The Polyadenylate Polymerases from Yeast*

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SUMMARY

Poly(A) polymerase activity was first detected in yeast extracts, primarily in association with the ribosomal fraction, by TWU AND BREITHAUER in 1971 (TWU, J. S., AND BREITHAUER, R. K. (1971) Biochemistry 10, 1576-1582). This activity has now been separated into three distinct enzymes by chromatography on DEAE-cellulose. Each of the three enzymes can catalyze the incorporation of adenylate residues from ATP into a polyadenylate (poly(A)) tract at the 3' terminus of a primer RNA. Enzyme I elutes at 0.07 M ammonium sulfate from the DEAE-cellulose column, utilizes the mixed polynucleotide poly(A,G,C,U) or ribosomal RNA most efficiently in vitro, and may be responsible in vivo for the initiation of the poly(A) tracts found on yeast messenger RNA. Enzyme II elutes from the column at 0.20 M ammonium sulfate, requires poly(A) itself or an RNA primer containing a 3'-oligo(A) tract, and may be responsible in the nucleus for the elongation of tracts initiated by enzyme I. Enzyme III elutes from the column at 0.56 M ammonium sulfate and is present in low amounts in nuclear extracts. It may be involved in adding poly(A) tracts to messenger RNA in mitochondria. These enzymes also have the intrinsic capacity for the incorporation of cytidylate residues from CTP, which correlates with the finding of cytidylate residues in the poly(A) tracts present in the yeast RNA, which is rapidly labeled in vivo. About 75% of the total poly(A) polymerase activity of yeast is enzyme I, most of which is present in the soluble protein fraction of the whole yeast extract. About 20% of the total poly(A) polymerase is enzyme II, and 1 to 5% is enzyme III.

All three of the yeast poly(A) polymerases require an RNA primer with a free 3'-hydroxyl group, show no requirement for a DNA template, require Mn²⁺ for optimal activity, have pH optima of 8.5, and are inhibited by GTP, CTP, UTP, and native yeast DNA. Polymerases I and II have similar molecular weights by gel filtration.

In 1960, Edmonds and Abrams described a poly(A) polymerase from calf thymus nuclei which catalyzed the synthesis of poly(A) from ATP in a reaction which was not dependent on DNA (1). The significance of this discovery was not appreciated for many years until poly(A) tracts were found (2-5) at the 3' terminus of mRNA and heterogeneous nuclear RNA in various eukaryotic organisms. These tracts are added to the RNA after transcription (6). It is generally considered that the poly(A) polymerases found in eukaryotes (7) are responsible for the addition of these poly(A) tracts. The calf thymus polymerase is localized in the nucleus (1, 7) where it can carry out this function. Distinct poly(A) polymerases also have been detected in mitochondria (8) and in chloroplasts (9). They presumably act to modify the organellar mRNAs. The presence of distinct nuclear and organellar poly(A) polymerases could explain the finding of more than one poly(A) polymerase in whole extracts of certain organisms (7, 10). In yeast, however, when two distinct poly(A) polymerases were discovered, a different explanation for the occurrence of two enzymes was suggested (11). Both enzymes were found in the nuclear extract, and the two enzymes had very different primer requirements. Enzyme I could add a short oligo(A) sequence to RNA of mixed composition and therefore could be responsible for initiation of the poly(A) tract. Enzyme II preferred poly(A) itself, which suggested it could add a long poly(A) tract onto an RNA already containing a short 3'-poly(A) tract. A sequential mechanism involving the two enzymes was proposed which could explain the existence of long poly(A) tracts. Poly(A) tracts about 50 adenylate residues in length have been detected on yeast mRNA by McLaughlin et al. (12).

In an extension of our previous work on yeast poly(A) polymerases (11), a third polymerase now has been separated from the other two, and studies have been made on the comparative properties of all three yeast poly(A) polymerases.

EXPERIMENTAL PROCEDURES

Materials

Poly(A) was obtained from Miles Laboratories. All other synthetic ribopolynucleotides were the gift of Dr. Leon Heppel (Cornell). Yeast ribosomal RNA was extracted from a ribosomal pellet by a phenol-sodium dodecyl sulfate treatment (13). The rRNA was precipitated by 10% NaCl from the RNA isolated from the ribosomes.

Native yeast DNA was obtained by the freeze-thaw method described by Smith and Halvorson (14) from 10 pounds of Anhauser-Busch yeast obtained locally. Calf thymus DNA from Worthington Biochemical Corp. was deproteinized by phenol extraction at room temperature (13). All DNA preparations were used free of detectable protein and RNA. DNA samples were denatured by exposure of native DNA to 0.1 M NaOH for 5 min followed by adjustment of the pH to 7 with HCl and Triton X-100.
All radioactive compounds were the products of Schwarz-Mann. Actinomycin D was from Sigma, and α-amanitin was from Calbiochem. DEAE-cellulose was obtained from Schleicher and Schuell.

Yeast strain S288C (alpha M (α)) from Dr. R. Mortimer, University of California, Berkeley, was grown in YPD medium, which contains 2% glucose, 2% Bacto-peptone (Difco), and 2% Bacto-yeast extract (Difco). In some cases Ardamine Z (Yeast Products, Inc.) was substituted for Bacto-yeast extract.

Methods

Chemical Analyses—DNA concentrations were determined by the diphenylamine reaction as described by Dische (15). RNA concentrations were determined by the ultraviolet absorption of the samples. Protein was determined by the method of Lowry et al. (16). The chain length of labeled oligo(A) tracts on RNA was determined as described by Hoff and Keller (11).

Poly(A) Polymerase Assay—the assay contained the following in a volume of 0.25 ml: 0.5 mM [γ-32P]ATP (4 μCi per μmol), 50 mM Tris-HCl (pH 8.5), 5 mM mercaptoethanol, 1 mM MnCl₂, 1 mg per ml of primer RNA (as indicated), and enzyme. Incubation was for 30 min at 30°C. The labeled product was precipitated with 5% trichloroacetic acid containing 5 mM sodium pyrophosphate, collected on a glass fiber filter membrane (type E, Gelman), washed, dried, and counted in a toluene-based scintillation fluid in a Packard scintillation counter with an efficiency of 40%. A zero-time blank tube was always run, as the blank increases with increasing salt concentration, and varies a great deal with different primer RNAs. A unit of enzyme is defined as the amount of enzyme which catalyzes the incorporation of 1 nmol of AMP (from modified rRNAs) into acid-insoluble material in 30 min.

Preparation of RNAs Containing Unlabeled Oligo(A) Tracts—Modified rRNA was prepared using unlabeled ATP in a reaction volume 10-fold larger than the usual assay. Samples were incubated for 0, 1, 2, or 3 hours. A small aliquot containing labeled ATP was run in parallel in each case to determine the chain length of the labeled oligo(A) tract formed as indicated above. The modified rRNAs were isolated by phenol extraction (15).

RESULTS

Purification of Yeast Poly(A) Polymerases

Twu and Bretthauer reported in 1971 (17) that they isolated a poly(A) polymerase from yeast which could utilize rRNA as primer. They indicated that the best source of enzyme with high specific activity was the high salt wash of the ribosomal fraction, although about 25% of the total enzyme appeared in the soluble protein fraction. We attempted to purify this enzymatic activity from the high salt extract of the ribosomal fraction further, and resolved this activity into three distinct poly(A) polymerases by chromatography on DEAE cellulose. The first of the three enzymes eluted from this column was called enzyme I. Enzyme I was present in 3-fold greater quantity in the soluble protein fraction of the whole yeast extract compared to the ribosomal fraction. Enzymes II and III were found only in the ribosomal fraction. A scheme for the separation and purification of these three enzymes is given in Fig. 1. A preliminary report dealing with enzymes I and II has been published (11).

All solutions used for enzyme purification were prepared in Buffer A (20 mM Tris-HCl (pH 8.0), 0.5 mM EDTA, 0.1 mM dithioerythritol), and all procedures were carried out at 0–4°C unless otherwise stated. All enzymes were stable at 0–4°C prior to DEAE-cellulose chromatography. Enzymes purified beyond the DEAE-cellulose step were not stable at 0–4°C, but were stable indefinitely when stored at −70°C or at −20°C lyophilized. Lyophilization of the enzymes caused only small losses of activity.

Preparation of Crude Yeast Extract and Separation of Bulk of Poly(A) Polymerase I from II and III—The yeast was grown in YPD medium at 30°C (see “Materials”) and harvested at a cell density of about 4 × 10⁶ cells per ml to purify polymerase I, II, or III at a cell density of about 8 × 10⁶ to purify only polymerase I. In a typical preparation, 350 g of cells were harvested, washed three times in Buffer B (Buffer A containing 5 mM MgCl₂), suspended in 350 ml of Buffer B, and passed through a Manton-Gaulin motorized French press at 8,500 p.s.i. to break the cells and most of the cellular organelles. The broken cells were centrifuged in a Sorvall GSA rotor at 10,000 rpm for 10 min. The supernatant fluid was centrifuged in a Beckman 30 rotor at 30,000 rpm for 20 min, and the pellet was discarded. The crude extract was centrifuged in the same rotor at 30,000 rpm for 18 hours to pellet the ribosomes. The ribosomal pellet, which contained most of poly(A) polymerases II and III and a fraction of the total polymerase I, was saved. The ribosome-free supernatant, which contained a majority of polymerase I, also was saved.

Separation of Three Poly(A) Polymerases from Yeast Ribosomal Fraction—The ribosomal pellets were homogenized in Buffer B containing 0.25 M sucrose and 50 mM NH₄Cl and layered over 5 ml of buffer which was identical except that it contained 0.5 M sucrose. This stepwise gradient was centrifuged in a Beckman 50 Ti rotor at 50,000 rpm for 2 hours. The upper layer was discarded. The pellet and bottom layer of the gradient containing the washed ribosomal fraction were homogenized in an equal volume of Buffer B containing 1 mM NH₄Cl (resulting in final concentrations of 0.25 M sucrose and 0.5 M NH₄Cl) and layered into each of two Beckman 50 Ti tubes over 5 ml of Buffer D containing 0.5 M sucrose and 0.5 M NH₄Cl. This stepwise gradient was centrifuged as described before, and the upper layer, the 0.5 M NH₄Cl wash, was retained. In subsequent experiments, substitution of KCl for NH₄Cl often improved the yield of enzyme. The 0.5 M NH₄Cl ribosomal wash was chromatographed on a Sephadex G-150 column (2.8 × 30 cm) equilibrated and eluted with Buffer A. One-milliliter fractions were collected and assayed for poly(A) polymerase activity with 1 mg per ml of poly(A) plus 1 μg per ml of rRNA as primer. The enzyme activity appeared as a single peak with maximal activity at the exclusion volume (Vₐ) of the column. The fractions containing maximal activity were combined and chromatographed on DEAE-cellulose as described in Fig. 2. Three peaks of [γ-32P]AMP incorporation activity appeared and were termed poly(A) polymerase I, II, and III in the order of the increasing molarity of ammonium sulfate required to elute the enzyme. The fractions in each peak were combined, dialyzed against 100 volumes of Buffer A for 4 hours, and then lyophilized. Although enzyme I can be isolated by this procedure, we routinely used for the isolation of enzyme I the procedure described below in which the enzyme is purified from the soluble fraction of the whole yeast extract. Enzyme I isolated by either procedure appeared to have identical properties. In most of the following experiments we used enzyme I isolated from the soluble fraction.

Polymerase II isolated by this procedure could be further purified on Sephadex G-150 as described in the legend of Fig. 3. A single broad peak of enzyme activity was found which eluted with a maximum activity at 1.4 times the Vₐ of the column. The elution position of enzyme II on the gel column apparently was changed as a result of the DEAE-cellulose chromatography; before DEAE-cellulose chromatography the enzyme eluted from the gel column at the Vₐ (see above). This shift suggested that the molecular weight of the DEAE-cellulose-purified enzyme II was lower than that of the crude enzyme. DEAE-cellulose chromatography also removed nucleic acid from the enzyme sample, and the enzyme became primer-dependent. The apparent shift in molecular weight produced by DEAE-cellulose chromatography may be due to removal of natural primer from
Fig. 1. Flow diagram of isolation of yeast poly(A) polymerases.

Fig. 2. Elution of poly(A) polymerases from DEAE-cellulose. A 0.5 M NH₄Cl ribosomal wash had been prepared and passed through a Sephadex G-150 column (see “Purification of Yeast Poly(A) Polymerases” under “Results”). This enzyme preparation (76 ml, 1.1 mg per ml of protein) was passed through a 75-ml DEAE-cellulose column equilibrated with Buffer A (see “Results”). The enzyme was eluted with 100 ml of a gradient of ammonium sulfate from 0 to 1 M in Buffer A. Two-milliliter fractions were collected. Each fraction was assayed as described under “Methods” using a mixture of 0.5 mg per ml of poly(A) and 0.5 mg per ml of rRNA as primer. ---, poly(A) polymerase activity; —-—, molarity of ammonium sulfate determined from conductivity measurements.

Fig. 3. Sephadex G-150 chromatography of poly(A) polymerase I and II. This figure is a composite plot of two separate experiments with the two enzymes. Each enzyme preparation, lyophilized after elution from DEAE-cellulose as in Fig. 2, was dissolved in 0.1 ml H₂O and chromatographed on a Sephadex G-150 column (1.2 x 10 cm) equilibrated and eluted with Buffer A (see “Results”). Fractions of 0.4 ml were collected and assayed with 1 mg per ml of rRNA for poly(A) polymerase I or 1 mg per ml of poly(A) for poly(A) polymerase II. ——, polymerase I; ——, polymerase II; $V_o$, exclusion volume of column determined with blue dextran (Pharmacia); $B_o$, bed volume of column determined from the column dimensions.

The three purified enzymes showed no shift in elution position when each was separately chromatographed a second time on DEAE-cellulose.

Purification of Poly(A) Polymerase I from the Ribosome-free Crude Extract—The ribosome-free crude extract previously described was concentrated from 200 to 10 ml by ultrafiltration through an Amicon XM-50 membrane under nitrogen gas at 25 p.s.i. of pressure. The concentrated sample was chromatographed on a Sephadex G-150 column (2.8 x 30 cm) equilibrated with Buffer A. One-milliliter fractions, eluted with Buffer A, were collected and assayed for poly(A) polymerase activity with 1 mg per ml rRNA plus 1 mg per ml poly(A) as primers. The
enzy~tic activity appeared as a single peak at the exclusion volume (V₀) of the column.

The active fractions from the Seph"ade~x column were combined, and the sample was chromatographed on a DEAE-cellulose column (2 × 20 cm) using a 60-ml gradient from 0 to 1 M ammonium sulfate in Buffer A. Fractions of 1.0 ml were colled and assayed for polyl(A) polymerase activity with 1.0 mg per ml of rRNA as primer. A single peak of enzyme activity eluting at 0.07 M ammonium sulfate was found corre~sponding to Peak 1 in Fig. 2. The combined enzyme fractions were dialyzed against 100 volumes of Buffer A overnight and then lyophilized.

Enzyme I could be purified further by chromatography on Seph"ade~x G-150. Just as with enzyme II, the single peak of enzyme I activ"y was found to elute at a position corresponding to 1.4 times the V₀ of the column (Fig. 3). The purification procedure for enzyme I is summar"zed in Table II.

The enzyme was isolated from 50 g of yeast (see "Results"). Enzyme activity is given as the higher of the two values obtained by assaying (see "Methods") with rRNA (1 mg per ml) as primer or no added primer.

| Enzyme fraction | Enzyme concentration | Volume | Protein | Total enzyme | Specific activity | Purification |
|-----------------|----------------------|--------|---------|--------------|------------------|--------------|
|                 | units/mg             | ml     | mg/ml   | units/μg     | units/mg         |             |
| Crude extract   | 0.86                 | 357    | 58      | 307          | 0.014            | 6            |
| Ribosome-free crude extract | 0.92 | 206 | 70 | 190 | 0.13 | 1 |
| First Seph"ade~x G-150 peak | 20.6 | 6 | 70 | 250 | 0.34 | 2.0 |
| DEAE-cellulose peak | 4.0 | 37 | 5.8 | 148 | 0.68 | 5.3 |
| Second Seph"ade~x G-150 peak | 150 | 1 | 65 | 150 | 2.3 | 18 |
|                 | 10                  | 1.3    | 1.3     | 150          | 12               | 102          |

* The values obtained on the crude extract are not considered in estimating purification, since they include polyl(A) polymerases II and III as well as I and are decreased by inhibitors in the crude extract.

Polymerase I was stimulated 10-fold by the addition of 1 mg per ml of rRNA after the first Seph"ade~x G 150 chromatography and developed an absolute primer dependence after purification on DEAE-cellulose. The crude extracts were maximally active with no additional primer.

**Determination of Contaminating Activities in Poly(A) Polymerase Preparations**—The DEAE-cellulose-purified enzymes I and II were tested for ribonuclease contamination by incubating each at the level of 100 μg per ml of protein with yeast tRNA at pH 7.0 in the presence of 2.5 mM MgCl₂. No hyperchromicity of tRNA developed during 1 hour at 30°. When polymcrases I and II were tested for contamination by DNA-dependent RNA polymerase (18), the incorporation of [α-32P]ATP was indistinguishable from that in the nonenzymatic control. Enzymes I and II also were tested for contamination by RNA nucleotidyl-transferase (EC 2.7.7.25) by incubating each with [γ-32P]ATP and commercial baker's yeast tRNA which lacks the terminal adenine (10). The value obtained for the incorporation of [γ-32P]ATP was indistinguishable from the nonenzymatic control value. This was true regardless of whether Mg²⁺ or Mn²⁺ was present in the assay. tRNA nucleotidyl-trasferase should be absent from the DEAE-cellulose peaks of polyl(A) polymerase, since this enzyme from yeast does not bind to DEAE-cellulose even at low salt concentrations (20).

**Primer Requirements of Yeast Poly(A) Polymerases**

Crude yeast extracts usually did not require added primer for maximal polyl(A) polymerase activity; often added primer was inhibitory. Primer RNA always was required after DEAE-cellulose chromatography. RNAs treated with periodate to oxidize the 3'-hydroxyl group under conditions minimizing side reactions (21) were rendered completely inactive with all three enzymes. This demonstrated that all three polylases act by adding adenylic residues to the 3'-hydroxyl end of the primer. Each enzyme also was tested under conditions in which an average of less than one labeled A residue was incorporated per molecule of primer. The label after alkaline hydrolysis (11) was over 90% adenosine and less than 10% adenylic in each case.

As reported by the authors previously (11), there is a striking difference between the primers which can be used by polymerase I and II. Polymerase I prefers a polynucleotide with mixed base composition, such as poly(A,G,C,U) or tRNA. This enzyme is less active with poly(G), poly(A), and poly(C), with decreasing activity in that order. Its activity with poly(U) is very low, less than 2% of that with poly(A,G,C,U). Polymerase II has significant activity only with poly(A). The primer specificity of polymerase III is very similar to polymerase I.

**Priming Ability of RNA with 3'-Oligo(A) Tracts Added**—As the previous report indicated (11), both the kinetics of polymerization and the primer preferences of polymerases I and II sugges~ted that the two enzymes are involved in a sequential mechanism for natural polyl(A) formation. Polymerase I could initiate on heteropolymer RNAs but formed only short polyl(A) se~quences before the reaction rate slowed down. Since polymerase II could utilize polyl(A) as a primer, it might be able to utilize the product of polymerase I as a primer and thereby extend the length of the polyl(A) tract. An experiment was devised to test this hypothesis. Various preparations of polyl(A) polymerase (see legend of Fig. 4) were used to add different numbers of adenylate residues onto rRNA. This modified rRNA was isolated, the oligo(A) chain length was determined (see "Methods"), and the modified RNA then was tested for primer activity. According to the above hypothesis, the presence of the additional aden-
tracts of the indicated length (see "Methods"), or poly(A). The rRNA containing 2
using 0.4 mg per ml of rRNA, rRNAs containing added oligo(A)
with other primers is given as a per cent of the maximal activity.

Three polymerases (1.2 units per mg) prepared from the yeast ribosomal fraction. Maximal activity of an enzyme means the activity obtained with the best primer for that enzyme. Activity
more residues was prepared with a less purified mixture of all three polymerases (1.2 units per mg) prepared from the yeast ribosomal fraction. Maximal activity of an enzyme means the activity obtained with the best primer for that enzyme. Activity

As shown in Fig. 4, ribosomal RNA containing no extra adenylate residues was the most efficient primer for polymerase I; the polymerization rate declined substantially with a modified rRNA primer containing only 2 added adenylate residues per chain. Enzyme II did not use rRNA efficiently except when an average of more than 6 adenylate residues had been added per chain. When the added poly(A) segment had an average of 9 residues or more, the rRNA became almost as good a primer for enzyme II as poly(A) itself. The results from this experiment agree with the previous data on primer specificity and the hypothesis presented (11). However, rRNA is not a normal primer for poly(A) polymerase in vivo, so this experiment can be considered only as a model of what might occur in vivo.

As can be seen in Fig. 4, the curve for polymerase III paralleled the curve for polymerase I. We suggest that enzyme III, like I, is responsible for adding only a few adenylate residues to mixed RNAs like mRNA. Although enzyme III resembles I in this and other respects (see below), there was never any indication in this study that enzyme III could be derived from I during the isolation and purification procedures.

Other Properties of Yeast Poly(A) Polymerases

Kinetics of Poly(A) Polymerase-A characteristic feature of polymerase I is a decrease in reaction rate with time using rRNA as a primer (11). This is presumably due to the primer becoming more poly(A)-like as the added oligo(A) segment becomes longer (see above). For the rest of this work, polymerase I was assayed within the linear portion of the reaction time curve when less than one adenylate residue was added on the average per chain of rRNA (11). Polymerase II gave a constant reaction rate with time using its preferred primer, poly(A). Polymerase III had kinetics similar to enzyme I. There was no observable lag period in the time course of reaction of any of the polymerases.

The initial rate of [PH]AMP incorporation from [PH]ATP was directly proportional to enzyme concentration for all combinations of enzymes and primers tested.

Effect of Ionic Strength, pH, and Divalent Ions-Polymerase I was relatively unaffected by addition of 100 mM KCl with rRNA as primer, but inhibited 40% by 100 mM KCl with poly(A) as primer. All three polymerases have nearly identical pH optima of 8.5. They all require Mn2+ as a divalent cation for maximal activity (Table III). As shown in Fig. 5, polymerase II required 2 mM Mn2+ for optimal activity compared to 0.5 mM Mn2+ for polymerase I. The standard assay contained 1 mM Mn2+ which was satisfactory for each enzyme although optimal for neither. Mg2+ was a poor substitute for Mn2+ for all three enzymes regardless of the primer used or the concentration of Mg2+ (Fig. 5). Poly(A) was a poor primer at high salt and magnesium concentrations, possibly because our preparation of poly(A) appeared to aggregate under these conditions.

Effect of Inhibitors—All of the polymerases were relatively unaffected by the DNA-dependent RNA polymerase inhibitors

| Relative polymerization rates |
|-----------------------------|
| Complete system: | 1.00 | 1.00 | 1.00 |
| - Mn2+, substitute 2 mM Mg2+ | 0.64 | 0.35 | 0.45 |
| + 10 μg per ml of α-amanitin | 1.12 | 1.06 | 1.15 |
| + 100 μg per ml of actinomycin | 0.98 | 1.25 | 0.88 |
| - Mercaptoethanol | 0.88 | 0.79 | 0.54 |
| + 1 mM p-hydroxymercuribenzoate | 0.04 | 0.07 | 0.09 |
| + 1 mM CTP | 0.48 | 0.78 | 0.48 |
| + 1 mM GTP | 0.07 | 0.09 | 0.15 |
| + 1 mM UTP | 0.44 | 0.70 | 0.56 |

Fig. 5. Divalent cation requirement for poly(A) polymerases I and II. The enzymes, purified on DEAE-cellulose, were assayed as described under "Methods" except for variation as indicated in the divalent cation concentration, [M2+]. All primers were present at a concentration of 1 mg per ml. -O--O, polymerase I with Mn2+, rRNA as primer; -O--O, polymerase I with Mg2+, rRNA as primer; -O--O, polymerase II with Mn2+, poly(A) as primer; -O--O, polymerase II with Mg2+, poly(A) as primer.

Fig. 4. Priming ability of rRNA with added oligo(A) tracts of varying lengths at the 3'-hydroxyl terminus. Poly(A) polymerases I, II, and III were assayed as described under "Methods" using 0.4 mg per ml of rRNA, rRNAs containing added oligo(A) tracts of the indicated length (see "Methods"), or poly(A). The rRNA containing 2 adenylate residues was prepared using a purified polymerase I preparation (2 units per mg). rRNA with more residues was prepared with a less purified mixture of all three polymerases (1.2 units per mg) prepared from the yeast ribosomal fraction. Maximal activity of an enzyme means the activity obtained with the best primer for that enzyme. Activity

TABLE III

Comparative properties of three yeast poly(A) polymerases

The enzymes purified as in Tables I and II were assayed (see "Methods") using the best primer for each enzyme as indicated in parentheses. The complete system gave with poly(A) polymerases I, II, and III about 1000, 500, and 200 cpm of AMP incorporation, respectively.
The enzymes were purified as in Fig. 2 and assayed as described under "Methods." All the poly(A) polymerases probably contain essential sulfhydryl groups, because mercaptoethanol is stimulatory and sulfhydryl-attacking reagents such as p-hydroxymercuribenzoate are inhibitory (Table III). All of the enzymes are inhibited strongly by GTP and less strongly by CTP and UTP (Table III, and see below). Sodium pyrophosphate, 1 mM, produced about 75% inhibition of each polymerase, probably because the enzymes produce pyrophosphate as an end product of reaction (cf. Ref. 17). Potassium phosphate at 1 mM produced less than 10% inhibition.

Addition of 1 mM ADP produced only a slight inhibition (less than 10%), which proved that the substrate of the polymerization reaction was really ATP and not ADP. Any [P]ADP formed during the incubation from the labeled 0.5 mM ATP would be diluted largely by the added unlabeled ADP. If the polymerization had been a nucleotide phosphorylase type reaction, using ADP, it would have been strongly inhibited by the added cold ADP.

Incorporation of Other Nucleoside Triphosphates by Yeast Poly(A) Polymerases—Only CTP substitutes significantly for ATP in the polymerization reactions catalyzed by polymerases I and II (Table IV). If poly(A) was substituted for rRNA as the primer with polymerase I, the rate of incorporation of CMP became as high as the rate of AMP incorporation (Table V). Enzyme III also showed a significant rate of CMP incorporation from CTP (Table V).

Because CMP incorporation was relatively high in our polymerase preparations, it was important to determine whether this incorporation was due to a number of distinct cytidylate-polymerizing enzymes contaminating the poly(A) polymerases, or due to intrinsic activities of the poly(A) polymerases. We separated and located the three poly(A) polymerases on a DEAE-cellulose column as described in the legend of Fig. 2, and then assayed all column fractions for CMP incorporation. Three peaks of CMP-incorporating activity were found, and these peaks corresponded well with the three peaks of poly(A) polymerase activity (Fig. 6). Polymerase I had a relatively high ratio of CMP:AMP incorporation with rRNA plus poly(A) as primers, in agreement with the data in Table V. Polymerases II and III produced smaller CMP incorporation peaks relative to the AMP incorporation peaks. Because the CMP and AMP peaks of the polymerases coincide in both position and shape, we have concluded tentatively that the observed CMP incorporation is an intrinsic property of the three poly(A) polymerases of yeast. This substrate activity of CTP could explain why the incorporation of labeled ATP was inhibited by unlabeled CTP, as demonstrated in Table III.

Our most purified preparations of polymerases I and II had very low rates of incorporation of UMP and GMP from the corresponding triphosphates (Table IV). Nevertheless, we assayed the fractions from the column described above (Fig. 6) for UMP and GMP incorporation. None of the fractions indicated any [P]UMP incorporation, but three or possibly four peaks of [P]GMP incorporation were found. The largest of these peaks had its maximal value at Fraction 16, and was definitely distinct from any poly(A) polymerase. A mixture of primers was used in this GMP incorporation assay. We included poly(G) since Burkard and Keller (9) had previously characterized a poly(G) polymerase from wheat which preferred poly(G) as a primer. Reassay of Fraction 16 from the above DEAE-cellulose column with many different primers separately showed that only poly(G) was active as primer. This distinctive primer specificity together with the fact that this GMP incorporation peak is distinct from any of the poly(A) polymerase peaks suggests that yeast has a

**TABLE IV**

| Assay conditions                        | Relative polymerization rates |
|-----------------------------------------|-------------------------------|
|                                         | Polymerase I with poly(A) primer | Polymerase II with poly(A) primer |
| Complete system, [P]ATP, 1 mM           | 1.00                          | 1.00                             |
| Complete system, [P]CTP, 1 mM           | 0.15                          | 0.13                             |
| Complete system, [P]UTP, 1 mM           | 0.03                          | 0.01                             |
| Complete system, [P]GTP, 1 mM           | 0.05                          | 0.03                             |

* [P]ATP omitted. The other nucleoside triphosphates substituted for ATP in the standard assay all had the same specific activity as ATP, 4 μCi per μmol.

**TABLE V**

| Enzyme       | Primer  | CMP:AMP incorporation |
|--------------|---------|------------------------|
| Polymerase I | rRNA    | 0.58                   |
| Polymerase I | poly(A) | 1.04                   |
| Polymerase II| rRNA    | 0.20                   |
| Polymerase III | rRNA  | 0.89                   |

**Fig. 6.** AMP, CMP, and GMP incorporation from nucleoside triphosphates by enzymes in DEAE-cellulose column fractions. A 0.5 mM NH₄Cl ribosomal wash was prepared and treated on a Sephadex G-150 column (see "Results"). The enzyme preparation (16 ml, 1.5 mg per ml of protein) was passed through a DEAE-cellulose column (1.25 X 15 cm) and eluted with a 30-ml gradient of 0 to 1 mM (NH₄)₂SO₄ in Buffer A. Fractions of 0.75 ml were collected and assayed (see "Methods") separately with each of the three [P]labeled nucleotide triphosphates at 1 mM (20 μCi per μmol) and with 0.5 mg per ml of each of the indicated primers. [P]UMP incorporation from [P]UTP also was measured, but it was very low in all fractions and was not plotted. O---O, [P]AMP incorporation with poly(A) plus rRNA as primers; □---□, [P]CMP incorporation with the same primers; O---O, [P]GMP incorporation with poly(A) plus poly(G) plus rRNA as primers.
specific poly(G) polymerase which is similar to the wheat chloroplast enzyme (9, 22). Further work will be necessary to establish this point.

There were two other smaller peaks of GMP incorporation in Fig. 6. Although they were located under the AMP incorporation peaks of poly(A) polymerases I and III, their shapes did not correspond well with the shapes of the AMP incorporation peaks. They may indicate the presence of G-polymerizing enzymes in addition to the major enzyme with a peak at Fraction 16.

The incorporation of CMP residues by poly(A) polymerase I was analyzed in greater detail. It was found that the $K_m$ for ATP (0.14 mm) was actually higher than that for CTP (0.04 mm). However, the $V_{max}$ for ATP was about 4 times higher than the $V_{max}$ for CTP. The actual proportion of C residues incorporated into poly(A) tracts in vivo therefore would depend on the concentrations of ATP and CTP in the cell. Our analysis of poly(A) tracts synthesized by a yeast culture under certain defined conditions suggested that adenyate and cytidylate residues had been incorporated in the ratio of about 3:1 (23).

An experiment was devised using polymerase I with both ATP and CTP as substrates to see if a mixed poly(A, C) tract would be formed or if separate poly(A) and poly(C) tracts would be formed. Poly(G) was chosen as primer since it is not attacked by pancreatic ribonuclease (24) and is the best primer with this property (11). Poly(A) polymerase I was incubated with poly(G), labeled ATP, and unlabeled CTP. The labeled poly(A) tracts formed on the poly(G) primer should be resistant to pancreatic ribonuclease treatment unless C residues (not resistant to the nuclease) were inserted into the poly(A) tract. It was found, however, that the presence of unlabeled CTP made the labeled poly(A) tracts sensitive to pancreatic ribonuclease (data not shown). Therefore, some of the CMP residues, although not necessarily all of them, must have been incorporated into the poly(A) tract.

Noncompetitive Inhibition of Poly(A) Polymerase I by GTP — The strong inhibition of enzyme I by GTP (Table III) was analyzed by the graphical method of Hunter and Downs, as modified by Dixon and Webb (25). The concentrations of both inhibitor and substrate were varied over a wide range and the data were plotted as described in the legend of Fig. 7. When data are plotted by this method, a horizontal line is generated with a noncompetitive inhibitor, and an inclined line (slope = $K_i/K_a$) is generated with a competitive inhibitor. The ordinate intercept in either case is numerically equal to the $K_i$ of the inhibitor. As shown in Fig. 7, GTP appears to be fully noncompetitive in its inhibition of polymerase I with a $K_i$ of 0.14 mm. The same $K_i$ value was obtained whether rRNA or poly(A) was used as the primer. Although the inhibition by GTP was noncompetitive, it was reversible. This was demonstrated by adding GTP to a final concentration of 10 mm to 0.2 ml of polymerase I (5 units per mg, 5 units per ml) and incubating the resulting solution for 30 min at 30° as in the usual assay. Then the enzyme was passed through a Sephadex G-25 column at 4° to free it of GTP. An enzyme assay showed that this procedure caused the enzyme to regain its initial specific activity.

Not only GTP but GDP and also 5'-GMP were inhibitory to polymerase I. GDP was a weaker inhibitor than GTP, and 5'-GMP was weaker still (Table VI). Neither guanosine nor 3'-GMP were inhibitory (Table VI).

GTP also inhibited enzymes II and III (Table III). The $K_i$ with enzyme II and poly(A) as primer was 0.14 mm. The $K_i$ with enzyme III and rRNA as primer was 0.25 mm. Whether this inhibition was competitive or noncompetitive was not determined.

The inhibition of polymerase I by CTP and UTP (Table III), analyzed on the Hunter and Downs plot (25), showed that these nucleoside triphosphates exhibited competitive inhibition (inclined slopes), with $K_i$ values of 0.50 mm for CTP and 0.20 mm for UTP (Fig. 7). The competitive inhibition by CTP could be explained by the fact that it is a competitive substrate (see above). Competitive inhibition by UTP cannot be due to the same cause, as there is practically no incorporation of UMP onto primers.

Native Yeast DNA Is Inhibitor of Three Yeast Poly(A) Polymerases—Addition of deoxyribonuclease to crude preparations of poly(A) polymerase always resulted in an apparent stimulation of polymerase activity (40 to 100%). DEAE-cellulose-purified enzyme I, which was free of nucleic acids, was not stimulated by addition of deoxyribonuclease. These observations led to experiments which demonstrated that purified poly(A) polymerase was inhibited by low concentrations of native yeast DNA. The $K_i$ for native yeast DNA, obtained by the graphical method of Dixon (25), was 0.2 μg per ml. The inhibition was surprisingly specific, since alkaline-denatured yeast DNA and native or alkaline-denatured calf thymus DNA produced no inhibition at concentrations up to 2 μg per ml. Polymerase II, assayed with poly(A) as primer, was inhibited by native yeast DNA to the same degree as enzyme I, but polymerase III required 2 to 3 times as much DNA to produce the same amount of inhibition seen with poly-

![Fig. 7. Graphical determination of inhibitor constants for GTP, CTP, and UTP with poly(A) polymerase I (23). Polymerase I (5 units per mg) was incubated with 1 mg per ml of rRNA as primer and with varying concentrations of [H]ATP and of one of the other three nucleoside triphosphates. CTP and UTP were added at 0.19, 0.38, and 0.96 mm. GTP was added at 0.074, 0.185, 0.290, and 0.56 mm. $i = inhibitor concentration (mm); v_i = reaction rate (cpm) in the presence of inhibitor; v_o = reaction rate (cpm) in the absence of inhibitor; $K_i = inhibitor constant. O — — O, assays with GTP added; C — — C, UTP added; □ — — □, CTP added. Reaction rates are given as counts per min of AMP incorporation in a 30-min incubation.]

| Additions (1 mm) | Relative poly(A) polymerase activity |
|-----------------|-------------------------------------|
| None            | 100                                 |
| Guanosine       | 97                                  |
| GMP (3')        | 98                                  |
| GMP (5')        | 78                                  |
| GDP (5')        | 24                                  |
| GTP (5')        | 7                                   |
erase I. The inhibition of enzyme I with native yeast DNA nearly could be eliminated by diluting the enzyme plus DNA 20-fold during assay, proving that this inhibition was reversible.

This strong and specific inhibition of polymerases I and II by native yeast DNA is strange in view of the location of these substances together in the nucleus. The histones present on yeast DNA (26) might be able to prevent this strong inhibition in vivo.

**Discussion**

The three yeast poly(A) polymerases we have separated, purified, and characterized probably have distinct roles in the addition of poly(A) tracts to RNA in the cell. The isolation of polymerases I and II from nuclei and the possible role of both of these enzymes in a two-stage synthesis of poly(A) tracts on nuclear RNAs have been previously discussed (11). Polymerase III activity was nearly absent in nuclear extracts. Preliminary unpublished data indicate that a mitochondrial fraction isolated from spheroplasts of galactose-grown yeast are enriched for polymerase III activity. Further work will be necessary to show if this enzyme is cytochrome c or a mitochondrial enzyme.

Mitochondrial messenger RNA from an animal tissue has been shown to contain poly(A) (27), and a poly(A) polymerase has been found in animal mitochondria (8). Mitochondrial messenger RNA from an animal tissue has been shown to contain poly(A) (27), and a poly(A) polymerase has been found in animal mitochondria (8).

The properties of the poly(A) polymerases described in this paper are consistent with their postulated role of adding poly(A) to newly transcribed messenger RNA. Each purified enzyme is dependent on added RNA primer. Polymerase I in vitro can best utilize primers that are messenger-like, in the sense of having a mixed nucleotide composition. Polymerase I exhibits a relatively slow rate with poly(G), poly(A), and poly(C) and a very slow rate with poly(U) (11). There is evidence now that the 3'-terminus of the RNA to which the poly(A) tract is attached in vitro does have a mixed sequence; the common sequence -GCAAU- was found adjacent to the poly(A) tract in the rapidly labeled RNA of yeast (23).

The fact that polymerase I can utilize yeast tRNA in vitro deserves some discussion since yeast tRNAs do not contain poly(A) tracts in vitro (12). The absence of poly(A) tracts in vitro could be explained if individual tRNAs, when released from the single large precursor tRNA by nuclelease action (28), interact immediately with ribosomal proteins. Also, poly(A) polymerase may be absent from the nucleus, where RNA synthesis occurs (28).

Poly(A) polymerase II is specific in vitro for a poly(A) primer, or for an RNA primer with an oligo(A) tract at the 3'-terminus. We propose that it functions in the nucleus to elongate a poly(A) tract which had been initiated by polymerase I.

The fact that tRNA is a very poor primer for all three yeast poly(A) polymerases in vitro may indicate that these polymerases require in a primer a longer 3'-terminal single-stranded region than is provided by the hydrogen-bonded tRNA structure. The lack of this priming ability even with tRNA lacking the 3'-terminal adenosine also indicates that these polymerases are distinct from the tRNA nucleotidyltransferase present in yeast (20).

The three yeast poly(A) polymerases differ from the poly(A) polymerases from other organisms so far reported in the literature in their specificity for nucleoside triphosphates. The three yeast enzymes apparently all have the intrinsic capacity to catalyze a significant amount of incorporation of CMP residues from CTP in addition to AMP incorporation from ATP. In vitro, these polymerases can catalyze the formation of a mixed tract of As and Cs on the primer. This result is consistent with the role of these enzymes in vivo, since we found that the poly(A) tracts on rapidly labeled RNA formed in yeast under certain conditions contain about 30% C residues (23). In our experience, the poly(A) tracts of rapidly labeled yeast RNA cannot be isolated on poly(U) membranes if pancreatic ribonuclease (which can cleave at C residues) is present along with ribonuclease T1 (which can cleave only at G residues). 1 Also in our experience, the poly(A) tracts of yeast do become partially resistant to pancreatic ribonuclease at high concentrations of salt (i.e. 100 mM ammonium sulfate), but higher yields of the poly(A) tracts are always obtained if ribonuclease T1 is used alone. 1 Most reports dealing with poly(A) tracts in other organisms state that a combination of ribonuclease T1 and pancreatic ribonuclease can be used to isolate the poly(A) tracts (6). The poly(A) tracts from these organisms contain almost exclusively adenylic residues, and the poly(A) polymerases of these same organisms are specific for AMP incorporation (7).

Because each of the three yeast poly(A) polymerases has the ability to incorporate significant amounts of CMP, it might appear that the name poly(A) polymerase for these enzymes is inappropriate. It seems best to retain the name poly(A) polymerase, however, not only because of the preponderance of AMP incorporation in vivo, but also because these yeast enzymes are members of a class of enzymes with this name which occur in various eukaryotic organisms and have a common function in these organisms.

The consistency between CMP incorporation by the yeast poly(A) polymerases in vitro and the presence of cytidylate residues in the yeast poly(A) tracts in vivo supports the idea that these enzymes synthesize the poly(A) tract in vivo. However, there is a discrepancy between our results and those obtained by McLaughlin et al. (12) who did not find cytidylate residues in poly(A) tracts. The reason for this discrepancy is not yet apparent, but it could be due to the fact that different labeling and isolation procedures were used. We isolated poly(A)-containing RNA from the total cell RNA after labeling whole yeast cells with 32P, for less than 1 min (23). McLaughlin et al. (12) labeled spheroplasts in a medium containing 0.5 M MgSO4 for 10 min and isolated the poly(A) tracts from polysomal RNA. These poly(A) tracts were found to be resistant to pancreatic ribonuclease digestion in 25 mM ammonium sulfate, so they must have contained few cytidylate residues. Further study will be needed to understand the cause of the differences between their results and our results.

The three yeast poly(A) polymerases were all inhibited by GTP, CTP, and UTP. The inhibition by CTP, which was competitive, could be explained because CTP is a competitive substrate. The inhibition by UTP, which was also competitive, cannot be so explained, as there was negligible incorporation from UTP. The inhibition by GTP was noncompetitive with ATP and entirely reversible; GTP could be an allosteric effector, which suggests it could have some regulatory significance in the cell in controlling the rate of synthesis of poly(A) tracts.

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