Ribosomal Ribonucleic Acid Synthesis by Isolated Yeast Ribonucleic Acid Polymerases†

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ABSTRACT: RNA products have been synthesized with isolated yeast (*Saccharomyces cerevisiae* S41) RNA polymerase IB, II, and III on purified yeast nuclear DNA and the percentage of ribosomal RNA (rRNA) in the purified products has been determined by competition hybridization experiments. RNA polymerase IB product contained 3%, polymerase II product contained 4%, and polymerase III product contained 6–8% rRNA. The RNA products contained at least 33 (IB-RNA), 21 (II-RNA), and 48% (III-RNA) symmetric RNA as determined by ribonuclease resistance after extensive self-annealing. The RNA products made in a polymerization reaction that was stimulated twofold by the π factor, a small heat-stable protein that stimulates yeast RNA polymerases, contained the same levels of rRNA as products made in the absence of π factor. The presence of π factor led to a higher level (62%) of symmetric transcription by polymerase II but did not affect the asymmetry of transcription for polymerases IB and III. Under the conditions used yeast RNA polymerases IB and II synthesized the proportions of rRNA expected for random transcription of both strands and the enzyme III produced a level two–three times higher than that. The π factor did not influence the reading of rRNA genes of any of the enzymes.

It has been shown in *in vitro* bacterial transcription systems that the ribosomal RNA genes can be transcribed selectively (Haseltine, 1972; Pettijohn, 1972).

Yeast is a eukaryotic organism whose DNA harbors only a few times more genetic information than a bacterium (Ogur *et al.*, 1952; Bicknell and Douglas, 1970). It was an interesting question whether in this simple eukaryote such a strong preferential transcription exists for one of the multiple polymerases (Adman *et al.*, 1972; Brogt and Planta, 1972) present in this organism. Measurement of the level of rRNA[1] synthesis by each of the yeast RNA polymerases *in vitro* could give the answer. Moreover, if the levels of rRNA made in an *in vitro* reaction are known, it is possible to determine whether these levels are affected by the presence of the π factor (Di Mauro *et al.*, 1972), a low molecular weight protein fraction that stimulated yeast RNA polymerases.

Under conditions used in this paper none of the yeast polymerases had the high preference for rRNA transcription displayed by the bacterial system. In contrast to what we expected, polymerase III product contained a two- to threefold higher proportion of rRNA than did *in vitro* products synthesized by polymerase IB and II. The π factor had no influence on the levels of rRNA products.

Methods

RNA Polymerases. Yeast RNA polymerases IB, II, and III were isolated essentially as described (Adman *et al.*, 1972). Late log phase cells (40 g) (*Saccharomyces cerevisiae* strain S41) were disrupted by agitation for 2 min in a Braun homogenizer in a mixture of 1 part cells, 1 part glass beads (0.45 mm diameter), and 1 part extraction buffer containing 0.1 M Tris-HCl (pH 7.4 at 4°), glycerol (20%, v/v), 20 mM $MgCl_2$, 0.8 M $(NH_4)_2SO_4$, 1 mM EDTA, 1 mM dithiothreitol, and phenylmethylsulfonyl fluoride (0.6 mg/ml, freshly added from stock solution containing 6 mg/ml in ethanol). The resulting supernatant was sonicated for 1 min at setting 50 in a Branson J17-V sonicator and then centrifuged at 48,000 rpm for 90 min in a Spinco type 60 Ti rotor. The resulting crude extract was diluted with 7 vol of TGED buffer (50 mM Tris-HCl (pH 7.4 at 4°), glycerol (25%, v/v), 0.5 mM EDTA, 0.5 mM dithiothreitol, and phenylmethylsulfonyl fluoride (0.3 mg/ml) and applied on a DEAE-Sephadex column (A-25, 2.5 cm × 37.5 cm) equilibrated with 0.05 M $(NH_4)_2SO_4$ in TGED buffer. The column was washed with 250 ml of 0.05 M $(NH_4)_2SO_4$ in TGED buffer and eluted with a 2.4-l. linear gradient of 0.05–0.45 M $(NH_4)_2SO_4$ in TGED buffer. The flow rate was 50 ml/hr. Enzymes were concentrated on small DEAE-Sephadex columns.

Template DNA. Yeast nuclear DNA was isolated from protoplasts of petite mutant RD1B (Hollenberg *et al.*, 1972) lacking mitochondrial DNA by a slightly modified method of Smith and Halverson (1967). According to boundary sedimentation analysis (Studier, 1965; Eigner and Doty, 1965) in a Spinco Model E the DNA consisted of three classes: 25% (by weight) with a double-stranded mol wt of 100×10^6 daltons, 50% of 2.3×10^6 daltons, and 25% of 2.3×10^6–100×10^6 daltons. The single-stranded molecular weights, determined by alkaline sedimentation, were lower by a factor of 10.

Ribosomal RNA. Cells were grown in 100 ml of low-phosphate medium to which 0.5 ml of carrier-free $^{32}PO_4$ (in 0.2 M HCl) and 0.4 ml of 0.2 M NaOH were added. Low-phosphate medium contained (per liter): 6 g of $(NH_4)_2SO_4$, 0.5 g of $MgSO_4 \cdot 7H_2O$, 0.5 g of $CaCl_2 \cdot 6H_2O$, 20 g of glucose, 1.2 mg of KH_2PO_4, and a mixture of 5 g of peptone and 2.5 g of yeast extract from which phosphate was removed by the following

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[1] Abbreviations used are: rRNA, ribosomal ribonucleic acid; TGED buffer, 50 mM Tris-HCl, glycerol (25%, v/v), 0.5 mM EDTA, and 0.5 mM dithiothreitol; SSC, standard saline-citrate.

treatment. The pH of 1 l. of H_2O containing 66 g of peptone, 34 g of yeast extract, and 15 g of $MgSO_4 \cdot 7H_2O$ was adjusted to pH 8 with concentrated NH_4OH. The precipitate was removed by centrifuging for 10 min at 10,000*g* and 2 M HCl was added until pH 7 was obtained.

Ribosomes and subunits were isolated according to Martin *et al.* (1970). RNA was isolated from separated 40S and 60S subunits by three phenol extractions and precipitated overnight at −20° in the presence of 0.1 M sodium acetate by 2 vol of ethanol. The precipitate was washed with 70% ethanol until free of phenol. The dried RNA pellet was solubilized in 0.1 × SSC and the salt adjusted to 2 × SSC. The solution was layered on a 10–30% sucrose gradient containing 10 mM sodium acetate (pH 5.2), 0.1 M NaCl, and 1 mM EDTA and centrifuged for 21 hr at 26,000 rpm in a SW-27 Spinco rotor at 4°. The 25S and 17S peak fractions from, respectively, the 60S and 40S subunit RNAs containing gradient were pooled separately and dialyzed against 2 × SSC. For hybridization experiments the two RNA species were mixed in equimolar quantities.

Unlabelled rRNA was isolated by the same procedure from cells grown on YEP–2% glucose medium.

Hybridization. Hybridization was performed according to the method of Gillespie and Spiegelman (1965). DNA (1 μg/ml) was denatured in 0.25 M NaOH for 15 min at room temperature, cooled, neutralized, and adjusted to 3 × SSC. The solution was slowly passed through Millipore filters (47 mm, GS 0.22 μ, prewashed with 100 ml of 3 × SSC) that were then washed with 40 ml of cold 3 × SSC. Filters were dried overnight at room temperature and heated for 2 hr at 80°. Discs at 8 mm diameter were used for hybridization. More than 80% of the loaded DNA was retained on the filters. Before adding the filters, the hybridization solution (RNA in 3 × SSC) was heated for 5 min at 100°. During the hybridization 30–40% of the DNA was lost from the filters. After 18 hr at 65° the filters were batchwise washed with cold 3 × SSC and incubated with 5 μg of pancreatic ribonuclease and 0.5 unit of ribonuclease T_1 per ml for 30 min at 37°. Filters were washed extensively with 3 × SSC, dried, solubilized in 1 ml of ethyl acetate, and counted after the addition of 10 ml of Liquiflor (New England Nuclear Corp.) in toluene. Assays were performed in duplicate and did not differ more than 10%. In most series of assays standard r[^{32}P]RNA was hybridized to record hybridization efficiency.

RNA Products. Polymerase IB (3.4 mg of protein), II (2.5 mg of protein), or III (2 mg of protein) was incubated at 30° with 50 mM Tris-HCl (pH 7.4 at 4°), 0.5 mM ATP, 0.5 mM CTP, 0.5 mM GTP, 0.1 mM [^{3}H]UTP (100 Ci/mol), 1.6 mM $MnCl_2$, 4 mM $MgCl_2$, yeast nuclear DNA (47 μg/ml) in 4 ml for, respectively, 35, 60, or 35 min ($(NH_4)_2SO_4$ concentrations in reactions IB, 50 mM, II, 60 mM, and III, 180 mM).

After cooling, 10 mM $MgCl_2$, *Escherichia coli* 4S RNA (25 μg/ml), and deoxyribonuclease (50 μg/ml) were added to the mixture and incubated 15 min at 37°. At 0°, 1% Sarkosyl and 0.2 M NaCl were added and the RNA mixture was extracted with 4 ml of phenol saturated with 2 × SSC. The phenol phase was reextracted with 1 ml of 2 × SSC. The combined water layers were applied on a Sephadex G-50 column (2.5 cm × 50 cm) in 0.1 × SSC. The RNA in the excluded volume was concentrated by evaporation and stored in 2 × SSC. Yields were 14 μg of IB-RNA, 12 μg of II-RNA, and 30 μg of III-RNA. Recoveries were over 80%.

The RNA product reactions *plus* π factor contained 0.5 ml of polymerase IB (2.1 mg of protein), 0.3 ml of polymerase II (0.8 mg of protein), or 0.3 ml of polymerase III (1.5 mg of protein), π factor (300 μg/ml), yeast nuclear DNA (78 μg/ml), and $(NH_4)_2SO_4$: 60 mM for polymerase IB, 90 mM for polymerase II, and 120 mM for polymerase III in a final volume of 5 ml. Other conditions are as in the reaction performed in the absence of the π factor; reaction 30 min at 30°. RNA was isolated as described above. Yields were 3 μg of IB-RNA, 3 μg of II-RNA, and 6 μg of III-RNA. Recoveries were 30% for IB-RNA and II-RNA and 40% for III-RNA.

The amounts of RNA product were determined on the basis of ^{3}H radioactivity.

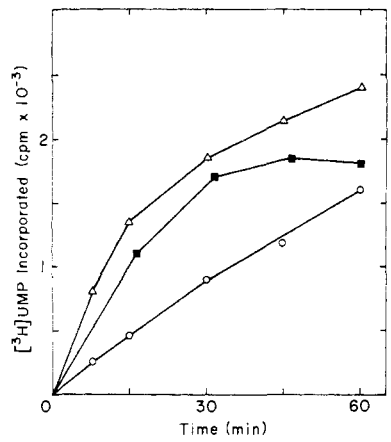

FIGURE 1: Time curves of RNA synthesis by yeast RNA polymerases. Isolated yeast RNA polymerases, 20 μl of IB (4.2 mg of protein/ml), 20 μl of II (2.5 mg of protein/ml), or 10 μl of III (4.9 mg of protein/ml) were incubated at 30° with 50 mM Tris-HCl (pH 7.4 at 4°), 0.5 mM ATP, 0.5 mM CTP, 0.5 mM GTP, 0.1 mM [^{3}H]UTP (100 Ci/mol), 1.6 mM $MnCl_2$, 4 mM $MgCl_2$, and yeast nuclear DNA (58.5 μg/ml). $(NH_4)_2SO_4$ was present in the following concentrations: IB, 50 mM; II, 60 mM; III, 180 mM. The final volume was 0.1 ml. At indicated times 20-μl aliquots were cooled and 100 μg of bovine serum albumin and 10% trichloroacetic acid were added. The precipitates were washed on glass fiber filter and counted: (■) polymerase IB; (○) polymerase II; (▽) polymerase III.

Results

RNA Polymerase Products. In preliminary assays performed in the presence of 1.6 mM $MnCl_2$, we found that polymerase IB did not show any activity on the yeast DNA template, while it was active on calf thymus DNA and polymerases II and III were fully active on this yeast template. The addition of 4 mM $MgCl_2$ to the polymerase IB reaction gave an activity normal compared to the other enzymes. In the presence of $MgCl_2$, $MnCl_2$ was not required. In order to get RNA products made under identical conditions, the polymerization reactions of all enzymes were performed in the presence of both Mn^{2+} and Mg^{2+}.

Figure 1 shows the kinetics of RNA synthesis by yeast RNA polymerases IB, II, and III under the conditions used for preparing *in vitro* RNA products for hybridization experiments. The product reactions were run for 35 min for polymerases IB and III and 60 min for polymerase II. Product RNA was isolated (see Methods) with a yield of 80% or higher.

To get an impression of whether the transcription is symmetric or asymmetric, the products were self-annealed and subjected to ribonuclease digestion (Figure 2). Enzymes IB, II, and III synthesized, respectively, at least 34, 21, and 43% symmetric RNA. These figures should be regarded as rough minimum estimates for the extent of symmetric transcription, since studies of percentage ribonuclease resistance *vs.* RNA concentrations and annealing time were not made.

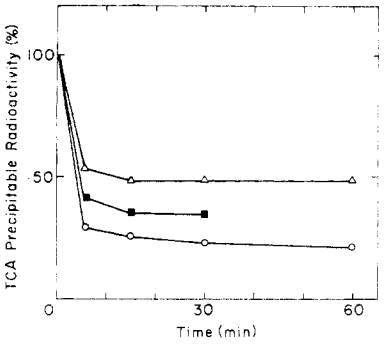

FIGURE 2: Ribonuclease sensitivity of annealed RNA products. Isolated RNA products were self-annealed for 18 hr at 65° in 2 × SSC at the following concentrations: IB-RNA, 14 μg/ml; II-RNA, 12 μg/ml; and III-RNA, 30 μg/ml. After annealing, pancreatic and T_1 ribonuclease (5 μg/ml and 0.5 U/ml, respectively) were added and the samples were incubated at 30°. At different times aliquots were cooled and the acid precipitable counts determined (see Methods). An aliquot of each RNA product was heated for 5 min at 100° and incubated for 45 min with the ribonuclease mixture. The nuclease resistant single-stranded RNA (IB-RNA, 3%; II-RNA, 9%; III-RNA, 4%) was subtracted as background: (■) IB-RNA; (○) II-RNA; (▽) III-RNA.

Hybridization Saturation Curve of Nuclear DNA and r[^{32}P]-RNA and Competition between r[^{32}P]RNA and Unlabeled rRNA. Figure 3 shows the hybridization of yeast nuclear DNA with increasing amounts of a mixture of 17S and 25S (equimolar amounts) r[^{32}P]RNA. The curve levels off at ~1.5% DNA saturation, a plateau value lower than those reported in the literature (Retèl and Planta, 1968; Schweizer *et al.*, 1969) (see Discussion).

At an RNA/DNA ratio of 0.1 the DNA was just saturated. Addition of increasing amounts of cold rRNA to such a hybridization mixture led to a monotonic decrease in the percentage of DNA in hybrid with r[^{32}P]RNA due to the isotope dilution (Figure 3). The addition of as little as 20 ng of unlabeled rRNA resulted in a detectable decrease in r[^{32}P]RNA hybridized.

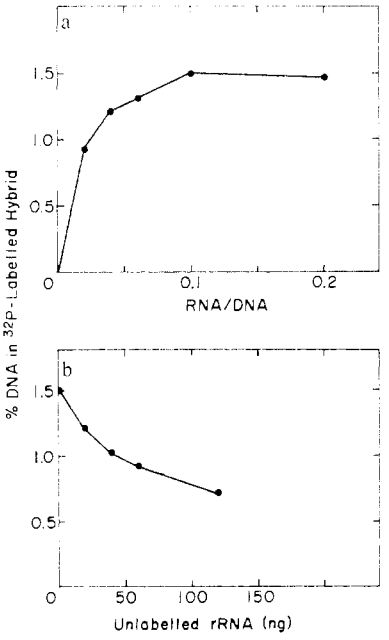

FIGURE 3: (a) Hybridization of yeast nuclear DNA with increasing amounts of *in vivo* rRNA. Conditions: 1.2 μg of [^{3}H]DNA (7400 cpm/μg) per filter incubated with purified r[^{32}P]RNA (equimolar mixture of 17S and 25S; spec act. 258 cpm/ng) in 0.2 ml of 2 × SSC for 18 hr at 65°. Other details are given under Methods. (b) Hybridization competition between r[^{32}P]RNA and cold rRNA. [^{3}H]DNA (1.2 μg) was hybridized with 120 ng of r[^{32}P]RNA *plus* indicated amounts of unlabeled *in vivo* rRNA (equimolar mixture of 17S and 25S) under the conditions of Figure 3a.

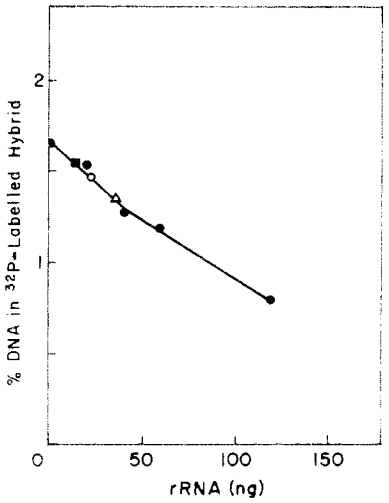

FIGURE 4: Competition of RNA products with hybridization of r[^{32}P]RNA. [^{3}H]DNA (1.2 μg) was hybridized under standard conditions with 120 ng of r[^{32}P]RNA (285 cpm/ng) *plus* indicated amounts of unlabeled rRNA (●), 490 ng of IB-RNA (■), 520 ng of II-RNA (○), or 480 ng of III-RNA (△). Other details are given under Methods.

Competition Power of RNA Products with Ribosomal [^{32}P]-*RNA.* Hybridization competition experiments were used to determine the amount of rRNA present in the RNA products. In hybridization reactions at an RNA/DNA ratio of 0.1, the competition by increasing amounts of cold rRNA or product RNAs was determined. The extent of competition obtained with each level of unlabeled rRNA was used to construct a standard curve (Figure 4), relating competition to input rRNA. Competition values obtained with a given amount of *in vitro* RNA (490 ng for IB product, 520 ng for II product, 480 ng for III product) were placed on the standard curve to determine the apparent amount of rRNA present. The IB, II, and III products gave extents of competition consistent with rRNA contents of 14, 22, and 38 ng of rRNA, respectively. Therefore, the proportion of rRNA transcription appeared to be 3% for enzyme IB, 4% for enzyme II, and 8% for enzyme III. *E. coli* 4S RNA up to 20 μg did not compete with hybridization of r[^{32}P]RNA.

To exclude the possibility that competition by the RNA products was due to aspecific inhibitors of the hybridization reaction, polymerase III product RNA (III-RNA) was hybridized to nuclear DNA and the amount of the hybridized RNA which could be competed out by added unlabeled rRNA was determined. In this assay r[^{32}P]RNA, which was used as an internal marker, was hybridized with 40% efficiency in the absence of competitor. With 250 ng of competitor added, the quantity of rRNA competed out (Figure 5) is 70% of that hybridized, or 28% (70 × 40%) of the input r[^{32}P]RNA. The competition curve obtained with unlabeled rRNA *vs.* III-[^{3}H]RNA can be corrected for the known efficiency of rRNA (from the ^{32}P data) to calculate the percentage of r[^{3}H]RNA present. The amount of [^{3}H]RNA competed out by 250 ng of rRNA is 5 ng (sp act. is 50,000 cpm/μg). If one assumes that r[^{3}H]RNA and r[^{32}P]RNA behave identically, then this 5 ng of r[^{3}H]RNA competed out should represent 19 ng of the input r[^{3}H]RNA. Thus, of a total input of

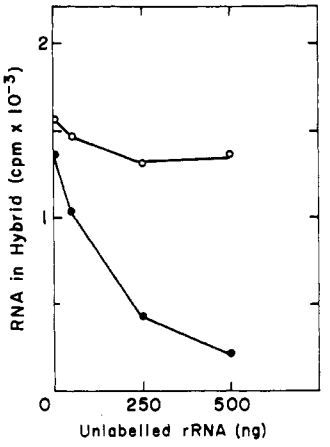

FIGURE 5: Enzyme III RNA product hybridization challenged by rRNA. Unlabeled yeast nuclear DNA (2.5 μg) was hybridized under standard conditions with 20 ng of r[^{32}P]RNA (174 cpm/ng), 310 ng of III-RNA (50 cpm/ng), *plus* indicated amounts of unlabeled rRNA. Other details are given under Methods: (●) ^{32}P cpm; (○) ^{3}H cpm.

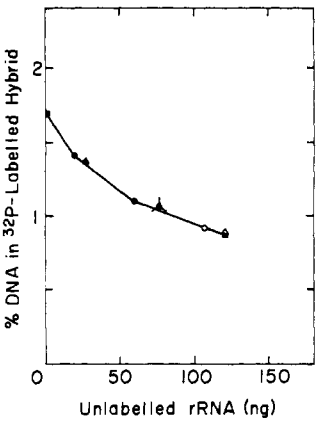

FIGURE 6: Competition of enzyme products made in the presence of the π factor with the hybridization of r[^{32}P]RNA. [^{3}H]DNA (1.2 μg) was hybridized with 120 ng of r[^{32}P]RNA (165 cpm/ng) *plus* the indicated amounts of unlabeled rRNA (●), 410 ng of IB-RNA (■), 440 ng of II-RNA (○), 385 ng of III-RNA (△), 450 ng of III-RNA made *minus* the π factor (▲), or 1350 ng of III-RNA made *minus* the π factor (▲). Other details are described under Methods.

310 ng of III-RNA, 6% (19 ng) appears to be rRNA. The fact that the value of 6% rRNA determined in this more critical assay (Figure 5) does not differ significantly from the 8% assayed in the first type of assay (Figure 4) indicated that competition of III-RNA, and most likely of the other identically isolated products IB-RNA and II-RNA in Figure 4, is mostly due to rRNA.

Product RNA Made in the Presence of π Factor. Product RNAs were made with the yeast RNA polymerases in the presence of a twofold stimulating amount of π factor. Results of the annealing reactions in Table I show that the π factor increased the self-complementarity of RNA made by polymerase II considerably and that of the other products slightly. If *in vivo* transcription is asymmetric these results indicated that the π factor not only did not increase the reading specificity, but actually decreased it in the case of polymerase II.

rRNA Content of RNA Products Made in the Presence of π Factor. Purified products made by yeast RNA polymerases in the presence of the π factor were added to a hybridization reaction of yeast DNA and r[^{32}P]RNA to determine the rRNA content (Figure 6). As a control, the competition of a concentration series of unlabeled rRNA and two concentrations of III-RNA made in the absence of the π factor and previously tested for rRNA content (Figure 4) was determined. Surprisingly, the RNA products made with π factor had a competing power which indicated they contained as much as 25–32% rRNA. For both concentrations of III-RNA made in the absence of π and used in this experiment 6% rRNA was calculated, a figure in good agreement with previous determinations (Figures 4 and 5). To determine whether the π-stimulated *in vitro* RNA products actually contained the high level of rRNA indicated by their competing ability (Figure 6), the labeled product RNAs were hybridized to nuclear DNA while challenging the hybridization of each of them with increasing amounts of rRNA. The results in Figures 7 and 8 showed that products IB, II, and III contain, respectively, 4, 4.5, and 10% rRNA. This suggests that the competition observed with π-factor stimulated RNAs in Figure 6 can only partially be due to the presence of rRNA. Most of the competition is probably an aspecific inhibition of the hybridization reaction by DNA or π factor present in the RNA products (see Discussion).

We conclude that under the experimental conditions used in this study the π factor does not preferentially stimulate rRNA transcription. Stimulation of transcription of other classes of RNA by the presence of the π factor cannot be excluded.

TABLE I: Self-Complementarity of Yeast RNA Polymerase Products.[a]

Enzyme	Cl_3CCOOH Precipitable Counts (%)	
	$-\pi$	$+\pi$
IB	33	35
II	21	62
III	48	60

[a] See Figure 2 for conditions of annealing and ribonuclease treatment. RNA synthesized in the presence of the π factor was annealed at the following concentrations: IB-RNA, 8 μg/ml; II-RNA, 10 μg/ml; III-RNA, 16 μg/ml.

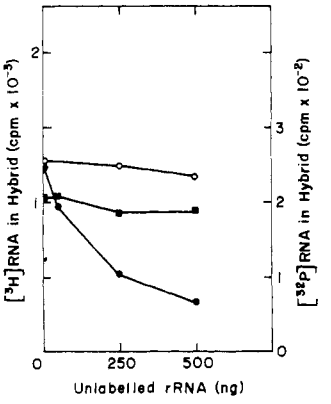

FIGURE 7: Hybridization of polymerases IB- and II-RNA, synthesized in the presence of the π factor, challenged by rRNA. Present are 40 ng of r[^{32}P]RNA (26 cpm/ng), *plus* 205 ng of IB-[^{3}H]RNA (■), 220 ng of II-[^{3}H]RNA (○); (●) [^{32}P]RNA in hybrid. Other details are as described in Figure 5.

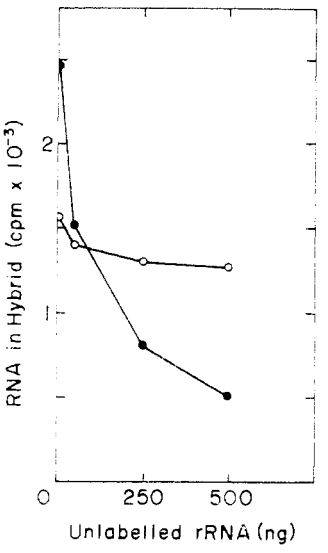

FIGURE 8: Hybridization of polymerase III-RNA, synthesized in the presence of the π factor, challenged by rRNA. Present are 40 ng of r[^{32}P]RNA (164 cpm/ng) and 230 ng of III-[^{3}H]RNA (○); (●) [^{32}P]RNA in hybrid. Other details are as described in Figure 7.

Discussion

One of the aims of this study was to determine the effect of the π factor on transcription specificity of yeast RNA polymerases. The only yeast RNA species that can readily be assayed for are the stable RNAs: rRNA, 5S RNA, and tRNA. We decided to look for the effect of π factor on the synthesis of rRNA since this would include an investigation of the level of the transcription of these rRNA genes by the different RNA polymerases.

When *E. coli* DNA and RNA polymerase are incubated under the proper conditions, the rRNA genes are preferentially transcribed (Haseltine, 1972; Pettijohn, 1972). Most likely this means that these genes are equipped with a very strong promotor. By determining the amount of rRNA synthesized by each of the isolated yeast polymerases we could establish whether yeast as one of the more simple eukaryotes would have preserved the strong rRNA promotor–polymerase interaction present in bacteria and whether a particular polymerase would be involved in this.

The results indicate that none of the yeast polymerases has the very high preference for rRNA transcription shown by the *E. coli* system. As compared to the level of 2% rRNA expected for uniform transcription of all regions of yeast DNA, polymerases IB and II synthesized rRNA preferentially by a factor of 1 to 2 and, surprisingly, polymerase III did so by a factor of 3 to 5. These data suggest that polymerase III could be involved in rRNA synthesis *in vivo*. Although the corresponding polymerase I is located within the nucleolus of rat liver cells (Roeder and Rutter, 1970) it should be stressed that there is no direct proof that it is responsible for rRNA synsynthesis. Of course we realize that our results depend strongly on the experimental conditions used.

In another eukaryote system, *Xenopus laevis*, RNA polymerases I and II did not show any preferential transcription of ribosomal DNA *vs.* bulk DNA. With purified *Xenopus* ribosomal DNA template, the homologous polymerases I and II produced RNA that hybridized to separated rDNA strands in the same ratio as did the product made by *E. coli* polymerase on a denatured rDNA template. Roeder *et al.* (1970) concluded that accurate *in vitro* synthesis of rRNA in *Xenopus* may require additional specificity factors in addition to only DNA and RNA polymerases.

The self-complementarity of the RNA products can give only an approximation of the degree of symmetric transcription since the efficiency of the annealing reaction is not known. In comparing the levels of ribonuclease resistance of the annealed products, the different RNA concentrations in the annealing reaction should be considered.

In our system the level of DNA saturation with rRNA (17S + 25S) was 1.5–1.7%, a value lower than reported in the literature (1.9–2.4%) (Retèl and Planta, 1968; Schweizer *et al.*, 1969). We found this level for DNA preparations from the same yeast strain purified in different ways and for DNA preparations from different strains. Two likely explanations are that (1) we isolated our rRNA from purified subunits, while others purified rRNA from crude ribosomal pellets; (2) we always observed a considerable loss of DNA from the filters during hybridization. Selective loss of hybrid would reduce the saturation level. In attempts to prevent the DNA from coming off the filter, we tried other types of filters (SS B-6; Millipore HA 0.45 μ), we denatured the DNA by heat or heat *plus* alkali, we used DNA preparations isolated by different methods, and we hybridized in 30% formamide at 37°, but none of these changes gave improvement. The DNA loss from filters made from the same large filter was always identical. As our DNA was labeled, we were able where needed to measure how much DNA was present on the filter.

The strong competition observed with the RNA products made in the presence of the π factor (Figure 6) could only partially be attributed to the presence of rRNA (Figures 7 and 8). Since this strong competition was not observed with the RNA products made in the absence of the π factor (Figure 4) and the products were isolated by identical procedures, this competition must be caused by the presence of the π factor. The π factor could lead to two artifacts. (1) During the product isolation the DNA template is broken down by deoxyribonuclease. The π factor binds to DNA and could protect it against complete degradation, so that undegraded DNA ends up in the RNA product. Prior to hybridization the RNA mixture is heated for 5 min at 100° to melt out annealed symmetric RNA and bring all RNAs to equal molecular weight. In this heating step, contaminating DNA would be denatured, subsequently acting as a competitor for rRNA hybridization. (2) The π factor could bind also to RNA and not be removed during the purification. In the heating step the protein is released and interferes directly with hybridization.

Acknowledgments

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References

Adman, R., Schulz, L. D., and Hall, B. D. (1972), *Proc. Nat. Acad. Sci. U. S. 69*, 1702.

Bicknell, J. N., and Douglas, H. C. (1970), *J. Bacteriol. 101*, 505.

Brogt, Th. M., and Planta, R. J. (1972), *FEBS (Fed. Eur. Biochem. Soc.) Lett. 20*, 47.

Di Mauro, E., Hollenberg, C. P., and Hall, B. D. (1972), *Proc. Nat. Acad. Sci. U. S. 69*, 1702.

Eigner, J., and Doty, P. (1965), *J. Mol. Biol. 12*, 549.

Gillespie, D., and Spiegelman, S. (1965), *J. Mol. Biol. 12*, 829.

Haseltine, W. A. (1972), *Nature (London) 235*, 329.
Hollenberg, C. P., Borst, P., and Van Bruggen, E. F. J. (1972), *Biochim. Biophys. Acta 277*, 35.
Hollenberg, C. P., and Hall, B. D. (1972), 17th Annual Meeting of the Biophysical Society, Columbus, Ohio, Abstract 118.
Martin, T. E., Bicknell, J. N., and Kumar, A. (1970), *Biochem. Genet. 4*, 603.
Ogur, M., Minckler, S., Lindegren, G., and Lindegren, C. C. (1952), *Arch. Biochem. Biophys. 40*, 175.
Pettijohn, D. E. (1972), *Nature (London), New Biol. 235*, 204.
Retèl, J., and Planta, R. J. (1968), *Biochim. Biophys. Acta 169*, 416.
Roeder, R. G., Reeder, R. H., and Brown, D. D. (1970), *Cold Spring Harbor Symp. Quant. Biol. 35*, 727.
Roeder, R. G., and Rutter, W. J. (1970), *Proc. Nat. Acad. Sci. U. S. 65*, 675.
Schweizer, E., MacKechnie, C., and Halverson, H. O. (1969), *J. Mol. Biol. 40*, 261.
Smith, J. D., and Halverson, H. O. (1967), *Methods Enzymol. 12*, 538.
Studier, F. W. (1965), *J. Mol. Biol. 11*, 373.

A Carbon-13 Nuclear Magnetic Resonance Study of Binding of Copper(II) to Purine Nucleotides†

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ABSTRACT: The influence of paramagnetic Cu^{2+} ions on the proton-decoupled ^{13}C nuclear magnetic resonance spectra of purine nucleotides has been studied. For 5′-AMP and 2′-AMP, the C-4 and C-5 resonances broaden first, followed by the broadening of the C-8 resonance upon the addition of Cu^{2+} ions. The C-2, C-6, and ribose resonances are unaffected over the metal ion concentration range studied. These results indicate that the metal ion is held near the N-7 position of the base, irrespective of the position of the phosphate group on the ribose ring. Possible models for a complex between 5′-AMP or 2′-AMP with Cu^{2+} are discussed. With 5′-GMP and 5′-IMP the C-4, C-5, and C-8 resonances are nearly equally affected by the Cu^{2+} ions, indicating that the metal ion is also held near the N-7 position of the base.

The interactions between nucleosides, nucleotides, and polynucleotides with Cu^{2+} ions have been extensively studied and more experimental work has been reported for the interaction of these ligands with Cu^{2+} than with any other single metal ion (Izatt *et al.*, 1971). Cu^{2+} ions have an influence on the melting temperature of DNA (Eichhorn, 1962) and the effect of Cu^{2+} ions on DNA denaturation has now been extensively studied (Eichhorn and Clark, 1965; Hiai, 1965; Coates *et al.*, 1965; Venner and Zimmer, 1966; Eichhorn and Shin, 1968; Liebe and Stuehr, 1972a, b; Holman and Jordan, 1972; Richard *et al.*, 1973) and occurs because the Cu^{2+} ions sever the bonds between the double helix. Recent work on Cu^{2+}–DNA denaturation (Liebe and Stuehr, 1972a) implies that Cu^{2+} ions bind to both phosphate and base moieties of DNA simultaneously. Cu^{2+} ions have significant and different effects on polyribonucleotides which are degraded into small oligonucleotides when heated with copper ions by the cleavage of phosphate bonds (Butzow and Eichhorn, 1965).

^{31}P Nuclear magnetic resonance (nmr) studies confirm the binding of Cu^{2+} ions to the phosphate portion of the nucleotides (Cohn and Hughes, 1962; Eichhorn *et al.*, 1966; Kan and Li, 1972; Missen *et al.*, 1972). Metal interactions with the base portion of the nucleotides have also been extensively studied. Proton magnetic resonance studies indicate that the H-8 resonance is much more strongly affected than the H-2 resonance for D_2O solutions of ATP (Cohn and Hughes, 1962; Schneider *et al.*, 1964), 5′-dAMP (Eichhorn *et al.*, 1966), 5′-AMP (Berger and Eichhorn, 1971a; Missen *et al.*, 1972), and 3′-AMP (Berger and Eichhorn, 1971a) indicating that the metal ion interacts with the adenine base at the N-7 position.

In 1958, Frieden and Alles (1958) proposed that Cu^{2+} ions form five-membered ring complexes with guanosine derivatives by coordinating with N-7 and the carbonyl oxygen O-6. The H-8 proton resonance is broadened in 5′-dGMP by Cu^{2+} ions indicating that the metal ions are bound at N-7 (Eichhorn *et al.*, 1966). Tu and Friederich (1968) by means of conductometric, potentiometric, and spectrophotometric titrations found that Cu^{2+} ions combined with guanosine, 5′-GMP, inosine, and 5′-IMP on a mole to mole basis. Infrared spectra indicate that the oxygen atom at C-6 was also involved so the pentacyclic complex involving the C-6 oxygen and the N-7 nitrogen was favored. Sletten (1971), however, has shown by X-ray analysis of the 9-methylhypoxanthinecopper crystal that the N-7 nitrogen is the only binding site and the chelate structure involving N-7 and O-6 is not observed.

Cu^{2+} ions broadened the H-8 and H-2 resonances of 5′-IMP equally and simultaneously (Berger and Eichhorn, 1971b) indicating either chelation to N-7 and the O-6 positions or binding of Cu^{2+} ions to nitrogen atoms on both rings. In the polymer poly(I), the H-8 proton resonance is preferentially broadened indicating that the metal binds to N-7 only (Berger and Eichhorn, 1971b). These studies were extended (Berger and Eichhorn, 1971c) to Cu^{2+} ion interactions with

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