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Characterization of Purified Poly(adenylic acid)-Containing Messenger Ribonucleic Acid from *Saccharomyces cerevisiae*†

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ABSTRACT: Yeast poly(adenylic acid)-containing messenger RNA was isolated from total cellular RNA by affinity chromatography on poly(uridylic acid)-cellulose. The relative complexity of the isolated yeast mRNA was assessed by hybridization analysis with complementary DNA synthesized from the isolated messenger RNA (mRNA) with viral reverse transcriptase. Approximately 25% of the mRNA hybridized at an apparent $C_rt_{1/2}$ of 5×10^{-3} mol s $l.^{-1}$, while the remainder hybridized at an average $C_rt_{1/2}$ of 10^{-1} mol s $l.^{-1}$. Poly(adenylic acid)-containing yeast mRNA was translated in vitro in a wheat germ cell-free extract, and the major polypeptides synthesized have the same molecular weight as the major proteins present in the cell. Four of these proteins were identified by coelectrophoresis and immune precipitation to be pyruvate kinase, enolase, aldolase, and glyceraldehyde-3-phosphate dehydrogenase. These data demonstrate in agreement with the hybridization results that yeast contains major mRNA species and that some of the glycolytic enzyme mRNAs make up part of the major fraction. A procedure is outlined for the preparation of yeast mRNA which is essentially free of ribosomal RNA contamination and is further enriched in the major mRNAs present in the cell.

Yeast is an appealing eucaryotic organism for the study of transcriptive mechanisms, because it shares many of the molecular components found in animal cells. Yeasts contain multiple forms of RNA polymerase which are similar in structure and properties to those of higher eucaryotic cells (Roeder, 1969; Ponta et al., 1971; Adman et al., 1972; Dezelee and Sentenac, 1973; Buhler et al., 1974; Hager et al., 1976; Chambon, 1975 (review)). Yeast mRNA contains poly(A)[1] residues at its 3′ terminus (McLaughlin et al., 1973; Groner et al., 1974) and has been shown to direct the in vitro synthesis of polypeptides which coelectrophorese with those synthesized in the cell (Gallis et al., 1975). Biochemical studies of transcriptional mechanisms are facilitated in yeast because the genetic complexity of the nonreiterated portion of yeast DNA is only a few times that of *Escherichia coli* DNA (Ogur et al., 1952). Yeast is amenable to rigorous genetic analysis (Mortimer and Hawthorne, 1969) and yeast cells are available in large quantity.

It is well known that yeasts are highly specialized metabolically. When grown aerobically or anaerobically on a fermentable carbon source, yeasts maintain a very high intracellular level of the glycolytic enzymes. When yeasts are grown on glucose, for example, the glycolytic enzymes comprise 25–65% of the total cellular protein (Hess et al., 1968). The kinetics of induction of the glycolytic enzymes have been studied in cells shifted from growth on acetate to glucose (Maitra and Zobo, 1974) and the levels of the enzymes have been measured in cells grown on increasing concentrations of glucose (Hommes, 1966). In each case dramatic induction (ranging from 3- to 200-fold) of the glycolytic enzymes was observed. These observations demonstrate that the synthesis of glycolytic enzymes in yeast cells results from regulated differential gene expression rather than constitutive production of these proteins. The study of transcription of these genes both in vivo and in vitro seems feasible and attractive.

In this paper we describe the isolation and characterization of yeast poly(A) containing mRNAs with respect to the number and relative abundance in vegetative cells. We have

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[1] Abbreviations used: poly(U), poly(uridylic acid); poly(A), poly(adenylic acid); cDNA, complementary deoxyribonucleic acid; tRNA, mRNA, and rRNA, transfer, messenger, and ribosomal ribonucleic acids, respectively; YNB, yeast nitrogen base; Hepes, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; CM, carboxymethyl; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

shown that a very limited number of mRNAs (approximately 10) make up 25% of the cellular poly(A)-containing mRNA. Messenger RNAs which code for pyruvate kinase, enolase, aldolase, and glyceraldehyde-3-phosphate dehydrogenase belong to this abundant population, suggesting that the high intracellular levels of these glycolytic enzymes are the result of control at the transcriptive level.

Materials and Methods

Materials. Uracil, adenine, heparin (grade I), cycloheximide, Hepes, and enolase (type III: from yeast) were purchased from Sigma Chemical Co. Actinomycin D and dithiothreitol (A grade) were obtained from Calbiochem. Creatine phosphate and creatine phosphokinase were from Boehringer Mannheim. Nucleotide triphosphates, oligo[dT(12-18)], and poly(U) were purchased from P-L Biochemicals. L-[^{35}S]Methionine (200 Ci/mmol), [5-^{3}H]dCTP (23 Ci/mmol), and [5,6-^{3}H]uridine (40 Ci/mmol) were obtained from New England Nuclear. Yeast nitrogen base (YNB) with and without amino acids, casamino acids, and Freund's complete adjuvant were from Difco. Goat anti-rabbit IgG was purchased from Miles and purified as described for rabbit sera before use. Pyruvate kinase was a generous gift from Dr. C. Seulter. Aldolase and glyceraldehyde-3-phosphate dehydrogenase were prepared by the methods of Richards and Rutter (1961) and Holland and Westhead (1973), respectively. Wheat germ was supplied by the "bar-Rav" Mill, Tel Aviv, Israel.

Cells. Messenger RNA was isolated from early-mid-log-phase yeast cells (*Saccharomyces cerevisiae,* strain F1) kindly donated by the Red Star Corp. (Division of United Foods) Oakland, Calif. Cells were washed twice in distilled water and suspended in breaking buffer. The cell suspension contained 85 g of wet weight cells, 10 ml of glycerol, and 5 ml of buffer containing 1 M Tris, pH 7.5, 0.1 M $MgCl_2$, and 0.2 M iodoacetate. The suspension was frozen in small pellets by dripping into liquid nitrogen and stored in liquid nitrogen until use.

Saccharomyces cerevisiae strain a364A (Gal-1, Ade-2, Ura-1, His-7, Lys-2, Tyr-1), supplied by Dr. Leland Hartwell, was used for labeling cellular RNA and protein. Cellular RNA was pulse labeled at 30 °C in cultures containing 0.7% yeast nitrogen base (YNB) with amino acids (20 μg/ml each of histidine, lysine, and tyrosine), 2% glucose, 20 μg/ml adenine, 1 μg/ml uracil, and 10 μCi/ml [^{3}H]uridine (60 Ci/mmol). [^{3}H]Uridine was added during mid-log-phase growth 10 min before harvesting. Cellular proteins were labeled in the same medium described above with the following changes: 0.7% yeast nitrogen base (YNB) without amino acids, 20 μg/ml uracil, and 7 μCi/ml [^{35}S]methionine (200 Ci/mmol). [^{35}S]Methionine was added during early log-phase growth 3–4 h before harvesting.

Preparation of RNA. Liquid-nitrogen-frozen cells were disrupted in a 200-ml capacity Eaton press at 9000 psi. The extract was then suspended in 2 volumes of a solution containing 10 mM Tris buffer, pH 7.5, 5 mM $MgCl_2$, 10 mM iodoacetate, 100 μg/ml heparin, and 100 μg/ml cycloheximide. Iodoacetate and heparin were present to inhibit ribonuclease activity and cycloheximide was employed to stabilize polysomes. Nuclear material and cellular debris were removed by centrifugation at 10 000*g* for 15 min. Cellular RNA was precipitated by adjusting the supernatant to pH 5.0 by dropwise addition of 1 N acetic acid. The precipitate was removed by centrifugation at 10 000*g* for 5 min and resuspended in sufficient 1 mM EDTA such that the A_{260nm} was less than 50 absorbance units/ml. Once dissolved the suspension was adjusted to 0.1 M Tris, pH 9.0, 0.1 M NaCl, 0.5% sodium docecyl sulfate, and 1 mM EDTA and immediately extracted with an equal volume of phenol–chloroform–isoamyl alcohol (50:49:1). After centrifugation at 15 000 *g* for 10 min, the aqueous phase was reextracted with phenol–chloroform–isoamyl alcohol. Excess phenol was removed from the final aqueous phase by two extractions with an equal volume of anhydrous ether. The RNA solution was then adjusted to 0.2 M sodium acetate and precipitated with 2 volumes of ethanol at −20 °C. The recovery of total RNA was routinely 9–12 mg/g (wet weight) of cells.

It is essential that early or mid-log phase cells be used to ensure the isolation of intact RNA. Greater than 90% of cellular RNA, pulse labeled with [^{3}H]uridine, can be recovered with this procedure. We have not been successful in isolating intact RNA from stationary cells or commercially available cake yeast.

Poly(U)-Cellulose Chromatography. Poly(U)-cellulose was prepared as described by Sheldon et al. (1972). Utilizing their conditions 2–5 mg of poly(U) were bound per g of cellulose (Whatman CF-11). Poly(U)-cellulose colums (10 × 1 cm) were washed with a solution containing 10 mM Tris, pH 7.5, 1 mM EDTA, and 90% formamide before use to ensure removal of unbound poly(U) and finally equilibrated in buffer containing 10 mM Tris, pH 7.5, 1 mM EDTA, and 0.1 M NaCl. RNA to be chromatographed was routinely suspended at 2 mg/ml in buffer containing 10 mM Tris, pH 7.5, 1 mM EDTA, and 0.1 M NaCl, heated at 50 °C for 2 min followed by rapid cooling in an ice bath, and loaded on the column at a flow rate of 0.5 ml/min. After loading, the column was washed with 3 column volumes of equilibration buffer and finally the poly(A)-containing mRNA was eluted with buffer containing 10 mM Tris, pH 7.5, 1 mM EDTA, and 90% formamide. Up to 50 ml of cellular RNA (2 mg/ml) could be applied to the column (10 × 1 cm) with greater than 90% retention of poly(A)-containing mRNA. Conditions for exceeding the capacity of the poly(U)-cellulose column are as described in the text.

Sucrose Density Gradient Centrifugation. RNA was sedimented in sucrose density gradients containing 15–30% sucrose (w/v), 20 mM Tris, pH 7.5, 20 mM NaCl, and 1 mM EDTA. Sucrose solutions were treated with 0.2% diethyl pyrocarbonate for 2 h and then placed in a boiling water bath for 30 min to remove the diethyl pyrocarbonate prior to use. The procedure ensured the inactivation of ribonucleases in the gradient solutions. Centrifugation was carried out in a Beckman SW 41 rotor at 39 000 rpm for 16 h at 4 °C. A_{260nm} profiles of the gradients were monitored via a flow cell attached to a Gilford 2000 recording spectrophotometer.

Formamide Gel Electrophoresis. RNA samples were electrophoresed in the presence of formamide according to Pinder et al. (1974). RNA samples (2.5 μg) were lyophilized, suspended in 10 μl of 0.02 M diethyl barbiturate in deionized formamide, and applied directly to 0.6 × 7 cm 4% acrylamide gels. Samples were initially electrophoresed at 25 V for 1 h and electrophoresis was continued at 60 V. The gels were fixed in 10% acetic acid, stained with 0.1% pyronine Y overnight, and destained with 10% acetic acid. The gels were scanned with a Gilford 2000 recording spectrophotometer equipped with a Gilford constant speed gel scanning device.

Translation in a Wheat-Germ Cell-Free Extract. Wheat germ S-30 fractions were prepared as described by Roberts and Paterson (1973). In vitro translations were carried out at 25 °C for 90 min in 75-μl reaction mixtures containing: 30 μl of S-30 fraction, 20 mM Hepes buffer, pH 7.6, 2 mM di-

thiothreitol, 1 mM ATP, 0.2 mM GTP, 8 mM creatine phosphate, 40 μg/ml creatine phosphokinase, 80 mM KCl, 3 mM magnesium acetate, 75 μCi of [^{35}S]methionine (200 Ci/mmol), and 30 μM of each of the unlabeled amino acids. Reaction mixtures contained 0.5–1 μg of mRNA (estimated in the case of total yeast RNA). Magnesium and potassium optima were determined for each preparation of wheat germ S-30. At the end of the incubation, aminoacyl-tRNAs were hydrolyzed by adjusting the reaction mixture to 0.1 N NaOH and incubating for an additional 15 min at 25 °C. The reaction was finally adjusted to 0.3% in casamino acids, precipitated with 10% trichloroacetic acid in the presence of 10 μg of bovine serum albumin. The precipitate was collected on GF/C filter disks and counted in toluene Omnifluor.

Polyacrylamide Gel Analysis. Polyacrylamide gel analysis of proteins was carried out using the discontinuous buffer system described by Laemmli (1970). Disc gel columns were 0.6 × 9 cm with a 1-cm stacking gel and a 10% acrylamide running gel. Slab gel electrophoresis was carried out in a Hoeffer slab gel apparatus. Slab gels were 1.5-mm thick containing a stacking gel and 10% acrylamide running gel. Disc gels were electrophoresed initially at 30 V and electrophoresis was continued at 90 V. Slab gels were preelectrophoresed at 90 V and electrophoresis was continued at 180 V. Slabs to be analyzed by autoradiography were fixed and washed overnight in a solution of 10% trichloroacetic acid and 25% 2-propanol to remove unincorporated [^{35}S]methionine. Coomassie blue stained gels were cut and scanned at 660 nm in a Gilford scanning device. Autoradiograms were analyzed by cutting lanes from the exposed film and scanning the film strips as described above.

Immune Precipitation with Enolase Antisera. Enolase antibodies were prepared in 8–10-lb New Zealand white rabbits by a single injection of 5 mg of purified enolase in Freund's complete adjuvant into the two rear leg muscles, rear toe pads, and subcutaneously along both flanks. After 4–5 weeks rabbits were bled by cardiac puncture. IgG was purified from the sera by ammonium sulfate precipitation and DEAE-cellulose–CM-cellulose chromatography as described by Palacios et al. (1972). The equivalence point for the enolase IgG was 40 μg of IgG/μg of enolase. The second antibody (Goat anti-rabbit IgG) had an equivalence point of 14 μg of goat IgG/μg of rabbit IgG.

Enolase was precipitated from in vitro translation reaction mixtures which were precentrifuged at 100 000*g* for 2.5 h in a SW 39 Beckman rotor equipped with 0.8-ml tube adaptors in order to remove polysomes which would become trapped in the immunoprecipitate. To 20 μl of the supernatant was added 10 μg of rabbit anti-enolase IgG (18 mg/ml) and the reaction was allowed to proceed overnight at 4 °C. An equivalent amount of goat anti-rabbit IgG (5 mg/ml) was then added and the precipitate was allowed to form at 4 °C overnight. The resultant precipitate was removed by centrifugation at 5000*g* for 30 min. Both the supernatant and the precipitate were analyzed by polyacrylamide gel electrophoresis as described above.

Preparation of Complementary DNA. Complementary DNA (cDNA) was synthesized according to Efstratiadis et al. (1975) with reverse transcriptase isolated from avian myeloblastosis virus (supplied by Dr. Joseph Beard). Reaction mixtures (50 μl) contained: 50 mM Tris buffer, pH 8.3, 6 mM $MgCl_2$, 60 mM NaCl, 10 mM dithiothreitol, 100 μg/ml actinomycin D, 20 μg/ml oligo[dT(12–18)], 50 μM each of dATP, dTTP, and dGTP, 130 μCi of [^{3}H]dCTP (23 Ci/mmol), 50 μg/ml poly(A)-containing mRNA, and 0.6 μg of reverse transcriptase. Reaction mixtures were incubated for 60 min at 37 °C, adjusted to 1 ml with buffer containing 0.1 M Tris, pH 9.0, 0.1 M NaCl, 1 mM EDTA, 0.5% sodium dodecyl sulfate, and extracted with an equal volume of phenol–chloroform–isoamyl alcohol (50:49:1). After centrifugation at 10 000*g* for 5 min the aqueous phase was chromatographed on a 1 × 25 cm column of Sephadex G-50 to remove unincorporated nucleotides. Fractions containing cDNA (void volume of the column) were adjusted to 0.2 M sodium acetate and precipitated in the presence of 10 μg of poly(U) carrier with 2 volumes of ethanol at −20 °C. Alkaline density gradient centrifugation of the cDNA was carried out in 5–10% sucrose gradients containing 0.9 M NaCl and 0.1 NaOH in a Beckman SW 50.1 at 35 000 rpm for 17 h at 20 °C. At this stage of purification cDNA was completely digestible with the single strand specific nuclease S1.

Reverse transcriptase assays of poly(U)-cellulose columns and sucrose gradients were carried out in 50-μl reaction mixtures as described for the preparation of cDNA except that the reaction mixtures contained 4 μCi of [^{3}H]dCTP, 0.01 μg of reverse transcriptase, and 0.01–0.5 μg of poly(A)-containing mRNA.

Hybridization. Hybridization reactions were carried out in sealed capillary tubes at 68 °C in 5–50-μl volumes containing 0.2 ng of cDNA, 10 mM Tris, pH 7.5, 0.3 M NaCl, 1 mM EDTA, and 0.25% sodium dodecyl sulfate and a vast excess of either poly(A)-containing mRNA or cellular DNA. The extent of hybridization was measured by dispensing the contents of the capillary tubes into 1 ml of digestion buffer containing 0.3 M NaCl, 30 mM sodium acetate, pH 4.5, 0.5 mM $ZnCl_2$, 10 μg/ml denatured calf thymus DNA, and S1 nuclease (prepared as described by Vogt, 1973). Digestion was carried out for 60 min at 37 °C. Mixtures were precipitated with 25% trichloroacetic acid in the presence of 100 μg/ml of bovine serum albumin. The precipitate was collected on GF/C filters, washed with sodium pyrophosphate (45 g of sodium pyrophosphate and 90 ml of concentrated HCl per l.), and counted in toluene Omnifluor.

T_m analysis was carried out in sealed capillary tubes containing cDNA–mRNA hybrid, 10 mM Tris, pH 7.5, 1 mM EDTA, 30 mM NaCl, and 0.25% sodium dodecyl sulfate. Capillaries were incubated at the indicated temperatures and digested with S1 nuclease as described above.

Globin mRNA and cDNA. Chick globin mRNA was isolated from phenol-extracted chick reticulocyte polysomal RNA by poly(U)-cellulose affinity chromatography and 15–30% sucrose density gradient centrifugation (Holland and Rutter, unpublished). The 10–12S globin mRNA fraction obtained contains all of the chick globin chain mRNAs as determined by translation of the mRNA in the wheat-germ cell-free extract and analysis of the protein synthesized on CM-cellulose chromatography in the presence of 8 M urea (Holland and Rutter, unpublished). cDNA was prepared from globin mRNA as described for yeast cDNA synthesis and hybridizations were carried out under the conditions described above.

Results

Preparation of Yeast Poly(A)-Containing mRNA. Since poly(A)-containing mRNA makes up only a small percentage of total yeast RNA, it is desirable in studies utilizing yeast mRNAs to have a large scale procedure for preparing RNA. Intact yeast RNA can be isolated as described in Materials and Methods rapidly and in large quantity. This contrasts with reported methods which involve the preparation of polysomes

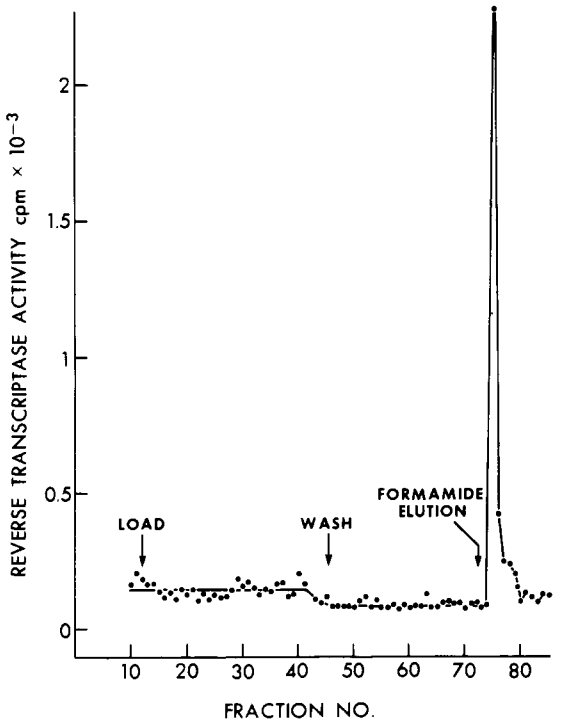

FIGURE 1: Fractionation of total yeast RNA by affinity chromatography on poly(U)-cellulose. Total yeast RNA (200 mg) was chromatographed on a poly(U)-cellulose column and poly(A)-containing mRNA was assayed on the basis of reverse transcriptase template activity as described in Materials and Methods. Reverse transcriptase assays contained 5 μl of fractions 1–73 and 1-μl aliquots of fractions 74–85.

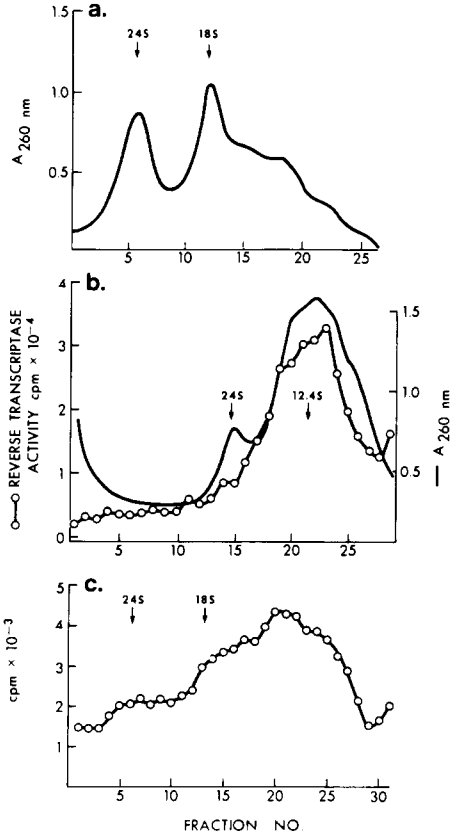

FIGURE 2: Sedimentation of yeast RNA which bound to poly(U)-cellulose. (a) RNA fractionated from total yeast RNA. (b) RNA fractionated from total yeast RNA which was heated at 50 °C for 2 min prior to poly(U)-cellulose chromatography. The reverse transcriptase template activity of 2-μl aliquots of each fraction was determined as described in Materials and Methods. (c) RNA fractionated from [^{3}H]uridine pulse labeled total yeast RNA. Sucrose density gradient centrifugation was carried out as described in Materials and Methods in a Beckman SW 41 rotor for 60 h at 39 000 rpm (a and c) or 30 000 rpm (b).

from yeast protoplasts, which is time consuming and difficult to scale up.

Poly(A)-containing mRNA was separated from the bulk of the cellular RNA by affinity chromatography on poly(U)-cellulose. Poly(A)-containing mRNA was monitored on the basis of its template activity for reverse transcriptase in the presence of an oligo(dT) primer. Approximately 0.5% of the cellular RNA is bound to the column in the presence of 0.1 M NaCl at 4 °C. Most of this RNA remains bound to the column when the NaCl concentration is dropped to zero at 4 °C, suggesting that the length of poly(A) residues on the yeast mRNA is sufficient to form stable hybrids with poly(U) residues on the column. Bound RNA was eluted with 90% formamide; as shown in Figure 1, greater than 95% of the reverse transcribable RNA binds to the column and is removed by the procedure.

A sucrose density gradient profile of the RNA eluted from the poly(U)-cellulose column is shown in Figure 2a. A heterogeneous distribution of mRNAs is observed along with substantial amounts of 24S and 18S rRNA when cellular RNA is chromatographed on poly(U)-cellulose directly. This rRNA contamination is not removed by rechromatography on poly(U)-cellulose; however, most of the rRNA contamination can be eliminated if the cellular RNA is preincubated at 50 °C for 2 min in 10 mM NaCl before chromatography on poly(U)-cellulose. The sucrose density gradient profile of 50 °C-treated RNA that binds to poly(U)-cellulose (Figure 2b) shows a markedly reduced level of rRNA contamination. Since free rRNA does not bind to poly(U)-cellulose, the rRNA which is retained on the column is probably associated with poly(A)-containing RNA. It is possible that the mild heating procedure disrupts these aggregates leading to a reduced level of rRNA contamination in the bound fraction. Preincubation at 50 °C does not completely eliminate rRNA contamination. As shown in Figure 2b the reverse transcriptase activity profile closely follows the A_{260nm} profile except in the portion of the gradient which contains rRNA. Figure 2c shows a sucrose density gradient profile of pulse-labeled RNA which binds poly(U)-cellulose. This profile is very similar to the reverse transcriptase activity profile in panel b and represents the actual size distribution of yeast poly(A)-containing mRNAs.

In Vitro Translation of Yeast mRNA. In an attempt to determine whether a portion of yeast mRNA does not bind to poly(U)-cellulose, total cellular RNA and poly(U)-bound RNA were translated in a wheat-germ cell-free extract and the polypeptides synthesized were compared. Messenger RNAs which do not contain poly(A) should be detected since poly(A) residues are not required for translation of a mRNA in wheat-germ extracts (Davies and Kaesberg, 1973). Autoradiograms of the polyacrylamide slab gel electrophoretic patterns of [^{35}S]methionine-labeled polypeptides synthesized under the direction of total yeast RNA and poly(U)-cellulose fractionated poly(A)-containing mRNA are shown in Figure 3. The patterns are strikingly similar with only minor differences in the relative amount of each polypeptide synthesized. Within the detection limits of the translation assay, the translatable mRNAs of yeast bind to poly(U)-cellulose; there is not a significant redistribution of relative mRNA concentrations after poly(U)-cellulose chromatography. These data suggest strongly that the major yeast mRNAs contain poly(A).

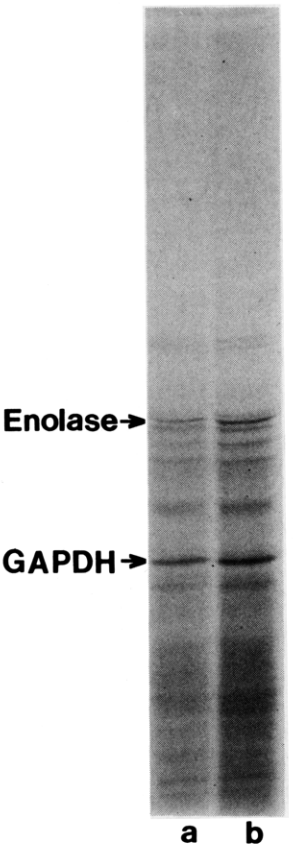

FIGURE 3: Autoradiogram of a sodium dodecyl sulfate–polyacrylamide slab gel of [^{35}S]methionine-labeled polypeptides synthesized in vitro in a wheat-germ cell-free extract in response to (a) total yeast RNA and (b) poly(U)-cellulose bound RNA. The mobilities of purified yeast enolase and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) markers are indicated by arrows.

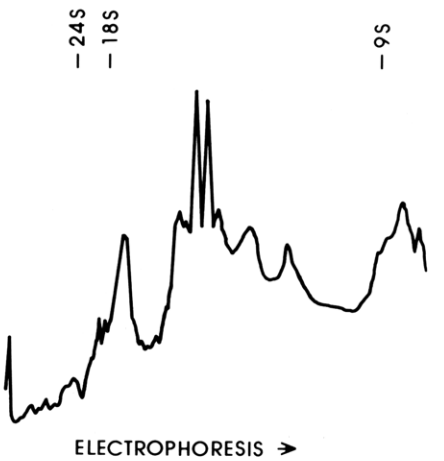

FIGURE 4: Formamide–polyacrylamide gel electrophoretic profile of RNA which bound poly(U)-cellulose under conditions of poly(A)-containing mRNA excess. Pyronine Y stained gels were scanned at 540 nm as described in Materials and Methods.

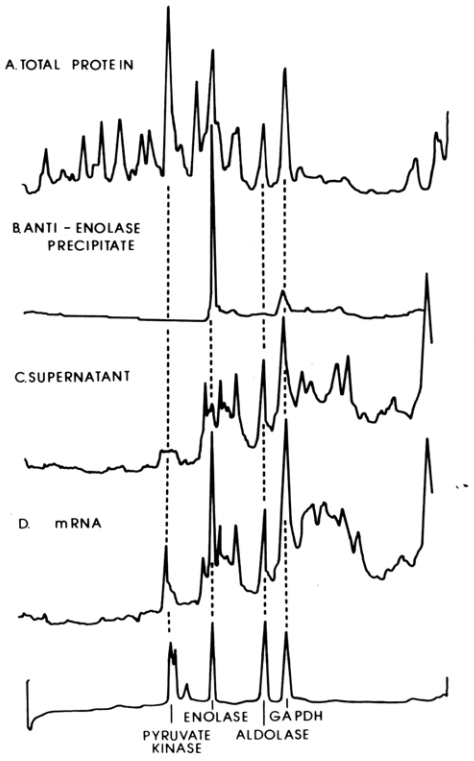

FIGURE 5: Densitometer tracings of autoradiograms of [^{35}S]methionine-labeled polypeptides synthesized in vivo and in vitro from yeast poly(A)-containing mRNA fractionated by sodium dodecyl sulfate–polyacrylamide electrophoresis. (A) Total [^{35}S]methionine-labeled yeast soluble polypeptides synthesized in vivo. (B) Anti-enolase immunoprecipitate of polypeptides synthesized in vitro from yeast poly(A)-containing mRNA. (C) Polypeptides synthesized in vitro from yeast poly(A)-containing mRNA. Purified yeast pyruvate kinase, enolase, aldolase, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) markers were stained with Coomassie blue and the polyacrylamide gel was scanned at 660 nm (bottom panel).

The two major polypeptides synthesized in both extracts shown in Figure 3 coelectrophorese with enolase and glyceraldehyde-3-phosphate dehydrogenase. These two glycolytic proteins make up 10–15% of yeast cellular proteins (Krebs, 1953; Westhead and McLain, 1964).

Preparation of Yeast Poly(A)-Containing mRNA Free of rRNA Contamination. The possibility that residual rRNA present in poly(A)-containing mRNA preparations binds to the poly(U)-cellulose column more weakly than free mRNA was tested by carrying out poly(U)-cellulose chromatography under conditions where the capacity of the column was exceeded by 10–20 times. Figure 4 shows a formamide gel electrophoretic analysis of the RNA which bound to the column under mRNA excess conditions. In contrast to the preparations described in Figure 2, there is little or no rRNA contamination in these preparations. This observation can be interpreted in two ways: that a fraction of rRNA forms a weak hybrid with the poly(U)-cellulose column and that excess free mRNA prevents these hybrids from being formed, or that a rRNA–mRNA complex has less affinity for poly(U) than free mRNA. Yeast mRNA prepared from overloaded poly(U)-cellulose columns can be resolved into a discrete number of species on polyacrylamide gels ranging in size from 7 to 18 S. Other mRNA species, that are either obscured by the major RNA's or are at relatively low concentration, may be present in the gel. Nevertheless it appears that these preparations of yeast mRNA are highly purified and contain predominantly a small number of RNA species.

Identification of the Major Polypeptides Synthesized in Vitro under the Direction of Highly Purified Yeast Poly(A)-Containing mRNA. In order to test the integrity of the isolated mRNA, highly purified yeast poly(a)-containing mRNA (free of rRNA contamination) was translated in a wheat-germ cell-free extract, and the polypeptides synthesized in vitro were compared with those synthesized in vivo. Figure 5, panel A, shows a densitometer tracing of an autoradiogram of a sodium

dodecyl sulfate–polyacrylamide gel of [^{35}S]methionine labeled proteins synthesized in vivo. Approximately 40 discrete polypeptides, of widely differing concentrations, were resolved. Panel D is a similar densitometer tracing of an autoradiogram of polypeptides synthesized in the wheat-germ cell-free extract under the direction of highly purified poly(A)-containing mRNA. There is a very good correspondence between the molecular weights of the polypeptides synthesized in vivo with those made in vitro. Quantitatively there is over-translation in vitro of polypeptides with molecular weights less than 30 000, and a corresponding under-production of polypeptides larger than 60 000. We attribute this anomaly to differential efficiency of protein synthesis in the wheat-germ extract as a function of polypeptide size rather than selective loss or degradation of large mRNAs. This conclusion is supported by the fact that, in the range of 30 000–60 000, there is very good qualitative and quantitative correlation of molecular weight and relative amount of polypeptides synthesized in vitro and in vivo. Four of the polypeptides synthesized in vitro and in vivo were identified by coelectrophoresis with glycolytic enzymes present at the designated percentages of cellular protein: pyruvate kinase (4–8%) (Hunsley and Suelter, 1969), enolase (5%) (Westhead and McLain, 1964), aldolase (2–5%) (Richards and Rutter, 1961), and glyceraldehyde-3-phosphate dehydrogenase (5–10%) (Krebs, 1953). The mobilities of the marker proteins (shown in the lower panel) correspond with the polypeptides synthesized in vitro (within the detection limits of the electrophoretic technique). These data do not rule out, however, the possibility that these proteins are synthesized in vitro as a slightly larger precursor molecule. Translation of enolase in vitro was further established by precipitation of newly synthesized enolase from the cell-free extract with purified IgG prepared against yeast enolase. Quantitative precipitation of newly synthesized enolase was achieved by incubating the cell-free extract with an excess of the anti-enolase IgG followed by incubation with an equivalent amount of antibody directed against the enolase IgG. Figure 5, panels B and C, show densitometer tracings of autoradiograms of the anti-enolase immune precipitate and the nonprecipitable portion of the extract, respectively. Enolase is the major species precipitated and is clearly depleted from the nonprecipitable portion of the extract. In panel C it appears that pyruvate kinase is also depleted along with enolase. This is due to the large amount of IgG heavy chain (unlabeled) present in the nonprecipitable extract which comigrates with pyruvate kinase and obscures the pyruvate kinase band. The fact that the principle polypeptides synthesized in vitro and in vivo correspond to the subunits of these glycolytic enzymes suggests that the messenger RNAs which code for these proteins are present at high intracellular concentration.

Preparation of DNA Complementary to Yeast Poly(A)-Containing mRNA. DNA complementary to yeast poly(A)-containing mRNA was synthesized by AMV reverse transcriptase acting on total yeast RNA or poly(U)-cellulose fractionated RNA. The reverse transcriptase reaction was carried out as described in Materials and Methods to ensure maximal copying of the mRNA. The size of the cDNA was determined by sedimentation in alkaline sucrose gradients and was similar for all three preparations. The sedimentation profile for cDNA synthesized from highly purified mRNA (free of rRNA) is shown in Figure 6a. The average size of the cDNA, 7–8 S, is smaller than the average size of the mRNA template (12 S); however, the broad distribution of cDNA size observed suggests that full copies of some of the yeast mRNAs were synthesized. The ability of this cDNA to form stable

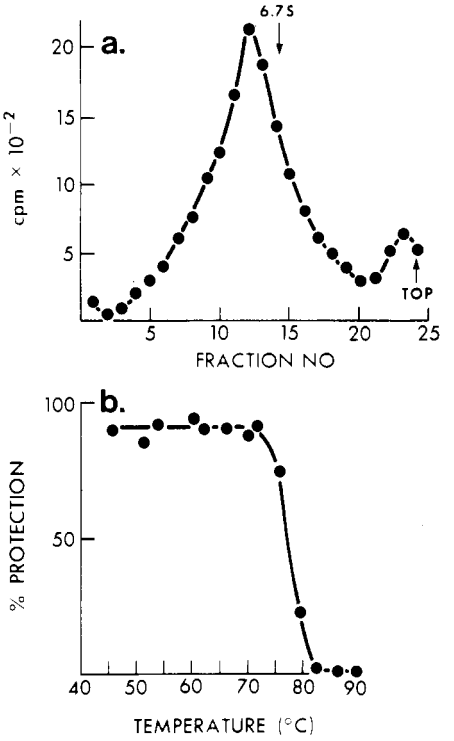

FIGURE 6: Physical properties of complementary DNA (cDNA) synthesized from yeast poly(A)-containing mRNA with reverse transcriptase. (a) Alkaline sucrose gradient sedimentation analysis of [^{3}H]cDNA. Gradients were centrifuged in a Beckman SW 50.1 at 35 000 rpm for 17 h at 20 °C. Complementary DNA was synthesized from yeast poly(A)-containing mRNA fractioned by poly(U)-cellulose chromatography under conditions which exceed the capacity of the column. (b) T_m analysis of hybrids formed between [^{3}H]cDNA and poly(A)-containing mRNA. Aliquots of the hybrid were incubated for 20 min at the indicated temperatures and the remaining hybrid was measured by digestion with S1 nuclease as described in Materials and Methods.

hybrids with yeast mRNA was determined by measurement of the T_m of the cDNA–mRNA hybrids. As shown in Figure 6B, the T_m of the hybrids, formed under the stringent hybridization conditions described in Materials and Methods, is 77 °C in 0.03 M NaCl. This high T_m value and the fact that 90% of the cDNA can be protected by mRNA against S1 nuclease digestion suggest a low level of mismatching in the hybrids. The cDNA should therefore be reliable for quantitative determinations of mRNA concentration and complexity.

Complexity Analysis of Yeast mRNA. In order to determine the relative sequence complexity of purified yeast poly(A)-containing mRNA, cDNA was hybridized with a vast excess of mRNA from which it was synthesized and the kinetics of hybridization were analyzed as described by Bishop (1972). Under RNA excess conditions, the rate of hybridization of each cDNA molecule is proportional to the concentration of mRNA which is complementary to it. Thus cDNAs which are complementary to mRNAs which are present at high concentration will hybridize more rapidly than those complementary to mRNAs present at low concentration. Figure 7 presents the hybridization curve obtained when cDNA prepared from yeast mRNA (isolated by poly(U)-cellulose chromatography under conditions which do not exceed the capacity of the column) is hybridized with mRNA from which it was synthesized. The hybridization curve is biphasic indicating that yeast mRNAs differ widely in relative concentration. Approximately 25% of the cDNA hybridized at a $C_rt_{1/2}$ of approximately 5×10^{-3} mol s l.$^{-1}$, while the remainder

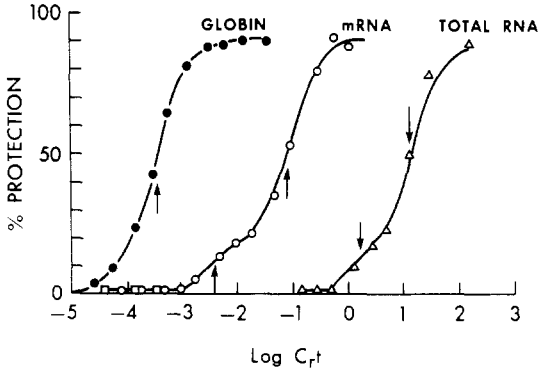

FIGURE 7: Complexity analysis of yeast poly(A)-containing mRNA. Kinetics of hybridization of [^{3}H]cDNA (0.2 ng) with 10–40 μg of poly(U)-cellulose fractionated mRNA from which it was synthesized (○). Kinetics of hybridization of [^{3}H]cDNA (0.2 ng) with 200–400 μg of unfractionated total yeast RNA (△). Kinetics of hybridization of globin [^{3}H]cDNA (0.2 ng) with 0.1–2 μg of purified globin mRNA (●). Hybridization reactions were carried out in a total volume of 50 μl at 68 °C as described in Materials and Methods.

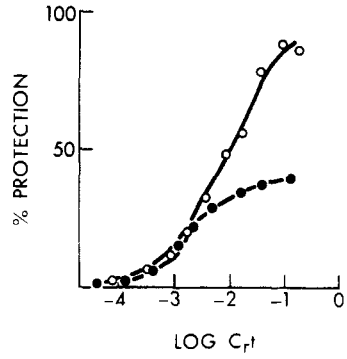

FIGURE 8: Complexity analysis of yeast poly(U)-cellulose bound mRNA isolated under conditions of mRNA excess. Poly(A)-containing mRNA was fractionated on poly(U)-cellulose under conditions which exceed the capacity of the column (see text) and hybridized with [^{3}H]cDNA synthesized from this RNA (○), or with [^{3}H]cDNA synthesized from total yeast RNA (●).

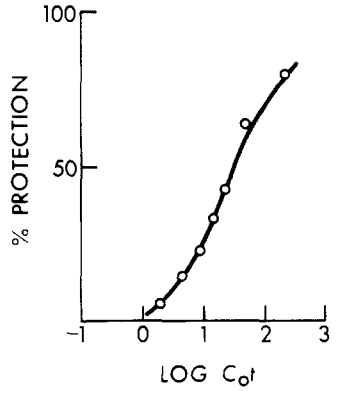

FIGURE 9: Kinetics of hybridization of [^{3}H]cDNA synthesized from total yeast RNA with yeast cellular DNA. Hybridization reactions contained 0.2 ng of [^{3}H]cDNA and 100 μg of sheared and denatured yeast DNA in a total volume of 400 μl. Hybridization was carried out at 65 °C under the conditions described in Materials and Methods.

hybridizes at a $C_rt_{1/2}$ of 10^{-1} mol s l.$^{-1}$. By comparing these apparent $C_rt_{1/2}$ values for yeast mRNA with that observed for the hybridization of chick globin cDNA with purified chick globin mRNA (5×10^{-4} mol s l.$^{-1}$), it is possible to estimate the complexity of the yeast mRNA preparation. Since the rapidly annealing fraction of yeast mRNA is 25% of the total mRNA, then the true $C_rt_{1/2}$ for the rapidly annealing fraction is approximately $5 \times 10^{-3}/4 = 1.2 \times 10^{-3}$. Thus the complexity difference between yeast mRNA and chicken globin is two- to threefold. Since the globin mRNA preparation is estimated to have a complexity of 800–900 nucleotides, the information in the rapidly annealing yeast mRNA fraction is sufficient to code for only a few polypeptides. The more slowly annealing fraction contains sufficient information for several hundred polypeptides. Assuming random copying of yeast poly(A)-containing mRNAs by reverse transcriptase, these data indicate that yeast contain a limited number of mRNA species which make up approximately 25% of the total poly(A)-containing mRNA population.

When cDNA was hybridized with an excess of unfractionated total yeast RNA, the same biphasic hybridization curve was observed (Figure 7). This curve is shifted to higher $C_rt_{1/2}$ values due to the presence of rRNA and tRNA in the total RNA extracts. By comparing the $C_rt_{1/2}$ values from the total RNA and purified RNA hybridization curves, we estimate that poly(A)-containing RNA comprises approximately 0.2% of total yeast cellular RNA. Hybridization curves identical with those shown in Figure 7 were obtained when cDNA prepared from unfractionated total yeast RNA was employed. Again 25% of the cDNA annealed at a $C_rt_{1/2} = 5 \times 10^{-3}$ mol s l.$^{-1}$ and the cDNA hybridized to 90% protection from S1 nuclease digestion. These data demonstrate that most, if not all, of the reverse transcribable mRNA binds to poly(U)-cellulose and that the relative concentration distribution of cellular poly(A)-containing mRNAs is not substantially altered after poly(U)-cellulose chromatography.

In contrast to the hybridization results described above, cDNA, synthesized from purified poly(A)-containing mRNA prepared by poly(U)-cellulose chromatography under conditions of mRNA excess, hybridized with the mRNA from which it was synthesized at a $C_rt_{1/2}$ value of 7×10^{-3} mol s l.$^{-1}$ (Figure 8). This lower $C_rt_{1/2}$ value suggests that this mRNA population is dramatically enriched in the most abundant cellular mRNA species. This conclusion is supported by the observation that mRNA prepared by this method protects cDNA synthesized from total yeast RNA to a maximal extent of 40% at $C_rt_{1/2}$ values in excess of 10^{-1} mol s l.$^{-1}$ (Figure 8). These data are not consistent with enrichment of a small number of mRNAs from the slowly annealing portion of yeast mRNA since the extent of hybridization of cDNA synthesized from total yeast mRNA is too high to be accounted for by a limited number of these mRNAs. Since the major proteins translated in response to this enriched fraction of yeast mRNAs are pyruvate kinase, enolase, aldolase, and glyceraldehyde-3-phosphate dehydrogenase, it is highly likely that the major glycolytic enzymes are coded for by mRNAs which are present at high intracellular concentration.

Titration of cDNA with Yeast DNA. In order to determine whether the sequences complementary to yeast poly(A)-containing mRNA are transcribed from the repeated or unique regions of the yeast genome, the kinetics of hybridization of cDNA synthesized from total yeast RNA with a vast excess of sheared and denatured DNA were analyzed (Figure 9). The curve is apparently monophasic with an observed $C_0t_{1/2}$ of 25 mol s l.$^{-1}$. This value is close to the expected value for renaturation of yeast DNA (Ogur et al., 1952). This suggests that most, if not all, of the poly(A)-containing mRNAs are transcribed from nonreiterated portions of the yeast genome.

Discussion

The complexity analysis of yeast poly(A)-containing mRNA described in this paper demonstrates marked differences in the intracellular concentration of yeast mRNA's. Approximately 25% of the isolated poly(A)-containing mRNA is coded for by less than 0.05% of the yeast genome. The intracellular concentration of the most abundant yeast mRNAs is significantly higher than the major mRNAs found in vegetative *Dictyostelium discoideum* (Verma et al., 1974), mouse L-cells (Ryffel and McCarthy, 1975a), and HeLa cells (Bishop et al., 1974). Based on the complexity of poly(A)-containing mRNA isolated from each of these cells, the most abundant mRNAs in these cells are present at five- to tenfold lower concentration than those found in yeast. In the case of L-cell mRNA it appears that the most abundant mRNAs are synthesized from the reiterated portion of the genome (Ryffel and McCarthy, 1975b). In contrast, yeast poly(A)-containing mRNAs appear to be synthesized from the nonreiterated portion of the DNA. Our observations indicate extraordinarily selective gene expression in vegetative yeast cells.

The estimate for the number of mRNAs present in yeast cells is a lower limit since mRNAs which do not contain poly(A) or are not reverse transcribed would not be reflected in this determination. This consideration may be significant since it has been suggested that a portion of yeast mRNA may not contain poly(A) (McLaughlin et al., 1973). In addition the relatively short stretches of poly(A) found in yeast mRNA (Groner et al., 1974) suggests that some species of yeast mRNA may be less efficiently reverse transcribed than others. Our data show that the major mRNAs of yeast do contain poly(A) since the polypeptides synthesized in response to either total cellular RNA or fractionated poly(A)-containing mRNA are qualitatively and quantitatively similar. This observation complements those of Gallis et al. (1975) who reported that fractionated yeast poly(A)-containing mRNA directs the synthesis in vitro of all of the major polypeptides found in the cell.

The possibility that the observed distribution of yeast poly(A)-containing mRNA concentrations results from an artificial redistribution of mRNA concentration during isolation is unlikely since such a redistribution should have been reflected in the translation of total yeast RNA vs. poly(A)-containing mRNA. The actual percentage of yeast poly(A)-containing mRNA made up by the most abundant mRNA species cannot be absolutely ascertained since the 25% value would be increased or decreased depending on whether or not these mRNAs were more or less efficiently reverse transcribed relative to other poly(A)-containing mRNAs.

An enrichment of the most abundant yeast poly(A)-containing mRNAs as well as removal of rRNA contamination was achieved by carrying out poly(U)-cellulose chromatography under conditions which exceed the capacity of the column. Ribosomal RNA and/or rRNA–mRNA aggregates are removed by this procedure presumably because they have a lower affinity for the column than free mRNA. The enrichment of the abundant mRNAs under these conditions remains unexplained but is probably related to differential affinity of yeast mRNAs for poly(U). Yeast mRNAs contain 20–60 residues of adenine (Groner et al., 1974) and this length distribution might be expected to give rise to differential stability of the poly(A)–poly(U) hybrids.

Pyruvate kinase, enolase, aldolase and glyceraldehyde-3-phosphate dehydrogenase messenger RNAs appear to be present at high intracellular concentration since these polypeptides are the major species synthesized in vitro under the direction of mRNA which is enriched in the most abundant poly(A)-containing species in the cell. Based on the predicted complexity of these mRNAs, they could comprise all of the rapidly annealing portion of poly(A)-containing mRNA. Since these are the most abundant proteins in the cell, these data suggest that their high cellular concentration is under transcriptional control. The high intracellular concentration of glycolytic mRNA in yeast will facilitate the isolation of each of these mRNAs in pure form and the subsequent synthesis of message-specific cDNAs. Acquisition of these materials will facilitate further studies on in vivo and in vitro transcription of these genes.

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Transcription of Yeast DNA by Homologous RNA Polymerases I and II: Selective Transcription of Ribosomal Genes by RNA Polymerase I†

Michael J. Holland,‡ Gordon L. Hager,§ and William J. Rutter*

ABSTRACT: Purified yeast DNA was transcribed by homologous RNA polymerases I and II and *Escherichia coli* RNA polymerase. Transcripts synthesized in vitro were analyzed by molecular hybridization with complementary DNA (cDNA) synthesized from yeast poly(A)-containing mRNA with viral reverse transcriptase and ribosomal DNA labeled in vitro by nick translation with *E. coli* DNA polymerase I. RNA synthesized by polymerase I and II in the presence of Mn^{2+} contained sequences complementary to cDNA and rDNA at a frequency consistent with random transcription of the template. Similarly, *E. coli* RNA polymerase synthesized an apparently random transcript in the presence of either Mn^{2+} or Mg^{2+}. In contrast to these results, RNA polymerase I but not polymerase II transcripts were markedly enriched in sequences complementary to rDNA when transcription was carried out in the presence of Mg^{2+}. The observed enrichment was 15–30-fold higher than observed for polymerase II or *E. coli* polymerase transcripts and is consistent with the transcript being comprised of 6–10% ribosomal sequences. These data strongly suggest that RNA polymerase I plays a critical role in selective transcription of ribosomal cistrons.

Eucaryotic cells contain multiple forms of RNA polymerase which differ in subunit structures, cellular location, and sensitivity to the toxin α-amanitin. Utilizing the differential sensitivity of the eucaryotic polymerases to α-amanitin, it was shown in isolated nuclei that polymerase I synthesizes ribosomal RNA (Blatti et al., 1970; Reeder and Roeder, 1972), polymerase II synthesizes heterogeneous nuclear RNA (Egyhazi et al., 1972; Blatti et al., 1970) and certain viral mRNAs (Price and Penman, 1972; Wallace and Kates, 1972), and polymerase III is involved in the synthesis of 5S rRNA and tRNA (Weinmann and Roeder, 1974). These results suggest that the eucaryotic polymerases like the bacterial enzymes may play a role in selective transcription of the eucaryotic genome.

Attempts to demonstrate selective transcription with purified polymerases and defined templates have not yielded convincing evidence concerning the ability or inability of the enzymes to specifically transcribe a eucaryotic gene. Polymerase I and II symmetrically transcribe purified *Xenopus laevis* ribosomal cistrons (Roeder et al., 1970); however, this result may have been due to a template of insufficient size. It has been reported that polymerase I synthesizes a greater amount of ribosomal sequences than polymerase II when high-molecular-weight *X. laevis* nucleolar DNA is used (Beebee and Butterworth, 1974a,b). Hollenberg (1973) reports that yeast polymerase I does not preferentially transcribe ribosomal cistrons in purified cellular DNA, while Cramer et al. (1974) report that there is strand selectivity by polymerase I when γ DNA, a fraction of yeast DNA highly enriched in ribosomal cistrons, is transcribed. In the latter study the degree of strand selectivity is not pronounced and the actual amount of ribosomal RNA synthesized by each polymerase was not determined. The results of studies utilizing chromatin as template have also been equivocal. Both polymerase I and II symmetrically transcribe ribosomal and 5S ribosomal cistrons in *X. laevis* (Honjo and Reeder, 1974). Using strand selectivity as the only criterion for selective transcription, the authors conclude that the isolated polymerases play no role in selective transcription. Transcription of reticulocyte chromatin by polymerase II resulted in a twofold higher production of globin mRNA sequences than synthesized by *E. coli* polymerase (Steggles et al., 1974).

The inability to convincingly demonstrate selective transcription with the eucaryotic polymerases is related to observations on the activity of the isolated eucaryotic polymerase

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Abbreviations used: rDNA and cDNA, DNA enriched in ribosomal cistrons and complementary DNA, respectively; rRNA, mRNA, and tRNA, ribosomal, messenger, and transfer RNA, respectively; poly(U), poly(uridylic acid); EDTA, ethylenediaminetetraacetic acid; DEAE, diethylaminoethyl; Tris, tris(hydroxymethyl)aminomethane.