Vol. 96, No. 3, 1980 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
October 16, 1980 Pages 1150-1155

AN mRNA DECAPPING ENZYME FROM RIBOSOMES OF SACCHAROMYCES CEREVISIAE*

Audrey Stevens

Biology Division, Oak Ridge National Laboratory,
Oak Ridge, Tennessee 37830

Received August 26,1980

SUMMARY: By use of [^{3}H]methyl-5'-capped [^{14}C]mRNA from yeast as a substrate, a decapping enzyme activity has been detected in enzyme fractions derived from a high salt wash of ribosomes of Saccharomyces cerevisiae. The product of the decapping reaction is [^{3}H]m^{7}GDP. That the enzyme is not a non-specific pyrophosphatase is suggested by the finding that the diphosphate product, m^{7}GpppA(G), and UDP-glucose are not hydrolyzed.

Nucleotide pyrophosphatase activities which cleave a pyrophosphate bond of m^{7}GpppN and of 5'-capped mRNA have been described in tobacco (1) and potato (2). While the enzyme from tobacco cleaves intact capped mRNA, endonuclease contamination of the enzyme from potato makes its cleavage of intact mRNA uncertain. m^{7}GMP is the product of the decapping reaction with these enzymes. An enzyme from HeLa cells (3, 4) specific for m^{7}G has been shown to hydrolyze m^{7}GpppN and m^{7}G-capped oligonucleotides up to ten in length, but not capped mRNA. Again, m^{7}GMP is the product.

When S. cerevisiae enzyme fractions containing a 5'→3' exoribonuclease (5) were examined for their activity by use of [^{3}H]methyl-5'-capped [^{14}C]mRNA from yeast as a substrate, it was found that some hydrolysis occurred, yielding acid-soluble ^{3}H and ^{14}C. Further investigations showed that the mRNA was decapped by an enzyme which was largely separated from the exoribonuclease on hydroxylapatite chromatography. This report describes the detection of the decapping enzyme and its product, m^{7}GDP.

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*Research sponsored by the Office of Health and Environmental Research, U.S. Department of Energy, under contract W-7405-eng-26 with the Union Carbide Corporation.

†Adenosine (75%) and guanosine (25%) were found by Sripati et al. (6) as the nucleosides linked to the triphosphate-capped region. Our electrophoresis did not give separation of m^{7}GpppA and m^{7}GpppG.

0006-291X/80/191150-06$01.00/0

METHODS AND MATERIALS

[^{3}H]methyl-5'-capped [^{14}C]mRNA was prepared from a 150-ml culture of S. cerevisiae A364A, mutant ts368, essentially as described by Sripati et al. (6). No advantage was taken of the fact that the mutant is unable to convert rRNA precursor to rRNA after extended incubation at 37°. Rather, labeling of spheroplasts was carried out for 10 min at 37° following a 3-min preincubation period at 37°. Labeling was done with 33 μCi of [^{3}H]uridine (specific activity = 58 mCi/mmol) and 1.5 mCi of [methyl-^{3}H]methionine (specific activity = 18 Ci/mmol). Total RNA was isolated as described by Sripati et al. (6). After the first oligo(dT)-cellulose column, the poly(A)-containing RNA was heated at 80° for 2 min following the addition of sodium dodecyl sulfate to 0.2%. It was then applied to a second oligo(dT)-cellulose column (0.5 X 3.0 cm) and eluted and precipitated in the manner described by Sripati et al. (6). About 40 nmol of poly(A)-containing mRNA with 390 cpm of ^{3}H and 1070 cpm of ^{14}C per nmol of nucleotide were obtained.

The poly(A)-containing mRNA thus obtained was characterized in the manner described by Sripati et al. (6). In one reaction mixture, 860 pmol were incubated with Penicillium (P_1) nuclease and Escherichia coli alkaline phosphatase in sequence. In the second, venom nucleotide pyrophosphatase was included with the alkaline phosphatase. The products were examined by paper electrophoresis at pH 3.5 as described below. Ninety percent of the ^{3}H label was found in m^{7}GpppA(G)$^+$ in the first reaction mixture, and the same percentage in m^{7}G in the second mixture. In both cases 10% of the ^{3}H label was found about equally distributed among the four ribonucleoside peaks after electrophoresis. The latter finding suggests that 10% of the ^{3}H label was in contaminating rRNA. About 75% of the ^{14}C label was in uridine and 25% in cytidine in both cases.

The enzyme fractions used in the experiments described below were obtained during the purification of a 5'→3' exoribonuclease from S. cerevisiae S288C (5). One difference in the procedure was that the hydroxylapatite column was first washed with 15 ml of buffer containing 0.5 M potassium phosphate (pH 7.5) before the overnight wash with buffer containing 0.05 M phosphate. (The exoribonuclease eluted from the column several fractions sooner with the high salt-washed column.)

High voltage paper electrophoresis was on Whatman 3 MM paper (20–30 x 50 cm) at 2000 volts for 75 min in pyridine-acetate buffer (pH 3.5).

RNA was determined by UV absorbance at 260 nm at an E_M = 10,000, and its concentration is expressed as micromoles of nucleotide. Protein was determined by UV absorbance at 280 nm.

[^{3}H]m^{7}GpppA(G) was prepared by treatment of 8 nmol of [^{3}H]methyl-5'-capped [^{14}C]mRNA with 50 μg of P_1 nuclease in 0.2 ml of 50 mM sodium acetate (pH 5.4). After 45 min at 37°, 40 μl of 1 M Tris buffer (pH 8.3) and 10 μg of E. coli alkaline phosphatase were added. After 30 min at 37°, the reaction mixture was diluted to 1 ml with 10 mM Tris (pH 7.8) and applied to a 1 x 2 cm column of DEAE-cellulose equilibrated with the Tris buffer. Nucleosides were eluted with 6 ml of 10 mM Tris (pH 7.8), and the [^{3}H]m^{7}GpppA(G) was eluted with 0.3 M NH_4HCO_3. The [^{3}H]m^{7}GpppA(G) fractions were lyophilized to remove the NH_4HCO_3.

m^{7}G and m^{7}GDP were obtained from P. L. Biochemicals, Inc. [^{3}H]UDP-glucose (glucose-1-^{3}H) was obtained from New England Nuclear Corp. Venom nucleotide pyrophosphatase and P_1 nuclease were from Sigma Chemical Company, and E. coli alkaline phosphatase from Worthington. S. cerevisiae A364A, mutant ts368, was a gift from Dr. Jonathan Warner. [Methyl-^{3}H]methionine and [^{14}C]-uridine were from Schwarz/Mann.

RESULTS

Detection of an mRNA Decapping Enzyme Yielding [^{3}H]m^{7}GDP in a Hydroxylapatite Fraction Derived from a High Salt Wash of Ribosomes of S. cerevisiae. By use of [^{3}H]methyl-5'-capped [^{14}C]mRNA of yeast as a substrate, it was found that a hydroxylapatite fraction obtained during the purification of a 5'→3' exoribonuclease from S. cerevisiae (5) degraded the RNA to ^{3}H- and ^{14}C-containing acid-soluble products. Reaction mixtures were examined by paper electrophoresis at pH 3.5 both before and after treatment with E. coli alkaline phosphatase. Figure 1 shows the results of the study. About two-thirds of the ^{3}H label is found in m^7 GDP before alkaline phosphatase treatment (Fig. 1A),

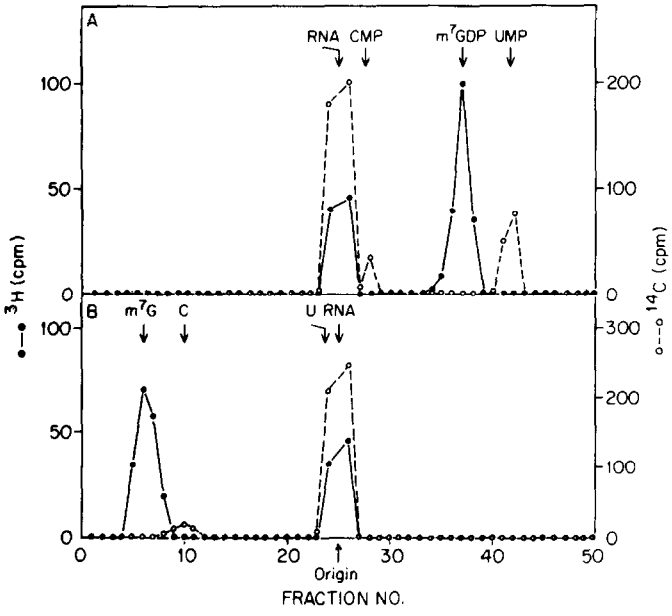

Fig. 1. Hydrolysis of [^{3}H]methyl-5'-capped [^{14}C]mRNA by a hydroxylapatite fraction derived from ribosomes of S. cerevisiae. 1 nmol of labeled mRNA was incubated in each of two reaction mixtures (50 µl) containing 50 mM Tris buffer (pH 8.3), 1 mM $MgCl_2$, 50 mM NH_4Cl, and 0.45 µg of a hydroxylapatite fraction (fraction 16) obtained during the purification of a 5'→3' exoribonuclease from ribosomes of S. cerevisiae (5). Prior to use, the hydroxylapatite fraction (1 ml) was dialyzed for 8 hr at 0° with 500 ml of 20 mM Tris buffer (pH 7.8) containing 10% glycerol, 50 mM NH_4Cl, and 0.2 mM dithiothreitol. After 15 min at 37°, both reaction mixtures were heated for 2 min at 80°. To the second mixture (B), 5 µg of E. coli alkaline phosphatase were added, and the mixture was incubated for 30 min at 37°. Both reaction mixtures were then applied to paper along with marker compounds (0.2 µmol each) and electrophoresed as described in Methods and Materials. The paper was dried, and marker compounds were detected by UV absorbance (UMP, CMP, cytidine, uridine) or UV fluorescence (m^{7}G and m^{7}GDP). The paper was cut into 1-cm strips, which were allowed to stand for 10 min at 50° in 0.5 ml of water. Radioactivity was then determined. The first reaction mixture (no alkaline phosphatase) is shown in (A), the second (alkaline phosphatase present) in (B). (A reaction mixture containing the dialysis buffer rather than enzyme was used as a control; all label was found at the origin.)

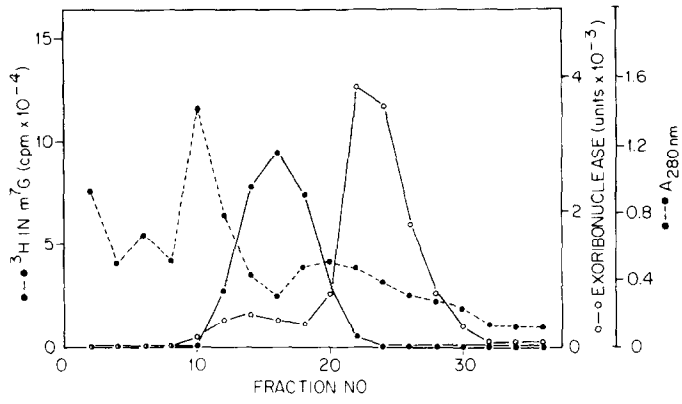

Fig. 2. Hydroxylapatite chromatography and measurement of decapping and exoribonuclease activities. Hydroxylapatite chromatography of a DEAE-cellulose fraction was carried out as described previously (5). All hydroxylapatite fractions were dialyzed as described in Fig. 1. Exoribonuclease activity was measured as described previously (5). Decapping enzyme activity was measured as described for the second reaction mixture (Fig. 1B). Marker m^{7}G was applied to the paper together with the reaction mixtures. After electrophoresis the m^{7}G spots (fluorescent) were cut in two, and each piece was eluted and counted. Activity is expressed as cpm of m^{7}G per total enzyme fraction.

and in m^{7}G after alkaline phosphatase treatment (Fig. 1B). Some ^{14}C label (25%) is found in UMP and CMP before treatment, and in uridine and cytidine after treatment. The degradation of ^{14}C label to UMP and CMP results from the low level of exoribonuclease present in the enzyme fraction.

Products of a reaction similar to that in Fig. 1A also were examined by DEAE-cellulose chromatography in the presence of 7 M urea in a manner described previously (7). All of the ^{3}H label that eluted from the column was found in m^{7}GDP, which elutes just past the peak of GMP. ^{14}C label was found in the mononucleotide peak. That no ^{3}H or ^{14}C label eluted in the higher oligonucleotide region suggests that a capped oligonucleotide is not an intermediate in the reaction.

Purification of the Decapping Enzyme. Other enzyme fractions obtained during the purification of the 5'→3' exoribonuclease as described previously (5) were examined for their decapping activity; the formation of m^{7}G following alkaline phosphatase treatment of reaction mixtures was used as an assay. The results are shown in Table I and Fig. 2. Slightly more than half of the decapping activity was detected in the ribosome fraction of S. cerevisiae extracts, and the activity was purified in the manner described previously for the exoribonuclease (5). As shown in Fig. 2, the decapping activity separated from the major portion of the exoribonuclease on hydroxylapatite chromatography. A minor peak of exoribonuclease activity, identified previously (5), elutes just prior to the peak of decapping activity. The peak decapping activity in fraction

TABLE I. Purification of the mRNA Decapping Activity[a]

Fraction	Volume (ml)	Protein (mg/ml)	Total activity (units x 10^{-3})	Specific activity (units/mg)
Ribosomes	60	68	278	68
High salt wash following dialysis and centrifugation	40	8.5	138	406
DEAE-cellulose after $(NH_4)_2SO_4$ precipitation and dialysis	2.1	21.0	168	3818
Hydroxylapatite, summation of active fractions	18.0	0.24	662	153,000
Hydroxylapatite, peak fraction 16	2.0	0.15	103	343,000

[a]All enzyme fractions were assayed as described in Figs. 1B and 2. Units = cpm of ^3H label converted to $[^3H]m^7G$.

16 from the hydroxylapatite column is purified 5000-fold. There is a fourfold increase in activity upon hydroxylapatite chromatography (Table I). It is possible that inhibitors of the decapping activity are removed.

Evidence that the Decapping Enzyme is Not a Non-specific Pyrophosphatase. That the enzyme described above does not hydrolyze the product, m^7GDP, to m^7GMP means that it does not hydrolyze all pyrophosphate bonds. As a further examination of the enzyme's specificity, $m^7GpppA(G)$ and UDP-glucose were tested as substrates. $m^7GpppA(G)$ (1 pmol, 600 cpm) and UDP-glucose (10 pmol, 15,000 cpm) were incubated under the same conditions as described in Fig. 1, except with 3 times more enzyme. The reaction mixtures were then treated with alkaline phosphatase and examined by electrophoresis for $[^3H]m^7G$ and $[^3H]$glucose. Neither was detected. With venom nucleotide pyrophosphatase (20 μg), 100% hydrolysis of both substrates was obtained. A previously described nucleotide pyrophosphatase from yeast (8) readily hydrolyzes UDP-glucose.

DISCUSSION

The finding of the decapping enzyme described here suggests that its action may be the first step in the degradation of capped mRNA. That the enzyme purifies closely with a 5'→3' exoribonuclease that would readily hydrolyze the mRNA chain following decapping suggests that the hydrolysis may be the second step in mRNA degradation. It will be interesting to see what factors may be involved in the control of these two activities.

It cannot yet be stated that decapping of intact mRNA takes place, although little degradation of RNA can be detected other than that caused by exoribonuclease action following decapping. It is still possible that a capped oligonucleotide is an intermediate and that it is decapped very rapidly. ^{14}C-labeled decapped oligonucleotides would not contain sufficient ^{14}C label to be detected in these studies.

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