Properties of a Purified Nucleolar Ribonuclease from Ehrlich Ascites Carcinoma Cells†

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ABSTRACT: A nucleolar ribonuclease specific for single-stranded ribonucleic acid (RNA) has been isolated and extensively purified from Ehrlich ascites carcinoma cells. The enzyme is optimally active at neutral pH and degrades RNA via a 2′,3′-cyclic intermediate leaving 3′- or 2′,3′-cyclic terminated oligonucleotides. The ribonuclease has an apparent molecular weight of 38 500 as judged by sedimentation equilibrium and is a basic protien having an isoelectric point greater than 9.0. The enzyme preferentially cleaves poly(C) over poly(U), poly(A), or poly(C)·poly(I). Limit digestion products of poly(C) degradation are on the average tri-, tetra-, and pentanucleotides. In the partial digestion of yeast 5.8S rRNA, the nucleolar ribonuclease cleaves only CpA phosphodiester bonds. Spermidine, spermine, and histone I inhibit the activity of nucleolar ribonuclease. Antibodies directed toward pancreatic RNase do not cross-react with the Ehrlich nucleolar ribonuclease.

Mature functionally competent eucaryotic RNA molecules such as ribosomal, messenger, and transfer RNA are all derived from larger precursors (Perry, 1976; Abelson, 1979), suggesting that enzymes which are involved in altering the structure of newly synthesized RNA may well be important in controlling cell growth (Perry, 1976; Abelson, 1979; Crick, 1979). We have, therefore, initiated a detailed study of the properties of nuclear ribonucleases in order to identify the molecular events in the biogenesis of RNA.

Over the past 10 years there have been numerous reports (Mirault & Scherrer, 1972; Prestayko et al., 1973; Winicov & Perry, 1974; Boctor et al., 1974) of nucleolar ribonuclease activities from various tissue sources. Because of the apparent nucleolar compartmentalization, these ribonucleases have been implicated in the maturation process of ribosomal RNA (Mirault & Scherrer, 1972; Prestayko et al., 1973; Winicov & Perry, 1974; Boctor et al., 1974). As part of an effort to define the role of ribonucleases in ribosomal RNA maturation, we have developed a new and simplified purification procedure that leads to a highly purified nucleolar ribonuclease. This report provides the first detailed analysis of the physical and enzymatic properties of the purified enzyme.

Experimental Procedure

Materials

Chemicals. Unlabeled and ^{3}H-labeled poly(C) (29.7 mCi/mmol), poly(A) (58 mCi/mmol), and poly(U) (512 mCi/mmol) were obtained from Miles Chemical Corporation [^{3}H]Uridine (47.5 mCi/mmol) and [γ-^{32}P]ATP (1500 to 2000 Ci/mmol) were purchased from New England Nuclear. Ampholytes (Pharmalyte pH 3–10 and pH 8.5–10) were from Pharmacia. Sperimine, spermidine, bovine serum albumin, and histone I were all obtained from Sigma Chemical Co. Hydroxylapatite was purchased from Bio-Rad Laboratories, agarose-5′-(4-aminophenylphosphoryl)uridine 2′(3′)-phosphate was from P-L Biochemicals, and DEAE-Sephadex A-25 was from Pharmacia.

Enzymes. Ribonucleases T_1 and U_2 and pancreatic ribonuclease were purchased from Sigma. Bacterial alkaline phosphatase and T_4 polynucleotide kinase were purchased from Bethesda Research Laboratories.

RNAs. Bacteriophage f_2 lysate was kindly provided by Dr. Patrice Zamenhof (University of California at Los Angeles). ^{32}P-Labeled f_2RNA was prepared esentially according to the procedure of Glitz (1968) for MS2 RNA. The specific radioactivity was 3×10^4 cpm per nmol. Yeast 5.8S RNA 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).

Methods

RNase Assay. The assay measures conversion of [^{32}P]f_2RNA to acid-soluble nucleotides. The reaction mixture (0.15 mL) contained 50 mM Tris-HCl, pH 7.5, 75 mM NaCl, 0.1 mg/mL bovine serum albumin, 4.5 nmol of labeled f_2RNA, 1mM β-mercaptoethanol, and 0.25 to 1.5 units of enzyme. After 30 min at 45 °C, the mixture was chilled at 0 °C in an ice bath and 0.025 mL of 12.5 mg/mL transfer RNA and 0.075 mL of 25% perchloric acid were added. Acid-insoluble material was removed by centrifugation at 8730*g* at 4 °C in a Beckman Microfuge for 2 min. The radioactivity of 0.2 mL of supernatant fluid was determined by mixing with 4 mL of Triton X-100 scintillation fluid (1 L of Triton X-100, 8 g of OmniFluor (New England Nuclear), 2 L of toluene). One unit of enzyme is that amount which produces 1 nmol of acid-soluble nucleotide in 30 min.

Sedimentation Equilibrium. Sedimentation equilibrium using a Beckman airfuge was carried out according to the procedure of Pollet et al. (1979). Sedimentation equilibrium was performed in 0.05 M Tris-HCl, pH 7.5, 5% glycerol, 0.05 M NaCl, 5 mg/mL bovine serum albumin, 1 mM β-mercaptoethanol, and 1 mM EDTA at 4 °C. The time of the run was 24 h at 32 800 rpm in a Beckman airfuge. Duplicate 0.1-mL samples were run and 10-μL fractions were withdrawn from the meniscus using 10-μL capillary pipets guided by a micromanipulator. Each 10-μL fraction was diluted with 10 μL of 50 mM Tris-HCl, pH 7.5, 10% glycerol, 1 mM β-mercaptoethanol, and 1 mM EDTA. Duplicate aliquots (5 μL) were assayed and processed as described for the standard assay. Data were expressed as a plot of the ln of enzyme activity as a measure of the enzyme protein concentration (ln *C*) vs. the square of the distance from the axis of rotation.

Extraction and Purification of Ribonuclease from Whole Nucleoli. Nuclei (2.2×10^{10}) were isolated from Ehrlich cells as described in the text. Nucleoli were isolated from these

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nuclei (suspended in 0.25 M sucrose, 0.01 M Tris-HCl, pH 7.5, and 3.3 mM $CaCl_2$) by the sonication procedure of Busch (1967). Isolated nucleoli were suspended in 300 mL of extraction buffer C (0.2 M KPO_4, pH 8.2, 1 mM EDTA, and 1 mM β-mercaptoethanol), and ribonuclease purification was then carried out by the same procedure as described in the text for whole nuclei as starting material.

Preparation of Duplex RNA. [^{3}H]Poly(C)·poly(I) was prepared by mixing 9.5 μL of 43.5 mM poly(I) with 9.0 μL of 45 mM [^{3}H]poly(C) (3 cpm/pmol) in 0.1 mL of 0.01 M sodium phosphate buffer, pH 7.0, and 0.1 M NaCl for 2 h at room temperature. [^{3}H]Poly(C) (3 cpm/pmol), which was used as a substrate for measurement of single-stranded RNase activity, was treated identically with the exception that poly(I) was omitted.

Assay for the Formation of 2′,3′-Cyclic CMP. The reaction mixture (0.05 mL) contained 0.025 M Tris-HCl, pH 7.5, 0.5 mM EDTA, 0.5 mM β-mercaptoethanol, 5% glycerol, 1.75 nmol of [^{3}H]poly(C) (specific activity 6.2×10^4 cpm/pmol), and 8 units of purified nucleolar RNase. The reaction was run at 45 °C, and 2-μL aliquots were spotted on a poly(ethylenimine) impregnated cellulose thin-layer chromatography plate (Merck) at 0-, 30-, 60-, 90-, and 120-min intervals. The thin-layer plate was developed with 0.3 M LiCl, and the areas that ran with the 2′,3′-cyclic CMP standards were cut from the plate and counted in 8 mL of toluene scintillation fluid.

Preparation of Antipancreatic Ribonuclease Antibody. Antisera to bovine pancreatic ribonuclease and to brain ribonuclease (Elson & Glitz, 1975) were provided by Dr. Dohn G. Glitz (University of California at Los Angeles) and further purified as follows: The antisera (2-mL aliquots) were brought to 40% saturation with solid ammonium sulfate at 0 °C. After 30 min, the precipitate was collected by centrifugation at 13800*g* for 10 min, dissolved in 1 mL of 0.1 M NaCl, 0.05 M Tris-HCl, pH 7.5, and 1 mM EDTA, and applied to a 1 × 30 cm Sephadex G-75 column equilibrated against this same buffer. The protein material in the void volume was pooled and found to be essentially free of serum RNase activity, as determined in our standard assay reaction mixture.

Polyacrylamide Digestion Patterns of 5.8S [5′-^{32}P]RNA. The 5′-^{32}P-labeling of yeast 5.8S RNA, the partial digestion by T_1, U_2, pancreatic and nucleolar RNase, and alkali, and the subsequent sequence analysis by gel electrophoresis were done according to the procedure of Donis-Keller et al. (1977).

Other Methods. Protein was determined by the method of Schaffner & Weissman (1973). Sodium dodecyl sulfate–polyacrylamide (12%) gels were formulated and run as described by Laemmli (1970). Staining was with 0.05% coomassie brilliant blue R (Bio-Rad Laboratories) in 50% methanol plus 10% acetic acid at 37 °C for 2 h, and destaining was done in 7.5% acetic acid. Isoelectric focusing in a glass J tube was carried out as described by Wang et al. (1974) using a 5 to 50% glycerol gradient containing 2% Pharmalyte in the pH 3–10 range and 0.5% Pharmalyte in the pH 8.5–10 range.

Results

Purification of Nucleolar RNase. All steps for the purification of the RNAse were carried out at 0–4 °C unless otherwise indicated. The Ehrlich tumor cells were carried 7 days in ICR mice which were inoculated intraperitoneally with 0.2 mL of undiluted ascites. For a typical harvest, 40 tumor-bearing mice were sacrificed and Ehrlich cells were collected by centrifugation at 270*g* for 5 min. The cell pellet was resuspended in 5 volumes (w/v) of buffer A (0.15 M NaCl, 5 mM Tris-HCl, pH 7.5, and 1 mM β-mercaptoethanol) and collected by centrifugation as described above. This washing step was repeated twice more, and the final cell pellet representing approximately 3×10^{10} cells was suspended in 9 volumes (w/v) of hypotonic buffer B (5 mM Tris-HCl, pH 7.5, 2 mM $CaCl_2$, 1 mM β-mercaptoethanol, 1 mM EDTA, 0.3% Triton X-100, 1 mM phenylmethylsulfonyl fluoride, and 1% 2-propanol). The cells were allowed to swell for 10 min and were then disrupted with a type A pestle in a glass Dounce homogenizer; typically, three strokes were sufficient to achieve greater than 98% breakage. The nuclei were collected by centrifugation at 2300*g* for 5 min. The nuclear pellet was resuspended in 9 volumes of buffer B and pelleted by centrifugation. This step was repeated twice to ensure a thorough washing of the nuclei. The final nuclear pellet was either frozen and stored at −20 °C or directly extracted as described below.

Nuclear Extraction. The Ehrlich cell nuclei (7×10^{10}) were suspended in buffer C (0.2 M potassium phosphate, pH 8.0, 1 mM EDTA, and 1 mM β-mercaptoethanol) at 1×10^8 nuclei/mL. The suspension (700 mL) was mixed gently for 2 h, and the supernatant was removed by decantation. The supernatant was saved, and the chromatin was suspended in one-half the previous volume (350 mL) of buffer C. After 1 h of gentle mixing, the chromatin was collected by centrifugation and the supernatant was pooled with that obtained from the first extraction. The pooled extract was centrifuged at 13800*g* for 20 min followed by a high-speed centrifugation at 105000*g* for 30 min. Pellets from both centrifugation steps were discarded. The supernatant was dialyzed against seven changes of 4 L of buffer D (1 mM potassium phosphate, pH 6.8, and 1 mM β-mercaptoethanol) over a 24-h period and then clarified by centrifugation at 105000*g* for 30 min. The supernatant represents fraction I (1064 mL).

Hydroxylapatite Chromatography. A hydroxylapatite column (2.5 × 32 cm) was equilibrated against buffer D. Fraction I was loaded at 40 mL/h. The column was washed with 150 mL of buffer D, then 450 mL of 5 mM potassium phosphate, pH 6.8, and 1 mM β-mercaptoethanol. The peak activity fractions which eluted with the 3 M KCl step (Figure 1A) were pooled and dialyzed against two changes of 1 L of buffer E (0.02 M sodium acetate, pH 5.5, 1 mM β-mercaptoethanol, and 1 mM EDTA) (fraction II, 175 mL).

Agarose–5′-(4-Aminophenylphosphoryl)uridine 2′(3′)-Phosphate Chromatography. A 5-mL (0.7 × 12 cm) column was equilibrated against buffer E and fraction II was loaded at 20 mL/h. The column was washed with 5 bed volumes of buffer E and then 5 bed volumes of 0.2 M acetic acid, and the enzyme was eluted with 1.5 M KCl in buffer F (50 mM Tris-HCl, pH 7.5, 1 mM β-mercaptoethanol, 1 mM EDTA, and 10% glycerol) (Figure 1B). The active fractions were pooled and dialyzed against buffer F (fraction III, 8.7 mL).

A summary of the purification and yield of the ribonuclease is presented in Table I. Fraction III represents a 500-fold purification over fraction I. The purity of fraction III, however, was difficult to quantitate, since the yield of coomassie stainable material on sodium dodecyl sulfate–polyacrylamide gels was signficantly lower than expected for the amount (20 μg) of protein loaded.

Properties of Purified Enzyme Fraction III. The activity of the purified enzyme was relatively stable, and the enzyme could be stored on ice at 0 °C in buffer F over several months without appreciable loss of activity. The enzyme was optimally active in Tris buffer at pH 7.2–7.5. The enzyme was insensitive to 10 mM *N*-ethylmaleimide and was active when assayed in the presence of 7 M urea, but 0.5% sodium dodecyl

Table I: Purification of Ehrlich Nucleolar Ribonuclease from Nuclei[a]

purification step	vol (mL)	units/mL	mg/mL	units/mg	yield (%)
(1) 0.2 M potassium phosphate, pH 8.2, extract[b]	1064				
(2) dialyzed, high-speed supernatant (fraction I)	1064	91	0.92	98	100
(3) hydroxylapatite chromatography (fraction II)	175	208	0.044	4730	38
(4) AG2′(3′)-5′-UDP chromatography[c] (fraction III)	8.7	1200	0.024	50000	11

[a] Purification was carried out as described in the text, starting from 7.0×10^{10} nuclei. One unit of enzyme is equivalent to 1 nmol of nucleotide solubilized in acid in 30 min at 45 °C in standard assay conditions with [^{32}P]f_2 RNA as the substrate. [b] A nonlinear assay with respect to enzyme concentration did not allow a reliable estimate of activity in the crude extract. [c] AG2′(3′)-5′-UDP is the abbreviation for agarose-5′-(4-aminophenylphosphoryl)uridine 2′(3′)-phosphate.

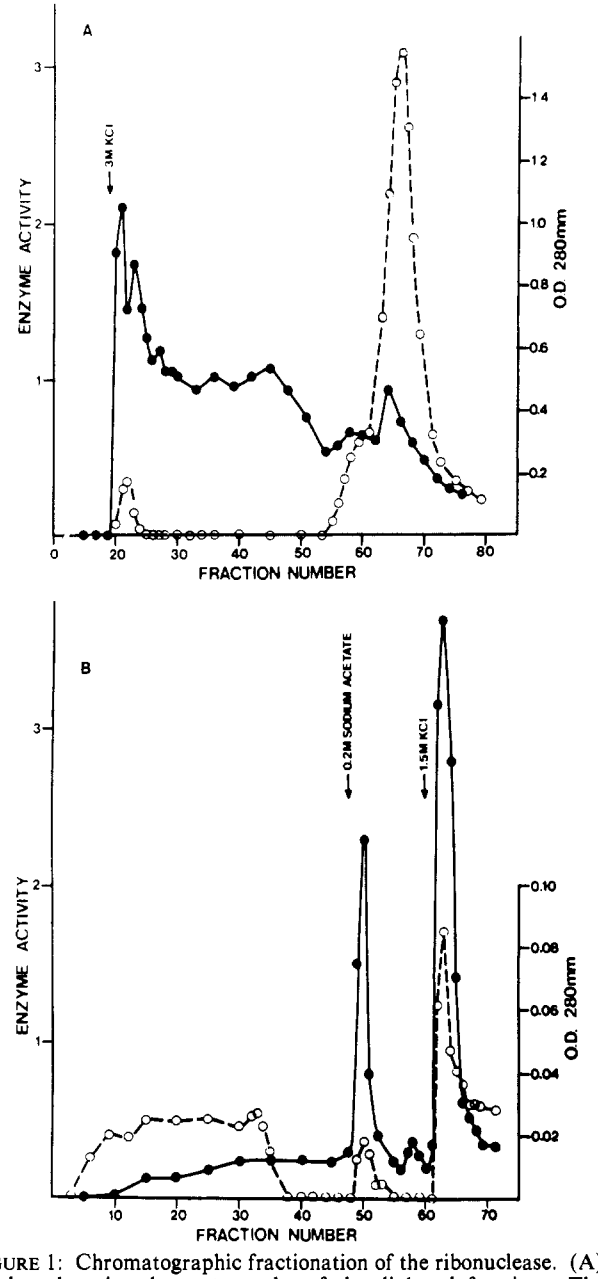

FIGURE 1: Chromatographic fractionation of the ribonuclease. (A) Hydroxylapatite chromatography of the dialyzed fraction. The dialyzed fraction was applied to hydroxylapatite column as described in the text. Aliquots (5 μL) were assayed for RNase activity, and activity is expressed in nanomoles of acid-soluble material liberated: (●) RNase activity; (○) OD 280 nm. (B) Agarose–5-(4-aminophenylphosphoryl)uridine 2′-(3′)-phosphate chromatography of hydroxylapatite fraction. This step was carried out as described in the text; activity was measured as described in A.

Table II: Purification of Ehrlich Nucleolar Ribonuclease from Isolated Nucleoli[a]

purification step	vol (mL)	units/mL	mg/mL	units/mg	yield (%)
(1) fraction I	365	36	1.0	36	100
(2) fraction II	65	72	0.11	656	34
(3) fraction III	6.5	223	0.035	6400	11

[a] Purification was carried out from nucleoli as described for nuclei in the text.

sulfate completely inactivated the enzyme. The RNase activity was detected at a pH greater than 9 when isoelectrically focused in a glycerol gradient (Wang et al., 1974). Monovalent cation salts such as NaCl, KCl, and NH_4Cl stimulated activity at concentrations up to 100 mM, after which some inhibition was observed; at 200 mM approximately 40% of the activity remained. $CaCl_2$ or $MgCl_2$ (10 mM) decreased the RNase activity to 60% of control. EDTA had essentially no effect on enzyme activity.

Molecular Weight. Sedimentation equilibrium experiments with the nucleolar RNase were done according to the procedure of Pollet et al. (1979) using a Beckman airfuge. A partial specific volume of 0.73 was assumed, and in each case (six runs) a straight-line relationship between ln C, based on ribonuclease activity, vs. the square of the distance from the axis of rotation was found. A molecular weight of 38 500 ± 2% was determined.

Nucleolar Localization. Fractionation procedures were employed to investigate the subnuclear distribution of the RNase. Extraction and purification of the RNase from isolated Ehrlich cell nucleoli were carried out in an identical fashion as described in the text for nuclei. As can be seen in Table II, the RNase activity extracted from nucleoli purified in a similar manner as that extracted from nuclei, although the extent of purification for the enzyme was considerably less than for nuclei as the starting material (see Table I). Sonic disruption of nuclei in the preparation of nucleoli appeared to decrease the initial specific activity in fraction I, which could not be overcome by subsequent purification steps. For this reason whole nuclei were the preferred source for starting material.

Cleavage of Native f_2RNA by Nucleolar RNase. Bacteriophage f_2RNA, having a sedimentation coefficient of 27 S, was incubated with the purified nucleolar RNase fraction, and at various times an aliquot was removed from the reaction mixture and treated with sodium dodecyl sulfate (0.5%) and EDTA (1 mM). The RNA products from each aliquot were analyzed by sedimentation through a gradient of 5 to 20% sucrose. Significant reduction of the f_2RNA occurred, and at the earliest time point the RNA was converted to a heterogeneous population (Figure 2). Under the conditions

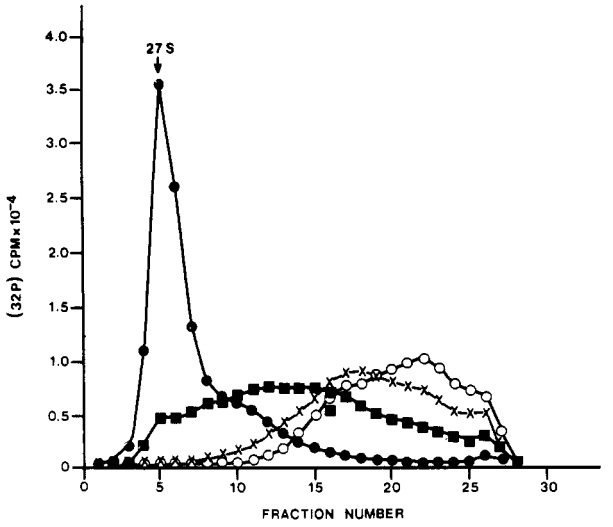

FIGURE 2: Sucrose density gradient analysis of ^{32}P-labeled f_2RNA after incubation with the nucleolar ribonuclease. The reaction mixture (1 mL) contained 0.05 M Tris-HCl, pH 7.5, 0.1 mg/mL bovine serum albumin, 1 mM β-mercaptoethanol, 34 nmol of [^{32}P]f_2RNA (specific activity 37 cpm/pmol), and 0.33 unit of enzyme. At the indicated times, aliquots (0.18 mL) were added to 0.02 mL of 5% sodium dodecyl sulfate and 0.1 M EDTA and mixed. These aliquots were then layered onto a 4.4-mL 5 to 20% sucrose gradient made up in 0.02 M sodium acetate, pH 5.4, 0.1 M NaCl, and 1 mM EDTA. Centrifugation was carried out in an SW60 rotor at 50000 rpm for 4 h at 23 °C. Fractions (0.2 mL) were collected from the bottom of the tube and counted in 4 mL of Triton X-100 scintillation fluid: (●) 0 min; (■) 5 min; (×) 15 min; (○) 30 min.

employed, less than 2% acid-soluble material was detected after 30 min of incubation. No cleavage of the f_2RNA was observed when the RNA was incubated with the nucleolar RNase in the presence of 0.5% sodium dodecyl sulfate. These results indicated that the RNase acts as an endonuclease on the f_2RNA.

Specificity for Degradation of Homoribopolynucleotides. RNases with a marked apparent preference for poly(C) have been reported from nucleoli of various cells (Prestayko et al., 1973; Winicov & Perry, 1974). In view of these reports, the preference of the Ehrlich nucleolar RNase was tested using single-stranded ^3H-labeled poly(C), poly(U), and poly(A) as substrates. As shown in Figure 3, essentially no degradation of [^3H]poly(A) or [^3H]poly(U) was observed under conditions which rendered [^3H]poly(C) 60% acid soluble.

Digestion of Single vs. Double-Stranded RNAs by Nucleolar RNase. The nucleolar RNase was assayed using either [^3H]poly(C) or [^3H]poly(C)·poly(I) as a substrate in order to determine the effect of secondary structure on enzyme activity. Less than 4% of the [^3H]poly(C)·poly(I) was degraded under the same conditions which resulted in 65% degradation of [^3H]poly(C) (Figure 4).

Products of [^3H]Poly(C) Degradation Formed after the Action of Nucleolar RNase. The products of [^3H]poly(C) treated with nucleolar RNase were analyzed by DEAE-Sephadex A-25 chromatography in 7 M urea (pH 7.5) (Figure 5). When the digestion was allowed to go to completion, approximately 90% of the substrate was acid soluble, and the radioactivity was predominantly found in the tri-, tetra-, and pentanucleotide range.

[1] These units are defined under standard assay conditions using f_2RNA as the substrate, except that 0.2% uranyl acetate was included in the 25% perchloric acid during the precipitation of acid-insoluble material, and as a consequence they are approximately 3.5 times lower than than those defined when uranyl acetate is not used.

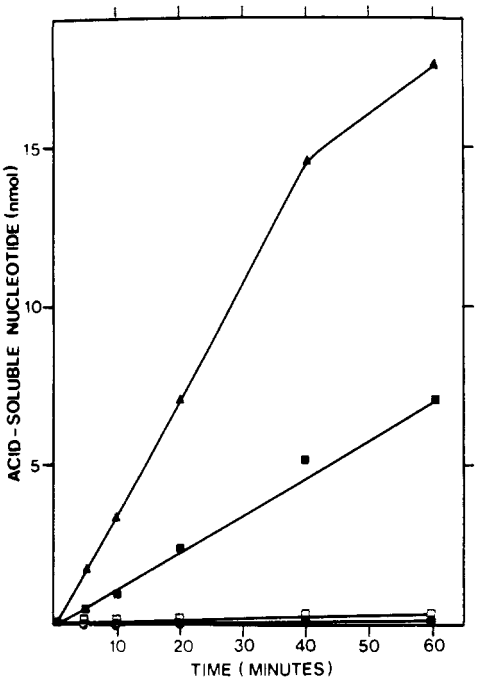

FIGURE 3: Hydrolysis of synthetic homopolyribonucleotides and f_2RNA by purified nucleolar RNase. The reaction mixtures (0.75 mL) contained 0.05 M Tris-HCl, pH 7.5, 0.075 M NaCl, 0.1 mg/mL bovine serum albumin, 1 mM β-mercaptoethanol, 3 units[1] of RNase, and 24 nmol of either (▲) [^3H]poly(C) (3 cpm per pmol), (●) [^3H]poly(A) (1.5 cpm per pmol, (□) [^3H]poly(U) (5 cpm per pmol, or (■) [^{32}P]f_2RNA (14.5 cpm per pmol). Aliquots (0.1 mL) were removed at the times indicated and added to 0.075 mL of 4 mg/mL transfer RNA and 0.57 mg/mL bovine serum albumin, to which 0.075 mL of 25% perchloric acid and 0.2% uranyl acetate was added at 0 °C. The samples were then processed and counted as described for a standard assay (Methods).

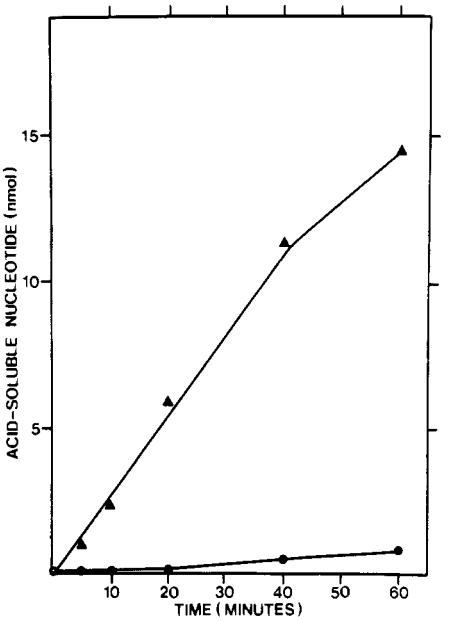

FIGURE 4: Degradation of [^3H]poly(C) vs. [^3H]poly(C)·poly(I) by Ehrlich nucleolar RNase. Reaction mixtures (0.75 mL) contained 0.05 M Tris-HCl, pH 7.5, 0.1 M NaCl, 0.1 mg/mL bovine serum albumin, 1 mM β-mercaptoethanol, 3 units[1] of RNase, and 24 nmol of either (▲) [^3H]poly(C) (4 cpm per pmol), or (●) [^3H]poly(C)·poly(I) (4 cpm per pmol). Reactions were run at 37 °C and started by the addition of enzyme. Aliquots (0.1 mL) were removed at the times indicated and added to 0.075 mL of 4mg/mL transfer RNA and 0.67 mg/mL bovine serum albumin, to which 0.075 mL of 25% perchloric acid and 0.2% uranyl acetate was added at 0 °C. The samples were then processed and counted as described for a standard assay (Methods).

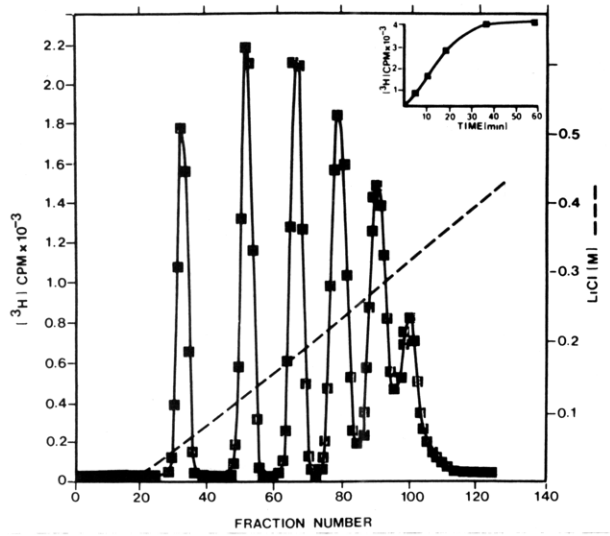

FIGURE 5: Separation by DEAE-Sephadex A-25 chromatography of oligonucleotides formed by nucleolar RNase. A reaction mixture (2 mL) containing 60 nmol of [^{3}H]poly(C) (specific activity 4 cpm per nmol), 50 mM Tris-HCl, pH 7.5, 0.1 mg/mL bovine serum albumin, 1 mM β-mercaptoethanol, and 8 units[1] of purified nucleolar RNase was incubated at 45 °C. Aliquots (0.05 mL) were taken at the designated times (see insert) into 0.125 mL of 2.5 mg/mL transfer RNA and 0.4 mg/mL bovine serum albumin, precipitated with 0.075 mL of 25% perchloric acid and 0.2% uranyl acetate at 0 °C. After 10 min, the aliquots were processed and counted as described for a standard assay (Methods). The remaining reaction material was heated at 100 °C in a boiling water bath for 2 min and then brought to 0.1 N HCl, incubated 1 h at 37 °C, and neutralized with NaOH. The mixture was diluted 10-fold with 5 mM Tris-HCl, pH 7.5, and 7 M urea and equilibrated at 65 °C. The sample was loaded onto a 3-mL (0.7 × 8 cm) DEAE-Sephadex A-25 column jacketed at 65 °C and equilibrated against the same buffer. The loaded column was then washed with 6 mL of buffer and a 150-mL gradient from 0.0 to 0.4 M LiCl was run. Fractions (1.5 mL) were collected and 0.5-mL aliquots of each fraction were counted in 8 mL Triton X-100 scintillation fluid.

To determine whether the endonuclease cleavage was on the 3′ or 5′ side of the phosphodiester linkage, [^{3}H]poly(C) was extensively hydrolyzed by the RNase, and the products were separated on poly(ethylenimine)-impregnated cellulose acetate plates. The radioactivity in the 2′,3′-cyclic CMP spot was determined by cutting the spot from the plate and counting it. Although only a small percentage of the total counts migrated with the 2′,3′-cyclic mononucleotide spot, the radioactivity increased linearly as a function of time from 0 to 120 min, demonstrating that the enzyme cleaves on the 5′ side of the phosphodiester bond.

Specificity of Degradation of Yeast 5.8S RNA Using RNA Sequence Analysis. The partial hydrolysis of 5′-^{32}P-labeled 5.8S RNA by the nucleolar RNase and the subsequent sequence analysis on a polyacrylamide gel are shown in Figure 6. Under conditions where the RNA has no secondary structure, the nucleolar RNase preferentially cleaved on the 3′ side of cytidylic residues adjacent to adenylic acid. Under similar conditions bovine pancreatic RNase cleaved at both CpA and UpA phosphodiester bonds.

Effects of Polyamines and Histone I on the Hydrolysis of RNA. It has been shown elsewhere that polyamines can significantly affect the hydrolytic activity of a number of ribonucleases (Bolton & Kearns, 1978; Levy et al., 1973; Igarashi et al., 1975). The relationship between variation of either polyamine or histone on the ribonuclease activity is depicted in Figure 7. It can be seen that the purified nucleolar RNase was inhibited by each of the basic constituents tested and that the extent of inhibition varied considerably. Nonbasic

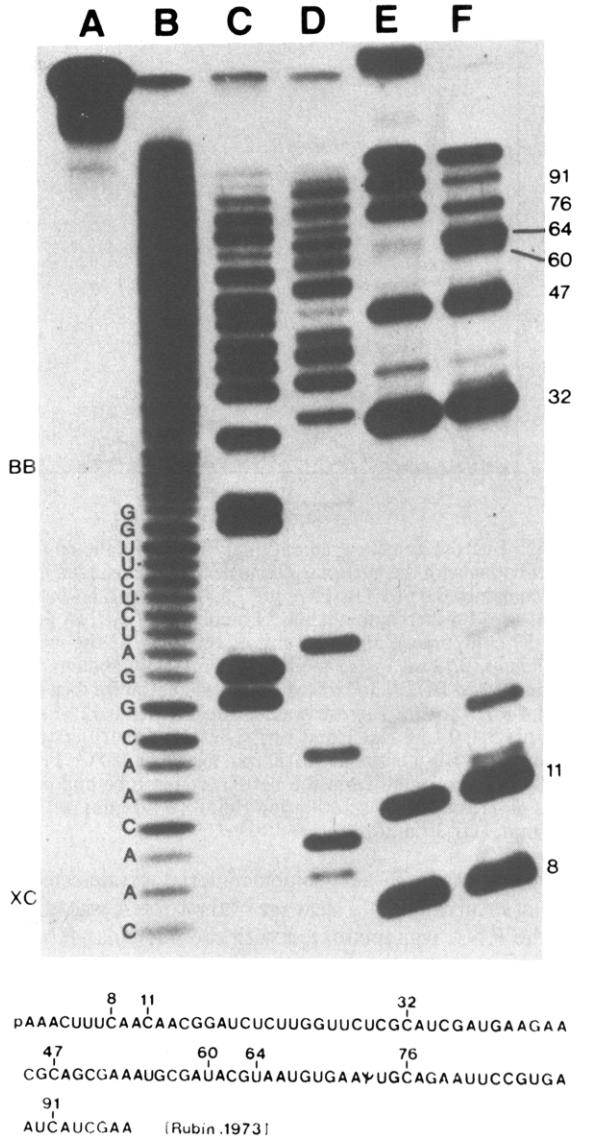

FIGURE 6: Autoradiograph of partial digestion products from 5′ end-labeled *Saccharomyces cerevisiae* 5.8S ribosomal RNA separated by size on a polyacrylamide gel slab and the partial nucleotide sequence at the 5′ end as determined by Rubin (1973). Five 20-μL samples (A, C–F), each containing approximately 1.5 × 10^5 cpm of 5.8S [5′-^{32}P]RNA, 0.5 mg/mL unlabeled transfer RNA, 20 mM sodium citrate, pH 5.0, 1 mM EDTA, 7 M urea, 0.02% xylene cyanol (XC), and 0.02% bromophenol blue (BB), were heated at 50 °C for 5 min, chilled, and handled as follows: (A) control with no enzyme; (C) 0.8 unit of RNase T_1; (D) 6.0 unit of RNase U_2; (E) 0.5 unit of Ehrlich nuclear RNase; (F) 1.25 pg of pancreatic RNase. All enzyme-treated samples and the control (no enzyme) were then incubated for 15 min at 50 °C, chilled, and layered on a 20% polyacrylamide and 7 M urea gel. Sample B, the partial alkali digest, was prepared as follows: a 10-μL sample containing 3.2 × 10^5 cpm of 5.8S [5′-^{32}P]rRNA, 0.5 mg/mL unlabeled transfer RNA, 50 mM $NaHCO_3/Na_2CO_3$, pH 9.0, and 1 mM EDTA was incubated for 12 min at 95 °C, and chilled, and then 10 μL of 10 M urea, 0.04% xylene cyanol, and 0.04% bromophenol blue was added and layered on the gel. Electrophoresis was carried out at constant power, 15 W, for 5 h, and the gel was exposed to X-ray film (Kodak X-Omat R-film) plus an intensifying screen (Dupont Cronex screen) for 14 h at −20 °C.

proteins such as bovine serum albumin, myoglobin, and rabbit γG globulin had no inhibitory effect on activity (data not shown).

Immunology. Antibodies raised against bovine pancreatic RNase did not inhibit the activity of the Ehrlich nucleolar RNase (Figure 8). Likewise, antibodies directed against bovine brain ribonuclease (Elson & Glitz, 1975) did not affect

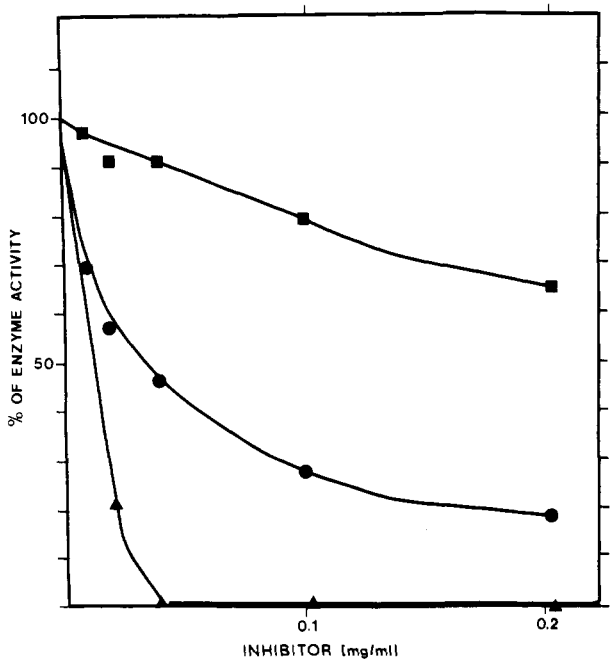

FIGURE 7: Effect of polyamines and histone I on hydrolysis of f_2RNA by Ehrlich nucleolar RNase. The reaction mixtures (0.15 mL) contained 0.05 M Tris-HCl, pH 7.5, 0.075 M NaCl, 0.1 mg/mL bovine serum albumin, 4.5 nmol of [^{32}P]f_2RNA (3.9×10^3 cpm per nmol), 1 mM β-mercaptoethanol, and basic protein or polyamine as indicated. Reactions were started by the addition of 1.3 units of enzyme, and samples were processed as described for the standard assay (Methods): (●) spermine; (■) spermidine; (▲) histone I.

the nucleolar RNase under conditions where they partially inhibited pancreatic RNase.

Discussion

The relatively simple purification procedure that we have devised leads easily and reproducibly to a highly purified nucleolar endoribonuclease in reasonable yield. Our studies with the purified RNase confirm previous reports which suggested that there are such activities associated with the nucleolus (Mirault & Scherrer, 1972; Prestayko et al., 1973; Winicov & Perry, 1974; Boctor et al., 1974). Although we find this RNase associated with the nucleolus, we cannot unequivocally exclude its presence in the nucleoplasmic or cytoplasmic fractions.

The molecular weight of the nucleolar endoribonuclease is estimated to be 38 500 on the basis of sedimentation equilibrium studies, and differs considerably from the 7000 molecular weight reported for a partially purified nucleolar endoribonuclease from Novikoff hepatoma cells (Prestayko et al., 1973). The molecular weight for the nucleolar ribonuclease activities from mouse L cells (Winicov & Perry, 1974), Hela cells (Mirault & Scherrer, 1972), and at hepatocytes (Boctor et al., 1974) were not reported.

The purified nucleolar RNase cleaved preferentially the synthetic homoribopolymer poly(C) over poly(U) and poly(A). These results are similar to those described for a nucleolar RNase extract from mouse L cells (Winicov & Perry, 1974). Exhaustive degradation of poly(C) by the nucleolar ribonuclease yielded tri-, tetra-, and pentanucleotides, unlike pancreatic RNase, which is capable of hydrolyzing poly(C) almost completely to mononucleotides. On the basis of these results, we found that the nucleolar RNase appears to prefer larger oligonucleotide substrates. The release of 2′,3′-cyclic CMP from the degradation of poly(C) shows that the ribonuclease is a member of the phosphotransferase or cyclizing

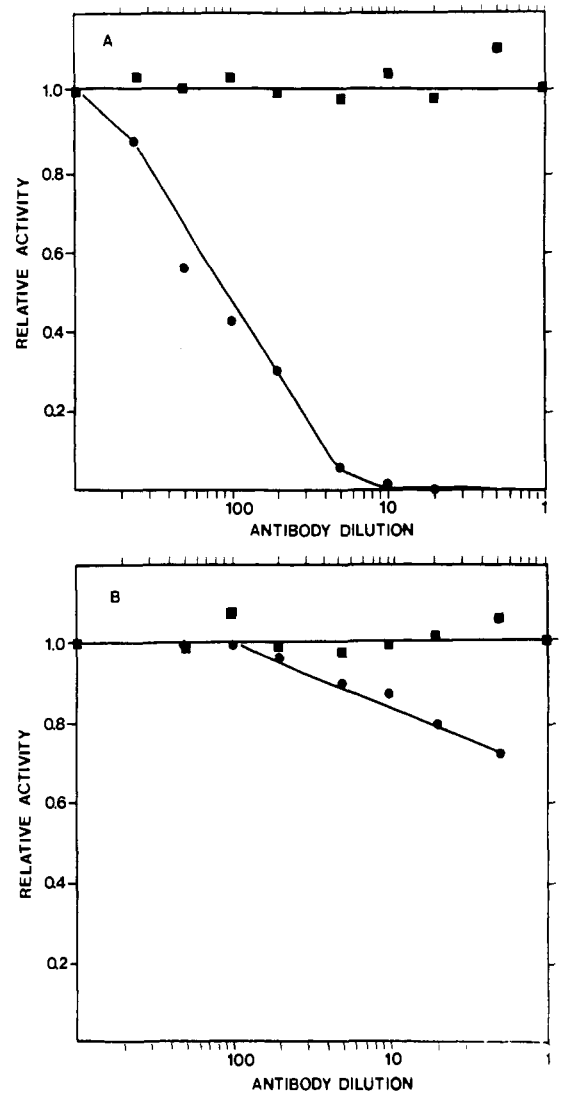

FIGURE 8: Effect of anti-bovine pancreatic RNase and anti-brain ribonuclease antibodies on nucleolar RNase. The reaction mixtures (0.15 mL) contained 0.05 M Tris-HCl, pH 7.5, 0.1 M NaCl, 4.5 nmol of [^{32}P]f_2RNA (15×10^3 cpm per nmol), 0.1 mg/mL serum albumin, and 1 mM β-mercaptoethanol. The amount of antibody varied from 4.3 to 0.0043 mg per reaction mixture as indicated. The reaction was started by the addition of 3.5 units of RNase and run at 37 °C. Reaction mixtures were stopped and processed as described for the standard assay (Methods): (A) anti-pancreatic RNase antibody; (B) anti-brain RNase antibody; (●) bovine pancreatic RNase; (■) Ehrlich nucleolar RNase.

type of RNA depolymerases (Barnard, 1969). The basic feature of this group of RNases is the use of 2′-OH group in an intramolecular attack at the adjacent phosphodiester bond. In line with this conclusion is the independence of divalent cation requirement by the nucleolar RNase, which is also a characteristic by cyclizing RNases (Barnard, 1969). This nucleolar RNase also exhibits an apparent preference for single-stranded structure. Double-stranded poly(C)·poly(I) was not degraded under conditions which rendered poly(C) 65% acid soluble.

The significantly higher rate of poly(C) vs. poly(U) hydrolysis by nucleolar RNase extracts has previously been taken to indicate a similar preference for cleavage of phosphodiester bonds adjacent to cytidine residues in RNA (Prestayko et al., 1973; Winicov & Perry, 1974). However, the fact that bovine pancreatic RNase is 25-fold more active against poly(C) than poly(U) and, yet is known to liberate comparable quantities of cytosine- and uracil-terminated fragments from RNA

(Bardon et al., 1976) has lessened the validity of this argument. Therefore, the possible specificity of the Ehrlich nucleolar RNase for cleavage adjacent to cytidine residues in RNA was directly tested. Yeast 5.8S RNA was used as the RNA substrate because the primary sequence is known (Rubin, 1973), and the analysis of the initial products of RNase digestion by the sequencing procedure of Donis-Keller et al. (1977) permitted a direct determination of specificity. Our results show that the Ehrlich nucleolar RNase preferentially attacked pyrimidine–adenylic acid phosphodiester bond linkages, and the degree of specificity was more stringent than for bovine pancreatic RNase (Richards & Wyckoff, 1971). Only cytidine–adenylic linkages were recognized by the nucleolar RNase, whereas pancreatic RNase recognized both cytidine– and uridine–adenylic acid linkages (see Figure 6).

It is well-known that polyamines can affect the degradation of RNA and synthetic homoribopolymers by ribonucleases (Levy et al., 1973; Igarashi et al., 1975). When using native f_2RNA as a substrate, the Ehrlich nucleolar RNase was found to be inhibited by the presence of polyamines. In contrast, pancreatic RNase was reported to be stimulated by polyamimes under similar conditions (Igarashi et al., 1975). Thus, the mechanism underlying the effects of polyamines appears complex and is probably related to the ability of polyamines to bind RNA (Bolton & Kearns, 1978). Nevertheless, the sensitivity of the nucleolar RNase to the presence of basic proteins and polyamines may, in part, account for the difficulty in assaying the enzyme in crude nuclear extracts (see Table I) as well as offer an explanation for the variation of properties previously described for the nucleolar RNase extracts of mouse L cells (Winicov & Perry, 1974) and Novikoff hepatoma cells (Prestayko et al., 1973).

A high degree of antigenic cross-reactivity has been demonstrated for pancreatic-like RNases of different species (Morikawa et al., 1968) and within different tissues in the same species (Elson & Glitz, 1975; Morikawa, 1967). Since the nucleolar ribonuclease appears to share some enzymatic resemblance to bovine pancreatic ribonuclease, it was of interest to determine whether the Ehrlich enzyme might also share some common antigenic structure. Antibodies elicited against pancreatic RNase, however, did not inhibit the nucleolar RNase activity. Similarly, antibodies directed against beef brain ribonuclease (24 000 molecular weight) (Elson & Glitz, 1975) also did not cross-react with the nucleolar RNase under conditions where they partially inhibited pancreatic RNase.

In summary, we have extensively characterized a highly purified nucleolar endoribonuclease which exhibits a high degree of specificity. Although its natural substrate(s) and physiological role have not yet been identified, its localization and specificity suggest a role in the processing of ribosomal RNA. Further studies will be necessary to establish its precise role in the maturation process.

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