Purification and Characterization of an Endoribonuclease from Nucleoplasm and Nucleoli of HeLa Cells*

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An endoribonuclease has been isolated from HeLa cell nuclei. Approximately 70% of the enzyme appears to be nucleolar bound; 30% is in the nucleoplasm.

Studies of the purified enzyme reveal that the enzyme is an endonuclease of estimated molecular weight 16,000. It produces oligonucleotides bearing 5'-phosphate end groups. The enzyme degrades poly(C) and poly(U), as well as RNA and heterogeneous nuclear RNA. Poly(A), double-stranded RNA, and DNA are not cleaved. The enzyme is heat-labile and is inhibited by 10 mM Mg²⁺ and 50 mM NaCl. The enzyme is probably distinct from previously described nuclear endonucleases.

MATERIALS AND METHODS

Cell Fractionation—HeLa cells (S₁₀) were grown in Joklik modified Minimal Essential Medium plus 10% fetal calf serum (Grand Island Biological Co.) in spinner bottles. Cells were collected at a density of 4 × 10⁹ cells/ml.

For each cell fractionation, 3 × 10⁹ cells were used. All steps were carried out at 0-4°C. Cells were swollen in 60 ml of low salt buffer (0.01 M Tris, pH 7.4, 0.01 M NaCl, 0.0015 M MgCl₂) for 10 min; 2 M sucrose and 10% Triton X-100 were then added to final concentrations of 0.25 M and 0.025%, respectively. The mixture was given 10 strokes in a Dounce homogenizer and was then centrifuged at 200 × g for 5 min to yield a pellet of nuclei. The nuclear pellet was washed by resuspending in 40 ml of high salt buffer (0.01 M Tris, pH 7.4, 0.01 M NaCl, 0.0015 M MgCl₂) for 10 min with gentle homogenization. Then, 1 ml of Tris buffer (1 M, pH 8.1) was added together with 6 ml of 2 M NaCl and 0.2 mg EDTA. After homogenization for 10 min, the mixture was centrifuged (30 min, 20,000 × g) and the pellet was re-extracted with 1/3 volume of the above solutions and centrifuged again. The combined supernatant is designated the crude nucleolar extract.

Both nucleoplasmic and nucleolar crude extracts were dialyzed against low salt buffer overnight. After dialysis, some insoluble material formed. It was removed by centrifugation for 10 min at 5000 × g and discarded.

Enzyme Assay—A reaction mixture of 80 μl contained 0.08 μg of [³²P]poly(C) (Miles 8.2 μg/μl), 0.01 M Tris, pH 7.4, 0.01 M NaCl, 0.0015 M MgCl₂, and enzyme. After incubation at 37°C, 200 μl of 10% trichloroacetic acid were added together with 100 μg of yeast RNA or 50 μg of albumin as carriers in a volume of 10 μl. The mixture was left on ice for 30 min and then centrifuged for 20 min at 6000 × g. A 200-μl portion of the supernatant was removed into 2.5 ml of counting fluid (Research Products International Corp., 3a 70B prebubbled liquid scintillation mixture) and the radioactivity determined. The substrates usually contained between 2 and 5% acid-soluble radioactivity except for poly(C) which has 50% acid-soluble radioactivity. One unit of enzyme is defined as that amount of enzyme which converts 1 μg of poly(C) into acid-soluble oligonucleotides.

To determine further the extent of fragmentation of a substrate, 200 μl of 55% ethanol was added to a reaction mixture together with carrier RNA. The precipitated nucleotides were dissolved in SDS.

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1 The abbreviations used are: rRNA, ribosomal RNA; hnRNA, heterogeneous nuclear RNA; albumin, bovine serum albumin; SDS, sodium dodecyl sulfate; poly(A) and poly(C), polymers of adenylic and cytidylic acids, respectively; rG.rC, double-stranded polymer of guanylic acid and cytidylic acid.
buffer and analyzed by polyacrylamide gel electrophoresis (16). The gels were sliced and dissolved in NH₄OH and the radioactivity determined as above.

Thin Layer Chromatography of Poly(C) Hydrolysis Products—Reactions were stopped by adding SDS to 0.4% and 50 µl of the reaction mixtures that were applied to PEI sheets (Brinkmann Instruments, Inc., Westbury, N. Y.) together with 10 µg each of 2'(3')-CMP and 5'-CMP as ultraviolet markers. The sheets were developed with 0.3 M LiCl for 90 to 120 min. The sample lanes were then cut into 1-cm pieces. Each piece was placed into a toluene-based scintillation mixture and the radioactivity was determined.

Size Determination of Oligonucleotides after Endonuclease Digestion—[3H]Poly(C) was incubated with endonuclease under standard condition. SDS was added to 0.1% to stop the reaction. The reaction mixture was fractionated on a P-30 Bio-Gel column (0.4 x 20 cm) previously washed with low salt buffer. After the reaction, the samples had passed through, the column was eluted batch-wise, first with 5 ml of the dialysis buffer and then 5 ml each of gradient buffer range of 1.0 to 1.5 for both nucleoplasmic or nucleolar extracts.

RESULTS

Purification

DEAE-cellulose Fractionation—Fig. 1 shows the DEAE-fractionation of crude nucleoplasmic (a) and nucleolar (b) extracts. For this, 50 ml (50 to 100 mg of protein) of nucleoplasmic or 10 ml (10 to 20 mg of protein) of nucleolar extract were placed on a DEAE-cellulose column (2 x 10 cm or 0.9 x 10 cm) previously washed with low salt buffer. After the samples had passed through, the columns were eluted with a linear gradient of 50 ml of low salt buffer and 50 ml of low salt buffer containing 0.2 M NaCl; 3.3-ml fractions were collected. NaOH was added to each fraction to 0.3 N and the mixtures digested overnight at 37°. HCl was then added to adjust the pH to between 6 and 7. The samples were applied to DEAE-Sephadex A-25 column (0.5 x 4 cm). Nucleosides were eluted with two 1-ml portions of low salt buffer and nucleotides were eluted with two 1-ml portions of low salt buffer containing 0.5 M NaCl, followed by two 1-ml portions of low salt buffer containing 0.3 M NaCl. The ratio of total counts to the nucleoside counts was used to calculate the average chain length in each Bio-Gel fraction.

Substrates and Other Materials—Synthetic polynucleotides were purchased from Miles Laboratories Inc., except that poly(U) was from Schwarz Biochemicals. [3H]RNA and [3H]poly(G)-poly(C) were gifts of Dr. Nina Federoff. [3H]DNA from Escherichia coli was a gift of Dr. Donald Kaplan. Snake venom phosphodiesterase and spleen phosphodiesterase were from Worthington Biochemicals.

Protein Determination—Protein concentration was determined by the method of Lowry et al. (17).

nucleic acid as indicated by A₂₆₀/A₂₈₀ ratios, which were in the range of 1.0 to 1.5 for both nucleoplasmic or nucleolar extracts.

Phosphocellulose Fractionation—The pooled fraction from the DEAE-cellulose column was dialyzed against 0.01 M Tris (pH 7.4), 0.01 M NaCl, and 1 mM dithiothreitol. A portion of the nucleolar material, containing 107 units of enzyme in 4 ml, was placed on a phosphocellulose column (0.9 x 4 cm). After the samples had passed through, the column was eluted batch-wise, first with 5 ml of the dialysis buffer and then 5 ml each of potassium phosphate buffer (pH 7.2) at concentrations of 0.1, 0.2, 0.3, 0.5, and 1.0 M, each containing 1 mM dithiothreitol. Fractions of 5 ml were collected. After dialyzing all fractions against low salt buffer, the fractions containing enzymatic activity were pooled (Fraction II).

The enzyme was eluted from phosphocellulose with 0.2 M potassium phosphate buffer, although occasionally some activity was found in the 0.3 M phosphate buffer eluate. Larger volumes of sample (up to 25 ml of Fraction I) were also used on identical columns with similar results.
material in 7.5-ml portions was layered onto a column (2 x 45 cm) was further purified by Sephadex G-100 gel filtration. The 7134 Endoribonuclease from HeLa Cell Nuclei Nucleoli by its increased mobility. In addition, the size of the oligonucleotides after enzymatic digestion was examined by fractionation on a DEAE-cellulose column.

Gel Filtration - The phosphocellulose fraction (Fraction II) was further purified by Sephadex G-100 gel filtration. The material in 7.5-ml portions was layered onto a column (2 x 45 cm) previously equilibrated with low salt buffer. Elution was carried out with the same buffer. Fractions of 6.5 ml were collected at 15 min intervals. At this stage, the enzyme eluted as a single symmetrical peak of activity.

Calibration of the column with human γ-globulin, ovalbumin, and cytochrome c gave an estimated molecular weight of the enzyme of 16,000. To stabilize the activity, 1 mg/ml of albumin was added to each of the active fractions. Enzyme preparations from nucleoplasm and nucleolar extracts showed identical elution patterns on Sephadex. Although it is not possible to precisely determine the distribution of the enzyme between nucleoplasm and nucleoli because of the presence of other activities in the crude fractions, we have made an estimate based on the recovery of the purified enzymes (Table II). This indicates that at least 70% of the enzyme is associated with the nucleolus.

### Enzymatic Properties

Pured enzyme fractions from nucleoplasm and nucleoli have identical properties in all cases.

Endonucleolytic Cleavage - When using [3H]poly(C) as substrate, the enzyme gave increasing amounts of alcohol- or acid-soluble radioactivity with increasing times of incubation (Fig. 2). However, the alcohol-soluble counts were considerably lower. This is because only oligonucleotides of length 6 or smaller are soluble in alcohol while oligonucleotides up to 20 nucleotides long are soluble in trichloroacetic acid (18). No mononucleotides were detectable even when nearly 100% of the substrate was made acid-soluble. These results show that initially the enzyme cleaves poly(C) into oligonucleotides of relatively large size. This is shown directly in Fig. 3, where the poly(C) was examined directly by gel electrophoresis before and after enzymatic cleavage. After 5 min of incubation with a low level of enzyme, when no alcohol-soluble counts can be detected, the treated substrate was fragmented as indicated by its increased mobility. In addition, the size of the oligonucleotides after enzymatic digestion was examined by fractionation on a P-30 Bio-Gel column. Individual fractions were hydrolyzed with NaOH. The average size of each fraction was determined by measurement of the nucleotide to nucleoside ratio (see "Materials and Methods"). The substrate, [3H]poly(C), had an average size of 40 nucleotides, while after endonuclease digestion the oligonucleotides were from 25 to 11 nucleotides in length. The presence of nucleosides in the NaOH digests of the product indicates that the oligonucleotides are terminated with 3'-OH groups. The validity of this conclusion was confirmed by demonstrating the absence of phosphatase in the purified enzyme (see below).

**Effect of Ionic Strength** - The enzyme was sensitive to increasing concentrations of NaCl. NaCl at 0.04 M gives 50% inhibition. NaF at 1 mM EDTA has almost no effect on enzymatic activity. When dialyzed enzyme is assayed in the presence of increasing amounts of Mg²⁺, enzymatic activity is reduced: 10 mM Mg²⁺ inhibits by 50%.

**Effect of pH** - The enzyme shows a broad pH profile with an optimum at approximately pH 7.2. The enzymatic activity is identical in potassium phosphate buffer and in Tris buffer.

**Effect of Mg²⁺ Concentration** - Dialysis against 1 mM EDTA has almost no effect on enzymatic activity. When dialyzed enzyme is assayed in the presence of increasing amounts of Mg²⁺, enzymatic activity is reduced: 10 mM Mg²⁺ inhibits by 50%.

**Effect of Ionic Strength** - The purified enzyme was sensitive to increasing concentrations of NaCl. NaCl at 0.04 M gives 50% inhibition. NaF at 10 mM had no effect on enzymatic activity although 3 mM NaF has been shown to be inhibitory to a nuclear exonuclease (19). Potassium ion at similar concentrations (10 mM) had no effect on the enzyme, nor did phosphate buffer.
were determined. At each time point, 200 μl of trichloroacetic acid were added to stop the reaction and the trichloroacetic acid-soluble counts per min were determined.

The presence of increasing amounts of purified endonuclease in reaction mixtures containing poly(C) and spleen phosphodiesterase give inhibition of mononucleotide released by the spleen phosphodiesterase (19). Fig. 5 shows that when poly(C) is incubated first with the purified endonuclease and then with spleen venom phosphodiesterase, it is hydrolyzed much more rapidly than without prior endonuclease treatment. This indicates that the cleavage by the endonuclease is such as to produce 3'-hydroxyl and 5'-phosphate end groups.

Experiments were also performed using spleen phosphodiesterase. This enzyme's mode of action is complementary to that of the venom enzyme; a 5'-hydroxyl group is necessary (19). The presence of increasing amounts of purified endonuclease in reaction mixtures containing poly(C) and spleen phosphodiesterase give inhibition of mononucleotide released by the spleen enzyme. By contrast, comparable amounts of pancreatic ribonuclease A had a slight stimulatory effect (Fig. 6). This result is consistent with the cleavage of the poly(C) to yield fragments bearing 5'-phosphate end groups.

Long term incubation (18 h) with the nucleolar endonuclease gave only 5'-CMP on thin layer chromatography plates (Fig. 7). No cytidine can be detected even after 18 h of incubation indicating the absence of any contaminating phosphatase.

**DISCUSSION**

Multiple peaks of enzymatic activities were found by DEAE-cellulose fractionation of either the nucleoplasmic or nucleolar extracts (Fig. 1). Because of this, the extent of purification (Table II) is difficult to quantitate. Moreover, there seemed to be an inhibitor of endonuclease activity in nucleoli. I detected only very low poly(C) degradative activity in crude nucleoli, while the activity of enzyme increased greatly after the DEAE-cellulose chromatography. No comparable increase of activity occurred with nucleoplasmic fractions.

We have begun the purification of nuclear ribonucleases because of the potential role in RNA processing (13). Based on the character of various intermediate rRNAs produced by in vivo maturation of ribosomal RNA and on data determined in the electron microscope, Winicov and Perry (5) have postulated four distinct endonucleolytic cleavage sites in the rRNA precursor molecules. These sites are common in several animal species. HeLa cells and mouse L-cells share the same cleavage sites, although the order of cleavage is different. Therefore, since processing endonucleases may be universal, it is of interest to compare the HeLa enzyme to that of other enzymes already implicated in processing.

The endonuclease described here is very similar to one described by Winicov and Perry (5) from mouse L-cells. Both enzymes cleave poly(C) but not poly(A), are inhibited by Mg2+, and are found bound to nucleoli. However, two important differences exist. The HeLa enzyme produces oligonucleotides having a 5'-phosphate end group, while the enzyme previously described produced oligonucleotides with 3'-phosphate end groups. It is of course possible that since the mouse enzyme was not highly purified, the termini observed were due to a contaminating activity. However, the latter enzyme does not cleave poly(U) while that from HeLa does. The HeLa endonuclease can also be compared to the enzyme isolated from pro-
ribosomal particles by Prestayko et al. (9). The cellular location and substrate specificities are similar, however, this enzyme has been shown to be extremely stable to heating while our enzyme is heat-labile.

Cordis et al. (10) have isolated an endonuclease from rat liver nuclei. This enzyme, like the HeLa enzyme reported here, produces oligonucleotides with 5'-phosphate end groups. However, the HeLa enzyme does not cleave poly(A) as does that from rat liver. Heppel isolated an endonuclease from pig liver (7), similar to the enzyme of Cordis et al. (10). It also attacks poly(A) to give oligonucleotides bearing 5'-phosphate end groups. It appears possible based on the above discussion as well as our experimental results (Fig. 1) that a number of nuclear endonucleases exist. However, some of the apparent differences may be due to the method of purification used and the purity obtained, since the presence of inhibitors or other nuclease may influence the observed characteristics.

The properties of the endonuclease described here make it a good candidate for a processing enzyme. The enzyme produces oligonucleotides bearing 5'-phosphate termini which are good substrates for the 3'-OH specific exonuclease of Sporn et al. (6). The endonuclease by itself or in combination with the exonuclease could cleave and trim the precursor to create the proper intermediates and the mature rRNA. Moreover, the fact that the enzyme is inhibited by NaCl and Mg²⁺ is consistent with the stability of precursor 45 S RNA in the high salt preparative method of Penman (15, 20).

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