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

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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.

on various DNA templates. It has been suggested in a number of studies that the eucaryotic polymerases do not initiate on double-stranded DNA but rather prefer nicks, gaps, and single-stranded regions in the DNA as initiation points (Mandel and Chambon, 1974a,b; Dezelee et al., 1974). Thus the observation that polymerases symmetrically transcribe templates and randomly transcribe cellular DNA can be explained on the basis of polymerases initiating at random sites in the template which are artificially generated during isolation. This conclusion is valid only if the polymerases which have been isolated are in fact the transcriptionally active forms present in the cell. There are also technical problems associated with quantitating the transcription of a gene or group of genes from genetically complex eucaryotic DNA. Many of the assays employed have involved hybridization competition utilizing filter-bound DNA that contains both repeated and nonrepeated sequences. Quantitative analysis of the transcription of a given gene by this method would obviously require careful monitoring of the efficiency of hybridization of both transcripts from repeated and nonrepeated sequences in the template. This problem is compounded in cases where genes are transcribed symmetrically since quantitative interpretation of hybridization kinetics requires that sequences in filter-bound DNA which are complementary to the transcripts be in excess.

To facilitate quantitative measurement of the in vitro synthesis of specific sequences from highly complex eucaryotic DNA, we have studied transcription of yeast DNA. This DNA is only three to five times more complex than the *E. coli* genome, allowing the measurement of in vitro transcription of unique genes in total cellular DNA. Hybridization assays were carried out with single-stranded DNA probes which are complementary to yeast 24S and 18S ribosomal RNA and yeast poly(A)-containing mRNA. The hybridization reactions take place in solution with a vast excess of transcript, allowing the quantitative measurement of the proportion of each transcript which contains mRNA and rRNA sequences. Utilizing this assay we have determined that, in the presence of Mg^{2+}, polymerase I alone is capable of preferential transcription of ribosomal cistrons in vitro.

Materials and Methods

Materials. Calf thymus DNA (grade I), α-amylase (type IV), and phenylmethylsulfonyl fluoride were purchased from Sigma Chemical Co. Nucleotide triphosphates and poly(U) were obtained from P-L Biochemicals Inc. Dithiothreitol (A grade) was from Calbiochem. Hydroxylapatite, HTP, and agarose A-5m were purchased from Bio-Rad Laboratories. Ammonium sulfate (enzyme grade) and [^{3}H]UTP (30 Ci/mmol) were from Schwarz/Mann. Pancreatic RNase and DNase I were obtained from Worthington. [^{3}H]dCTP (23 Ci/mmol) and [^{32}P]UTP (180 Ci/mmol) were purchased from New England Nuclear. *E. coli* DNA polymerase I was prepared according to Jovin et al. (1969). *E. coli* K12 and B were purchased from Grain Processing Corp., Muscatine, Iowa.

Preparation of RNA Polymerases. RNA polymerases were isolated from mid-log-phase yeast (*Saccharomyces cerevisiae,* strain F1) obtained from Red Star Corp. (Division of United Foods), Oakland, Calif. Cells were washed twice in water and suspended in a solution containing final concentrations of 50 mM Tris, pH 7.9 (4 °C), 0.1 mM EDTA, 5 mM $MgCl_2$, 0.5 mM dithiothreitol, 1% dimethyl sulfoxide, 0.5 mM phenylmethylsulfonyl fluoride, and 10% glycerol. The wet weight concentration of cells in the suspension was 85 g/100 ml. The cell suspension was frozen in small pellets by dripping the suspension into liquid nitrogen. Frozen cells were broken in a 200-ml capacity Eaton press at 9000 psi. Disrupted cells were suspended in an equal volume of buffer A (50 mM Tris, pH 7.9, 0.1 mM EDTA, 5 mM $MgCl_2$, 0.2 mM dithiothreitol, 10% glycerol), containing 1% dimethyl sulfoxide, 0.5 mM phenylmethylsulfonyl fluoride, and 0.7 M $(NH_4)_2SO_4$, sonicated for 30 min at a power input of 100 W to reduce viscosity, and centrifuged at 10 000*g* for 20 min to remove cell debris. The supernatant was adjusted to 0.1 M $(NH_4)_2SO_4$ final concentration by addition of 2.5 volumes of buffer A. This suspension was adjusted to 0.25% protamine sulfate by addition of 2% protamine sulfate and the precipitate which formed was removed by centrifugation at 10 000*g* for 10 min. The clear supernatant was adjusted to 80% of saturation with solid $(NH_4)_2SO_4$ and the precipitate containing RNA polymerases was removed by centrifugation at 10 000*g* for 30 min. The precipitate was suspended in buffer A to a final volume of 600 ml (1.5 kg preparation). The suspended $(NH_4)_2SO_4$ precipitate was chromatographed on a 55 × 10 cm column of agarose A-5m equilibrated with buffer A containing 0.1 M $(NH_4)_2SO_4$. Fractions containing polymerase activity were pooled and adjusted to 82% of saturation with solid $(NH_4)_2SO_4$ and the precipitate was allowed to form at 4 °C overnight. After centrifugation at 10 000*g* for 30 min, the precipitate was dissolved in buffer A. The final ammonium sulfate concentration was adjusted to 40 mM by rapid dialysis in a Bio-Rad Diaflo apparatus against buffer B (50 mM Tris, pH 7.9, 0.1 mM EDTA, 5 mM $MgCl_2$, 0.2 mM dithiothreitol, 25% glycerol). The dialyzed extract was chromatographed on a 5 × 75 cm column of DEAE-Sephadex A-50 and eluted with a 6-l. gradient containing buffer B and a 0.05–0.75 M $(NH_4)_2SO_4$ gradient. Fractions containing either polymerase I or II were pooled and concentrated in an Amicon concentrator over an XM-50 membrane until the optical density at 280 nm was greater than 1.0 unit/ml. The pools were then precipitated by dialysis against saturated $(NH_4)_2SO_4$ at 4 °C. The active precipitates were recovered by centrifugation at 10 000*g* for 30 min, suspended in buffer B, dialyzed in the Bio-Rad DiaFlo apparatus to 25 mM $(NH_4)_2SO_4$ and bound to a 10 × 2 cm column of DNA-cellulose prepared by the method of Litman (1968). After washing each column with suspension buffer, the enzymes were eluted with 0.75 M $(NH_4)_2SO_4$. Each enzyme fraction was concentrated by ultrafiltration over an XM-50 membrane in an Amicon 200 concentrator. Enzyme was stored in aliquots at −176 °C in buffer B. The subunit compositions of RNA polymerases I and II prepared by this procedure were analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (Figure 1). Gels contained 9% acrylamide and electrophoresis was carried out as described by Hager et al. (1976). RNA polymerase II was completely sensitive to α-amanitin (50 μg/ml), indicating that the preparation is not contaminated with either RNA polymerase I or III. Preparations of RNA polymerase I and II were analyzed for the presence of RNA polymerase III by chromatography on DNA-cellulose with a linear KCl gradient as described by Hager et al. (1976). Neither preparation contained RNA polymerase activity which cochromatographed with RNA polymerase III further suggesting that the preparations are free of RNA polymerase III activity.

Routine assays of yeast RNA polymerases I and II were carried out at 30 °C in 75-μl reaction mixtures containing 50 mM Tris buffer, pH 7.6 (30 °C), 2 mM $MnCl_2$, 0.6 mM GTP, CTP, and ATP, 6 mM NaF, 0.5 mM dithiothreitol, 50 mM $(NH_4)_2SO_4$, 50 μg/ml each of denatured and native calf thymus DNA, and 0.01 mM [^{3}H]UTP (specific activity 1

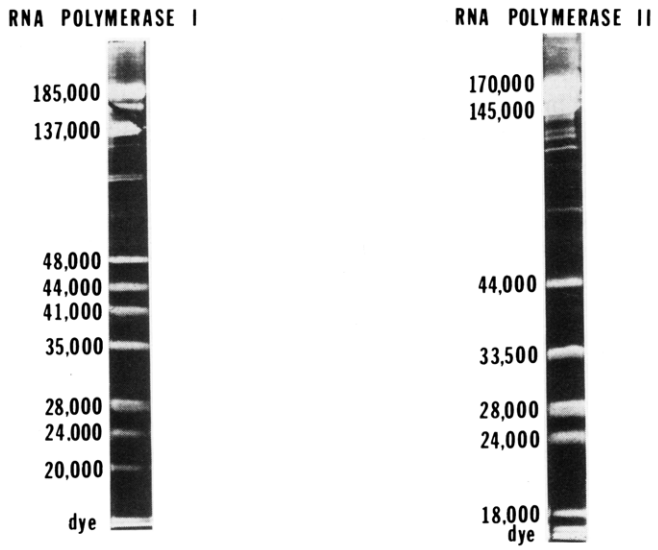

FIGURE 1: Subunit composition of yeast RNA polymerases I and II. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis was carried out in 9% acrylamide as described by Hager et al. (1976).

mCi/mmol). For polymerase specific activity measurements, 0.1 mM UTP was included in the assay. Assays employing yeast native DNA contained 80 μg/ml yeast DNA and either 2 mM $MnCl_2$ or 10 mM $MgCl_2$. One unit of polymerase activity is defined as the incorporation into RNA of 1 nmol of UMP in 10 min under the reaction conditions described above.

Deoxyribonuclease activity in polymerase preparations was determined by incubating aliquots of the enzymes with labeled supercoiled DNA and measuring conversion of supercoils (form I) to open circles (form II). Since this conversion requires only a single-strand nick in the DNA, this represents a very sensitive assay for endonuclease activity.

E. coli holo RNA polymerase was prepared by the method of Burgess (1969) with the modification that the DEAE-cellulose column was replaced by a DEAE-Sephadex A-25 with a 0.1–0.75 M linear KCl gradient.

Preparation of Yeast DNA. Early log phase yeast cells (*S. cerevisiae*) from Red Star Corp. were washed two times in water and suspended in buffer at a final concentration of 50 mM Tris, pH 7.5, 10 mM EDTA, 10% glycerol, and 85 g of cells (wet weight) per 100 ml of solution. The cell suspension was frozen in liquid nitrogen as described previously for the enzyme preparation. The cells were disrupted in a 200-ml capacity Eaton press at 9000 psi. The broken cells were suspended in 2 volumes of 50 mM Tris, pH 7.5, 10 mM EDTA, 0.1 M NaCl and centrifuged at 10 000*g* for 10 min. The pellet containing the nuclear material was suspended in twice the centrifuged volume in 50 mM Tris, pH 7.5, and 10 mM EDTA. To this suspension was added an equal volume of 5% sodium dodecyl sulfate followed by 2 volumes of phenol saturated with buffer containing 10 mM Tris, pH 7.5, and 1 mM EDTA. The mixture was gently swirled for 20 min at room temperature and finally centrifuged at 10 000*g* for 10 min. The aqueous fraction was then extracted with an equal volume of chloroform–isoamyl alcohol (24:1, v/v) for 20 min and centrifuged at 10 000*g* for 10 min and the aqueous phase was reextracted with Tris–EDTA buffered phenol as described above. The aqueous phase was overlayered with 2 volumes of 95% ethanol and yeast DNA was slowly wound out with a large glass rod. The DNA was dissolved in 0.1 mM EDTA without stirring. The DNA solution was adjusted to 10 mM Tris, pH 7.4, and 200 μg/ml of RNase (boiled for 10 min to inactivate DNase) and incubated at 37 °C for 1–2 h. A trace amount of labeled [^{3}H]rRNA (specific activity 20 000 cpm/μg) was included in the digestion and trichloroacetic acid precipitability of this [^{3}H]rRNA was used to monitor progress of the reaction. After RNase digestion the extract was adjusted to 0.5% sodium dodecyl sulfate and 20 μg/ml proteinase K and incubated at 37 °C for 1 h. After digestion the extract was adjusted to 5 mM $MgCl_2$ and 10 mM $(NH_4)_2SO_4$ and then extracted with an equal volume of Tris–EDTA buffered phenol. Addition of Mg^{2+} and $(NH_4)_2SO_4$ forms insoluble salts of polyphosphate. Polyphosphate has been shown to be a potent inhibitor of the template activity of yeast DNA (Ponta et al., 1974). Purified yeast DNA was dialyzed extensively against buffer containing 10 mM Tris, pH 7.4, and 0.1 mM EDTA and stored at 0 °C.

The molecular weight of the yeast DNA was determined by sedimentation velocity analysis in alkali in a Model E Beckman ultracentrifuge equipped with scanning optics. The average single-strand molecular weight of the purified DNA is approximately 10×10^6.

In Vitro Synthesis of RNA. RNA was synthesized in vitro at 30 °C in reaction mixtures containing 50 mM Tris, pH 7.6, 10 mM $MgCl_2$ or 2 mM $MnCl_2$, 6 mM NaF, 0.1 mM dithiothreitol, 0.1 mM EDTA, 50 mM $(NH_4)_2SO_4$, 0.3 mM each of ATP, GTP, CTP, and UTP, [^{32}P]UTP at a final specific activity of 2000 cpm/nmol in the reaction mixture, yeast DNA, and RNA polymerase. RNA was synthesized in vitro at fixed ratios of RNA polymerase to DNA. Transcripts for cDNA titrations were synthesized at RNA polymerase to yeast DNA ratios of 1:10 (w/w). For rDNA titrations synthesis was carried out at polymerase to DNA ratios of 1:100 (w/w). These ratios were chosen to prevent saturation of the DNA by RNA polymerase. With a tenfold mass excess of DNA over RNA polymerase, there is less than one polymerase molecule per yeast gene. At a 100-fold mass excess of DNA there are approximately ten polymerase molecules per ribosomal cistron. The specific activities of yeast RNA polymerases I and II varied with the preparation and a range of values is given in Table I. The specific activity of the *E. coli* RNA polymerase using yeast DNA as template was 150–300 units/mg in the presence of either Mg^{2+} or Mn^{2+}. RNA synthesis was linear for 20 min. After 20–30 min reaction mixtures were adjusted to 50 mM sodium acetate, pH 5.0, 1 mM EDTA, 5 μg/ml poly(U) carrier, 0.1 M NaCl, and 0.5% sodium dodecyl sulfate (the final solution was titrated to pH 5.0 with 1 N acetic acid). The reaction mixtures were then extracted with an equal volume of phenol (buffered with 50 mM sodium acetate, pH 5.0, 1 mM EDTA, and 0.1 M NaCl) with vigorous shaking at 55 °C for 10 min followed by rapid cooling to 4 °C. After centrifugation at 10 000*g* for 20 min, most of the yeast DNA template was present at the interface and was discarded. The aqueous phase was extracted with ether to remove phenol and adjusted to pH 7 with 1 M Tris, pH 9.0, diluted with 3 volumes of water to lower the ionic strength, adjusted to 10 mM $MgCl_2$ and 20 μg/ml RNase-free DNase, and finally incubated for 30 min at 30 °C. The mixture was reextracted with Tris–EDTA buffered phenol and precipitated with 2 volumes of ethanol at −20 °C. The large precipitate which was comprised mostly of nucleotides was collected by centrifugation at 15 000*g* for 5 min and suspended in the smallest possible volume of 1 mM EDTA. Nucleotides were removed by chromatography on Sephadex G-25 equilibrated in 1 mM EDTA. Newly synthesized RNA, present in the void volume of the column, was reprecipitated with 2 volumes of ethanol at −20

°C. The amount of RNA synthesized and recovered after purification was calculated from the amount of trichloroacetic acid precipitable [^{32}P]UMP present. Recoveries of 80% of the RNA synthesized were routinely achieved.

Preparation of 24S and 18S rRNA. Yeast 24S and 18S rRNAs were isolated from phenol extracted total yeast cellular RNA isolated as described previously (Holland et al., 1976). Poly(A)-containing mRNA was removed by chromatography on poly(U)-cellulose. Yeast 24S and 18S rRNA which do not bind the poly(U)-cellulose column were purified by density gradient centrifugation. Sucrose gradients (15–30%) containing 0.1 M NaCl and 1 mM EDTA were centrifuged in a Beckman SW 41 rotor at 35 000 rpm for 16 h at 4 °C.

Isolation of [^{3}H]rDNA. High-molecular-weight yeast DNA isolated as described above was sheared at 1000 psi in a French pressure cell to an average of 2000 base pairs. In order to obtain a fraction of DNA which is enriched in ribosomal cistrons, the sheared DNA was heat denatured and partially reannealed in 0.12 M potassium phosphate, pH 6.8, and 0.1 mM EDTA at 65 °C. Since ribosomal cistrons are repeated in yeast DNA (Schweizer et al., 1969), partial renaturation would give an enrichment in these repeated sequences. The extent of reannealing was measured by the ability of the DNA to bind to hydroxylapatite (1-ml columns equilibrated in 0.12 M sodium phosphate, pH 6.8) at 60 °C. A $C_0t_{1/2}$ = 0.1 mol s l.$^{-1}$ was observed for 10% annealing of these rather large fragments of yeast DNA. Preparative renaturation reactions were adjusted to 0.075 M potassium phosphate by diluting with water and applying to a hydroxylapatite column (20 μg of DNA per g of hydroxylapatite) equilibrated in 0.075 M potassium phosphate pH 6.8 at 40 °C. Single-stranded DNA was eluted from the column by increasing the column temperature to 50 °C and washing the 0.075 M potassium phosphate, pH 6.8. Double-stranded DNA was removed from the column by elution with 0.15 M potassium phosphate, pH 6.8, at 50 °C. The double-stranded DNA, which is enriched in ribosomal cistrons, was labeled by nick translation with *E. coli* DNA polymerase I (Rigby, P., Dieckmann, M., and Berg, P., manuscript in preparation). Nick translation reactions were carried out at 14 °C in 139-μl volumes containing 50 mM sodium phosphate, pH 7.4, 5 mM $MgCl_2$, 15 μM each of dATP, dGTP, dTTP, 50 μCi of [^{3}H]dCTP (25 Ci/mmol), 2 μg of reannealed yeast DNA, 0.5 ng of pancreatic DNase I, and 3 μg of *E. coli* DNA polymerase I. Reactions were terminated after 3–4 h at approximately 75% of maximum labeling by adjusting the mixture to 75 mM EDTA and heating at 68 °C for 10 min. Unincorporated nucleotides were removed by chromatography on a 0.5 × 10 cm Sephadex G-50 column equilibrated in 0.075 M potassium phosphate, pH 6.8. Labeled DNA was heat denatured and single-stranded and double-stranded DNA were separated on hydroxylapatite as described above. Approximately 5% of the nick translated DNA was nondenaturable and presumably contains intramolecular self-complementary sequences. The single-stranded DNA obtained by hydroxylapatite chromatography was hybridized with a 10 000-fold mass excess of purified 24S and 18S rRNA to a C_rt = 1 in the presence of 0.12 M potassium phosphate and 0.1 mM EDTA at 70 °C. The resultant RNA–DNA duplex was separated from single-stranded [^{3}H]DNA on hydroxylapatite by the same procedure described above. Free rRNA and rRNA–DNA duplexes elute with the 0.15 M potassium phosphate wash and resolve completely from single-stranded DNA. The mixture of rRNA and rRNA–DNA duplex was boiled for 10 min in 0.33 N NaOH to hydrolyze RNA, neutralized, and chromatographed on a Sephadex G-50 column equilibrated in 10 mM Tris, pH 7.4, and 0.1 mM EDTA. [^{3}H]rDNA was precipitated with 2 volumes of ethanol in the presence of 10 μg of poly(U) carrier at −20 °C. The size of the [^{3}H]rDNA probe was determined by sedimentation in alkaline sucrose gradients as described previously (Holland et al., 1976).

TABLE I: Activity of Yeast RNA Polymerases I and II.[a]

	Sp. Act.	
	Polymerase I	Polymerase II
Native calf thymus DNA (Mn^{2+})	50–150	100–200
Native yeast DNA (Mn^{2+})	18–55	7–14
Native yeast DNA (Mg^{2+})	11–38	2–4

[a] RNA polymerase specific activity is expressed as nanomoles of UMP incorporated per milligram of protein in 10 min under the assay conditions described in Materials and Methods.

Hybridization Conditions. All of the hybridization reactions were carried out in solutions containing 10 mM Tris, pH 7.5, 0.3 M NaCl, 1 mM EDTA, and 0.25% sodium dodecyl sulfate. Reaction mixtures were sealed in capillary tubes and incubated at the indicated temperatures: [^{3}H]cDNA vs. RNA, 68 °C; [^{3}H]rDNA vs. DNA, 68 °C; and [^{3}H]rDNA vs. RNA, 72 °C. Hybrid formation was monitored with single-strand specific S1 nuclease as previously described (Holland et al., 1976).

Results

Template Activity of Yeast RNA Polymerases I and II. Yeast RNA polymerases I and II prepared as described in the preceding paper (Hager et al., 1976) transcribe native yeast DNA with very low efficiency even though their activity on denatured calf thymus DNA is similar to that reported for most eucaryotic polymerases. The activities of yeast polymerases I and II prepared by the modified procedure described in Materials and Methods are shown in Table I. Yeast polymerase II activity is still quite low on native yeast DNA in the presence of either Mg^{2+} or Mn^{2+} and is similar to the activities reported in the previous paper (Hager et al., 1976). The activity of polymerase I in Mg^{2+}, however, is greater than tenfold higher than that of the previous preparations. DNA saturation curves in the presence of Mg^{2+} for polymerases I and II from the modified procedure are shown in Figure 2. RNA polymerase I activity does not plateau even at the highest DNA concentration tested. Polymerase II, however, reaches maximal activity at very low DNA concentration (5 μg/ml). These data suggest that there are fewer sites in the DNA from which polymerase I can productively initiate RNA synthesis than there are for RNA polymerase II. RNA polymerase I prepared as discussed in the previous paper (Hager et al., 1976) reaches maximal activity at 5–10 μg/ml yeast DNA and is inhibited as the DNA concentration is increased. The structural basis for this altered RNA polymerase I activity is at present not understood.

Analysis of mRNA Sequences in RNA Synthesized by Polymerases I and II from Yeast DNA. Complementary DNA, synthesized from total yeast poly(A)-containing mRNA (Holland et al., 1976), was titrated with poly(A)-containing mRNA and RNA synthesized by yeast polymerases I and II on yeast DNA in the presence of Mn^{2+} in order to determine the relative number and amount of cellular mRNA sequences synthesized in vitro (Figure 3). The titration curve of cDNA with in vivo synthesized mRNA (Holland et al., 1976) is distinctly biphasic, approximately 25% of the mRNA hybridizing at an apparent $C_rt_{1/2}$ of 0.005 mol s l.$^{-1}$, and the remainder

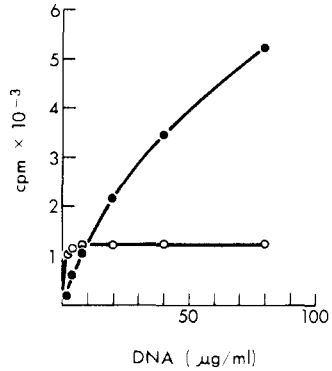

FIGURE 2: Saturation of purified yeast RNA polymerase I (●) and RNA polymerase II (○) with high-molecular-weight native yeast DNA. Assays were carried out in the presence of Mg^{2+} and saturating nucleotide triphosphates and terminated at 10 min as described in Materials and Methods.

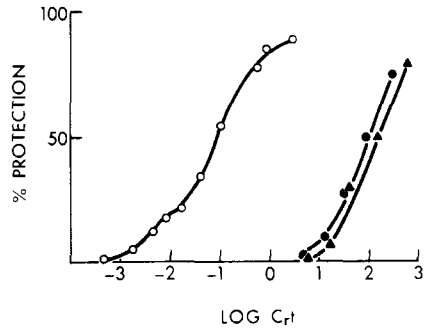

FIGURE 3: Hybridization of yeast cDNA with purified poly(A)-containing yeast mRNA (○). RNA synthesized in vitro from native yeast DNA by RNA polymerase II (●), and RNA synthesized in vitro from native yeast DNA by RNA polymerase I (▲). Hybridization reactions contained 0.2 ng of [^{3}H]cDNA and either 10–40 μg of purified poly(A)-containing yeast mRNA or 40 μg of in vitro synthesized RNA in a total volume of 5 μl. Hybridization conditions and analysis of hybrid function were as described in Materials and Methods.

at an average $C_rt_{1/2}$ of 0.1 mol s^{-1}. In contrast, the hybridization curves obtained with the in vitro synthesized RNAs are monophasic, with a $C_rt_{1/2}$ of 100 mol s l.$^{-1}$ for the polymerase I product and a $C_rt_{1/2}$ of 80 mol s l.$^{-1}$ for the polymerase II product. Thus, the distribution of mRNA species present in the growing cell is not reproduced in the in vitro transcripts, in terms either of qualitative distribution or quantitative concentration. Essentially, all of the cDNA was protected by the in vitro synthesized RNAs, indicating that most, if not all, of the sequences complementary to the cDNA are synthesized in vitro. The fact that the hybridization curves are monophasic suggest, however, that all of the sequences complementary to cDNA are present at the same relative concentration in the in vitro transcripts. Furthermore, given an observed $C_0t_{1/2}$ of 25 mol s l.$^{-1}$ for titration of cDNA with an excess of denatured total yeast cellular DNA (Holland et al., 1976), and the fact that the rate of hybridization of cDNAs with DNA is two- to sixfold more rapid than with mRNA (Verma et al., 1974; Bishop, 1972), the $C_rt_{1/2}$'s for the in vitro RNA products indicate that sequences complementary to cDNA in these transcripts are present at a frequency consistent with random transcription of yeast DNA. This observation is in marked contrast with the several thousand fold higher concentration of these sequences in cellular poly(A)-containing mRNA.

Preparation and Characterization of a cDNA Probe Specific for Ribosomal RNA Sequences. Preparation of [^{3}H]rDNA. The cDNA hybridization assay is useful for

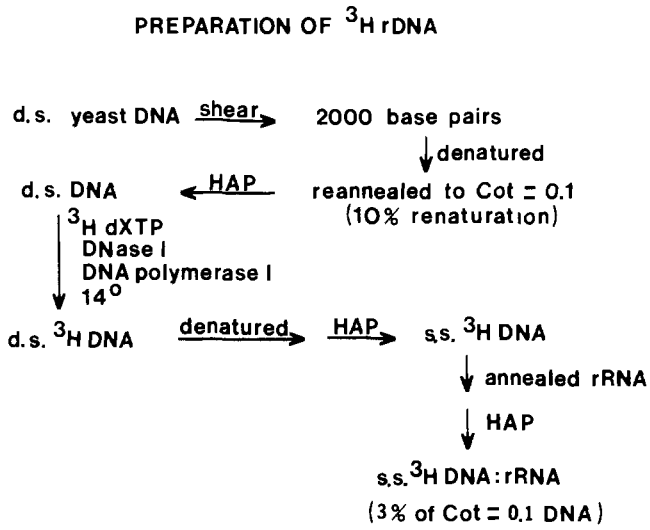

FIGURE 4: Schematic representation of the procedure for the in vitro labeling and isolation of yeast rDNA.

quantitatively measuring the proportion of a specific population of mRNA sequences in a mixture of RNAs. In order to make similar measurements of the amount of rRNA sequences synthesized in vitro, single-stranded rDNA (complementary to 24S and 18S rRNA) labeled to high specific radioactivity was isolated. Figure 4 shows a schematic representation of the procedure used for the labeling and isolation of yeast rDNA. Yeast DNA was first sheared to large-sized pieces to minimize the amount of nondenaturable DNA synthesized during the nick translation reaction. Sheared DNA was denatured and reannealed to 10% renaturation in order to enrich for ribosomal cistrons which are repeated in yeast DNA. Renatured DNA was isolated by thermal chromatography on hydroxylapatite and labeled by nick translation with *E. coli* DNA polymerase I in the presence of DNase I as described in Materials and Methods. Utilizing [^{3}H]dCTP (30 Ci/mmol) yeast DNA was labeled to a specific activity of 2–3 × 10^6 cpm/μg. This specific activity corresponds to 16–20% mass copy of the template by DNA polymerase I. A portion of the labeled DNA (approximately 5%) was nondenaturable, presumably because it contains intramolecular self-complementary sequences. Nick translated DNA was, therefore, denatured and the nondenaturable portion of the labeled DNA was removed by hydroxylapatite chromatography. Single-stranded labeled DNA was hybridized with a vast excess (10 000-fold) of purified 24S and 18S rRNA to a C_rt value of 1.0 mol s l.$^{-1}$. These conditions were chosen to avoid self-annealing of the DNA or hybridization of mRNA contaminants in the rRNA with the labeled DNA. The rRNA–DNA duplex was isolated by thermal chromatography on hydroxylapatite as described in Materials and Methods. Under these conditions, rRNA–DNA duplex and free rRNA elute together but are completely resolved from single-stranded DNA. Approximately 3% of the reannealed fraction hybridized with rRNA, resulting in an overall yield of 0.3% of total yeast DNA complementary to mature 24S and 18S rRNA. Given a combined complexity of 6600 nucleotide pairs for 24S and 18S rRNA (Udem and Warner, 1972) and a total complexity of 17 × 10^6 base pairs for the total haploid yeast genome (Ogur et al., 1952), the amount of rDNA isolated by this procedure corresponds to a copy number of 15 for ribosomal cistrons, as compared with a copy number of 30 derived below. The recovery of ribosomal-specific sequences, therefore, is estimated to be about 50%.

Size of [^{3}H]rDNA and T_m Analysis. The size of the

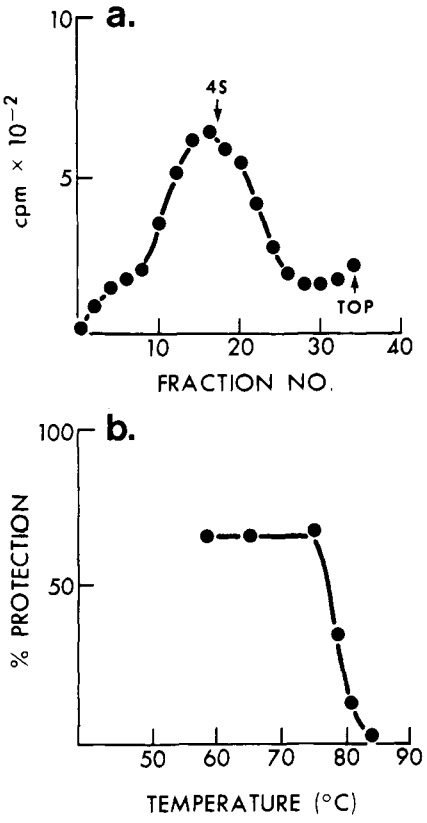

FIGURE 5: Physical properties of yeast [^{3}H]rDNA. (a) Alkaline sucrose gradient sedimentation analysis of [^{3}H]rDNA. Gradients containing 5–20% sucrose, 0.1 M NaOH, and 0.9 M NaCl were centrifuged in a Beckman SW 65T rotor at 40 000 rpm for 20 h at 20 °C. (b) T_m analysis of hybrids formed between [^{3}H]rDNA and yeast ribosomal RNA. Aliquots of hybrid formed between [^{3}H]rDNA and a vast excess of purified yeast 24S and 18S rRNA 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.

[^{3}H]rDNA probe was determined by sedimentation analysis in alkaline sucrose gradients (Figure 5). The average size of the rDNA fragments is 4–5 S (200 nucleotides). This value for rDNA size was observed when the nick translation reaction was terminated at 75% of maximal copying.

The T_m of hybrids formed between rRNA and [^{3}H]rDNA under the conditions described in Materials and Methods is 78 °C in 0.03 M NaCl. The high thermal stability of hybrids formed with this probe indicates that, like cDNA synthesized with reverse transcriptase, the [^{3}H]rDNA is a faithful copy of ribosomal sequences. The rDNA is protected against S1 nuclease digestion to a maximum of 70% over a wide range of C_rt values. The remaining 30% may represent contaminating sequences which are not complementary to rRNA or rDNA molecules which are of insufficient size to form stable hybrids.

Hybridization of [^{3}H]rDNA with rRNA and Total Cellular Yeast DNA. As shown in Figure 6, the $C_rt_{1/2}$ for hybridization of [^{3}H]rDNA with 24S and 18S rRNA is 3.2×10^{-3} mol s l.$^{-1}$. This value is sixfold greater than that observed for hemoglobin mRNA ($C_rt_{1/2} = 5 \times 10^{-4}$ mol s l.$^{-1}$, Holland et al., 1976). These values are internally consistent since the combined complexity of 24S and 18S rRNA (6600 nucleotides, Udem and Warner, 1972) is five to six times that of hemoglobin mRNA (Leder et al., 1974). The predicted complexity of the [^{3}H]rDNA combined with the thermal stability justifies the use of this probe to quantitate rRNA sequences in complex mixtures of RNA.

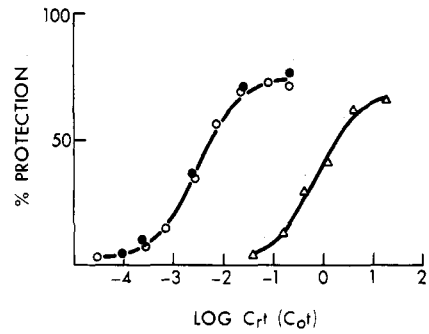

FIGURE 6: Kinetics of hybridization of rDNA with yeast and rRNA and yeast cellular DNA. Yeast [^{3}H]rDNA (1 ng) was hybridized with 1–5 μg of purified 24S and 18S rRNA (○, ●, duplicate experiments) and with 10 μg of sheared and denatured yeast cellular DNA (△). Hybridization reactions were carried out in a total volume of 100 μl and analyzed as described in Materials and Methods.

The [^{3}H]rDNA probe hybridized with a vast excess of sheared and denatured cellular DNA at a $C_0t_{1/2}$ value of 0.8 mol s l.$^{-1}$. The copy number (n) for ribosomal cistrons can be derived from the following relationship:

$$(C_0t_{1/2}, \text{ repeated sequence})(\text{copy number}) = (C_0t_{1/2}, \text{ unique sequence})$$

$$n = \frac{C_0t_{1/2},\ [^3\text{H}]\text{cDNA vs. total DNA}}{C_0t_{1/2},\ [^3\text{H}]\text{rDNA vs. total DNA}} = \frac{25 \text{ mol s l.}^{-1}}{0.8 \text{ mol s l.}^{-1}} = 30$$

where $C_0t_{1/2}$, [^{3}H]cDNA vs. total DNA (25 mol s l.$^{-1}$), was determined by hybridization of [^{3}H]cDNA with a vast excess of total yeast DNA (Holland et al., 1976). This copy number for the DNA preparations used in our experiments is lower than the range of 100–150 previously reported (Fukuhara, 1967; Retel and Planta, 1968; Schweizer et al., 1969; DeKloet, 1970; Retel and Van Keulen, 1975). Since these DNA preparations were used for the in vitro synthesis of RNA, we have used this value (30) for the ribosomal gene copy number in the quantitative interpretation of our data.

Analysis of mRNA and rRNA Sequences in RNA Synthesized by E. coli RNA Polymerase from Yeast DNA. RNA was synthesized from high-molecular-weight yeast DNA with *E. coli* RNA polymerase and analyzed with [^{3}H]cDNA and [^{3}H]rDNA. The [^{3}H]cDNA probe hybridized with RNA synthesized in the presence of Mg^{2+} or Mn^{2+} at $C_rt_{1/2}$ values of 70 and 150 mol s l.$^{-1}$, respectively (Figure 7). These $C_rt_{1/2}$ values are in the same range as those obtained for the hybridization of [^{3}H]cDNA with RNA synthesized by polymerases I and II in the presence of Mn^{2+} (Figure 3). As indicated in the discussion of the yeast polymerase transcripts, these values are consistent with random transcription by *E. coli* polymerase of the DNA template with respect to mRNA sequences represented in the cDNA probe.

The $C_rt_{1/2}$ values for titration of [^{3}H]rDNA with *E. coli* polymerase transcripts synthesized in the presence of Mg^{2+} or Mn^{2+} are 0.8 and 1.4 mol s l.$^{-1}$, respectively (Figure 7). We calculate the concentration of mature 24S and 18S rRNA sequences in a random transcript of both strands of yeast DNA as follows:

$$\frac{(\text{complexity of 24S + 18S rRNA})(\text{copy number})}{(\text{complexity of total DNA})} = \text{fraction of rRNA in random transcript}$$

$$\frac{(6600)(30)}{(17 \times 10^6)(2)} = 5.8 \times 10^{-3}$$

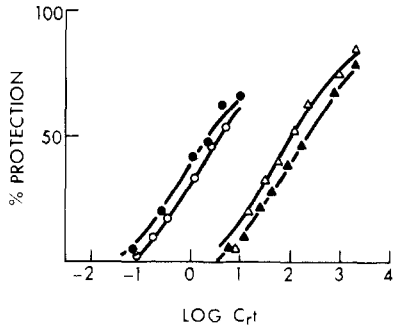

FIGURE 7: Kinetics of hybridization of yeast cDNA and rDNA with RNA synthesized in vitro from native yeast DNA by *E. coli* polymerase. Yeast [^{3}H]cDNA (0.2 ng) synthesized from purified poly(A) mRNA was hybridized with 50 μg of RNA synthesized by *E. coli* RNA polymerase in the presence of either Mg^{2+} (△) or Mn^{2+} (▲). Yeast [^{3}H]rDNA (0.6 ng) was hybridized with 5 μg of RNA synthesized by *E. coli* RNA polymerase in the presence of either Mg^{2+} (●) or Mn^{2+} (○).

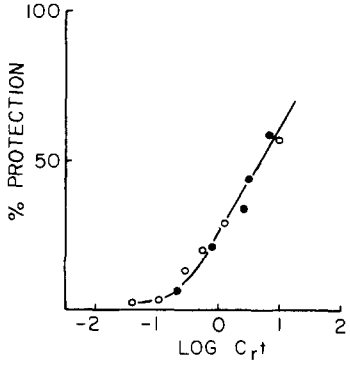

FIGURE 8: Kinetics of hybridization of yeast rDNA with RNA synthesized in vitro from native yeast DNA by RNA polymerase II. Yeast [^{3}H]rDNA (0.6 ng) was hybridized with 5 μg of RNA synthesized by purified RNA polymerase II in the presence of either Mg^{2+} (●) or Mn^{2+} (○).

therefore, the $C_rt_{1/2}$ for a completely random RNA transcript hybridized with the rDNA probe should be 3.2×10^{-3} mol s l.$^{-1}$/5.8×10^{-3} = 0.55 mol s l.$^{-1}$. The $C_rt_{1/2}$'s for [^{3}H]rDNA hybridization with RNA synthesized with *E. coli* RNA polymerase in the presence of Mg^{2+} and Mn^{2+} are 1.5- and 2.5-fold higher than predicted for a random transcript of the DNA. These data indicate that the concentration of ribosomal RNA in *E. coli* transcripts of yeast DNA, in the presence of either Mg^{2+} or Mn^{2+}, is not significantly lower than the level predicted for random transcription.

Analysis of rRNA Sequences in RNA Synthesized by Polymerases I and II from Yeast DNA. Titration of [^{3}H]rDNA with RNA Synthesized in Vitro by Yeast RNA Polymerase II. The kinetics of hybridization of [^{3}H]rDNA with RNA transcribed by RNA polymerase II in the presence of Mg^{2+} and Mn^{2+} are shown in Figure 8. The $C_rt_{1/2}$ value for both the Mg^{2+} and Mn^{2+} titration curves is 1.7 mol s l.$^{-1}$. Since the predicted $C_rt_{1/2}$ for random transcription of the DNA (derived above) is 0.55 mol s l.$^{-1}$, the observed $C_rt_{1/2}$ suggests that RNA polymerase II does not preferentially transcribe ribosomal cistrons in vitro regardless of the divalent metal ion present during transcription and may, in fact, undertranscribe these sequences.

Titration of [^{3}H]rDNA with RNA Synthesized in Vitro by Yeast RNA Polymerase I. The $C_rt_{1/2}$ value for the titration of [^{3}H]rDNA with RNA synthesized by RNA polymerase I in the presence of Mn^{2+} is 1.1 mol s l.$^{-1}$ (Figure 9). This value

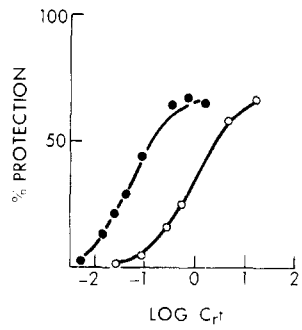

FIGURE 9: Kinetics of hybridization of yeast rDNA with RNA synthesized in vitro from native yeast DNA by RNA polymerase I. Yeast [^{3}H]rDNA (0.6 ng) was hybridized with 5 μg of RNA synthesized by purified RNA polymerase I in the presence of either Mg^{2+} (●) or Mn^{2+} (○).

is very similar to those reported above for [^{3}H]rDNA with RNA synthesized by *E. coli* polymerase in the presence of Mg^{2+} (0.80 mol s l.$^{-1}$) and Mn^{2+} (1.4 mol s l.$^{-1}$), and yeast polymerase II in the presence of Mg^{2+} and Mn^{2+} (1.7 mol s l.$^{-1}$). In marked contrast, the RNA synthesized by yeast RNA polymerase I in the presence of Mg^{2+} hybridized at a $C_rt_{1/2}$ of 5.5×10^{-2} mol s l.$^{-1}$. This value is 15–30-fold higher than those observed for either of the *E. coli* polymerase or polymerase II transcripts, or the polymerase I transcripts synthesized in the presence of Mn^{2+}. Thus the relative concentration of ribosomal sequences in products synthesized by polymerase I, specifically in the presence of Mg^{2+}, is 15–30-fold higher than the concentration of ribosomal sequences in the *E. coli* and polymerase II products. Comparison of the $C_rt_{1/2}$ obtained for hybridization of [^{3}H]rDNA with purified 24S plus 18S rRNA (3.2×10^{-3} mol s l.$^{-1}$) with that observed for RNA synthesized by polymerase I in the presence of Mg^{2+} shows that the absolute concentration of 24S plus 18S sequences in the transcript is 6%. Since the [^{3}H]rDNA probe is specific only to mature 24S and 18S rRNA sequences, the value of 6% may represent an underestimate of the total concentration of ribosomal sequences in the transcript. The possibility that the rRNA sequences measured are not newly synthesized but are derived from either the yeast DNA template or the polymerase is untenable since the same amount of RNA polymerase I and yeast DNA was used to synthesize RNA in the presence of Mn^{2+} and 5–10-fold higher amounts of yeast DNA were used to synthesize RNA with RNA polymerase II. Furthermore we have extracted RNA directly from the same quantities of RNA polymerase I and DNA utilized to synthesize the transcripts described above. We were unable to detect rRNA sequences in these extracts by the rDNA hybridization assay. We conclude, therefore, that, under the appropriate reaction conditions, RNA polymerase I preferentially transcribes ribosomal cistrons.

Discussion

The specific activities of purified eucaryotic RNA polymerases on intact DNA templates are significantly lower than those reported for bacterial polymerases (Chambon, 1975). Under suitable assay conditions (low-molecular-weight denatured DNA), the polymerizing activities of the eucaryotic enzymes do approach the values reported for the *E. coli* enzyme (Burgess, 1969). The template requirements for eucaryotic polymerases are similar to those of the *E. coli* core RNA polymerase. Analysis of the template requirements for the eucaryotic enzymes suggests that they initiate RNA synthesis at nicks and single-stranded gaps in the template and

that the enzymes are incapable of initiating on double-stranded DNA (Mandel and Chambon, 1974a,b; Dezelee et al., 1974). These observations suggest that purified enzymes are not themselves capable of interacting with specific initiation sites and that other factors are required.

Our data demonstrate that the activity of yeast RNA polymerase I on high-molecular-weight native yeast DNA in the presence of Mg^{2+} is highly dependent on the method of purification of the enzyme. RNA polymerase I prepared by the method described in this paper is not saturated by the same levels of DNA required for RNA polymerase II. This observation suggests that polymerases I and II do not initiate RNA synthesis from the same sites in the template. Furthermore, the specific activity of RNA polymerase I on high-molecular-weight yeast DNA is at least an order of magnitude higher than that of enzymes described previously (Dezelee et al., 1974; Hager et al., 1976; Valenzuela et al., 1976). We have not ascertained the nature of the anomalous activity of different preparations of yeast polymerase I on native DNA, but it should be pointed out that Goldberg et al. (1976) have shown that a polypeptide critical for the transcription of native DNA by polymerase I from rat liver and Krebs II ascites cells can be lost during purification. Similar polypeptides may be lost or inactivated during purification of the yeast enzyme.

The DNA utilized in these experiments was of high molecular weight, but initiation at nicks or single strand regions is not precluded. It does appear, however, that there are a considerably smaller number of polymerase I initiation sites in yeast native DNA than polymerase II sites. In light of the high level of ribosomal RNA sequences synthesized in vitro by polymerase I, some of these initiation sites may be ribosomal gene promoters.

Cellular mRNA and rRNA sequences present in transcripts synthesized by polymerases I and II were assayed by hybridization with cDNA and rDNA. These DNA probes were labeled to specific activities in excess of 2×10^6 cpm/μg in order to minimize the amount of in vitro transcript necessary for the analysis. The hybridization reactions were carried out in solution ensuring that all of the sequences in the reaction are available for hybrid formation, Finally, hybridizations were carried out under conditions of RNA excess (Bishop, 1972), making it possible to determine the number of newly synthesized sequences which are complementary to the DNA probes and the relative amount of these sequences synthesized based on the extent of hybridization and the kinetics of the hybridization reaction. Many of the studies purporting to show specific transcription of ribosomal cistrons by RNA polymerase I have employed competition hybridization assays utilizing filter-bound DNA which contains both repeated and nonrepeated sequences (Cramer et al., 1974; Beebee and Butterworth, 1974a,b). In these studies only a small percentage of the RNA synthesized in vitro formed hybrids and the relative efficiency of rRNA sequence hybridization was not determined, making it impossible to ascertain the actual amount of rRNA synthesized in vitro. Van Keulen et al. (1975) employ a competition assay using unlabeled newly synthesized RNA as the competitor. They report that a partially purified RNA polymerase I synthesizes high levels of rRNA sequences from high-molecular-weight native DNA. However, they have not demonstrated the absence of RNA polymerase III from their preparations. A small degree of contamination with this enzyme, as has been reported for a similar preparation of polymerase I (Valenzuela et al., 1976), can be significant because of the known ability of polymerase III to efficiently transcribe double-stranded DNA, and because of the interspersion of 5S genes and the large ribosomal cistrons in yeast DNA (Rubin and Sulston, 1973). Our polymerase I preparations are free of polymerase III, and our studies establish that polymerase I can selectively transcribe ribosomal cistrons. Van Keulen et al. (1975) have further shown that the size of the DNA crucially affects the extent of specific transcription. This implies that nicks or single-stranded regions contribute false starts. Thus the elimination of these in the template may increase the degree of selective transcription.

The distribution of cellular mRNA and rRNA sequences synthesized in vitro by RNA polymerases I and II in the presence of Mn^{2+} is consistent with the hypothesis that both enzymes randomly transcribe native DNA under these reaction conditions. We argue that random initiation is not an intrinsic property of RNA polymerase I since random initiation by this enzyme appears to be due to the presence of Mn^{2+} in the reaction mixture. Because the activity of these enzymes is low in Mg^{2+}, we have not yet synthesized the large quantities of RNA in the presence of Mg^{2+} needed to satisfy the excess requirements for the cDNA titration. The divalent metal ion effects described in this paper are an important consideration since almost all studies on in vitro transcription by eucaryotic RNA polymerases have been carried out with RNA synthesized in the presence of Mn^{2+} or a combination of Mn^{2+} and Mg^{2+}. It is possible that the failure of others to observe specific transcription of ribosomal cistrons by polymerase I (Roeder et al., 1970; Honjo and Reeder, 1974) may in part by related to the improper choice of divalent metal ion. The observation that Mn^{2+} does not alter RNA synthesis in isolated nuclei could be related to the fact that the bulk of the RNA synthesized in nuclei is derived from elongation of RNA chains which have already been initiated in vivo (Reeder and Roeder, 1972).

Selective initiation by *E. coli* RNA polymerase on bacteriophage T7 DNA is a function of the ratio of polymerase molecules to promoter sequences (Minkley, 1974). The titrations of rDNA reported here were carried out with RNA synthesized under conditions of approximately ten enzyme molecules per ribosomal cistron. This is in the range reported to be necessary for selective transcription of T7 DNA.

It seems clear that the preferential transcription of ribosomal cistrons by polymerase I in the presence of Mg^{2+} is a specific function of the polymerizing enzyme even though sites of initiation and termination have not been measured. Neither polymerase II nor *E. coli* polymerase preferentially synthesizes ribosomal sequences, suggesting that preferential transcription of ribosomal cistrons is a property of the polymerase I molecule. We conclude, therefore, that polymerase I does in fact play a critical role in the transcription of ribosomal cistrons. Since purified yeast DNA was used as a template, this implies that the interaction of RNA polymerase I with the template does not require chromosomal protein-dependent conformations of the DNA. This conclusion does not exclude the possibility that other cellular components play a role in the regulation of ribosomal RNA synthesis but does rule out the extreme hypothesis that chromosomal proteins alone are responsible for regulating selective transcription in the eucaryotic cell.

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