1982; Bowman et al., 1983).

For elucidation of the enzymes involved in ribosomal RNA processing, research in our laboratory has been directed toward the identification of ribonucleases that are associated with purified nucleoli. Thus far we have identified three distinct single-strand-specific ribonucleases. Two of these enzymes are single-strand-specific endonucleases which we have designated nucleolar RNase I and nucleolar RNase II, respectively (Eichler & Tatar, 1980; Eichler & Eales, 1982). In this report, we described a third single-strand-specific activity, nucleolar RNase III, which has been identified as a 5′→3′ exoribonuclease. Its properties, which distinguish it from the other nucleolar ribonucleases and imply a possible role in processing of ribosomal RNA, are presented in this paper.

Materials and Methods

Materials. Unlabeled and labeled [^{3}H]poly(C) (22.8 mCi/mmol), [^{3}H]poly(A) (42 mCi/mmol), [^{3}H]poly(U) (525 mCi/mmol), [^{3}H]poly(C)·poly(I) (9.8 mCi/mmol), and [^{3}H]poly(U)·poly(A) (19.6 mCi/mmol) were obtained from Miles Chemicals Corp. Cytidine 3′,5′-[5′-^{32}P]diphosphate (2000 Ci/mmol) was purchased from ICN. [γ-^{32}P]ATP (1000–2000 Ci/mmol) was prepared as described by Maxam & Gilbert (1977). Cytidine 3′,5′-diphosphate was purchased from P-L Biochemicals. The dye-binding protein assay kit was obtained from Bio-Rad. Poly(ethylenimine)-impregnated cellulose thin-layer chromatography plates were purchased from Brinkmann.

Proteins and Enzymes. Bacterial alkaline phosphatase and T_4 polynucleotide kinase were purchased from Bethesda Research Laboratories. S_1 nuclease and T_4 RNA ligase were obtained from P-L Biochemicals. Bovine serum albumin, ovalbumin, and yeast alcohol dehydrogenase were obtained from Sigma. Human placental ribonuclease inhibitor was prepared by the method of Blackburn (1979).

RNAs. ^{32}P-Labeled f_2 RNA (4×10^4 cpm/nmol) was prepared essentially according to the procedure of Glitz (1968) for MS2 RNA, and at the same time, modifications of this procedure also permitted the preparation of ^{32}P-labeled *Escherichia coli* RNA (2.5×10^4 cpm/nmol). Yeast 5.8S rRNA was prepared according to the procedure of Rubin (1975) from frozen yeast cells (*Saccharomyces cerevisiae* X2180-1B) generously provided by Dr. Jeremy Thorner (University of California at Berkeley). The 5′-^{32}P labeling of yeast 5.8S rRNA was carried out according to the procedure of Donis-

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Keller et al. (1977) using T_4 polynucleotide kinase and [γ-^{32}P]ATP. The 5′-^{32}P-end labeling was considered complete when an additional aliquot of enzyme resulted in no increased incorporation of label. The 3′-^{32}P labeling of yeast 5.8S rRNA was accomplished by following the procedure of Peattie (1979) using T_4 RNA ligase and [5′-^{32}P]pCp. End-labeled yeast 5.8S rRNA was purified by 8% polyacrylamide–urea gel electrophoresis (Maxam & Gilbert, 1977). Double-labeled poly(A) was prepared as follows: [^{3}H]poly(A) (6.4×10^3 cpm/nmol) was treated with bacterial alkaline phosphatase to remove terminal phosphate residues and then purified by phenol extraction and concentrated by ethanol precipitation. The 5′-^{32}P- and 3′-^{32}P-end labeling of [^{3}H]poly(A) was carried out in a similar fashion as described previously for the end labeling of yeast 5.8S rRNA.

DNA Substrates. T_7 [^{3}H]DNA was prepared according to the procedure of Richardson (1966). The specific radioactivity was 4×10^4 cpm/nmol. Heat-denatured T_7 [^{3}H]DNA was prepared by heating in 0.1 M NaCl–0.015 M sodium citrate for 5 min in a boiling water bath and quick chilling to 0 °C. The heat-denatured T_7 [^{3}H]DNA was 95% single stranded as determined by S_1 nuclease sensitivity.

RNase Assay. The assay measures the conversion of *E. coli* [^{32}P]RNA to acid-soluble nucleotides. The reaction mixture (0.05 mL) contained 50 mM tris(hydroxymethyl)aminomethane hydrochloride (Tris-HCl), pH 8.0, 0.025 M NaCl, 0.2 mM $MgCl_2$, 0.1 mg/mL bovine serum albumin, 5 nmol of labeled RNA, and 0.5–1.0 unit of ribonuclease activity. The reaction was incubated at 37 °C for 30 min, at which time 0.125 mL of 2.5 mg/mL tRNA and 2.0 mg/mL bovine serum albumin was added. The mixture was precipitated with 0.075 mL of 25% perchloric acid containing 0.75%-uranyl acetate at 0 °C. After 10 min, the material was centrifuged in a Beckman B microfuge for 2.5 min. An aliquot (0.2 mL) of the supernatant was counted in 4 mL of Triton X-100 scintillation fluid [1 L of Triton X-100 and 2 L of 3a20 toluene scintillation fluid (Research Products International Corp.)] and counted in a Beckman 7500 liquid scintillation counter.

Determination of Polarity. The reaction mixture (0.2 mL) contained 50 mM Tris-HCl, pH 8.0, 50 mM NaCl, 0.2 mM $MgCl_2$, 0.1 mg/mL bovine serum albumin, 20 units of RNase, and either 20 nmol of [5′-^{32}P,^{3}H]poly(A) or 20 nmol of [3′-^{32}P,^{3}H]poly(A). At 5, 15, 30, and 60 min, aliquots (0.01 mL) were taken and processed and counted. At 60 min, a second aliquot (0.05 mL) was taken and mixed with 1 mL of 5 mM Tris-HCl, pH 7.5, and 7 M urea and loaded onto a 1-mL DEAE-Sephadex A25 column (0.7 × 2.6 cm) jacketed at 65 °C and equilibrated against this same buffer. The loaded column was washed with 2 mL of buffer, and a 50-mL gradient from 0.0 to 0.4 M LiCl was run. Fractions (0.55 mL) were collected and counted.

Determination of the Nature of Termini. The reaction mixture (0.025 mL) contained 20 mM Tris-HCl, pH 8.0, 0.1 M NaCl, 0.2 mM $MgCl_2$, 52.6 nmol of [^{3}H]poly(C) (8.7×10^3 cpm/nmol), and 38 units of ribonuclease. At 0, 20, and 40 min, aliquots (5 μL) of the reaction mixture were spotted on a poly(ethylenimine)-impregnated cellulose thin-layer plate along with cytidine 3′- and 5′-mononucleotide UV markers. The thin-layer plate was developed with 1 M acetic acid to 4 cm and then with 0.3 M LiCl to 16 cm. Samples (1 cm^2) were cut from each lane and processed in a Packard sample oxidizer for counting.

Glycerol Gradient Centrifugation. An aliquot (0.1 mL) of fraction IV was layered on a 3.8-mL 10–30% (v/v) glycerol gradient containing 50 mM Tris-HCl, pH 7.5, 0.25 M NaCl, and 0.1 mM ethylenediaminetetraacetic acid (EDTA). The gradients were centrifuged 14 h at 5×10^4 rpm at 4 °C in a SW60 rotor with bacterial alkaline phosphatase (6.1 S), bovine serum albumin (4.3 S), and ovalbumin (3.55 S) as markers in parallel gradients. After centrifugation, fractions (0.13 mL) were collected from the bottom of the tube. Aliquots (5 μL) of the indicated fractions were assayed for ribonuclease activity. Protein standard peaks were determined by the Bio-Rad dye-binding protein assay of each fraction.

Preparation of Nucleoli. Nucleoli were prepared from Ehrlich ascites tumor cells according to the method of Muramatsu et al. (1974) with the following modifications. Nuclei prepared as described previously by Eichler & Tatar (1980) were resuspended in 0.25 M sucrose, 1 mM $MgCl_2$, and 0.5 mM dithiothreitol at approximately 1×10^8 nuclei per mL. This suspension (100 mL) was divided into four equal fractions, each of which was used to overlay 10 mL of 0.88 M sucrose, 10 mM Tris-HCl, pH 8.0, 5 mM $MgCl_2$, and 0.5 mM dithiothreitol in 50-mL polycarbonate centrifuge tubes. After centrifugation for 10 min at $860g_{av}$ in a Beckman JS-7.5 swinging bucket rotor, the nuclear pellets were suspended in 100 mL of 0.25 M sucrose buffer. Aliquots (10 mL) were sonicated at approximately 85 W of power by using a 0.5-in. disruptor horn on a Model W375 Heat Systems Ultrasonics, Inc., cell disrupter. Three 5-s pulses with 10-s pauses between yielded greater than 98% disruption of nuclei as judged by phase contrast microscopy. Aliquots (25 mL) of disrupted material were then layered over 10 mL of 0.88 M sucrose buffer. Centrifugation was carried out as described above. The nucleolar pellets were resuspended in a total of 40 mL of 0.25 M sucrose buffer and again sonicated in 10-mL aliquots but for only two 5-s pulses. As before, this sonicated suspension was collected by centrifugation through 0.88 M sucrose buffer. The nucleolar pellets were then combined in 0.25 M sucrose buffer, centrifuged, and stored at −20 °C.

Protein Determination. Protein was measured by the method described by Bio-Rad for their dye-binding protein assay using rabbit γ-globulin as the standard protein.

Purification of Nucleolar Exoribonuclease. All steps for the purification of the ribonuclease were carried out at 0–4 °C unless otherwise indicated.

(*A*) *Nucleolar Extraction.* Purified Ehrlich cell nucleoli (from approximately 5×10^{10} nuclei) were suspended in 100 mL of 0.2 M potassium phosphate, pH 8.2, 1 mM EDTA, and 1 mM β-mercaptoethanol. After 1.5 h of gentle mixing, the chromatin was collected by centrifugation at 20000*g* for 30 min. The supernatant was dialyzed against three changes of 2 L of buffer A (1 mM potassium phosphate, pH 6.8, and 1 mM β-mercaptoethanol) over a 20-h period. This material was then clarified by centrifugation at 20000*g* for 30 min and again at 105000*g* for 60 min. The supernatant represents fraction I (120 mL).

(*B*) *Hydroxylapatite Chromatography I.* A hydroxylapatite column (1.5 × 16 cm) was equilibrated against buffer A. Fraction I was loaded at a flow rate of 30 mL/h, and 6-mL fractions were collected. The column was washed with 40 mL of buffer A and then 90 mL of 5 mM potassium phosphate, pH 6.8, and 1 mM β-mercaptoethanol. The column was eluted with 120 mL of 5 mM potassium phosphate, pH 6.8, 3 M KCl, and 1 mM β-mercaptoethanol followed by a second step (120 mL) of 0.1 M potassium phosphate, pH 6.8, 3 M KCl, and 1 mM β-mercaptoethanol. The peak activity fractions which eluted with the second high salt step were pooled and dialyzed against two changes of 1 L of buffer B (50 mM Tris-HCl, pH 7.5, 10% glycerol, 1 mM EDTA, and 1 mM β-mercapto-

ethanol). The dialyzed material was clarified by centrifugation at 10000*g* for 15 min and represents fraction II (20.8 mL).

(*C*) *DEAE-cellulose Chromatography.* A DEAE-cellulose column (2.5 × 10.5 cm) was equilibrated against buffer B. Fraction II was loaded at 40 mL/h, and 6.5-mL fractions were collected. The column was washed with 100 mL of buffer B and a gradient (500 mL) of 0–0.5 M NaCl in buffer B used to elute the ribonuclease. Peak fractions eluting at approximately 0.1 M NaCl were pooled and represent fraction III (72 mL).

(*D*) *Hydroxylapatite Chromatography II.* A second hydroxylapatite column (1.5 × 3 cm) was equilibrated against buffer A. Fraction III was loaded at a rate of 30 mL/h, and 1.2-mL fractions were collected. The column was washed with 10 mL of buffer A and then with 20 mL of 5 mM potassium phosphate, pH 6.8, and 1 mM β-mercaptoethanol. The column was eluted with 20 mL of 5 mM potassium phosphate, pH 6.8, 3 M KCl, and 1 mM β-mercaptoethanol followed by a second step (20 mL) of 0.1 M potassium phosphate, pH 6.8, 3 M KCl, and 1 mM β-mercaptoethanol. The ribonuclease activity which eluted with the second high salt step was pooled and desalted by passage through a Sephadex G-25 column equilibrated against 0.05 M Tris-HCl, pH 7.5, 0.25 M NaCl, 10% glycerol, and 1 mM β-mercaptoethanol. The void peak material was pooled and represented fraction IV (9.5 mL).

Assay for Contaminating Activities. Degradation of 5.8S [5′-^{32}P]rRNA by the nucleolar exoribonuclease was monitored in order to detect contaminating phosphatase or endonucleolytic activity. The reaction mixture (0.16 mL) contained 50 mM Tris-HCl, pH 8.0, 50 mM NaCl, 0.2 mM $MgCl_2$, 0.25 mg/mL yeast RNA, 6.3 × 10^5 cpm of 5.8S [5′-^{32}P]rRNA, and 52 units of ribonuclease. At 5, 10, 30, and 60 min, four aliquots of the reaction mixture were taken. One aliquot (3 μL) was processed for acid-soluble material. The second (3 μL) aliquot was added to 0.1 mL of 0.1 N HCl to which 0.05 mL of a 20% suspension of Norite in 1 mM sodium pyrophosphate was added. After 10 min at 0 °C, the material was centrifuged 2.5 min in a Beckman microfuge, and a 0.1-mL sample of the supernatant was counted. The third aliquot (3 μL) was spotted onto a poly(ethylenimine)-impregnated cellulose plate. The plate was developed with 1 N HCOOH and 0.5 N LiCl. Positions of the UV standards were marked, and the plate was exposed to X-ray film (Kodak X-Omat R-film) plus an intensifying screen (Du Pont Cronex screen) for 24 h. The fourth aliquot (0.01 mL) of the reaction mixture was mixed with 0.015 mL of 10 M urea, 0.25 mg/mL yeast RNA, 0.02 M EDTA, 0.04% xylene cyanol (XC), and 0.04% bromphenol blue (BB). Samples were layered on a 20% polyacrylamide–7 M urea gel. Electrophoresis was carried out at 400 V for 4 h and the gel exposed to X-ray film (Kodak X-Omat R-film) plus an intensifying screen (Du Pont Cronex screen) for 14 h at −70 °C.

Results

Enzymatic Purity of Fraction IV. A summary of the partial purification of the nucleolar exoribonuclease is presented in Table I. Fraction IV was found to be stable for several months when stored at 0 °C; however, further attempts at purification resulted in considerable loss of activity. Since the assay procedure for ribonuclease activity was not selective for the nucleolar exoribonuclease, it was not possible to directly follow the purification of this nuclease until the DEAE chromatography step, at which point this material was essentially devoid of other ribonuclease activity.

The following experiments were performed in order to confirm that only a single nuclease species is present in fraction IV. First, the enzyme was incubated with 5.8S [5′-^{32}P]rRNA, and the release of counts was monitored either by acid solubilization or by Norite adsorption. All the acid-soluble label released as a function of time could be accounted for as Norite-adsorbable material. Second, timed aliquots of this same reaction mixture were chromatographed on poly(ethylenimine) thin-layer plates under conditions which would distinguish the mononucleotide, 5′-AMP, released from the 5′ end of yeast 5.8S rRNA (Rubin, 1973) from inorganic phosphate. The 5′-terminal label was released only as the mononucleotide. Finally, aliquots of this reaction mixture were subjected to electrophoresis on a 20% polyacrylamide–urea gel in order to detect endonucleolytic activity. Within the resolution of this gel system, the label released moved exclusively as a single band equivalent to the mononucleotide.

As another test, the ribonuclease material (fraction IV) was subjected to electrophoresis on a 7.5% polyacrylamide gel at pH 8.9. The gel was then sliced, and the slices were assayed for nuclease activity after elution with buffer. As shown in Figure 1, a single peak of ribonuclease activity was observed. In addition, both gel filtration chromatography (Figure 2) and

Table I: Partial Purification of Ehrlich Nucleolar Exoribonuclease[a]

purification step	vol (mL)	units/mL	mg/mL	units/mg
(I) 0.2 M K_2HPO_4, pH 8.2, extract	120	530	0.52	
(II) hydroxylapatite chromatography I	21	1640	1.02	
(III) DEAE-cellulose chromatography	72	360	0.10	3700
(IV) hydroxylapatite chromatography II	9.5	2200	0.36	6100

[a] Purification was carried out as described in the text, starting from nucleoli extracted from 5 × 10^{10} nuclei. One unit of RNase activity was defined as 1 nmol of nucleotide released into acid-soluble material in the standard reaction described under Materials and Methods.

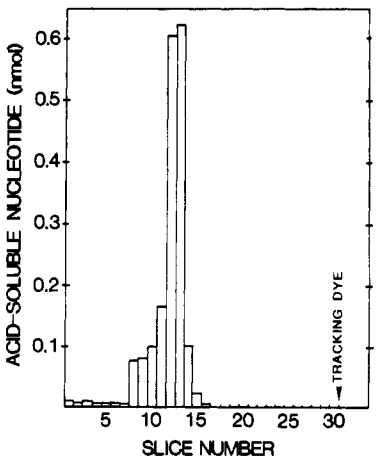

FIGURE 1: Polyacrylamide gel electrophoresis of the nucleolar ribonuclease. A 7.5% polyacrylamide gel was prepared according to the procedure of Eichler (1973), except that 20% glycerol was included in the gel. Spun column chromatography (Maniatis et al., 1982) was used to change the buffer in which the enzyme sample (0.1 mL) was dissolved to 0.125 M Tris–phosphate, pH 8.9, 1 mM β-mercaptoethanol, and 10% glycerol. An aliquot (1 μL) of 0.4% bromphenol blue was added to the sample (0.05 mL) before it was loaded onto the gel (0.5 × 10 cm). Electrophoresis was carried out for approximately 2.5 h at 2 mA/gel at 4 °C. The gel was sliced into 3-mm sections, and each section was eluted overnight at 4 °C with 0.25 mL of 0.1 M Tris-HCl, pH 8.0, 0.4 mM $MgCl_2$, 1 mg/mL bovine serum albumin, and 20% glycerol. An aliquot (0.015 mL) of each fraction was assayed in a reaction mixture (0.05 mL) containing 0.06 M Tris-HCl, pH 8.0, 0.2 mM $MgCl_2$, 0.1 mg/mL bovine serum albumin, 5 nmol of *E. coli* [^{32}P]RNA, and 6% glycerol. Samples were processed and counted as described under Materials and Methods.

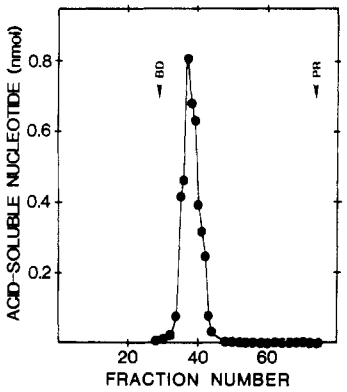

FIGURE 2: Gel filtration of nucleolar ribonuclease. A Sephadex G-200 column (1.5 × 84 cm) was equilibrated against 0.05 M Tris-HCl, pH 7.5, 0.25 M NaCl, 10% glycerol, 0.1 mM EDTA, and 1 mM β-mercaptoethanol. An aliquot (0.5 mL) of fraction IV was layered on the column, and 2.0-mL fractions were collected. The elution position of the ribonuclease was determined by monitoring enzyme activity as described under Materials and Methods. The fractional elution volumes (K_{av}) of various standard proteins used to calibrate the column were determined by either the Bio-Rad dye-binding protein assay or the OD_{280} of each 2.0-mL fraction. Standard proteins were yeast alcohol dehydrogenase (41.2 Å), bacterial alkaline phosphatase (33 Å), and ovalbumin (29 Å). BD, blue dextran; PR, phenol red.

sedimentation on a glycerol gradient showed only a single peak of ribonuclease activity. Taken together, these results strongly support the conclusion that the ribonuclease activity in fraction IV was devoid of endonucleolytic and phosphatase activity and was present in one molecular species.

Physical Properties. To determine the apparent molecular weight for the partially purified nucleolar ribonuclease, the enzyme was chromatographed on a Sephadex G-200 molecular sieving column that was standardized with proteins of known molecular weight. A single peak of ribonuclease activity eluted at a position close to alcohol dehydrogenase which has a molecular weight of 150000 (Siegel & Monty, 1966) (Figure 2), and a Stokes radius of 42 Å was determined from the elution volume of the nuclease (Laurent & Killander, 1964). A sedimentation coefficient of 4.3 s for the nucleolar ribonuclease was estimated according to the method of Martin & Ames (1961) using ovalbumin, bovine serum albumin, and bacterial alkaline phosphatase ($s_{20,w}$ = 3.6, 4.3, and 6.1 S, respectively) (LeMaire et al., 1980; Siegel & Monty, 1966) as markers on a glycerol gradient. When the values for the Stokes radius (42 Å) and the sedimentation coefficient (4.3 S) were substituted into the Svedberg equation (Siegel & Monty, 1966), an apparent molecular weight of 76000 was calculated by assuming a partial specific volume of 0.73.

Properties of Partially Purified Enzyme Fraction IV. The nucleolar ribonuclease was found to be optimally active in Tris buffer at pH 7.5–8.5. The enzyme requires $MgCl_2$ for optimal activity, with an optimum of 0.2 mM (Figure 3). This divalent cation requirement was not satisfied by either Mn^{2+} or Ca^{2+}. EDTA at a concentration of 4 mM completely inhibits enzyme activity. Sulfhydryl reagents such as dithiothreitol or β-mercaptoethanol had no effect on RNase activity. Human placental RNase inhibitor, a known inhibitor of nucleolar ribonuclease I (Eichler et al., 1981), had no effect on this enzyme.

Substrate Specificity. Base specificity of the nucleolar enzyme was tested by using single-stranded ^{3}H-labeled poly(C), poly(U), and poly(A) as substrates. As shown in Figure 4, the enzyme degraded these ribohomopolymers equally well, indicating a lack of base specificity. The effect of secondary structure was tested by using duplex ribopolymers. Assays

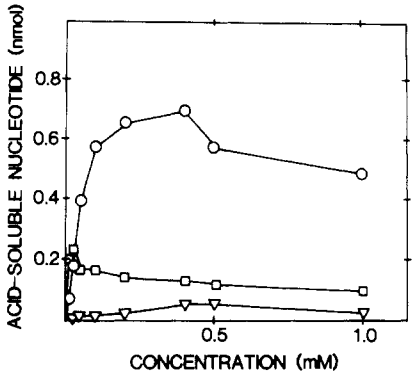

FIGURE 3: Effect of divalent cation on the hydrolysis of RNA by the nucleolar ribonuclease. The reaction mixture (0.05 mL) contained 0.05 M Tris-HCl, pH 8.0, 0.025 M NaCl, 0.1 mg/mL bovine serum albumin, 5 nmol of [^{32}P]RNA (3.9 × 10^3 cpm/nmol), and divalent cation as indicated. Reactions were started by the addition of 0.7 unit of ribonuclease, and samples were processed as described for a standard assay. (○) $MgCl_2$; (□) $MnCl_2$; (▽) $CaCl_2$.

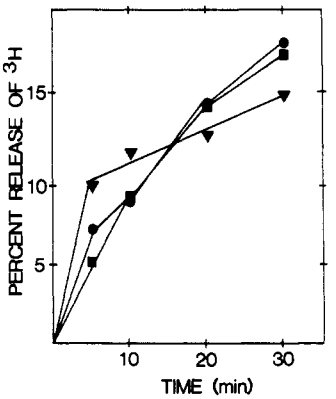

FIGURE 4: Specificity of nucleolar ribonuclease. The reaction mixtures (0.2 mL) contained 0.05 M Tris-HCl, pH 8.0, 0.025 M NaCl, 0.2 mM $MgCl_2$, 0.1 mg/mL bovine serum albumin, 16 units of ribonuclease, and 20 nmol of either (●) [^{3}H]poly(A) (3 × 10^3 cpm/nmol), (■) [^{3}H]poly(C) (9.8 × 10^3 cpm/nmol), or (▼) [^{3}H]poly(U) (3 × 10^3 cpm/nmol). Aliquots (0.025 mL) were removed at the indicated times and then processed and counted as described under Materials and Methods.

Table II: Substrate Specificity of Exoribonuclease[a]

substrate	rel act.
Single vs. Double Stranded[b]	
[^{3}H]poly(C)	1.00
[^{3}H]poly(C)·poly(I)	0.01
[^{3}H]poly(U)	1.00
[^{3}H]poly(A)·poly(U)	0.08
RNA vs. DNA[c]	
E. coli [^{32}P]RNA	1.00
T_7 [^{3}H]DNA, native	0.005
T_7 [^{3}H]DNA, heat denatured	0.054

[a] The complete reaction (0.25 mL) contained 0.05 M Tris-HCl, pH 8.0, 0.025 M NaCl, 0.2 mM $MgCl_2$, and 0.1 mg/mL bovine serum albumin. [b] Control substrates were the single-stranded complements of the duplex RNA assayed with 10.4 units of RNase and 25 nmol of RNA substrate. [c] Control substrate was *E. coli* [^{32}P]RNA. The assay contained 5 units of RNase and 25 nmol of RNA or DNA substrate.

using [^{3}H]poly(C)·poly(I) or [^{3}H]poly(U)·poly(A) showed that no significant degradation of the duplex ribopolymers occurred relative to their single-strand complements, [^{3}H]poly(C) or [^{3}H]poly(U) (Table II). Further analysis of the ribonuclease specificity was conducted to determine its ability to degrade DNA. As shown in Table II, the nucleolar ribonuclease showed no significant degradation of native or denatured DNA relative to an RNA substrate.

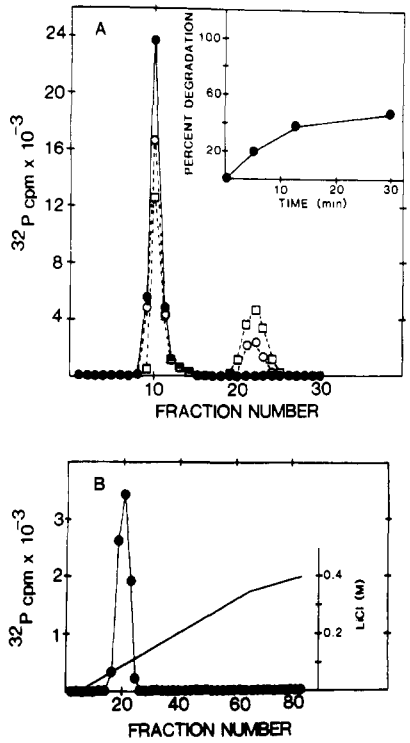

FIGURE 5: Gel filtration analysis of products formed by the nucleolar RNase. The reaction mixture (0.3 mL) contained 0.05 M Tris-HCl, pH 8.0, 0.025 M NaCl, 0.1 mg/mL bovine serum albumin, 0.2 mM $MgCl_2$, 30 nmol of [^{32}P]RNA (7.5 × 10^3 cpm/nmol), and 19 units of ribonuclease. (A) At the indicated times (see inset), aliquots (0.05 mL) were adjusted to 10 mM EDTA and 0.5% sodium dodecyl sulfate in a total volume of 0.15 mL. This material was then layered onto a Sephacryl S-200 column (1 × 19 cm) equilibrated against 50 mM sodium phosphate, pH 7.0, 0.15 M NaCl, 10 mM EDTA, and 0.5% sodium dodecyl sulfate. Fractions (0.6 mL) were collected and counted in 4 mL of Triton X-100 scintillation fluid: (●) 0 min; (○) 5 min; (□) 15 min. (B) The 30-min timed aliquot was brought to 1 mL in 5 mM Tris-HCl, pH 7.5, and 7 M urea and loaded onto a 1-mL DEAE-Sephadex A25 column (0.7 × 2.6 cm) jacketed at 65 °C and equilibrated against this same buffer. The loaded column was washed with 2 mL of buffer, and a 50-mL gradient from 0.0 to 0.4 M LiCl was run. Fractions (0.55 mL) were collected and counted in 4 mL of Triton X-100 scintillation fluid.

Mode of Attack. To determine whether the nucleolar ribonuclease acts as an endo- or exonuclease, timed aliquots of an RNA digestion were analyzed for size distribution on a Sephacryl S-200 molecular sieving column. Regardless of the extent of degradation, material from the void peak was converted solely to material which eluted at a position equivalent to that of the mononucleotide (Figure 5A). Analysis of the reaction material on a DEAE-Sephadex A25 column further supported these findings (Figure 5B), demonstrating that only mononucleotide product was released by the action of the nucleolar RNase. These results would be expected if the nuclease degrades the RNA exonucleolytically.

An exonuclease may digest a polymeric substrate in either a distributive or a processive fashion (Nossal & Singer, 1968; Thomas & Olivera, 1978). The distributive mode of attack results in a general shortening of the polymer as a function of the extent of the reaction. In contrast, the processive mode of attack results in the degradation of one RNA molecule before attack begins on another (Nossal & Singer, 1968). Because no intermediate-size material was observed during the time course of degradation (Figure 5A,B), it was concluded that the nucleolar exonuclease degrades RNA processively (Nossal & Singer, 1968).

The processive exonucleolytic degradation of RNA by the nucleolar RNase was confirmed by using 5′-^{32}P-labeled

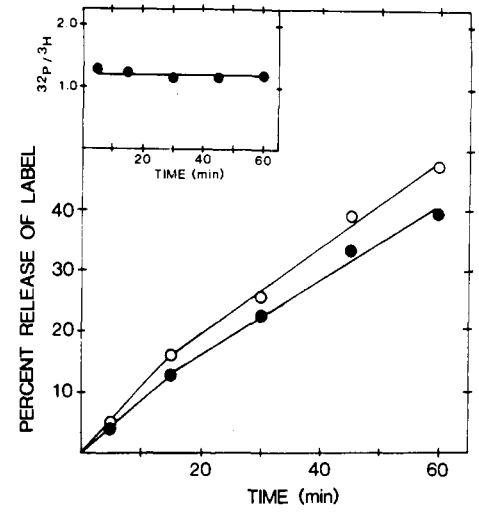

FIGURE 6: Digestion of double-labeled poly(A) by the nucleolar ribonuclease. The reaction mixture (0.2 mL) contained 50 mM Tris-HCl, pH 8.0, 0.025 M NaCl, 0.1 mg/mL bovine serum albumin, 0.2 mM $MgCl_2$, 16 units of RNase, and 20 nmol of [5′-^{32}P,^{3}H]poly(A). At the indicated times, aliquots (0.01 mL) were removed and then processed and counted as described under Materials and Methods. (○) ^{32}P label; (●) ^{3}H label. The *inset* shows the ratio of ^{32}P label to ^{3}H label released.

[^{3}H]poly(A) as a substrate. A distributive exonuclease will preferentially release one label over the other depending on the polarity of attack, whereas a processive exonuclease will release both labels at approximately the same rate (Thomas & Olivera, 1978). As shown in Figure 6, the rates of release of the 5′-^{32}P label and ^{3}H label were nearly identical, further demonstrating that the nucleolar exonuclease degrades RNA processively.

Mode of Phosphodiester Bond Cleavage. The phosphodiester bond of a polynucleotide chain can be cleaved in one of two ways by a nuclease. To determine whether the exoribonuclease cleavage occurs on the 3′ or 5′ side of the phosphodiester bond, [^{3}H]poly(C) was hydrolyzed by the ribonuclease. The products of degradation were separated on a poly(ethylenimine)-impregnated cellulose thin-layer chromatography plate under conditions that distinguish 5′- from 3′-mononucleotides. The radiolabel released by the action of the exonuclease moved exclusively with the cytidine 5′-mononucleotide marker. The nucleolar exonuclease, therefore, cleaves on the 3′ side of the phosphodiester bond, releasing 5′-mononucleotides.

Direction of Attack by the Exonuclease. To define the direction of attack by the nucleolar exoribonuclease, [^{3}H]-poly(A) containing either a 5′- or a 3′-^{32}P-end label was used as a substrate. The rationale for this approach was based on an earlier observation of Nossal & Singer (1968), who found that a processive exonuclease may leave a short oligonucleotide product which originates from the opposite end of attack. In other words, processivity may be lost as enzymatic degradation nears the opposite termini of the polymer chain.

When [5′-^{32}P,^{3}H]poly(A) was used as the substrate, the rates of release of the 5′-^{32}P label and ^{3}H label were essentially the same. Chromatography of this reaction material on a Sephadex DEAE A25 column showed that both labels were released as mononucleotide products. In contrast, when [3′-^{32}P,^{3}H]poly(A) was used as the substrate, the rate of release of the 3′-^{32}P label was significantly slower than that of the ^{3}H label. If the nucleolar exoribonuclease completely degraded the [3′-^{32}P,^{3}H]poly(A), the 3′-^{32}P label would be released as [5′-^{32}P]pCp and the ^{3}H label as 5′-AMP. However, chromatography of this reaction material on a Sephadex

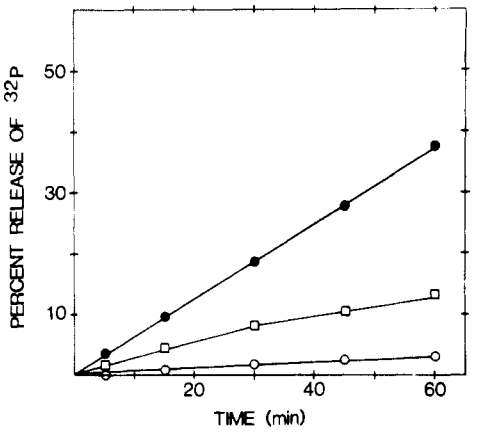

FIGURE 7: Degradation of end-labeled yeast 5.8S rRNA by the nucleolar exoribonuclease. The reaction mixture (0.05 mL) contained 2.5×10^5 cpm of either 5′-^{32}P- or 3′-^{32}P-end-labeled yeast 5.8S rRNA in 50 mM Tris-HCl, pH 8.0, 50 mM NaCl, 0.025 mg/mL tRNA, 0.2 mM $MgCl_2$, and 16 units of nucleolar exoribonuclease. At the indicated times, aliquots (0.003 mL) were taken and processed and counted as described under Materials and Methods. (●) 5.8S [5′-^{32}P]rRNA; (○) 5.8S [3′-^{32}P]rRNA; (□) 5.8S [3′-^{32}P]rRNA (3′-dephosphorylated).

DEAE A25 column showed that most of the ^{32}P label is released as oligonucleotides greater than a pentanucleotide in length. The ^{3}H label was released essentially as mononucleotide. These results indicate that the exoribonuclease degrades in a 5′ to 3′ direction and that the enzyme loses processivity as it approaches the 3′ terminus. Moreover, since the [^{3}H]poly(A) was terminally dephosphorylated before the addition of label to either the 5′ or the 3′ end, these results also indicate that the nucleolar exoribonuclease will initiate attack from either a 5′-phosphoryl or a 5′-hydroxyl end of the [^{3}H]poly(A) molecule.

To further support the findings for a 5′→3′ direction of attack by the exonuclease, yeast 5.8S rRNA was labeled at either the 5′ or the 3′ end with ^{32}P. Since the 5.8S rRNA has been shown to have considerable secondary structure (Nazar, 1982; Nazar et al., 1975), the complete processive degradation by the single-strand-specific exonuclease should be blocked. Therefore, preferential release of the 5′-^{32}P-end label would indicate polarity. As shown in Figure 7, nearly 40% of the 5′ label was released when less than 5% of the 3′ label was released. Although dephosphorylation of the 3′-end-labeled 5.8S rRNA affected the level of degradation, this increase is likely due to a small number of breaks introduced into the RNA during the reaction with bacterial alkaline phosphatase as was observed on a 20% polyacrylamide–urea gel. The observation that the 5′-end label was released preferentially over the 3′-end label was taken to support the findings that this exoribonuclease attacks RNA from the 5′ end.

Discussion

The processing of ribosomal RNA occurs almost exclusively within the nucleolus of eucaryotic cells (Perry, 1976). Although the details of the enzymatic activities responsible for this processing are still largely unknown, considerable effort in this area has led to the identification and characterization of several possible candidates (Eichler & Tatar, 1980; Eichler & Eales, 1982; Perry & Kelley, 1972; Boctor et al., 1974; Mirault & Scherrer, 1972; Winicov & Perry, 1973). To this end, we have identified and partially purified a unique nucleolar ribonuclease of 76 000 molecular weight which degraded single-stranded RNA exonucleolytically in a 5′ to 3′ direction. This exoribonuclease degraded RNA exclusively and would initiate attack at either a 5′-phosphoryl or a 5′-hydroxyl terminus, releasing 5′-mononucleotides. The nucleolar exoribonuclease did not dissociate from the RNA substrate after cleavage of the 5′-terminal mononucleotide but rather remained bound until nearly the entire polynucleotide chain was degraded. The population of small resistant oligonucleotide products which remain after digestion and originate from the 3′ end of the polynucleotide chain may suggest that the enzyme has a preference for longer oligomeric chains (Nossal & Singer, 1968).

The nucleolar exoribonuclease described herein shares its processive exonucleolytic mechanism with a nuclear exoribonuclease reported earlier by Lazarus & Sporn (1967) and a yeast ribosomal exoribonuclease reported by Stevens (1980). All three single-strand-specific exoribonucleases require a divalent cation for the degradation of RNA molecules; however, the nucleolar exoribonuclease can be clearly distinguished from both of the other exoribonuclease activities. Unlike the nucleolar exoribonuclease, the nuclear exoribonuclease described by Lazarus & Sporn (1967) degrades RNA in a 3′ to 5′ direction, requires a 3′-hydroxyl group to permit attack, and shows significant variation in its ability to degrade the various ribohomopolymers, poly(A), poly(U), and poly(C). Although the yeast 5′→3′ exoribonuclease described by Stevens (1980) shows a similar polarity of attack, the yeast enzyme demonstrates a preference for 5′-phosphorylated termini not observed with the nucleolar 5′→3′ exoribonuclease. In addition, the ability of the yeast exoribonuclease to use Mn^{2+} nearly as well as Mg^{2+} as the divalent cation clearly distinguishes this enzyme from the nucleolar enzyme. A comparison of the molecular size of the nuclear 3′→5′ exoribonuclease (Lazarus & Sporn, 1967) and the yeast 5′→3′ exoribonuclease (Stevens, 1980) to the 76 000 molecular weight nucleolar exoribonuclease described in this paper is not possible since their values are not available.

The properties of this nucleolar exoribonuclease evoke interesting possibilities as to its biological role in nucleolar RNA metabolism. The 5′ to 3′ polarity and processive mode of attack suggest a role for this enzyme as an exonucleolytic trimming activity. The sensitivity to secondary structure might provide a possible means for preventing the complete processive degradation of a precursor RNA molecule. Alternatively, the enzyme would be well suited for involvement in the turnover of discarded RNA products arising from endonucleolytic processing steps in order to replenish nucleotide pools.

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Registry No. Single-strand-specific 5′→3′-exoribonuclease, 50864-51-2.

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Ribulose-1,5-bisphosphate Carboxylase/Oxygenase Incubated with Cu^{2+} and Studied by Electron Paramagnetic Resonance Spectroscopy†

Rolf Brändén,* Thomas Nilsson, and Stenbjörn Styring

ABSTRACT: Ribulose-1,5-bisphosphate carboxylase/oxygenase binds Cu^{2+} at a specific site when stoichiometric amounts of Cu^{2+} are added at saturating concentrations of HCO_3^-. The presence of HCO_3^- (CO_2) is necessary to obtain specific binding of Cu^{2+}. The activating metals Mg^{2+} and Co^{2+} compete with Cu^{2+}, indicating a common site on the enzyme for these metals. Electron paramagnetic resonance (EPR) proves that at least one nitrogen ligand is involved in the binding of Cu^{2+} at this site. When ribulose 1,5-bisphosphate is added to the Cu^{2+}-incubated enzyme, a tight enzyme–Cu^{2+}–ribulose 1,5-bisphosphate complex is formed. In this ternary complex only oxygen atoms are directly coordinated to Cu^{2+} as seen by its characteristic EPR spectrum. A similar but distinct spectrum is obtained if 2-carboxyarabinitol 1,5-bisphosphate, an analogue to the six-carbon intermediate in the RuBP carboxylase reaction, is added to the Cu^{2+}-incubated enzyme. These results indicate that a metal–substrate complex is formed at the active site of the enzyme. A function for the metal in catalysis is thus implied.

Ribulose-1,5-bisphosphate carboxylase/oxygenase (EC 4.1.1.39) is a metal-activated enzyme that catalyzes two reactions (Calvin & Massini, 1952; Andrews et al., 1973):

$$\text{RuBP} + CO_2 + H_2O \rightarrow 2(\text{3-PGA}) + 2H^+ \quad (1)$$

$$\text{RuBP} + O_2 \rightarrow \text{3-PGA} + \text{phosphoglycolate} + 2H^+ \quad (2)$$

The active form of the enzyme contains bound CO_2 and a metal ion. The CO_2 molecule is bound as a carbamate and is stabilized by the metal ion (Lorimer et al., 1976; Lorimer & Miziorko, 1980). There is, however, no experimental evidence for a direct coordination of the metal to the carbamate.

The enzyme that in vivo is activated by Mg^{2+} has also been shown to be catalytically active in the presence of Mn^{2+}, Co^{2+}, Ni^{2+}, and Fe^{2+} (Christeller, 1981). Interaction with other transition metals has not been seriously studied, probably because they support very little activity.

In catalysis a molecule of CO_2, different from the CO_2 in the activating process, or O_2 reacts with RuBP[1] (reactions 1 and 2) (Lorimer, 1981). So far, it has not been proved that the metal is involved in the catalytic reactions, but some indications for a direct participation exist. The metal and the catalytic CO_2 are close enough to suggest a role for the metal in catalysis (Miziorko & Mildvan, 1974). The F^- inhibition of the Mg^{2+}- and Mn^{2+}-activated enzymes (Nilsson & Brändén, 1983) and the drastic drop of K_i for O_2 for the Mn^{2+}-activated enzyme, compared to the Mg^{2+}-activated enzyme (Christeller, 1981), indicate that the metal ion participates directly in catalysis.

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[1] Abbreviations: EPR, electron paramagnetic resonance; RuBP, ribulose 1,5-bisphosphate; HEPPS, *N*-(2-hydroxyethyl)piperazine-*N*′-propanesulfonic acid; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; CABP, 2-carboxyarabinitol 1,5-bisphosphate; 3-PGA, 3-phosphoglyceric acid.

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