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# EVIDENCE FOR A 5' → 3' DIRECTION OF HYDROLYSIS BY A 5' MONONUCLEOTIDE-PRODUCING EXORIBONUCLEASE FROM SACCHAROMYCES CEREVISIAE

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SUMMARY: [$^{3}$H]rRNA labeled at the 5' terminus with $^{32}$P and [$^{3}$H]rRNA labeled at the 3' end with [$^{14}$C] $(pA)_n$ have been degraded at 0° with a highly purified exoribonuclease from Saccharomyces cerevisiae. The results show that with the [$^{32}$P, $^{3}$H] substrate, the $^{32}$P label is rendered acid-soluble at a much faster rate than the $^{3}$H label. Both acid-soluble labels are found in 5' mononucleotide. With the [$^{14}$C, $^{3}$H]rRNA, the $^{3}$H label is hydrolyzed at a faster rate than the $^{14}$C label. The exoribonuclease hydrolyzes in the 5' → 3' direction.

Both transcription and translation of mRNA occur in a 5' → 3' direction, but the mode of mRNA degradation remains uncertain. No ribonuclease has been firmly established to hydrolyze from the 5' terminus with the production of 5' mononucleotides (readily utilizable products) so that the direction of mRNA degradation could be similar to that of transcription and translation. However, evidence suggestive of such a ribonuclease has been reported recently in certain eukaryotic systems. Furuichi et al. (1) reported that reovirus mRNAs with 5'-terminal $m^{7}GpppG^{m}$ or GpppG are more stable than mRNAs containing unblocked ppG at the 5' end when injected into Xenopus laevis oocytes or incubated with extracts of wheat germ and mouse L cells. Shimotohno et al. (2) showed that 5' terminally capped mRNAs are considerably more stable to degradation by wheat germ extracts than those with the cap structure removed. Both groups reported analyses of products of the hydrolyses which suggested the presence of 5' mononucleotide-producing exoribonuclease activity. Shimotohno and Miura (3)

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have extended these studies and briefly described a 20-fold purified enzyme fraction from the supernatant fraction of wheat germ extracts. The enzyme formed 5' mononucleotides and failed to hydrolyze 5' capped mRNA and mRNA lacking a 5' phosphate group. Furthermore, the authors stated that the enzyme degraded terminally labeled RNA in a manner that showed a 5' → 3' direction of hydrolysis. However, no data were presented on this important point.

A report (4) from my laboratory provided evidence that a 200-fold purified exoribonuclease from Saccharomyces cerevisiae might degrade from the 5' terminus with the formation of 5' mononucleotides; both 5' capped mRNA and poly(A) lacking a 5' phosphate group were hydrolyzed at a much slower rate than poly(A) with a 5' phosphate group. The present communication reports studies with RNAs having well-defined, labeled end groups that definitely confirm the 5' → 3' mode of hydrolysis.

## METHODS AND MATERIALS

The exoribonuclease was purified from the high-salt wash of ribosomes of S. cerevisiae by DEAE-cellulose and hydroxyapatite chromatography. The details of the procedure will be reported elsewhere (Stevens, unpublished). A brief account of the properties of the 200-fold purified enzyme fraction has been published (4). When the assay described previously (4) is used, with 0.15 μmol of [$^3$H]poly(A) as substrate, 1 μg of the enzyme would hydrolyze 72% of the poly(A) in 30 min at 37°.

[$^3$H]rRNA was prepared from E. coli grown in 100 ml of M9 medium (5) and labeled with 1 mg (0.5 mCi) of [$^3$H]uridine (Schwarz/Mann) during the logarithmic phase of growth. The cells were collected and washed with 20 ml of 10 mM Tris buffer (pH 7.5) containing 5 mM $MgCl_2$ and 10 mM NaCl. They were then suspended in 3 ml of the same buffer and frozen and thawed 3 times after the addition of 1 mg of lysozyme and 40 μg of DNase (Worthington Biochemical Corp.). Sodium dodecyl sulfate was added to a concentration of 0.05%, and after the suspension cleared it was extracted 3 times using equal volumes of phenol. NaCl was added to a concentration of 1 M, and then 3 volumes of ethanol were added. After sitting in ice for 1 hr, the precipitate was collected by centrifugation and dissolved in 0.6 ml of 10 mM Na acetate buffer (pH 5.2) containing 100 mM NaCl. Three 0.2-ml aliquots were layered on 5–20% sucrose gradients containing the same buffer and centrifuged for 18 hr at 21,000 rpm using a Spinco SW 39L rotor. Fractions containing the 23S rRNA and the 16S rRNA were pooled separately. Each was dialyzed against 3 mM Na acetate buffer (pH 5.2) and lyophilized to a volume of 1 ml.

For the preparation of [$^3$H]rRNA labeled at the 5' end with $^{32}$P, the $^3$H-labeled 23S rRNA was first treated with alkaline phosphatase to remove the 5'-terminal phosphate. The reaction mixture contained 3 μmol of [$^3$H]rRNA, 150 mM Tris buffer (pH 8.0), 1 mM $MgCl_2$, and 40 μg of alkaline phosphatase (Worthington Biochemical Corp.) in a volume of

1.5 ml and was incubated for 1 hr at 37°. It was extracted twice with phenol and dialyzed for 18 hr against 5 mM Na acetate buffer (pH 5.2). It was then treated with polynucleotide kinase and [$\gamma$ - $^{32}$P]ATP to label the 5' phosphate termini. The reaction mixture contained 1 µmol of [$^{3}$H]rRNA, 10 mM dithiothreitol, 50 mM Tris buffer (pH 7.5), 2 mM $MgCl_2$, 75 µM ATP, 0.1 mCi of [$\gamma$- $^{32}$P]ATP (Amersham), and 10 units of polynucleotide kinase (P-L Biochemicals) in a final volume of 1.3 ml. After 1 hr at 37°, the reaction mixture was applied to a 1.5 cm x 81 cm column of Bio-Gel A-5m equilibrated with 10 mM Na acetate buffer (pH 5.2) containing 100 mM NaCl. The column was eluted with the same buffer. Fractions of 2 ml were collected, and aliquots were counted. The chromatography showed that some degradation of the RNA had taken place during the dephosphorylation and $^{32}$P-labeling procedures. The excluded fraction from the column was rechromatographed on the Bio-Gel A-5m column as described above. Fig. 1 shows the elution pattern. The ratio of $^{32}$P to $^{3}$H label in the excluded fraction suggested an average molecular weight of 500,000. This was verified by centrifugation of the RNA in a sucrose gradient as described above. The major portion of the radioactivity sedimented the same as 16S rRNA used as a control. No labeled RNA smaller than molecular weight 250,000 was found. The excluded fraction (volume, 44–52 ml) from the column, shown in Fig. 1, was lyophilized to a volume of 1 ml and dialyzed against 3 mM Na acetate buffer (pH 5.2) for 5 hr. It was then used in the assays described below.

The 16S rRNA from the first gradient, as described above, was used for the preparation of [$^{3}$H]rRNA labeled at the 3' termini with [$^{14}$C] $(pA)_n$. Partially purified poly(A) polymerase was prepared from E. coli as described by Hardy and Kurland (6). Their studies of the enzyme showed that the major product of the enzyme activity was a short chain of poly(A) attached to the 3' terminus of a primer RNA (rRNA or tRNA). Reaction mixtures for the incorporation of [$^{14}$C]AMP contained, in a volume of 1 ml, 0.65 µmol of $^{3}$H-labeled 16S rRNA, 0.5 mM [$^{14}$C]ATP (Amersham) (79 x $10^6$ cpm), 10 mM Tris buffer (pH 8.0), 10 mM $MgSO_4$, 2 mM $MnCl_2$, and 20 µg of poly(A) polymerase. After 30 min at 30°, sodium dodecyl sulfate was added, to a concentration of 0.05%, and the mixture was extracted twice with an equal volume of phenol. Residual phenol was removed by extraction with ether, and the labeled RNA was applied to a Bio-Gel A-5m column as described above. About 50% of the label was found in the excluded fraction, which was then lyophilized to a 1-ml volume and dialyzed for 4 hr against 3 mM Na acetate buffer (pH 5.2). It was then used in the assays described below. Treatment of the labeled RNA with snake venom phosphodiesterase (Worthington Biochemical Corp.) showed that 16, 30, and 95% of the $^{14}$C label was rendered acid-soluble when 6, 12, and 40% of the $^{3}$H label was acid-soluble.

Protein was determined by UV absorbance at 280 nm. The concentrations of polyribonucleotide are expressed as µmol of nucleotide.

## RESULTS AND DISCUSSION

For determination of whether the exoribonuclease from S. cerevisiae attacked an RNA molecule at the 5' or 3' terminus, rRNA molecules bearing specific labels at these termini were used. Ribosomal RNA was used as a labeled substrate because it is hydrolyzed at nearly the same rate as poly(A) by the exoribonuclease (4). It has also been shown (4) that the exoribonuclease hydrolyzed [$^{3}$H] $(pA)_3$ [$^{14}$C] $(pA)_n$ with the simultaneous

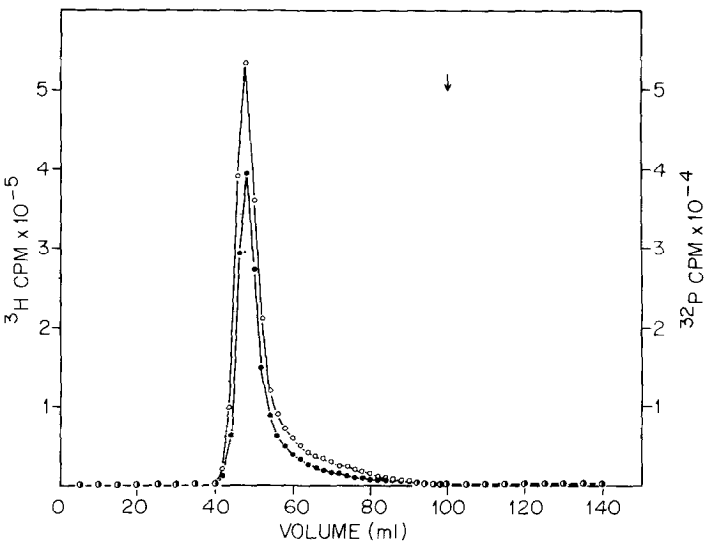


Fig. 1. Chromatography of [$^3$H]rRNA containing 5' terminal $^{32}$P on a Bio-Gel A-5m column. 0.5 μmol of [$^{32}$P, $^3$H]rRNA was applied to a 1.5 x 81 cm column of Bio-Gel A-5m equilibrated with 10 mM sodium acetate buffer (pH 5.2) containing 100 mM NaCl. The column was eluted with 140 ml of the same buffer. Fractions of 2 ml were collected, and 10-μl aliquots were counted with aqueous scintillation fluid {4 g of 2,5-bis[2-(5-tert-butyl)benzoxazolyl)]thiophene per liter of solvent containing toluene:Methyl Cellosolve, 2:1} in a Packard scintillation counter. The arrow marks the peak fraction where tRNA comes off the column. ●—●, $^3$H; ○—○, $^{32}$P.

release of both labels. The following conditions were employed to show a differential release of terminal label: a larger substrate RNA was used, the reactions were carried out with a large amount of enzyme to saturate as many ends as possible, and the reactions were carried out at 0° to slow the rate of hydrolysis.

The degradation of [$^3$H]rRNA labeled at the 5' terminus with $^{32}$P is shown in Fig. 2. At 0° (Fig. 2A), with a large amount of enzyme, the $^{32}$P label is rendered acid-soluble at a much faster rate than the $^3$H label. At 1 min, the ratio of acid-soluble $^{32}$P to acid-soluble $^3$H is 18. The reaction was also carried out at 37° with one-fifth the amount of enzyme to show the hydrolysis of a larger amount of $^3$H label. The solubilization of $^{32}$P is still slightly faster than that of the $^3$H (Fig. 2B).

The products of the hydrolysis of the [$^{32}$P, $^3$H]RNA at 0° were examined in two ways. First, the acid-soluble fractions from reaction mixtures incubated for 30 sec and

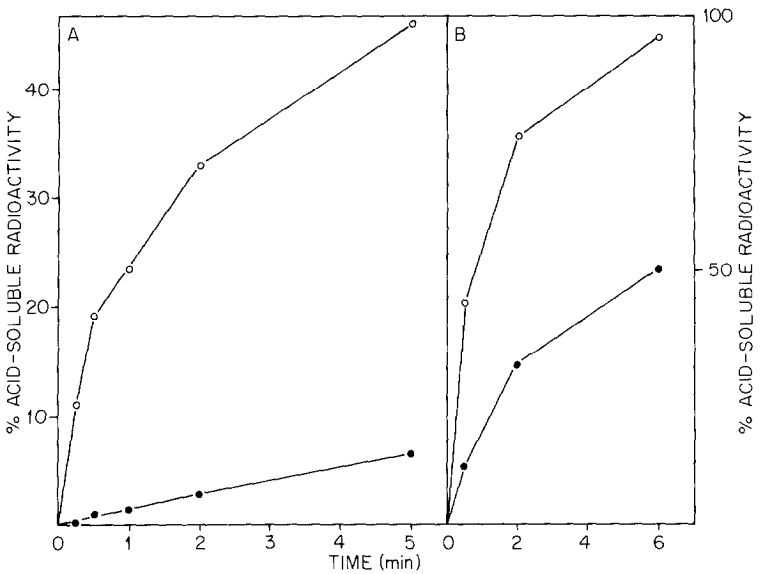


Fig. 2. Degradation of [$^3$H]rRNA containing 5' terminal $^{32}$P with the exoribonuclease from S. cerevisiae at 0° (A) and 37° (B). Reaction mixtures contained 7 nmol of [$^{32}$P, $^3$H]rRNA (31,700 cpm of $^3$H and 3850 cpm of $^{32}$P), 27 mM sodium glycinate buffer (pH 9.5), 0.2 mM $MgCl_2$, 5 mM $NH_4Cl$, 5 mM KCl, 50 µg bovine serum albumin and enzyme in a final volume of 0.12 ml. 5.3 µg of enzyme were used at 0° (A) and 1.1 µg of enzyme at 37° (B). Mixtures containing buffer instead of enzyme were used as controls. The reactions were stopped by the addition of 0.1 ml of 7% $HClO_4$ and 100 µg of bovine serum albumin. After sitting in ice for 10 min, the acid-insoluble material was sedimented by centrifugation for 10 min at 3000 x g. 100 µl of the supernatant solution were counted with aqueous scintillation fluid (described under Fig. 1) in a Packard scintillation counter. ●—●, $^3$H; ○—○, $^{32}$P.

5 min were subjected to descending paper chromatography with isopropanol:$NH_4OH$:0.1 M boric acid (60:10:30) as the solvent. This solvent separates $P_i$ from the 5' mononucleotides, besides separating the latter from 3' mononucleotides (4). All the $^{32}$P and $^3$H label in the acid-soluble fraction was found in the 5' mononucleotides. Second, the products of a 10-min hydrolysis were chromatographed on a Bio-Gel A-5m column similar to that used in Fig. 1. The results of the chromatography are shown in Fig. 3. No accumulation of $^{32}$P-labeled small polynucleotides occurs, the hydrolysis of which might explain the faster release of $^{32}$P label. There is some $^3$H label in small polyribonucleotides because some of the RNA chains are partially degraded.

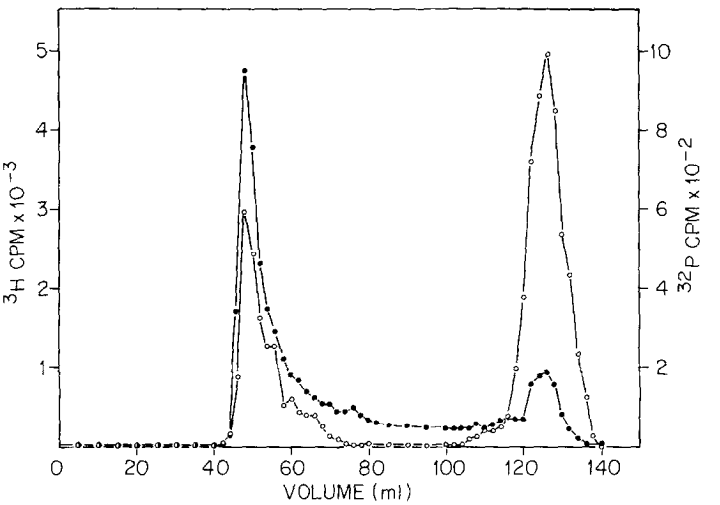


Fig. 3. Chromatography of the product of the reaction of [$^3$H]rRNA containing 5' terminal $^{32}$P with the exoribonuclease on a Bio-Gel A-5m column. A 10-min reaction was carried out as described under Fig. 2. The reaction was stopped by the addition of sodium dodecyl sulfate to 0.05%. The mixture was applied to a column of Bio-Gel A-5m and eluted as described under Fig. 1. 0.2-ml aliquots of the fractions were counted as described under Fig. 1. ●——●, $^3$H; ○——○, $^{32}$P.

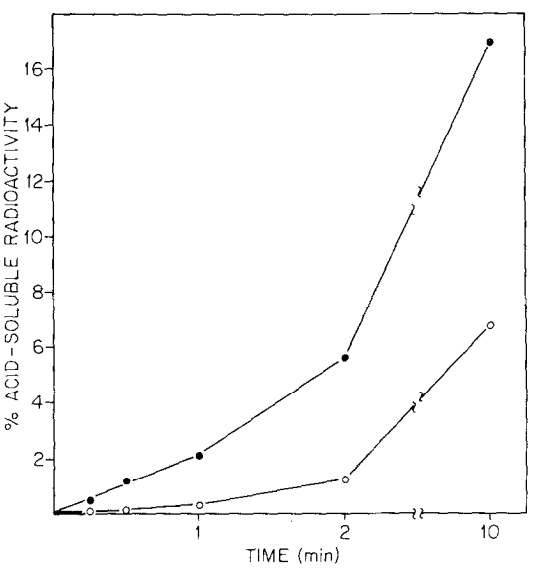


Fig. 4. Degradation of [$^3$H]rRNA, labeled at the 3' terminus with [$^{14}$C] $(pA)_n$, with the exoribonuclease at 0°. Reaction mixtures contained 4.1 nmol of [$^{14}$C, $^3$H]rRNA (7680 cpm of $^3$H and 12,800 cpm of $^{14}$C) and were set up and assayed as described for the 0° reactions under Fig. 2. ●——●, $^3$H; ○——○, $^{14}$C.

The degradation of [$^{3}$H]rRNA labeled at the 3' terminus with [$^{14}$C] $(pA)_n$ by a large amount of enzyme at 0° is shown in Fig. 4. In this case, the 3'-terminal $^{14}$C label is rendered acid-soluble at a slower rate than that of the total $^{3}$H label.

The pattern of degradation of the two terminally labeled RNAs demonstrates that the initial site of attack by the yeast ribonuclease is at the 5' terminus. Such an enzyme could be very important in the degradation of mRNA in eukaryotic systems. The investigations of Furuichi et al. (1) showing that capped mRNA is protected from hydrolysis in diverse systems suggest the possible widespread occurrence of such ribonucleases. Enzymes that remove 5' caps, and ribonucleases, as described here, may be very important in determining the functional lifetime of different mRNAs.

REFERENCES

1. Furuichi, Y., LaFiandra, A., and Shatkin, A. J. (1977) Nature 266, 235–239.

2. Shimotohno, K., Kodama, Y., Hashimoto, J., and Miura, K. (1977) Proc. Natl. Acad. Sci. USA 74, 2734–2738.

3. Shimotohno, K., and Miura, K. (1977) Proceedings of the 1977 Molecular Biology Meeting of Japan, pp. 83–85, Kyoritsu Shuppan Co., Ltd., Tokyo.

4. Stevens, A. (1978) Biochem. Biophys. Res. Commun. 81, 656–661.

5. Adams, M. H. (1959) Bacteriophages, p. 446, Interscience Publishers, Inc., New York.

6. Hardy, S. J. S., and Kurland, C. G. (1966) Biochemistry 5, 3668–3676.