Characterization of a Nucleolar Endonuclease Possibly Involved in Ribosomal Ribonucleic Acid Maturation†

Ilga Winicov* and Robert P. Perry

ABSTRACT: An endonuclease which may be involved in rRNA precursor maturation has been isolated and partially purified from L cell nucleoli. The enzyme has been characterized with respect to its activity toward the four ribohomopolymers poly(A), poly(C), poly(G), and poly(U), various cellular RNA species, and 80S ribonucleoprotein particles containing the 45S rRNA precursor. Poly(C) was found to be the only ribohomopolymer cleaved by the endonuclease. The enzyme also cleaves 45S pre-rRNA, mRNA, heterogeneous nuclear RNA, and, to a limited extent, 28S rRNA, 18S rRNA, and 45S pre-RNA which is in 80S ribonucleoprotein particles. The enzyme cleavage products are molecules with 3′-phosphate and 5′-OH termini. The implications of this specificity in ribosomal maturation are discussed.

The maturation process for eukaryotic rRNA, by which the initial products of transcription of the rRNA genes are converted to the rRNA components found in cytoplasmic ribosomes, involves the production of a readily observed series of intermediates (*cf.* reviews by Darnell, 1968, Perry, 1969, Burdon, 1971, and Maden, 1971) and the loss of certain portions of the precursor molecules. The linear arrangement of the conserved and nonconserved regions of the 45S ribosomal precursor molecules has been established (Weinberg *et al.*, 1967; Weinberg and Penman, 1970; Perry and Kelley, 1972; Maden *et al.*, 1972; Wellauer and Dawid, 1973), but the enzymatic aspects of the maturation process are still poorly understood.

Since both the 18S and 28S rRNA sequences are present in each 45S rRNA precursor molecule, the overall processing reaction necessarily requires at least one endonucleolytic cleavage step. Initial studies of processing in isolated nucleoli (Liau *et al.*, 1968; Vesco and Penman, 1968) suggested the presence of more than one ribonucleolytic activity in the nucleolus, and indeed both exonucleolytic (Kelley and Perry, 1971) and endonucleolytic activities (Mirault and Scherrer, 1972a; Prestayko *et al.*, 1972, 1973; Kwan, Gotoh, and Schlesinger, personal communication) have been found to be associated with nucleoli. In this work we describe an endonuclease that has been partially purified from L cell nucleoli and characterized with respect to its activity on synthetic ribohomopolymers, various cellular RNA species, and isolated nucleolar ribonucleoprotein particles containing the ribosomal precursor RNA.

Materials and Methods

Mouse L cells were grown at 37° in suspension cultures as described previously (Perry and Kelley, 1968a) and used for preparation of enzyme, ribonucleoprotein particles, and RNAs.

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[1] Abbreviations used are: poly(A), poly(C), poly(U), and poly(G), polymers of adenylic, cytidylic, uridylic, and guanylic acids, respectively; DOC, deoxycholate; DTT, dithiothreitol; BSA, bovine serum albumin; SDS, sodium dodecyl sulfate; TEA, triethanolamine; EDTA, (ethylenedinitrilo)tetraacetic acid; PTP-di-PFO, perfluorooctanoyl-*p*-acetamidophenyl-3′,5′-phosphothymidine; RNP, ribonucleoprotein.

[8-^{14}C]Poly(adenylic acid) (0.98 Ci/mol of P), [^{3}H]poly(uridylic acid) (58.9 Ci/mol of P), and [^{3}H]poly(cytidylic acid) (51.5 Ci/mol of P) were obtained from Miles Laboratories, Inc.; [^{3}H]poly(guanylic acid) (80 Ci/atom of P) was purchased from Nuclear Dynamics, Inc. Poly(cytidylic acid), poly(guanylic acid) and poly(uridylic acid) were subjected to chromatography on a Sephadex G-100 (Pharmacia) column, and material from the exclusion peaks was used for subsequent experiments. Perfluorooctanoyl-*p*-acetamidophenyl-3′,5′-phosphothymidine (PTP-di-PFO) (Sporn *et al.*, 1969a) was a generous gift from Dr. Michael Sporn. Crystallized bovine serum albumin (BSA) was purchased from Mann Research.

Preparation of RNA. 28S rRNA and 18S rRNA were extracted from ribosomal pellets of exponentially growing cells which had been labeled 48 hr with 0.1 μCi/ml of [2-^{14}C]uridine (56.7 Ci/mol) as described by Perry and Kelley (1968a). The rRNA was fractionated on 5–25% (w/w) sucrose gradients in 0.01 M Tris (pH 7.4), 0.1 M NaCl, 1 mM EDTA, and 0.5% sodium dodecyl sulfate (SDS buffer). The gradients were centrifuged in a Beckman SW27 rotor at 20° for 16 hr at 21,000 rpm, fractionated, and monitored by radioactive assay, and the appropriate fractions precipitated with ethanol. The RNA preparation was dissolved in 0.1 M NaCl–1 mM EDTA and stored at −20°.

mRNA, prepared from cells labeled 2 hr with 10 μCi/ml of [5-^{3}H]uridine (28.2 Ci/mmol), was extracted (Perry *et al.*, 1972) from polyribosomal pellets (Wettstein *et al.*, 1963) with 0.5% SDS–chloroform–phenol and precipitated with ethanol. The polyadenylated mRNA was purified by chromatography on an oligo(dT)-cellulose column (Nakazato and Edmonds, 1972; as modified by Aviv and Leder, 1972).

Nuclear heterogeneous RNA (HnRNA) was prepared (Perry *et al.*, 1972) from isolated nucleoplasm (Penman, 1966).

Ribosomal 45S precursor RNA was prepared from cells labeled 15 min with 10 μCi/ml of [methyl-^{3}H]methionine (6.3 Ci/mmol) in medium containing $^{1}/_{10}$ normal methionine concentration and 20 μM each of adenosine and guanosine. Nucleoli were isolated by the method described by Penman (1966). Nucleolar RNA was extracted (Perry *et al.*, 1972) and fractionated on two successive 15–30% (w/w) sucrose gradients in SDS buffer that were centrifuged at 20° in a Beckman SW 27 rotor for 16 hr at 21,000 rpm. The gradients were monitored by radioactive assay, the appropriate peak fractions were

precipitated with ethanol, and the RNA was dissolved in 0.05 M NaCl–1 mM EDTA and stored at −70°.

Preparation of Preribosomal Ribonucleoprotein Particles. Exponentially growing L cells were concentrated 8-fold and labeled 15 min at 37° with 5 μCi/ml of [5-^{3}H]uridine. The cells were quickly chilled and washed with balanced salts solution, and the cell pellet was suspended in 20 volumes of 10 mM triethanolamine (TEA) (pH 7.4), 10 mM NaCl, and 1 mM $MgCl_2$ (TNM buffer). After a 15-min incubation on ice, sucrose was added to a final concentration of 0.25 M and the cells were broken by homogenization with an Elvehjem-Potter homogenizer. The nuclei and unbroken cells were sedimented 3 min at 900*g* and suspended in 15 volumes of TNM buffer containing 0.25 M sucrose (TNMS buffer), and the homogenization was repeated. The nuclei were washed once with 10 volumes of TNMS buffer containing 0.05% Triton X-100, and once with 10 volumes of TNMS buffer containing 0.5% deoxycholate (DOC) and 1% Tween 40. The clean nuclei, as shown by Methyl Green Pyronine (Unna) stain (Perry and Kelley, 1972), were suspended in 7.5 volumes of 10 mM TEA (pH 7.4), 10 mM NaCl, 2 mM $MgCl_2$, and 0.5 M sucrose, stirred on a Vortex mixer for 30 sec after addition of DOC to a final concentration of 0.5%, and mixed by Vortex for 90 sec with 2 μg/ml of DNase [deoxyribonuclease I, electrophoretically purified, RNase free (Sigma)]. The nuclear lysate was incubated on ice for 10 min followed by an additional 10-min incubation with 1 mM PTP-di-PFO. Nucleoli were purified by centrifugation at 4° through a discontinuous 2.2 and 1 M sucrose gradient in 10 mM TEA (pH 7.4), 10 mM NaCl, and 2 mM $MgCl_2$ for 45 min at 27,000 rpm in a Beckman SW 27 rotor. The clean nucleoli were suspended in 10 mM TEA (pH 7.4), containing 10 mM KCl, 0.1 mM $MgCl_2$, and 20 mM dithiothreitol (DTT), and disrupted by homogenization for 10 min in a loose-fitting Dounce homogenizer at 25°. Nucleolar debris was sedimented at 20,000*g* for 15 min at 2° and the supernatant fraction containing the ribonucleoprotein (RNP) particles were layered onto 15–30% (w/w) sucrose gradients in 10 mM TEA (pH 7.4), 10 mM NaCl, 2 mM EDTA, and 1 mM DTT. The RNP particles were separated by centrifugation at 2° for 16 hr at 24,000 rpm in a Beckman SW 27 rotor. The gradient fractions were monitored by their radioactivity, and the fractions containing 80S preribosomal particles were pooled and used in subsequent assays or frozen in Dry Ice and stored at −70° for later use.

Endonuclease Preparation. Nucleolar endonuclease was extracted from nucleoli purified as described in the section on RNP particle preparation, with the exception that the PTP-di-PFO treatment was omitted. The extraction and purification procedure for the enzyme are similar to that described for the HeLa cell enzyme by Mirault and Scherrer (1972a). The extensively dialyzed $(NH_4)_2SO_4$ (55% saturated) supernatant fraction was adsorbed for 30 min at 4° with DEAE-cellulose (0.4 volume of DEAE-cellulose slurry in 50 mM TEA (pH 8.0), 50 mM KCl, and 1 mM DTT to 1 volume of dialysate). The fraction that failed to adsorb to DEAE-cellulose was concentrated by precipitation to 75% saturation with $(NH_4)_2SO_4$ using bovine serum albumin (BSA) as carrier. The $(NH_4)_2SO_4$ precipitate was dissolved in a small volume of 50 mM TEA (pH 8.0), 50 mM KCl, and 1 mM DTT, dialyzed overnight against 1 l. (changed once) of the same buffer at 4°, and stored at −70°. The final concentration of carrier BSA in various assay systems ranged from 100 to 300 μg per ml, and had no detectable effect on the enzyme activity. The BSA used in these experiments was previously demonstrated to be free from endonuclease activity (Perry and Kelley, 1972). This conclusion was verified in the present set of experiments by demonstrating that the presence of three times the normal amount of BSA in a reaction mixture which included RNA particles and a rate-limiting amount of enzyme did not produce any additional nucleolytic cleavage.

Ribonuclease Assay. The endonuclease activity was measured by production of acid-soluble products from [^{3}H]poly(cytidylic acid) ([^{3}H]poly(C)). The assays were done in 150-μl volumes containing the following reagents: 50 mM TEA (pH 7.4), 50 mM NaCl, 50 mM KCl, 1 mM DTT, 5.5 μg/ml of [^{3}H]poly(C), and varying amounts of the preparation containing ribonuclease activity. The assay mixtures were incubated at 37° for 30 min, after which time the reaction was chilled on ice, 50 μg of yeast tRNA was added, and the unhydrolyzed poly(C) precipitated by the addition of 75 μl of 25% perchloric acid–0.75% uranyl acetate. The sample was centrifuged at 900*g* for 30 min at 0° and the radioactivity of an aliquot of the acid-soluble fraction was counted in an aqueous fluor as described previously (Perry and Kelley, 1968b). A unit of enzyme is defined as that amount which solubilizes 1 μg (3.1 nmol of nucleotide) of poly(C) in 1 hr at 37°.

Polyacrylamide Gel Electrophoresis of the RNA. The nucleolar endonuclease activity was also monitored by electrophoresis of various cellular RNA species on 2.7% acrylamide gels (Perry and Kelley, 1968b). After exposure to the nuclease at 37° the RNA was extracted from the reaction mixture by SDS–phenol–chloroform (Perry *et al.*, 1972), precipitated with ethanol, dissolved in electrophoresis buffer containing 30% sucrose, and subjected to electrophoresis as described above. In some assays, the entire reaction mixture was made 1% in SDS–1 mM EDTA and subjected to electrophoresis.

Endonuclease hydrolysis of the homopolymers was monitored on 10 cm of 14% acrylamide–0.49% diacrylate gels in E buffer (0.04 Tris–0.02 M sodium acetate–1 mM EDTA, pH 7.2) overlaid with 1 cm of 2.7% gel. Electrophoresis was carried out for 4 hr at 5 mA/gel, after which the gels were sliced and dissolved by shaking for 48–72 hr in 10 ml of toluene containing Liquifluor and 3% Protosol (New England Nuclear), and the radioactivity was counted.

Determination of Apparent K_m Values for 28S and 18S rRNA. Incubations of steady-state labeled 28S or 18S rRNA with the endonuclease were carried out in total volume of 50 μl containing 2.5 μmol of TEA (pH 7.4), 2.5 μmol of NaCl, 2.5 μmol of KCl, 50 pmol of DTT, 0.5–6.0 μg of rRNA and ±2.1 μg of endonuclease preparation. After 20 min at 37°, the reaction was stopped by the addition of SDS and EDTA, heated at 65° for 5 min, and layered on 2.7% gels for electrophoresis as described above. The rRNA was monitored on gels, and the amount of RNA hydrolyzed by the enzyme was calculated from the amount of material displaced from the rRNA peak on the gel for each of the rRNA concentrations tested. The data were plotted as a standard reciprocal plot (as in Figure 5) and the apparent K_m values determined from the abcissa intercept.

Nucleotide Analysis by Thin-Layer Chromatography. Thin-layer chromatography was carried out on plastic backed cellulose impregnated with polyethyleneimine (PEI) (Brinkman Instruments) as described previously (Kelley and Perry, 1971). The chromatogram was cut in lengthwise strips, which were then cut into 1-cm pieces. The PEI-cellulose was removed by scraping, dissolved in 0.1 ml of distilled water and 0.3 ml of Protosol, and counted in 10 ml of Liquifluor–toluene scintillant.

Results

The purification steps used in preparing the endonuclease re-

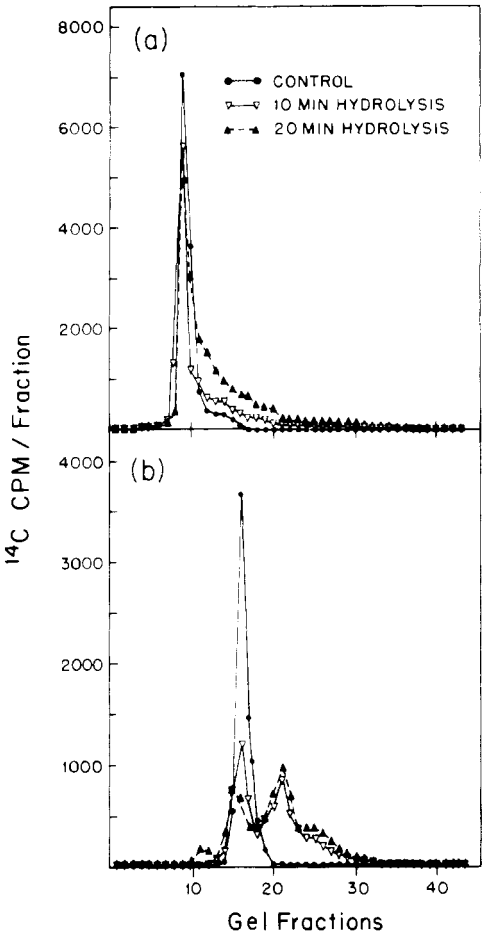

FIGURE 1: Endonuclease digestion of ^{14}C-labeled 28S and 18S rRNA. The 30-μl reaction volumes contained 3.52 pmol of 28S rRNA or 2.79 pmol of 18S rRNA, 1.5 μmol of TEA (pH 7.4), 1.5 μmol of NaCl, 1.5 μmol of KCl, and 30 pmol of DTT. The incubations were carried out at 37° in the presence of 1.6 μg of endonuclease preparation for 0 min (●—●), 10 min (▽—▽), and 20 min (▲ - - ▲). The assay mixtures were made 1% in SDS and 1 mM EDTA and the entire mixture was applied to 2.7% gel for electrophoresis as described in Methods. Gel electrophoresis patterns of the rRNA after enzymatic hydrolysis: (a) 28S rRNA, (b) 18S rRNA.

moved at least 95% of the protein in the nucleolar extract. The extent of purification is difficult to quantitate, however, because of the presence of other ribonucleases in the original extract. This was indicated by the fact that the original extract contains a poly(A) hydrolyzing activity that is lost during the course of purification. The final enzyme preparation obtained has no 3′-hydroxyexonuclease activity (Lazarus and Sporn, 1967), and does not produce detectable acid-soluble material from [^{14}Cthymidine-labeled single-stranded DNA (about 7 S) in 1 hr at 37°. The enzymatic activity is inhibited by Mg^{2+}

The substrate specificity of the nucleolar endonuclease was investigated by exposing a number of cellular RNA species to the enzyme. Although no acid-soluble products were obtained with either 28S or 18S rRNA, even after a 1-hr incubation at high enzyme to substrate ratios, the endonuclease does cleave purified rRNA to a limited extent as shown by analysis with gel electrophoresis (Figure 1). The endonuclease hydrolysis of 28S rRNA produces fragments of random size (Figure 1a). Reaction products that were maintained for 10 min at 70° in 10 mM EDTA before electrophoresis were indistinguishable from the unheated samples, thus indicating that the products do not contain additional "hidden breaks," and that the cleavages are largely confined to single-stranded regions of the molecules. In contrast to 28S rRNA, the 18S component shows a more restricted first cleavage product (Figure 1b). The extrapolated molecular weight of this product is 0.35×10^6, or one-half of the 18S rRNA. This cleavage product which can be seen to accumulate for some time is eventually broken down after prolonged incubation. Heterogeneous nuclear RNA and messenger RNA were also cleaved to random sized fragments by the endonuclease.

To learn more about the nucleotide specificity of the nucleolar endonuclease, we incubated it with each of the ribohomopolymers: poly(A), poly(G), poly(U), and poly(C). After a suitable incubation with the enzyme, acid-soluble products were produced from poly(C), but no acid-soluble products could be obtained from poly(A), poly(G), or poly(U), even after a 4-hr incubation. The extent of hydrolysis of the incubated homopolymers was also monitored by electrophoresis on 14% acrylamide–diacrylate gels (Figure 2). This assay would readily allow the detection of as little as one cleavage per molecule. The inset in Figure 2b shows the radioactivity profile for poly(G) after incubation at 37° with and without endonuclease. Despite purification on Sephadex G-100 columns the homopolymers showed a rather disperse pattern of migration on the 14% gels. Therefore, to obtain maximum sensitivity for detection of changes in the mean size of the molecules, the data were plotted as a cumulative sum of the percentage of counts along the gel. Thus, Figure 2b shows that 50% of the poly(G) molecules migrate with an apparent molecular weight of 1.7×10^4 (3.3 S) or greater, and that this value has not been changed by incubation with the nucleolar endonuclease. Similar patterns are observed in Figure 2a and 2c with poly(A) and poly(U), which also show no significant decreases in their mean molecular weight after extended exposure to the endonucleases. In contrast, poly(C) is readily hydrolyzed as seen in Figure 2d. The mean molecular weight of poly(C) shows a change from 3.5×10^4 to 0.31×10^4 (approximately 10 nucleotides) which indicates an average of 10 breaks/molecule. This extent of cleavage predicts that about 60% of the poly(C) substrate would be rendered acid soluble, which agrees with the values obtained under the standard assay conditions.

To determine whether the endonuclease cleavage is on the 3′ or 5′ side of the phosphodiester linkage, [^{3}H]poly(C) was extensively hydrolyzed with the enzyme and the acid-soluble products were separated by thin-layer chromatography as described in Methods. The radioactivity profile of the thin-layer plates in Figure 3 shows that, although only 4% of the acid-soluble material migrates with the mononucleotides, all of that which does migrate appears as 2′,3′-CMP, thus indicating that 3′-phosphates are the product of the nucleolar endonuclease cleavage. The relatively low proportion of mononucleotide product may be indicative of a low affinity of the enzyme for small oligonucleotides.

Further evidence for the 3′-phosphate product of the endonuclease cleavage was obtained by coupling the nucleolar endonuclease and the ascites 3′-OH-requiring exonuclease (Lazarus and Sporn, 1967) (Figure 4). Prior incubation of an RNA substrate with increasing amounts of the nuclear endonuclease can be seen to decrease proportionately the amount of acid-soluble product released by the exonuclease. This result is similar to a previous finding with micrococcal ribonuclease (Sporn *et al.*, 1969b) and indicates that the endonuclease cleavage produces molecules with 3′ ends that are resistant to subsequent attack by the exonuclease. These data together with those in Figure 3 lead us to conclude that the nucleolar endonuclease cleaves 5′-*O*-phosphate bonds, thus producing 5′-hydroxyl and 3′-phosphate groups.

Poly(C) hydrolysis was used to develop an assay system for

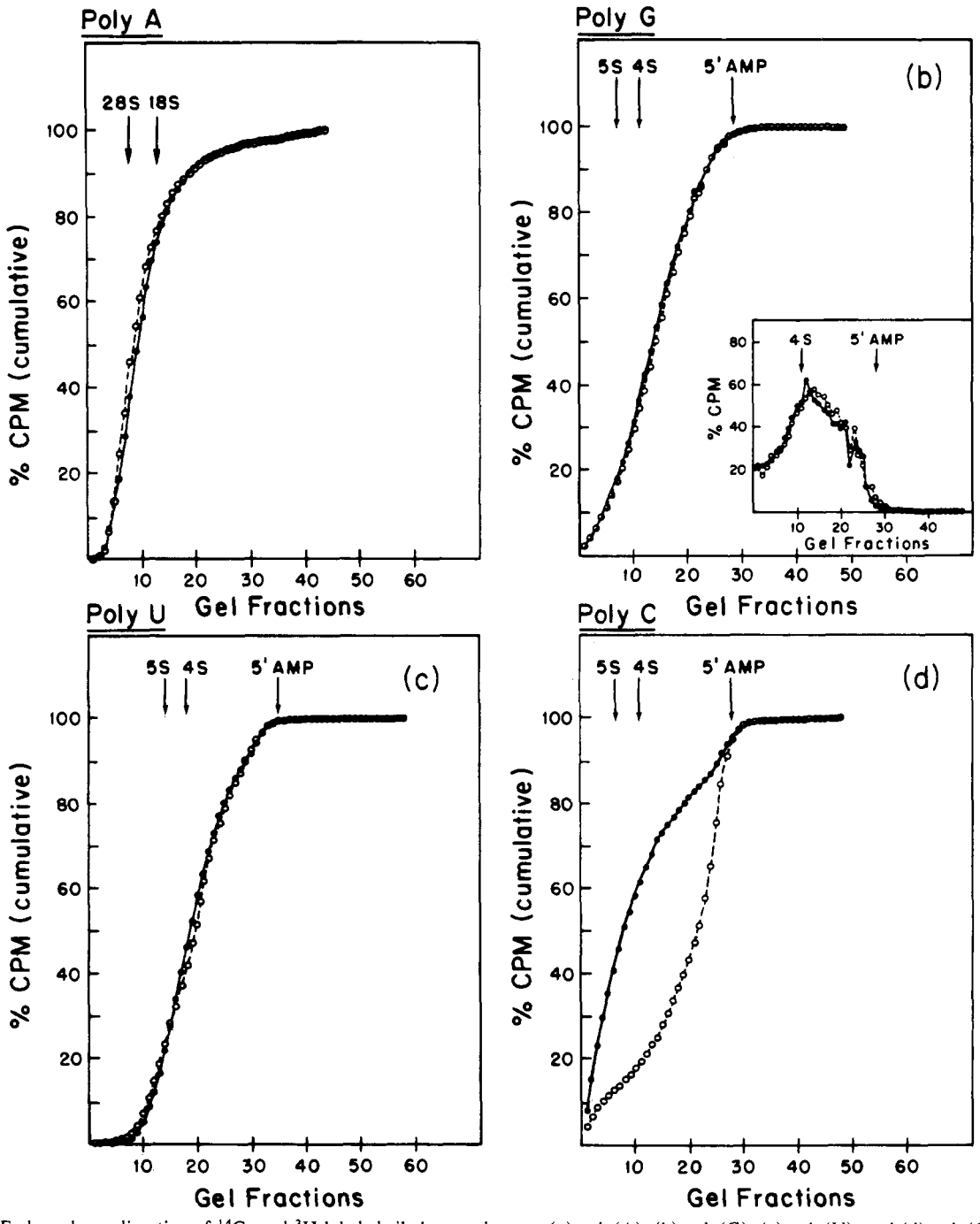

FIGURE 2: Endonuclease digestion of ^{14}C- and ^{3}H-labeled ribohomopolymers: (a) poly(A), (b) poly(G), (c) poly(U), and (d) poly(C). The 50-μl reaction volumes contained the following concentration of the ribohomopolymers and enzyme: [^{14}C]poly(A), 23 μg/ml, ±10 units of endonuclease/ml; or [^{3}H]poly(G), 1.0 μg/ml, [^{3}H]poly(U), 0.7 μg/ml, and [^{3}H]poly(C), 1.0 μg/ml, ±5 units of endonuclease/ml. The incubation was carried out for 30 min at 37° in the presence of 2.5 μmol of Tris (pH 7.4), 2.5 μmol of NaCl, and 50 pmol of DTT. The reaction was stopped as described in Figure 1, and electrophoresis was carried out on 14% gels for 4 hr with poly(G), poly(U), and poly(C), and 2.7% gels for 2.5 hr with poly(A). The arrows indicate positions of 28S, 18S, 4S, and 5S marker RNA and AMP. The data are plotted as per cent total counts per gel fraction in inset of b, and as the cumulative sum of this percentage along the gel for the four homopolymers in a, b, c, and d. (O - - O) Incubation in the presence of endonuclease; (●—●) incubation in the absence of endonuclease.

the nucleolar endonuclease as described in Methods. The assay was found to be linear with both time and enzyme concentration. Data obtained from assays with increasing substrate concentration, plotted reciprocally in Figure 5, show that the apparent K_m of the nucleolar endonuclease for poly(C) is 2.86 μg/ml or 8.85 μM (P).

A series of experiments were designed to obtain more detailed information about the nature of cleavage sites in cellular RNA species which can be recognized by the endonuclease. The obvious choice of substrate for this type of study is 45S precursor rRNA. However, difficulty in obtaining sufficient amounts of the precursor RNA for substrate concentration studies prompted us to use the more readily obtainable 18S and 28S rRNA species, which were observed to be hydrolyzed to a limited extent by the endonuclease. Since poly(C) is extensively hydrolyzed by the endonuclease, we investigated the possibility that the hydrolysis specificity of rRNA depends solely on its C residue composition. This idea was tested by determining the apparent K_m values for both 18S and 28S rRNA and comparing them with the previously determined value of 8.8 μM (P) for poly(C). Since no acid-soluble products could be detected from rRNA, the measurements were carried out using

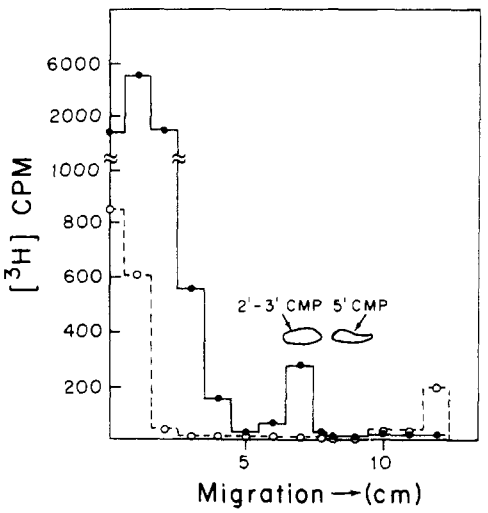

FIGURE 3: Thin-layer chromatography of acid-soluble products after extensive hydrolysis of [^{3}H]poly(C) with the endonuclease. The assay mixture contained 0.1 μg of [^{3}H]poly(C) in a total of 100-μl incubation buffer as in Figure 2. The incubation was carried out 16 hr at 37°, ±0.62 unit of endonuclease. At this point another 0.62 unit was added and the incubation was continued for another 24 hr. The entire mixture was then precipitated with perchloric acid and neutralized with KOH, the sediment was removed by centrifugation, and the acid-soluble material was treated as described in Methods. The radioactivity is plotted as a function of distance from the origin. The 2',3'-CMP and 5'-CMP markers were chromatographed with each sample and were visualized by ultraviolet absorption. CMP in sample: (●—●) incubation with endonuclease; (O - -O) incubation without endonuclease.

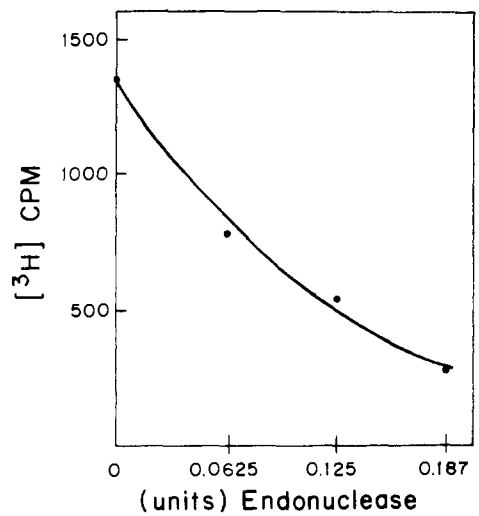

FIGURE 4: Acid-soluble product formation by 3'-OH-specific exonuclease as a function of nucleolar endonuclease concentration in the assay mixture. The assay mixture contained in 100-μl total volume: 0.16 μg of polyadenylated mRNA, 5 μmol of TEA (pH 7.4), 5 μmol of NaCl, 0.25 μmol of $MgCl_2$, 0.1 μmol of DTT, and 40 μg of BSA. After 10-min incubation at 37° in the presence of the indicated amount of endonuclease, 0.16 unit of the exonuclease was added and the incubation was continued for an additional 20 min. The reaction mixture was precipitated with 0.4 N perchloric acid (final concentration) and the acid-soluble radioactivity was measured.

gel electrophoresis of endonuclease hydrolysis products at various rRNA concentrations (see Methods). The apparent K_m values determined from the abcissa intercept of a standard reciprocal plot were 173 μM (P) for 28S rRNA and 133 μM (P) for 18S rRNA. These K_m values are at least 15–20-fold greater than that observed with poly(C). Since cytidylate comprises about one-fourth of the nucleotides in rRNA, only a fourfold increase in the K_m would be expected if the nucleolar endonuclease recognized all of the C residues in the ribosomal sequences. The fact that the K_m values are much greater than this suggests that the recognition process must involve either sequences of more than one C residue, or that the conformation of the rRNA markedly restricts the possibilities of enzymatic cleavage.

Since the natural substrate for the nucleolar endonuclease that is involved in ribosomal maturation is an RNP particle (Warner and Soiero, 1967; Liau and Perry, 1969) containing both ribosomal and non-ribosomal proteins (Warner and Soiero, 1967, Soiero and Basile, 1973), we studied the activity of the partially purified endonuclease on the RNP particles. The 80S preribosomal particles containing mostly 45S rRNA precursor molecules were stable when incubated at 37° in the assay mixture without enzyme. The addition of the nucleolar endonuclease preparation, however, led to the appearance of RNA intermediates (Figure 6) which correspond in their electrophoretic migration to the 36S, 32S, and 28S species observed *in vivo*. In contrast, incubation of a comparable amount of purified 45S pre-rRNA led to the rapid and nonspecific degradation shown in the inset of Figure 6. No accumulation of the intermediate pre-rRNA species can be observed even with lower levels of 45S rRNA hydrolysis. These observations indicate that the protein moiety of the RNP particles may be responsible for much of the specificity, as well as the resistance, observed in pre-rRNA cleavage.

Discussion

The endonuclease which we have purified from L cell nucleoli appears to have an obsolute requirement for cytidylate residues. This restricted specificity distinguishes this enzyme from other known nuclear ribonucleases (Lazarus and Sporn, 1967; Heppel, 1966; Prestayko *et al.*, 1973), all of which have a broader substrate specificity.

Recent reports have described nucleolar endonuclease activities in HeLa cells (Mirault and Scherrer, 1972a,b) and Novikoff hepatoma cells (Prestayko *et al.*, 1972, 1973). The specificity of the HeLa cell nucleolar endonuclease was characterized only to a limited extent, but like the L cell and Novikoff hepatoma enzymes it had no activity on poly(A) and was inhibited with high concentrations of Mg^{2+}. The endonuclease of Novikoff hepatoma nucleoli appears to differ from the L cell endonuclease in at least two of its properties. The Novikoff hepatoma enzyme appeared to be capable of hydrolyzing both poly(C) and poly(U) to acid-soluble fragments, whereas the L cell endonuclease has no detectable activity on poly(U). Another difference between the two enzymes appears to be in the degree of production of mononucleotides. The Novikoff hepatoma enzyme produces easily measurable amounts of CMP from 5S RNA, whereas the L cell endonuclease releases from poly(C) only 4% of acid-soluble material as CMP after extensive enzymatic hydrolysis.

The endonuclease can cleave a number of cellular RNA species, such as mRNA, HnRNA, 28S rRNA, and 45S pre-rRNA at multiple sites, indicating that there is probably a wide variety of nucleotide sequence(s) which can be recognized by the enzyme. The initial cleavage product observed from 18S rRNA suggests a particularly susceptible site in that molecule. The comparison of the apparent K_m values of the endonuclease for poly(C) and the 28S and 18S rRNA has given a further clue toward substrate specificity requirements for the enzyme. The data indicate that the C-residue recognition is not sufficient for hydrolysis, but that the substrate requirements must either involve more than one adjacent C residue or that in addition, conformational factors influence the cleavage site recognition in the RNA.

A possible role of the L cell endonuclease in the maturation of ribosomal precursor RNA was suggested by the finding that

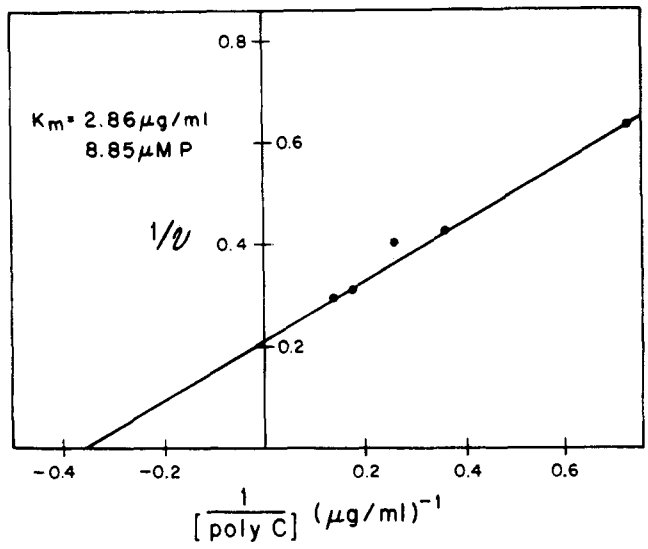

FIGURE 5: K_m determination for the nucleolar endonuclease action on poly(C). Endonuclease assays were carried out as described in Methods with [^{3}H]poly(C) concentrations ranging from 1.4 to 7.4 μg per ml and 0.35 unit of endonuclease. The acid-soluble radioactivity released after 30 min was determined, corrected for release of acid-soluble radioactivity in the absence of enzyme (*ca.* 15%), and used as a measure of the initial velocity, *v*. Each point on the reciprocal plot represents the average of two experiments.

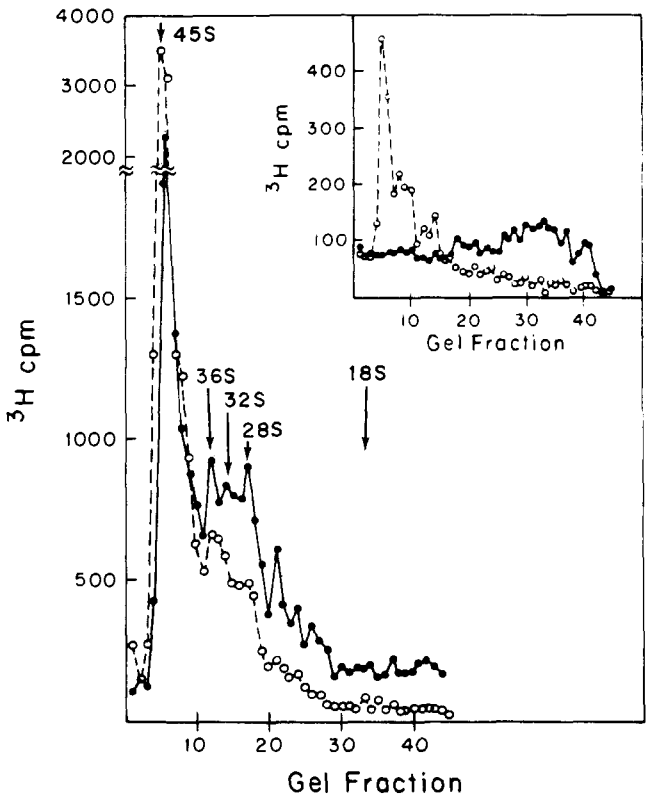

FIGURE 6: Hydrolysis of 80S RNP particles and 45S ribosomal precursor RNA with the nucleolar endonuclease. 80S RNP particles isolated on a sucrose gradient and containing 3.89 μg of precursor RNA were incubated in a total volume of 1.15 ml of incubation mixture ±0.83 unit of endonuclease for 8 min at 37°. The reaction was stopped by the addition of SDS to 0.5% and EDTA to a final concentration of 6 mM. The RNA was extracted with chloroform–phenol in the presence of 30 μg of tRNA carrier, precipitated with ethanol, and applied on 2.7% gels for electrophoresis. The purified 45S precursor rRNA, 1.57 μg, was incubated in a total volume of 85 μl of incubation mixture ±0.089 unit of endonuclease for 3.5 min at 37°. The reaction was stopped as above and the entire sample was applied on a 2.7% gel for electrophoresis. Final concentrations of the components for the incubation mixture for both RNP particles and the RNA are: 50 mM TEA (pH 7.4), 50 mM NaCl, 50 mM KCl, 5 mM $MgCl_2$, and 1 mM DTT. Gel electrophoresis was carried out 5.75 hr at 5 mA/gel as described in Methods. The ratio of RNA/enzyme/time of incubation is for 80S RNP particles: 0.58 μg/unit/min; 45S RNA; 0.59 μg/unit/min. Electrophoresis profile of RNA extracted from 80S RNP particles after incubation at 37° with endonuclease (●—●); without endonuclease (O - - O). Inset: electrophoresis profile of 45S RNA after incubation with (●) and without (O) endonuclease.

incubation of the isolated 80S ribonucleoprotein particles with the enzyme results in the production of RNA size classes coincident with the naturally occurring intermediate species. However, the correctness of the cleavage points, and consequently the definitive implication of this enzyme in pre-rRNA processing, still requires further verification. The difference in extent of cleavage when ribonucleoprotein particles were used as substrate as compared to when naked 45S pre-rRNA was used as substrate indicates that the protein components can contribute to the specificity of the cleavage reaction and modulate the extent of nuclease activity. Previous studies by Mirault and Scherrer (1972b), on pre-ribosomal processing in nucleolar extracts of HeLa cells, have shown that the processing property is not shared by pancreatic ribonuclease A. This observation indicates that the specificity of the nuclease as well as the nature of the ribonucleoprotein complex contributes to the maturation process.

If this enzyme is involved in ribosomal RNA maturation, then the finding that it produces 3′-phosphate groups has several implications. First, the 3′-phosphate ends left by the endonuclease would be resistant to the 3′-hydroxyl specific exonuclease activity (Lazarus and Sporn, 1967) that is relatively abundant in the nucleolus (Kelley and Perry, 1971) and can attack both ribosomal and the precursor RNA species (Perry and Kelley, 1972). Second, if endonuclease cleavages occurred at the 3′ termini of either 28S or 18S rRNA, then a phosphatase would be needed to remove the 3′-phosphate groups, since the mature 18S and 28S rRNA components are terminated by 3′-hydroxyl groups.

Finally, it is necessary to consider the possibility that more than one nuclease is involved in the maturation of rRNA. This has been found to be the case in tRNA maturation, where the endonuclease which correctly removes the 5′ nonconserved end *in vitro*, apparently cannot remove the other nonconserved segment at the 3′ end (Altman and Smith, 1971; Robertson *et al.*, 1972); an additional nuclease activity is presumably required for this cleavage. Analogously, an enzyme other than the endonuclease studied here may be implicated in the cleavage which produces the correct 5′ terminus of the 18S rRNA since that molecule has a phosphate group at its 5′ terminus (Lane and Tamaoki, 1967; Maden and Forbes, 1972). A similar situation may exist in rRNA maturation in *Escherichia coli*, where ribonuclease III has been implicated in rRNA processing (Nikolaev *et al.*, 1973). This enzyme is dissimilar to the L cell endonuclease in its substrate specificity and ion requirements, but also produces 3′-phosphate end groups upon cleavage of RNA (Robertson *et al.*, 1968), thus indicating that additional enzymatic activity is necessary for the final rRNA maturation.

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References

Altman, S., and Smith, J. D. (1971), *Nature (London), New Biol. 233*, 35.

Aviv, H., and Leder, P. (1972), *Proc. Nat. Acad. Sci. U. S. 69*, 1408.

Burdon, R. H. (1971), *Prog. Nucl. Acid Res. 11*, 331.

Darnell, J. E. (1968), *Bacteriol. Rev. 32,* 262.
Heppel, L. A. (1966), *in* Procedures in Nucleic Acid Research, Cantoni, G. L., and Davies, D. R., Ed., New York, N. Y., Harper & Row, p 31.
Kelley, D. E., and Perry, R. P. (1971), *Biochim. Biophys. Acta 238,* 357.
Lane, B. G., and Tamaoki, T. (1967), *J. Mol. Biol. 27,* 335.
Lazarus, H. M., and Sporn, M. B. (1967), *Proc. Nat. Acad. Sci. U. S. 57,* 1386.
Liau, M. C., Craig, N. C., and Perry, R. P. (1968), *Biochim. Biophys. Acta 169,* 196.
Liau, M. C., and Perry, R. P. (1969), *J. Cell Biol. 42,* 272.
Maden, B. E. H. (1971), *Progr. Biophys. Mol. Biol. 22,* 127.
Maden, B. E. H., and Forbes, J. (1972), *FEBS (Fed. Eur. Biochem. Soc.) Lett. 28,* 289.
Maden, B. E. H., Salim, M., and Summers, D. F. (1972), *Nature (London), New Biol. 237,* 5.
Mirault, M. E., and Scherrer, K. (1972a), *FEBS (Fed. Eur. Biochem. Soc.) Lett. 20,* 233.
Mirault, M. E., and Scherrer, K. (1972b), *FEBS (Fed. Eur. Biochem. Soc.) Lett. 28,* 197.
Nakazato, H., and Edmonds, M. (1972), *J. Biol. Chem. 247,* 3365.
Nikolaev, N., Silengo, L., and Schlessinger, D. (1973), *J. Biol. Chem. 248,* 7967.
Penman, S. (1966), *J. Mol. Biol. 17,* 117.
Perry, R. P. (1969), *in* Handbook of Molecular Cytology, Lima-de-Faria, A., Ed., Amsterdam, North-Holland Publishing Co., p 620.
Perry, R. P., and Kelley, D. E. (1968a), *J. Mol. Biol. 35,* 37.
Perry, R. P., and Kelley, D. E. (1968b), *J. Cell Physiol. 72,* 235.
Perry, R. P., and Kelley, D. E. (1972), *J. Mol. Biol. 70,* 265.
Perry, R. P., LaTorre, J., Kelley, D. E., and Greenberg, J. R. (1972), *Biochim. Biophys. Acta 262,* 220.
Prestayko, A. W., Lewis, B. C., and Busch, H. (1972), *Biochim. Biophys. Acta 269,* 90.
Prestayko, A. W., Lewis, B. C., and Busch, H. (1973), *Biochim. Biophys. Acta 319,* 323.
Robertson, H. D., Altman, S., and Smith, J. D. (1972), *J. Biol. Chem. 247,* 5243.
Robertson, H. D., Webster, R. E., and Zinder, N. D. (1968), *J. Biol. Chem. 243,* 82.
Soiero, R., and Basile, C. (1973), *J. Mol. Biol. 79,* 507.
Sporn, M. B., Berkowitz, D. M., Glinski, R. P., Ash, A. B., and Stevens, C. L. (1969a), *Science 164,* 1408.
Sporn, M. B., Lazarus, H. M., Smith, J. M., and Henderson, W. R. (1969b), *Biochemistry 8,* 1698.
Vesco, C., and Penman, S. (1968), *Biochim. Biophys. Acta 169,* 188.
Warner, J. R., and Soiero, R. (1967), *Proc. Nat. Acad. Sci. U. S. 58,* 1984.
Weinberg, R. A., Loening, U., Willems, M., and Penman, S. (1967), *Proc. Nat. Acad. Sci. U. S. 58,* 1088.
Weinberg, R. A., and Penman, S. (1970), *J. Mol. Biol. 47,* 169.
Wellauer, P. K., and Dawid, I. B. (1973), *Proc. Nat. Acad. Sci. U. S. 70,* 2827.
Wettstein, F. O., Staehelin, T., and Noll, H. (1963), *Nature (London) 197,* 430.