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A YEAST ENDORIBONUCLEASE STIMULATED BY NOVIKOFF HEPATOMA SMALL NUCLEAR RNAS U1 AND U2*

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Summary. Using [^{3}H]m^{7}Gppp[^{14}C]RNA-poly(A) from yeast as a substrate, an endoribonuclease has been detected in enzyme fractions derived from a high salt wash of ribonucleoprotein particles of Saccharomyces cerevisiae. The [^{3}H]m^{7}Gppp[^{14}C]RNA-poly(A) seems to be a preferred substrate since other polyribonucleotides are hydrolyzed more slowly, if at all. The enzyme is inhibited by ethidium bromide, but fully double-stranded polyribonucleotides are not hydrolyzed. The hydrolysis of [^{3}H]m^{7}Gppp[^{14}C]RNA-poly(A) is stimulated about 2.5-fold by the addition of small nuclear RNAs U1 and U2 of Novikoff hepatoma cells. Results show that the stimulation involves an interaction of the labeled RNA with the small nuclear RNA.

When [^{3}H]m^{7}Gppp[^{14}C]RNA-poly(A) from yeast was tried as a substrate for a partially-purified 5'→3' exoribonuclease from yeast (1), it was found that ^{3}H label bound by oligo(dT)-cellulose was converted to an unbound, acid-insoluble form. Further investigations showed the presence of an endoribonuclease. Certain properties of the enzyme and its stimulation by snRNA are described in this report.

METHODS AND MATERIALS

[^{3}H]m^{7}Gppp[^{14}C]RNA-poly(A) was prepared from a 600 ml log phase culture of Saccharomyces cerevisiae A364A, mutant ts 368, by labeling spheroplasts with [^{3}H]Me-methionine and [^{14}C]uridine, as described previously (2). Poly(A)-containing RNA labeled in the manner described (2,3) contains [^{3}H]methyl only in m^{7}G of the cap structure. Total RNA was isolated from the spheroplasts and poly(A)-containing RNA was separated by oligo(dT)-cellulose chromatography as described by Sripati et al. (3). After the first oligo(dT)-cellulose column, the poly(A)-containing RNA was heated in 90% formamide for 2 min at 68°C. Following dilution to 5% formamide with

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Abbreviations: snRNA, small nuclear RNA; SDS, sodium dodecylsulfate.

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10 mM Tris-HCl, pH 7.5, containing 10 mM EDTA, 0.2% SDS and 0.5 M LiCl (high salt buffer), it was applied to a second oligo(dT)-cellulose column and eluted and precipitated in the manner described by Sripati et al. (3). About 160 nmol of RNA were obtained containing about 800 cpm of ^{14}C and 300 cpm of ^{3}H per nmole of nucleotide.

The endonuclease was purified from ribonucleoprotein particles of yeast in the same manner as described for a 5'→3' exoribonuclease (1) up to the hydroxylapatite chromatography step. The chromatography on hydroxylapatite was modified by elution of the column with a linear gradient from 50 to 660 mM potassium phosphate. The peak fraction of exoribonuclease was eluted with 380 mM potassium phosphate, while the endonuclease was eluted with 380 to 500 mM potassium phosphate. The overall purification of the endonuclease is about 125-fold from the starting ribonucleoprotein particle fraction. For the studies reported here, peak hydroxylapatite fractions were used as the enzyme. The A_{280}/A_{260} of the fractions was 1.4.

The endonuclease was assayed by using [^{3}H]m^{7}Gppp[^{14}C]RNA-poly(A) as a substrate and measuring the % of ^{3}H label rendered non-retainable by oligo(dT)-cellulose. Reaction mixtures (50 µl) contained about 1.6 nmol of labeled RNA, 50 mM Tris-HCl, pH 8.3, 50 mM NH_4Cl, 25 mM EDTA, and enzyme. After incubation for 10 min at 37°C, 0.5 ml of high salt buffer was added and the solution was applied to a 0.2 ml column of oligo(dT)-cellulose. The effluent was collected and the column was washed with 1 ml of high salt buffer which was collected in the same vessel. The column was washed with 1 ml of high salt buffer which was discarded, and then the retained poly(A)-containing RNA was collected using a 1.5 ml wash with 10 mM Tris-HCl, pH 7.5. The ^{3}H label and ^{14}C label in the two RNA fractions were counted and the % of unbound ^{3}H label was calculated. Reaction mixtures lacking enzyme and plus enzyme with no incubation were used as controls. The usual value of non-bound ^{3}H label for these control mixtures was about 5–8%. Controls plus snRNAs were similar. It was found that phenol extraction of reaction mixtures prior to chromatography on oligo (dT)-cellulose did not alter the results, and that treatment was not routinely used.

SDS-polyacrylamide gel electrophoresis (3% gels) was carried out as described by Loening (4) using 0.5 × 6 cm cylindrical gels. Electrophoresis was at room temperature using 4 ma/gel for about 2 hr or until the bromphenol blue marker dye had migrated about 45 mm. The gels were cut into 2 mm slices using a Mickle gel slicer, and 0.5 ml of 1 N NaOH was added to each sample. After heating 30 min at 80°C, the samples were neutralized with HCl and counted. Eight µg of rRNA (16S and 23S) of E. coli were used as markers and electrophoresed on a separate gel. The gel was stained with 0.2% methylene blue (5) containing 0.2 M sodium acetate and 0.2 M acetic acid and then destained with water.

A preparation of 16S [^{3}H]rRNA from E. coli was made by gradient centrifugation as described previously (6). It contained a small amount of 23S RNA. 5S, 16S, and 23s rRNA and tRNA of E. coli were obtained from Boehringer Mannheim. All synthetic polyribonucleotides were from Miles Biochemicals. The concentration of each snRNA was between 30–100 µg per ml.

All polyribonucleotides were determined by UV absorbance at 260 nm using an E_M = 10,000, and the concentrations are expressed as nmol of nucleotide. Protein was determined by UV absorbance at 280 nm.

The S. cerevisiae mutant used here was a gift of Dr. Jonathan Warner. Oligo(dT)-cellulose was from Collaborative Research, Inc.

RESULTS AND DISCUSSION

Characterization of the [^{3}H]m^{7}Gppp[^{14}C]RNA-poly(A). [^{3}H]m^{7}Gppp[^{14}C]RNA-poly(A) from the second oligo(dT)-cellulose column has been examined by SDS-polyacrylamide gel electrophoresis, and gel profiles of absorbance of methylene blue stain and ^{3}H and ^{14}C labels are shown in Fig 1. The absorbance scan in Fig. 1 shows that the average size of the RNA is about

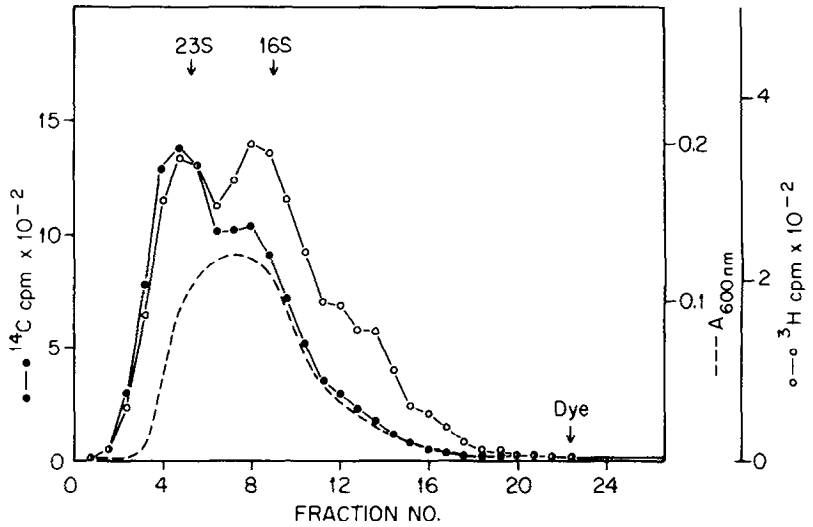

Fig. 1. SDS-polyacrylamide gel electrophoresis of [^{3}H]m^{7}Gppp[^{14}C]RNA-poly(A). Gel electrophoresis of 27 nmol of [^{3}H]m^{7}Gppp[^{14}C]RNA-poly(A) (Preparation B), followed by staining and destaining of the gel, was carried out as described under Methods and Materials. The gel was then scanned at 600 nm. Following scanning, the gel was sliced and counted as described under Methods and Materials.

18–20S. That the higher molecular weight RNA (>20S) has a higher ^{14}C specific activity than the smaller RNA suggests that it is probably nuclear poly(A)-containing RNA or pre-mRNA. An estimate of the amount of pre-mRNA from the gel profiles in Fig. 1 is about 30%. Groner and Phillips (7) have shown that nuclear poly(A)-containing RNA contains about 20% of the total label of poly(A)-containing RNA when S. cerevisiae spheroplasts are labeled with [^{3}H]adenine for 10 min. Characterization of the [^{3}H]m^{7}Gppp[^{14}C]RNA-poly(A) by enzyme digestion, as described previously (2), showed that it still contains a small amount of rRNA since about 12% of the ^{3}H label is in nucleosides (all four) other than m^{7}G.

Evidence for a Yeast Endoribonuclease. Using [^{3}H]m^{7}Gppp[^{14}C]RNA-poly(A) as a substrate with reaction mixtures and assay conditions as described under Methods and Materials, a hydroxylapatite enzyme fraction, obtained during the purification of a 5'→3' exoribonuclease from yeast (1), degraded the RNA to oligo(dT)-cellulose non-retainable products containing ^{3}H and ^{14}C label. No acid-solubilization of the RNA label occurred as determined by the assay described earlier (1). The reaction did not require Mg^{2+} and occurred in the presence of EDTA, so the Mg^{2+}-dependent exoribonuclease in the fraction did not interfere with the study of the reaction. Several [^{3}H]m^{7}Gppp[^{14}C]RNA-poly(A) preparations from the second oligo(dT)-cellulose column varied in their reactivity with the endoribonuclease, giving ^{3}H release values from 15 to 36%, using 0.9 µg of hydroxylapatite-purified enzyme. Two preparations, called A and B, were used in the studies described below. Using the

oligo(dT)-cellulose assay, the reaction is linear with time up to 10 min, and is linear with substrate concentration in the range of concentrations used in this study. An enzyme concentration curve using poly(A)-RNA preparation A as a substrate showed 17, 30, and 55% release of ^{3}H label from oligo(dT)-cellulose with 0.36, 0.9, and 2.7 µg of enzyme.

Polyacrylamide gel electrophoresis of the labeled RNA following incubation without and with the enzyme fraction is shown in Fig. 2A. A considerable reduction in the size of the RNA is obtained in the presence of the enzyme, providing evidence for endoribonuclease activity. Gel analysis of the [^{3}H]m^{7}Gppp[^{14}C]RNA-poly(A) still bound to oligo(dT)-cellulose following incubation without and with the enzyme is shown in Fig. 2B. The results with the ^{3}H label show that the high molecular weight RNA is preferentially hydrolyzed. (The ^{3}H label of hydrolyzed RNA molecules is removed on oligo(dT)-cellulose treatment.) In a reaction in which 35% of the total ^{3}H label was rendered non-retainable by oligo(dT)-cellulose, more than 50% of the high molecular weight RNA is hydrolyzed, while only about 10% of the smaller RNA is hydrolyzed.

Other single-stranded polyribonucleotides also were tested as substrates for the enzyme. The gel pattern of a preparation of [^{3}H]rRNA of *E. coli* is shown following incubation without and with the enzyme. Only about 10-15% of either the 23S or 16S RNA is hydrolyzed. The single-stranded synthetic polyribonucleotides, poly(A), poly(U), and poly(C), also were tested as substrates, using conversion to acid-soluble products as a measure of activity [as described in (1)]. No hydrolysis was detected using 15× the amount of enzyme used in the studies shown in Fig. 2. Pre-tRNA of yeast has been shown to be cleaved by an endonuclease to yield half molecules and the intervening sequence (8,9). The enzyme described here does not hydrolyze [^{32}P]pre-tRNA (Table I).

Stimulation of the Enzyme by snRNA. The intercalating agent, ethidium bromide, which has been shown to inhibit many of the reported RNases that hydrolyze dsRNA (10,11), inhibited the reaction set up as described under Fig. 2A, the inhibition being 100% at 0.5 mM, 95% at 0.1 mM, and 60% at 0.01 mM. However, no hydrolysis of fully dsRNA could be detected. As shown in Table I, no hydrolysis was found with reovirus dsRNA. No hydrolysis of poly(U)•poly(A) could be detected using acid-solubilization as an assay (1).

That some double-stranded regions in pre-mRNA and mRNA may be caused by their hybridization with snRNAs has been suggested in recent reports (12–16). It seemed possible that local double-stranded regions were features of the sites of hydrolysis by the endoribonuclease. Addition of the snRNAs U1 and U2 of Novikoff hepatoma cells stimulated the hydrolysis of each [^{3}H]m^{7}Gppp-[^{14}C]RNA-poly(A) preparation as shown in Table I. A concentration curve with

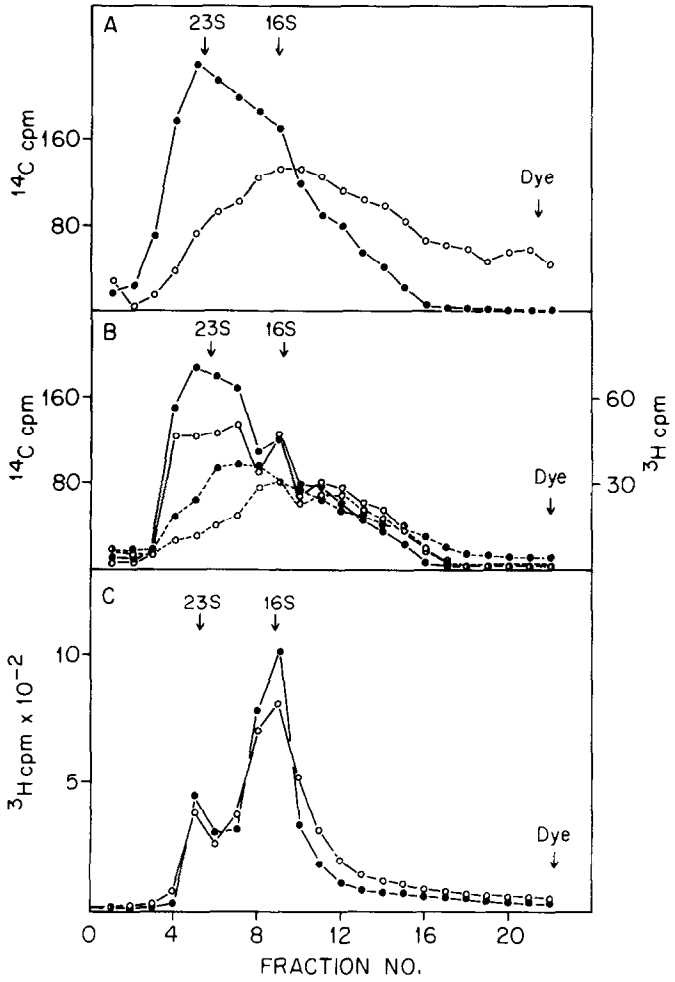

Fig. 2. Gel electrophoretic analysis of RNA following incubation without and with the yeast endonuclease. (A) 3.2 nmol of [^{3}H]m^{7}Gppp[^{14}C]RNA-poly(A) (preparation A) were incubated in a 50 µl reaction mixture with 0.9 µg of enzyme, along with a control minus enzyme, as described under Methods and Materials. After 10 min at 37°C, 0.1 ml of 10 mM Tris-HCl buffer, pH 7.5, containing 10% glycerol and 0.2% SDS was added to each reaction mixture. They were then examined by gel electrophoresis as described under Methods and Materials, and the gels were sliced and counted. ^{14}C label is plotted. ●——●, minus enzyme, ○——○, plus enzyme. (B) The same reaction mixtures as described in (A) were analyzed as described under Methods and Materials by oligo(dT)-cellulose chromatography, except the bound poly(A)-containing RNA was eluted with 0.5 ml of low salt buffer. The RNA was precipitated with 2.5 volumes of ethanol and left at -40°C, overnight. The precipitate was collected by centrifugation and the RNA was examined by electrophoresis as in (A). Both ^{14}C and ^{3}H labels are plotted. ●——●, ^{14}C label minus enzyme; ○——○, ^{3}H label minus enzyme: ●----●, ^{14}C label plus enzyme; ○----○, ^{3}H label plus enzyme. (C) 3.6 nmol of E. coli 16S [^{3}H]rRNA (containing a small amount of 23S rRNA) in reaction mixtures without and with enzyme were examined as in (A). ●——●, ^{3}H cpm minus enzyme, ○——○, ^{3}H cpm plus enzyme.

snRNAs U1 and U2 is given in Table I and a 2.4-fold stimulation is found with the highest amount of snRNA. The snRNA also stimulates slightly the hydrolysis of E. coli rRNA, as shown in Table I. The stimulation by the

TABLE I. - Hydrolysis of Different Polyribonucleotides by the Yeast Endoribonuclease and Its Stimulation by snRNA.

Polyribonucleotide	Addition	% Hydrolysis
(A) Reovirus [^{3}H]RNA, 1.5 nmoles (3000 cpm)	None	<1
E. coli [^{3}H]rRNA, 1.8 nmoles	None	10
	snRNAs U1 and U2, 1 µl	15
Yeast [^{32}P]pre-tRNA, 2.5 × 10^4 cpm	None	<5
(B) [^{3}H]m^{7}Gppp[^{14}C]RNA-poly(A), 1.8 nmoles (Preparation A)	None	29
	snRNAs U1 and U2, 1 µl	38
[^{3}H]m^{7}Gppp[^{14}C]RNA-poly(A), 1.8 nmoles (Preparation B)	None	15
	snRNA U1, 1 µl	25
	snRNA U2, 1 µl	22
	snRNAs U1 and U2, 1 µl	37
	E. coli 5S rRNA, 0.1 µg	13
	E. coli tRNA, 0.1 µg	16
[^{3}H]m^{7}Gppp[^{14}C]RNA-poly(A), 1.8 nmoles (Preparation B)	None	15
	snRNAs U1 and U2, 0.25 µl	23
	snRNAs U1 and U2, 0.50 µl	29
	snRNAs U1 and U2, 1.0 µl	35
[^{3}H]m^{7}Gppp[^{14}C]RNA-poly(A), 0.6 nmoles (Preparation B) (Not preincubated)	None	16
	snRNAs U1 and U2, 0.3 µl	14
[^{3}H]m^{7}Gppp[^{14}C]RNA-poly(A), 0.6 nmoles (Preparation B) (Preincubated)	None	14
	snRNAs U1 and U2, 0.3 µl	30

All the reaction mixtures contained the other components and 0.9 µg of enzyme as described under Methods and Materials. The % hydrolysis of reovirus [^{3}H]RNA was measured by gradient centrifugation (10–30% sucrose, 20 mM Tris-HCl, pH 7.5, 0.1 M NH_4Cl) of the reaction mixture along with a control mixture minus enzyme. The % hydrolysis of *E. coli* [^{3}H]rRNA was determined by gel electrophoresis as described in Fig. 2C. Hydrolysis of [^{32}P]pre-tRNA was measured by gel electrophoresis as described by Peebles *et al.* (8). A reaction mixture containing yeast pre-tRNA cleaving enzyme was used to check the electrophoresis profile of the cleaved pre-tRNA and one lacking enzyme served as a control. All the % hydrolysis values with [^{3}H]m^{7}Gppp[^{14}C]RNA-polyA were determined by the oligo(dT)-cellulose assay described under Methods and Materials. In the last two reaction mixtures, the labeled RNA (without and with snRNAs) was first preincubated for 5 min at 37°C in 10 µl with 20 mM Tris-HCl, pH 7.7, 10 mM $MgCl_2$, and 50 mM NH_4Cl.

snRNAs is also inhibited by ethidium bromide. In contrast to the snRNAs, 5S rRNA and tRNA of *E. coli* do not stimulate the endonuclease (Table I).

When the concentrations of both the [^{3}H]m^{7}Gppp[^{14}C]RNA-poly(A) and snRNAs are reduced to ~1/3 of that required for a 2.5-fold stimulation, no stimulation by the snRNAs is observed (Table I). However, when the labeled RNA and snRNAs are preincubated for 5 min (37°C) at five times that concentration in the presence of 50 mM NH_4Cl and 10 mM $MgCl_2$, and then diluted into reaction mixtures plus enzyme, more than a 2-fold stimulation is

found (Table I). The latter results suggest that the stimulation by the snRNAs involves an interaction with the labeled RNA.

Endoribonucleases that hydrolyze dsRNA have been suggested as possible candidates for enzymes necessary for processing of mRNA precursors (10,11). The enzyme described here may be involved in such processing. That it is stimulated by snRNA suggests that short double-stranded regions may be the sites of its hydrolysis.

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