# SYNTHETIC POLYNUCLEOTIDES AS MODEL SUBSTRATES FOR RIBOSOMAL RNA PROCESSING

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Received October 16, 1973

Summary: A nuclear exoribonuclease from Novikoff ascites cells was used to study the hydrolysis of single-stranded heteropolymers containing [$^{14}$C]adenylic acid and either uridylic acid or cytidylic acid and heteropolymers of [$^{14}$C]adenylic acid and one of the corresponding 2'-O-methylated nucleotides. The results of these studies indicate that both the rate and extent of hydrolysis are greatly inhibited by the presence of 2'-O-methylated nucleotides. Restriction of exonuclease activity by 2'-O-methylated nucleotides provides a possible mechanism for rRNA processing.

The ribosomal RNA of eukaryotic cells is synthesized in the nucleolus as a large (45S) precursor molecule which is subsequently cleaved through a series of intermediate steps to form the mature 28S and 18S rRNA molecules. The 45S precursor is modified at the time of synthesis by methylation at the 2'-OH of the ribose moiety at specific sites within the polynucleotide (1). During processing nearly one half of the nucleotides originally present in the 45S precursor molecule are lost, but the 2'-O-methylated regions of the molecule are conserved (2). Recently Perry and Kelley (3) have studied an exoribonuclease isolated from nucleoli which is believed to function in rRNA processing (4). This exoribonuclease is probably identical to an exoribonuclease which was isolated from whole nuclei by Lazarus and Sporn (5). Both nucleases require $Mg^{++}$ and produce 5'-mononucleotides from hydrolysis of RNA from the 3'-OH end. Further, Perry and Kelley (3) have shown that this nuclear exoribonuclease hydrolyzed 45S precursor molecules many times faster than either 28S or 18S mature rRNA. The decline in rate of hydrolysis of mature rRNA was postulated to be due to either secondary structure or post-transcriptional modification of the substrate, perhaps 2'-O-methylation.

Synthetic copolymers, which can be characterized both chemically and physically, are ideal model substrates for studying the restriction of nucleases potentially involved in rRNA processing. In this communication we report the results of studies with an isolated nuclear exoribonuclease on rates of hydrolysis of sets of co-

polymers which differ only in the presence or absence of 2'-O-methyl groups.

## Materials and Methods

The 3'-OH specific nuclear exoribonuclease was isolated from Novikoff ascites cells by the procedure outlined by Lazarus and Sporn (5). It was determined that rapid isolation of very high yields of endonuclease-free 3'-OH specific exoribonucleas could be obtained by utilizing an abbreviated method suggested in the same publication. The cells were washed and lysed with Triton N-101. Isolated nuclei were extracted at pH 6.4 and 8.0 and the enzymatically active protein purified through two ammonium sulfate precipitations. The endonucleolytic activity was then separated from the exoribonuclease using a DEAE-cellulose column. Enzyme preparations used in this study had specific activities of 800 to 1,000 units enzyme/mg protein. One unit of enzyme, as defined by Lazarus and Sporn (5), is that amount which liberates 1 μmole of AMP from poly(A)/hr at 37°.

The radioactive heteropolymers utilized in these experiments were synthesized using bacterial polynucleotide phosphorylase as described previously (6). The reaction mixtures were designed to produce polyadenylic acid[poly(A)] with random incorporation of relatively low levels of cytidylic acid[poly(A,C)], or uridylic acid[poly(A,U)] and poly(A) with low levels of 2'-O-methylcytidylic acid[poly(A,Cm)], 2'-O-methyluridylic acid[poly(A,Um)], or 2'-O-methyladenylic acid[poly(A,Am)]. Each reaction contained [$^{14}$C]ADP (25 μCi/mmole) and was made 40% in dimethylsulfoxide to enhance random incorporation of nucleotides (6,7). Before use each polymer was checked for protein contamination by the method of Lowry (8) and monomer contamination by descending paper chromatography in n-propanol-$NH_4OH$-water (55: 10: 35). The size range of the polymer preparations was determined by centrifugation through sucrose gradients (pH 9.0) using Escherichia coli 4S tRNA as an internal marker. The base composition of each polymer was determined by high pressure liquid chromatography after complete hydrolysis to nucleosides (9). The 2'-O-methyl content of poly(A,Am) was determined by quantitation of methanol released from 2'-methoxy groups by perchloric acid hydrolysis as described previously (10). Base ratios of the other 2'-O-methylated polymers were verified by this technique.

Table 1: Properties of heteropolymers used in hydrolysis studies.

| Polynucleotide | Composition | Size |
|---|---|---|
| (A) | 100% A | 4.3-7.2S |
| (A) | 100% A | 3.6-5.8S |
| (A,C) | 80% A<br>20% C | 3.4-3.8S |
| (A,U) | 80% A<br>20% U | 3.0-5.7S |
| (A,Am) | 84% A<br>16% Am | 4.2-7.6S |
| (A,Cm) | 82% A<br>18% Cm | 3.8-6.7S |
| (A,Um) | 72% A<br>28% Um | 3.0-4.6S |

A modification of the exonuclease assay suggested by Sporn (5) was employed. A typical reaction mixture contained: Tris-HCl, 0.1 M (pH 7.4); $MgCl_2$, 0.004 M; potassium phosphate, 0.025 M (pH 7.4); dithiothreitol, 0.3 mM; bovine serum albumin, 100 µg; nuclear exoribonuclease, 8 units; substrate, 1 µmole (nucleotide); and water to make 120 µl. The reaction was incubated at 37° and 15 µl samples were withdrawn at selected time intervals. Each sample was added to 60 µl (0.36 OD) yeast tRNA in a 1 ml conical centrifuge tube kept at 4°. When sampling was complete, 80 µl of ice cold 0.8 M perchloric acid was added to each tube with vigorous mixing. After centrifugation for 30 min at 24,000 x g, the entire supernatant was withdrawn and counted by liquid scintillation.

## Results

Characterization of nuclear exoribonuclease. The enzyme preparations were shown to be free of nuclear endoribonuclease activity by monitoring the change in substrate size during the course of hydrolysis. Figure 1 shows sucrose density gradient profiles of 4-7S [$^{14}$C]poly(A) after hydrolysis for various periods of time. The main polymer peak did not shift significantly even after 120 min incubation when

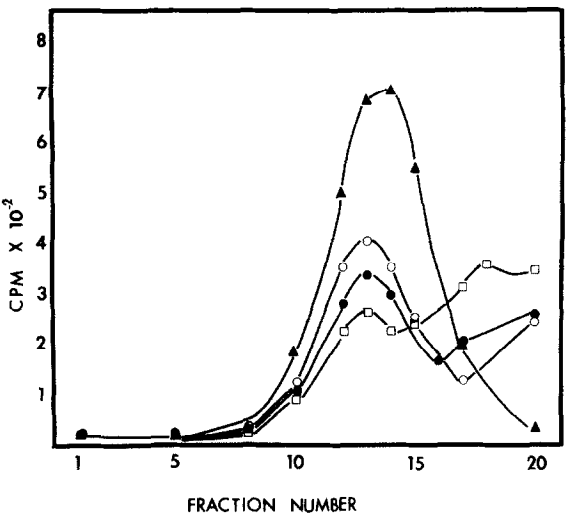


Figure 1: Sucrose density profiles of [$^{14}$C]-poly(A) after incubation with nuclear exoribonuclease for 0 min, ▲; 30 min, ○; 60 min, ●; and 120 min, □. Fractions were collected directly in scintillation vials containing 10 ml Triton-X-114-xylene counting fluid and counted by liquid scintillation.

over 30% of the polymer had been hydrolyzed. The products of hydrolysis were characterized by descending paper chromatography. All of the UV absorbing material either remained at the origin or moved to a position corresponding to 5'-AMP. The distribution of radioactivity on the chromatograms confirmed 5'-AMP as the exclusive product of [$^{14}$C]poly(A) degradation. It was concluded that the enzyme preparations were free of detectable endoribonuclease activity, since the presence of endonucleolytic activity would have resulted in rapid broadening of the main polymer peak and the appearance of oligonucleotides on the chromatograms.

Saturating levels of enzyme for poly(A) hydrolysis were determined to be 8 units enzyme per μmole (nucleotide) poly(A). This ratio of enzyme to polymer was kept constant in all reaction mixtures. Doubling the amount of enzyme per reaction mixture did not affect the rate of hydrolysis of any of the polymers.

Heteropolymer characterization. The properties and percentage composition of the heteropolymers employed are shown in Table 1. All polymer preparations were free of protein and monomer contamination and were of approximately the same size. The

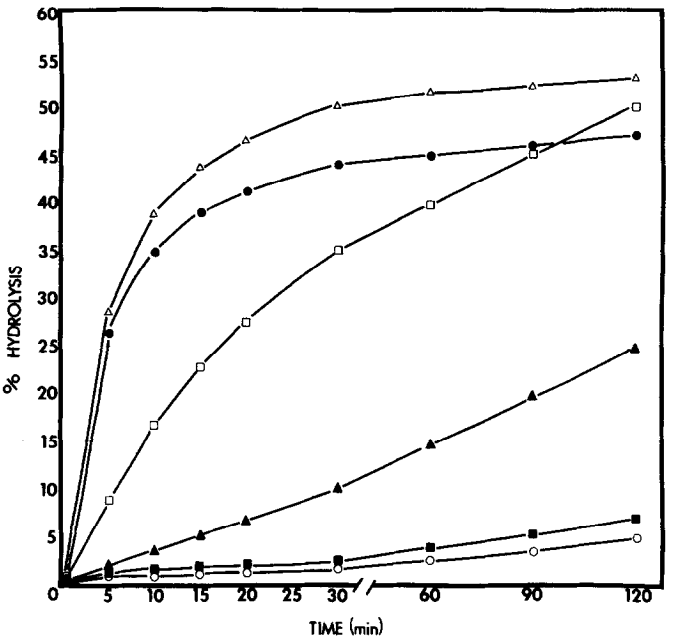


Figure 2: The time course hydrolysis of poly(A,C), △ ; poly(A,U), ● ; poly(A), □; poly(A,Am), ▲; poly(A,Um), ■ and poly(A,Cm), ○, by 3'-OH specific nuclear exoribonuclease. The ratio of enzyme to substrate was kept constant at 8 units enzyme/μmole (nucleotide) substrate.

heteropolymers were 70 to 80% adenylic acid and contained 20 to 30% cytidylic acid, uridylic acid, or one of the analogous 2'-O-methylated nucleotides.

Heteropolymer hydrolysis. Figure 2 shows comparative rates of hydrolysis of the heteropolymers. It can be readily seen that insertion of 2'-O-methylated nucleotides into the heteropolymer greatly reduced the rate and extent of the hydrolysis. Initial velocities for the hydrolysis of each polymer are shown in Table 2. Poly(A,C) was hydrolyzed 65 times faster than poly(A,Cm) and poly(A,U) was degraded 60 times faster than poly(A,Um). Poly(A), however, was hydrolyzed only 7 times more rapidly than poly(A,Am). In separate studies (data not shown) heteropolymers containing far lower levels of methylated nucleotides (8%) were equally restrictive to hydrolysis by 3'-OH specific nuclear exoribonuclease.

## Discussion

The insertion of 2'-O-methylated nucleotides into the polynucleotide chain

greatly affected the rate of hydrolysis by 3'-OH specific nuclear exoribonuclease. The most dramatic inhibition was observed when (Cm) and (Um) were incorporated into the polynucleotide. Previous kinetic characterization of this exoribonuclease has shown that poly(U) and poly(C) are degraded at 12% and 4% respectively, of the rate for poly(A) (5). Poly(A,C) and poly(A,U), however, were more rapidly hydrolyzed than was poly(A). The slower degradation of poly(A) may be due to its significant secondary structure at neutral pH (11).

Complete hydrolysis was not observed with any of the heteropolymers. When fresh enzyme was added to the incubation mixtures after 120 min, no significant increase in hydrolysis was observed. Degradation of polynucleotides by this specific nuclear exoribonuclease, like E. coli RNase II hydrolysis, results in release of 5'-mononucleotides until an oligonucleotide resistant to further hydrolysis is produced (12). Our data suggests that methylation, rather than any effect of substrate size, is responsible for the severely limited hydrolysis of these polymers.

Studies of rRNA processing using natural RNA molecules are limited by the difficulty in isolating large quantities of the 45S precursor. Use of heteropolymers has provided a tool with which to study the effects of 2'-O-methylation, base composition and sequence on the hydrolysis of polynucleotides by enzymes which have been implicated in rRNA processing. The results of Perry and Kelley (3) clearly demonstrated that 45S rRNA precursor was degraded by 3'-OH specific nuclear exoribonuclease but that 18S and 28S rRNA were hydrolyzed only to a very limited extent by this same enzyme. The molecular explanation, however, for this altered activity remained ambiguous. Our results indicate that the presence of 2'-O-methylated nucleotides significantly inhibits the hydrolytic activity of 3'-OH specific nuclear exoribonuclease and may therefore be the mechanism whereby the cell selectively stabalizes rRNA sequences. These results may also explain the observation that post-transcriptional 2'-O-methylation of the 45S precursor molecule is required for normal processing of rRNA (13). These synthetic polynucleotides should be useful model compounds to further probe the role of 2'-O-methyl groups in rRNA processing.

Table 2: Initial velocity of polymer hydrolysis by 3'-OH specific exoribonuclease.

| Polynucleotide | Vo(nmoles AMP/min) |
|---|---|
| (A) | 26.0 |
| (A,Am) | 3.8 |
| (A,C) | 85.0 |
| (A,Cm) | 1.3 |
| (A,U) | 84.0 |
| (A,Um) | 1.4 |

Acknowledgements

We thank Karen Friderici for skilled technical assistance and discussions during the course of this investigation. This work was supported by Public Health Service Research Grant No. CA 13175 from the National Cancer Institute. Michigan Agricultural Experiment Station Article No. 6575.

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