Transcriptional Role of Yeast Deoxyribonucleic Acid Dependent Ribonucleic Acid Polymerase III†

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ABSTRACT: Isolated yeast nuclei synthesize RNA via the endogenous RNA polymerases (nucleosidetriphosphate:RNA nucleotidyltransferase, EC 2.7.7.6) when incubated with ribonucleoside triphosphates in vitro. Electrophoretic analysis of the newly synthesized RNA on polyacrylamide gel slabs indicates that four discrete classes of low molecular weight RNA (less than 5.8 S) are synthesized. Two of the low molecular weight RNA species coelectrophorese with yeast 5S and 4S marker RNAs while the other two RNA classes have electrophoretic mobilities similar to those reported (Blatt, B., & Feldmann, H. (1973) *FEBS Lett. 37*, 129) for yeast 4S precursor RNAs (pre-4S RNA). The synthesis of all four classes of low molecular weight RNA is not detectably inhibited by α-amanitin concentrations as high as 2.4 mg/mL. Since yeast RNA polymerase III is resistant to high concentrations of α-amanitin, while polymerases I and II are inhibited, it appears that 5S RNA, 4S RNA, and pre-4S RNAs in yeast are synthesized by RNA polymerase III. In addition to the discrete low molecular weight RNA species synthesized in vitro, isolated yeast nuclei also synthesize a heterogeneous distribution of RNA species ranging in size from 7 S to greater than 25 S. The synthesis of more than half of this RNA, including species as large as 17–25 S, is resistant to very high concentrations of α-amanitin (2.4 mg/mL). These results suggest that yeast RNA polymerase III might also be responsible for the synthesis of some intermediate and high molecular weight RNA species.

Multiple forms of eukaryotic DNA-dependent RNA polymerase have now been found in many different species of plants, animals, and fungi (for a review see Roeder, 1976). The existence of three different classes of RNA polymerases suggests that each enzyme class has a specialized function in the transcription of genetic information. The existing data for vertebrate RNA polymerases indicate that this is indeed the case. RNA polymerase I appears to be responsible for the synthesis of the precursor to ribosomal 18S and 28S RNAs (Roeder & Rutter, 1970; Reeder & Roeder, 1972). RNA polymerase II is responsible for the synthesis of heterogeneous nuclear RNA and adenovirus-specific messenger RNA in adenovirus-infected KB (human) cells (Zylber & Penman, 1971; Weinmann et al., 1974a,b). The vertebrate nucleoplasmic RNA polymerase III appears to function in the synthesis of a variety of low molecular weight RNA species. A number of studies indicate that ribosomal 5S RNA and tRNA precursors are synthesized by polymerase III (Weinmann & Roeder, 1974; Weinmann et al., 1974a,b; Udvardy & Seifart, 1976; Weil & Blatti, 1976). RNA polymerase III is also responsible for the synthesis of several additional discrete low molecular weight cellular RNA species (Sklar & Roeder, 1977) as well as several small discrete RNA species from adenovirus-infected cells (Weinmann et al., 1976).

Yeast cells contain three classes of nuclear DNA-dependent RNA polymerases which are analogous in many respects to the class I, class II, and class III RNA polymerases isolated from vertebrate cells. Studies of salt dependence of enzyme activity, template specificity, and chromatographic behavior on DEAE[1]-substituted cellulose and Sephadex columns (Adman et al., 1972; Schultz & Hall, 1976; Valenzuela et al., 1976) and the comparisons that have been made between the protein subunit structures of the vertebrate and yeast RNA polymerases (Chambon, 1974; Valenzuela et al., 1976) all support the concept of a homology between yeast RNA polymerases I, II, and III and the class I, class II, and class III RNA polymerases, respectively, from vertebrate cells. In addition, as is seen for other eukaryotic systems (Chambon, 1974), polymerase II is the most sensitive of the three yeast RNA polymerases to inhibition by the mushroom toxin, α-amanitin (Buhler et al., 1974; Schultz & Hall, 1976; Valenzuela et al., 1976).

However, in contrast to the above similarities seen between the corresponding yeast and vertebrate RNA polymerases, the yeast enzymes I and III show a pattern of α-amanitin sensitivity that is the reverse of that seen for the analogous vertebrate RNA polymerases. Yeast polymerase I can be completely inhibited by high concentrations of α-amanitin, whereas yeast polymerase III is only slightly sensitive to concentrations exceeding 2 mg/mL (Schultz & Hall, 1976; Valenzuela et al., 1976). This contrasts sharply with the α-amanitin inhibition pattern seen for the RNA polymerases from HeLa cells, mouse plasmacytoma cells, and *Xenopus laevis* where the class I RNA polymerases are completely resistant to the drug while the class III RNA polymerases are completely inhibited by concentrations of α-amanitin in excess of 200 μg/mL (Weil & Blatti, 1976; Schwartz et al., 1974; Sklar & Roeder, 1975).

This significant incongruity between the properties of the yeast and vertebrate class I and class III RNA polymerases underscores the importance of determining the transcriptional

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[1] Abbreviations used are: DEAE, diethylaminoethyl; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid; Temed, tetramethylethylenediamine; $PhCH_2SO_2F$, phenylmethanesulfonyl fluoride; 4S RNA, low-molecular weight RNA fraction containing the transfer RNAs; pre-4S RNA, 4S RNA precursors.

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function of each yeast RNA polymerase. It is not clear which properties of the isolated RNA polymerases are most strongly correlated with their in vivo functions. Therefore, the possibility must be considered that yeast and vertebrate RNA polymerases which show similar catalytic properties might, nevertheless, exhibit differences in transcriptional function. In particular, the differences in ribosomal RNA gene organization between yeast and higher eukaryotes suggest the possibility that one of the yeast RNA polymerases might have functions in common with several of the vertebrate RNA polymerases.

Both in yeast and the higher eukaryotes, the ribosomal 5.8S, 18S, and 28S RNAs are transcribed together in a single large precursor molecule (Udem & Warner, 1972; Darnell, 1968). In most eukaryotes, the genes for the ribosomal precursor RNAs are tandemly repeated in the nucleolar organizer region of the genome (Wallace & Birnstiel, 1966; Birnstiel et al., 1971) while the 5S cistrons are arranged in one or more clusters of tandem repeats in regions of the genome which are unlinked to the nucleolar organizer (for references see Edstrom & Lambert, 1975). In the higher eukaryotes, the distinct chromosomal localizations of the 5S genes and ribosomal genes can be correlated with the observation that RNA polymerase III makes 5S RNA while a different enzyme, polymerase I, synthesizes the ribosomal RNA precursor.

In contrast, the 5S genes in yeast are interspersed in an alternating arrangement with the genes for the ribosomal precursor RNAs (Rubin & Sulston, 1973). Therefore, the possibility might exist that the same yeast RNA polymerase transcribes both types of genes. Since the results of the in vitro transcription experiments of Van Keulen et al. (1975) and Holland et al. (1977) suggest that yeast RNA polymerase I preferentially transcribes the ribosomal cistrons, this raises the possibility that yeast RNA polymerase I might also transcribe the 5S genes.

As an initial approach to the study of the functions of the yeast RNA polymerases, I have examined the properties of RNA synthesis by isolated yeast nuclei. In these experiments I have used the differential α-amanitin sensitivities of yeast RNA polymerases I, II, and III to determine the RNA polymerase(s) responsible for the synthesis of 5S RNA and transfer RNA precursors. By examining the RNA products made in yeast nuclei under increasing degrees of α-amanitin inhibition, I have shown that yeast RNA polymerase III appears to be responsible for the synthesis of ribosomal 5S RNA and transfer RNA precursors.

Materials and Methods

Biochemicals. Glusulase was obtained from Endo Laboratories, Inc. (Garden City, N.Y.). Ficoll 400 was obtained from Pharmacia. Tris-base, sucrose (RNase-free), urea, and ammonium sulfate were Schwarz/Mann Ultra Pure reagents. Sodium dodecyl sulfate and formamide (AnalaR grade) were from BDH Chemicals Ltd. Acrylamide and *N,N'*-methylenebis(acrylamide) were Bio-Rad Electrophoresis Purity reagents. 1-Ethyl-2-[3-(1-ethylnaphtho[1,2-*d*]thiazolin-2-ylidene)-2-methylpropenyl]naphtho[1,2-*d*]thiazolium bromide (Stains-All) was obtained from Eastman Organic Chemicals. Total yeast transfer RNA and pyruvate kinase (no. 15744) were obtained from Boehringer Mannheim GmbH. Unlabeled ribonucleoside triphosphates were purchased from P-L Biochemicals and [^{3}H]UTP (22–27.6 Ci/mmol) was from New England Nuclear. α-Amanitin and salmon sperm DNA were purchased from Calbiochem.

Yeast Strains and Growth Medium. The *Saccharomyces cerevisiae* strain Y55 is a wild-type homothallic diploid (Bhargava & Halvorson, 1971). *S. cerevisiae* strain +D4 is a heterothallic diploid described by Hartwell et al. (1970). Low phosphate medium is prepared as described by Hollenberg (1973) except that 20 μg/mL adenine and 20 μg/mL uracil are also present while KH_2PO_4 is not added. YEP medium is composed of 2% glucose, 2% Bacto-peptone (Difco), 1% yeast extract (Difco), 40 μg/mL adenine, and 40 μg/mL uracil.

Solutions. Buffer A was 18% (w/w) Ficoll and 0.5 mM $CaCl_2$, dissolved in 20 mM KH_2PO_4 (pH 6.5). Immediately before use, $PhCH_2SO_2F$ (34 mM stock solution in absolute ethanol) was added to a final concentration of 1.7 mM. Buffer B was 1 M sorbitol, 7% (w/w) Ficoll, 20% (w/w) glycerol, and 0.5 mM $CaCl_2$, dissolved in 20 mM KH_2PO_4 (pH 6.6). $PhCH_2SO_2F$ was added just before use to 1.7 mM. Buffer C was 0.05 M Tris-Cl (pH 7.5, 22 °C), 0.6 M sucrose, 1 mM $MgCl_2$, 10 mM NaCl, and 1 mM dithiothreitol. REX buffer was 0.05 M Tris-Cl, 0.1 M NaCl, 0.01 M EDTA, pH 7.4. TBE buffer was 90 mM Tris-borate (pH 8.3) and 2.5 mM Na_3EDTA.

Preparation of Yeast Nuclei. The procedure for the isolation of yeast nuclei from spheroplasts is a modification of a procedure developed by Lohr & Van Holde (1975) which is based on the method of Wintersberger et al. (1973). Cells of *Saccharomyces cerevisiae* strain Y55 were grown with aeration at 30 °C in YEP medium to a final density of 1–2 × 10^7 cells/mL. Cells were harvested by centrifugation, washed several times with glass-distilled water, and then converted to spheroplasts by the two-step procedure of Cabib (1971) except that glusulase digestion was carried out in an unbuffered solution containing 1 M sorbitol.

All the following steps were performed at 4 °C. Spheroplasts were harvested by centrifugation at 2000*g* for 10 min, washed once with 1 M sorbitol containing 1.7 mM $PhCH_2SO_2F$ (7.5 mL per g of original cells), and collected by centrifugation at 4000*g* for 10 min. The spheroplast pellet was then resuspended in at least 5 vol of buffer A. Spheroplasts lyse in this buffer while nuclei remain intact. Cells and unlysed spheroplasts were removed by centrifugation at 2000*g* for 5 min. Nuclei remained in the supernatant and were collected by centrifugation at 30 000*g* for 25 min. Membranous material at the top of the supernatant was removed by vacuum aspiration, and the remaining supernatant decanted. Nuclei in the resulting pellet were initially resuspended in 2 vol of buffer B. The volume was then increased to 10 vol with buffer B and contaminating cells and/or unlysed spheroplasts were removed by low-speed centrifugation (about 5 min at 3000*g*; however, exact conditions depend upon type of centrifuge employed). The supernatant was examined by phase contrast microscopy to determine the intactness and purity of the nuclear preparation. The low-speed centrifugation was repeated as many times as necessary to remove contaminating cells. Nuclei were collected from the final supernatant by centrifugation at 20 000*g* for 25 min, and the nuclear pellet was resuspended in 0.1 mL of buffer C per 10^{10} original cells. Yields by this procedure were 30–50% based on recovery of nuclear DNA (J. Bennetzen, personal communication). Stored frozen at −70 °C, the endogenous RNA polymerase activity of the isolated yeast nuclei was stable for several weeks.

Measurement of RNA Polymerase Activity. Assays for the endogenous RNA polymerase activity in isolated nuclei were performed in reaction mixtures of either 50 or 100 μL containing 50 mM Tris-Cl (pH 7.9, 22 °C), 10 mM $MgCl_2$, 0.5 mM each of ATP, CTP, and GTP, 0.05 mM [^{3}H]UTP (specific activity was 1.5 Ci/mmol), 5 mM phosphoenolpyruvate, 20 μg of pyruvate kinase per mL, 1 mM dithiothreitol, 0.2 M sucrose, appropriate quantities of ammonium sulfate or KCl,

and yeast nuclei. α-Amanitin, when present, was premixed with substrates at 0 °C prior to the addition of nuclei. Reaction mixtures were incubated 10 min at 25 °C and usually terminated by the addition of 10 μg of heat-denatured salmon sperm DNA and 1.5 mL of 10% Cl_3AcOH containing 0.12 M sodium pyrophosphate. Acid-precipitable radioactivity in RNA was determined as described by Adman et al. (1972). In some experiments, reactions were terminated by the addition of 0.5 vol of 1% (w/v) sarkosyl containing 0.05 M sodium pyrophosphate. The resulting mixtures were vortexed and an aliquot pipetted onto DEAE-cellulose disks (Whatman DE81) followed by immersion of the disks in 0.5 M Na_2HPO_4. The disks were washed and dried as described by Roeder (1974) and radioactivity was measured by liquid scintillation counting in Liquifluor–toluene (New England Nuclear Co.).

Isolation of in Vitro Synthesized RNA. High specific activity RNA was prepared from nuclei incubated as described above in a final volume of 100 μL except that the specific activity of the [^{3}H]UTP was increased to 15–20 Ci/mmol. After a 10-min incubation at 25 °C, a 10-μL aliquot was sampled for determination of cold Cl_3AcOH precipitable radioactivity while the remainder of the reaction mixture was terminated by the addition of 0.7 mL of a buffer containing 0.1 M sodium acetate (pH 5.1), 10 mM Na_3EDTA, and 0.5% sodium dodecyl sulfate (pH 5 RNA buffer). Yeast transfer RNA (50 μg) was added as carrier and the mixture extracted for 15 min at 23 °C with 1 vol of water-saturated phenol. The aqueous phase was collected by centrifugation for 10 min at 4500*g* and the phenol phase and interface were reextracted with 0.5 vol of pH 5 RNA buffer for 10 min at 23 °C. The two aqueous phases were combined and extracted with a phenol–chloroform (1:1) mixture for 10 min at 23 °C. The phases were separated by centrifugation and the organic phase removed with a Pasteur pipet, leaving the interface behind. The aqueous phase and interface were then extracted with 1 vol of chloroform for 10 min at 23 °C and centrifuged as before. The RNA in the aqueous phase was precipitated with 2 vol of absolute ethanol at −20 °C after addition of 0.1 vol of 2 M sodium acetate. After the solution was kept overnight at −20 °C, the RNA was collected by centrifugation at 25 000*g* for 60 min at −15 °C in a Sorvall HB-4 rotor. The RNA was dissolved in 30 μL of TBE buffer and stored frozen at −20 °C. The final yield of RNA was 65 to 85% of the Cl_3AcOH-precipitable counts.

Polyacrylamide Slab-Gel Electrophoresis of RNA. Slab-gel electrophoresis was performed using the vertical gel electrophoresis cell of Raymond & Nakamichi (1962) (purchased from E-C Apparatus Corp.) which provides a gel slab 12.5 × 17.5 × 0.3 cm with 16 sample wells. The buffer system employed was the Tris–borate–EDTA (TBE) buffer described by Peacock and Dingman (1968). The final concentrations of each of the stock reagents in the 10% running gel were 10% (w/v) acrylamide, 0.26% (w/v) *N,N'*-methylenebis(acrylamide), 1 × TBE buffer, and 7 M urea. The final composition of the spacer gel was 3% (w/v) acrylamide, 0.15% (w/v) *N,N'*-methylenebis(acrylamide), 1 × TBE buffer, and 7 M urea. The gel solutions were polymerized by the addition of *N,N,N',N'*-tetramethylethylenediamine (Temed) and 10% (w/v) ammonium persulfate to final concentrations of 0.05% (v/v) and 0.1% (w/v), respectively.

The gel slab was prepared according to the following procedure. The bottom of the electrophoresis column was occluded by polymerizing a 30-mL plug of the 10% running gel in place, with the cell supported at an angle of 45°. The apparatus was then placed vertically and filled with 10% running gel solution to a level 4.5 cm from the top of the gel column. Before the 10% acrylamide solution polymerized, the 3% spacer gel solution was gently layered on top of the running gel solution until the gel chamber was filled, and the sample-well former was then inserted. The slight mixing of the 3 and 10% phases that occurs prevents the formation of a sharp interface and subsequent stacking of RNA molecules. When polymerization was complete, the upper and lower buffer chambers were filled with 1 × TBE buffer. No preelectrophoresis was performed. RNA samples were diluted (1:1) with 2.3 M sucrose containing 0.008% (w/v) bromophenol blue, and 15-μL aliquots were applied to the sample wells. Electrophoresis was carried out for 5 h at 30 °C at 200 V (constant voltage). At the completion of the electrophoresis the gel slab was removed from the apparatus and stained overnight in the dark in a 0.005% (w/v) solution of Stains-All dissolved in 50% formamide in water (Dahlberg et al., 1969) and destained in running tap water in the dark.

Determination of Distribution of Radioactivity in Polyacrylamide Slab Gels. The sample slots to be counted were cut from the slab gel as lengthwise strips and cut into 1.1-mm slices as described by Dingman and Peacock (1968). Each gel slice was combined with 2 drops of distilled water (measured with a Pasteur pipet) and 0.7 mL of Protosol (New England Nuclear) in a tightly capped glass vial. Following incubation for 16 h at 37 °C with shaking, 5.0 mL of scintillation fluid (4 g of Omnifluor (New England Nuclear) per liter of toluene) was added and the samples counted in a Beckman LS-230 scintillation counter.

Preparation of in Vivo Marker RNAs. Cells of *S. cerevisiae* strain +D4 were grown in 250 mL of low phosphate medium at 30 °C to a density of 5 × 10^6 cells/mL and then labeled for two generations (3.5 h) with $^{32}PO_4$ (3.3 μCi/mL). Sodium phosphate buffer (0.5 M) (pH 6.8) was then added to a final concentration of 0.03 M and the cells were harvested by centrifugation after an additional 30-min incubation. Cells were washed twice with REX buffer and disrupted by agitation with glass beads (Adman et al., 1972). The RNA in the homogenate was purified by successive phenol extractions and ethanol precipitated twice (Andrew et al., 1976). Unlabeled total yeast RNA was prepared by a similar procedure except that the RNA was deproteinized by successive extractions with a (1:1) mixture of phenol and chloroform.

The ^{32}P-labeled ribosomal 5.8S and 5S RNAs and 4S RNA were further purified by continuous-elution preparative polyacrylamide gel electrophoresis. The elution cell employed, procedure of operation, and buffer system used were as described by Hagen & Young (1974). ^{32}P-Labeled total yeast RNA (800 μg) was electrophoresed on a 4-cm, 2.5% polyacrylamide gel with a constant current of 15 mA, elution flow rate of 0.1 mL/min, and fraction time of 10 min. The fractions containing 5.8S, 5S, and 4S RNAs were pooled, combined with 0.15 vol of 3 M NaCl, and precipitated by the addition of 2 vol of absolute ethanol. Following overnight storage at −20 °C, the precipitated RNA was collected by centrifugation at 25 000*g* for 1 h at −15 °C in a Sorvall HB-4 rotor. The RNA (100 μg) was dissolved in 0.4 mL of sterile distilled water and stored frozen at −20 °C.

Calculation of Inhibitory Effects of α-Amanitin on Synthesis of Various RNA Species. In order to quantitate the effect of α-amanitin on the synthesis of each class of RNA, the following calculations were employed for each incubation conducted in the presence of a different concentration of the drug. For each region of the gel (heterodisperse RNA, peak I, peak II, peak III, and peak IV) the total radioactivity present in that region was determined and the appropriate background subtraction made. For peaks I–IV, the backgrounds used in the calculations are represented by the dotted lines in panels

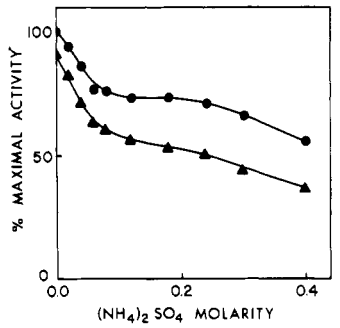

FIGURE 1: Effect of ammonium sulfate concentration on endogenous nuclear RNA polymerase activity. Isolated yeast nuclei were incubated in the presence (▲) or absence (●) of 100 μg/mL of α-amanitin in a final reaction volume of 50 μL. Other conditions were as described in Materials and Methods except that the ammonium sulfate concentration was varied as indicated. Incorporation of radioactivity into RNA was determined by pipetting aliquots onto DEAE-cellulose (DE-81) disks which were processed as described in Materials and Methods. The maximal activity (100%) was 77.4 pmol of UMP incorporated per mL.

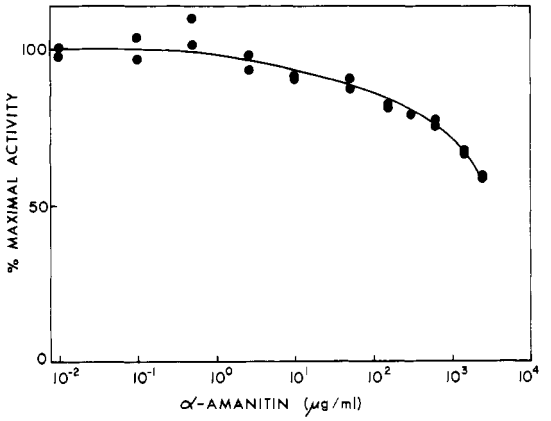

FIGURE 2: Effect of α-amanitin concentration on endogenous nuclear RNA polymerase activity. Nuclei were isolated as described in Materials and Methods, and 2.5-μL aliquots of the nuclear suspension were assayed for endogenous RNA polymerase activity in a final reaction volume of 50 μL. Conditions for the assay were as described in Materials and Methods except that the final concentration of ammonium sulfate was 0.02 M and the concentration of α-amanitin was varied as indicated. Incorporation of radioactivity into RNA was determined as described in Figure 1. Maximal activity (100%) represents 66 pmol of UMP incorporated per mL (●).

b and c of Figure 4 (background A). For the determination of the total radioactivity in the heterodisperse region as well as for the determination of the total radioactivity recovered from the gel, a background of 26 cpm was used (background B). (This background was determined by measuring the average radioactivity present in slices from a slot of the gel which had an unlabeled RNA sample.)

The fractional contribution of each class of RNA to the total radioactivity recovered from a particular gel slot was then calculated using the equation: fractional contribution = [(total ^3H cpm in particular gel region) − (appropriate background)]/[(total ^3H cpm recovered from gel slot) − (background B)]. Thus, for a particular concentration of α-amanitin, the fraction of the total radioactivity in RNA that is present in a particular size class can be calculated. To determine the amount of each RNA species synthesized in a reaction with a particular concentration of α-amanitin, the fractional contribution of each RNA species (determined by the equation above) is multiplied by the total ^3H cpm incorporated during the incubation. This yields a value in cpm for each type of RNA synthesized in a particular incubation.

Results

Isolation of Yeast Nuclei. Several procedures were tested for the isolation of yeast nuclei active for RNA synthesis. The nuclear isolation procedure based upon osmotic shock of yeast spheroplasts in the presence of Ficoll (Lohr & Van Holde, 1975; Wintersberger et al., 1973) yields a nuclear preparation which has very reproducible properties of RNA synthesis (Schultz, 1976). All three yeast DNA-dependent RNA polymerases (enzymes I, II, and III) are readily solubilized from the isolated nuclei; however, the yield of polymerase II relative to the yields of RNA polymerases I and III is several-fold lower than that expected based on relative recoveries from whole cells (Schultz, 1976). In a modification of the earlier procedures, I have included 1.7 mM phenylmethanesulfonyl fluoride ($PhCH_2SO_2F$) in the Ficoll-containing buffers. Nuclei prepared in the presence of $PhCH_2SO_2F$ appear morphologically more nearly intact by phase microscopy and are two- to threefold more active for RNA synthesis than nuclei prepared in the absence of $PhCH_2SO_2F$.

Conditions of Endogenous RNA Synthesis. In order to determine the optimal conditions for RNA synthesis by isolated yeast nuclei, the effects of a variety of parameters were investigated. The yeast nuclear system exhibits optimum or near optimum activity under the following conditions: 25 °C, 10 mM Mg^{2+}, pH 7.9. Although the presence of 3 mM Mn^{2+} was found to give slightly higher activity, 10 mM Mg^{2+} was used in our experiments as Mg^{2+} appears to give greater fidelity of transcription in other systems (Van Keulen et al., 1975; Holland et al., 1977). The temperature of the incubation has a very pronounced effect on the duration of RNA synthesis by isolated nuclei. At 25 °C, RNA synthesis occurs linearly for nearly 10 min and incorporation continues at a decreased rate for more than 30 min. At elevated temperatures (30 or 37 °C), the initial rate is greater, but the reaction is linear for less than 5 min and essentially complete by 15 min. The total amount of RNA synthesized is greater at lower temperatures.

The data in Figure 1 show the influence of ammonium sulfate concentration on the endogenous RNA polymerase activity of isolated yeast nuclei. RNA synthesis was measured in the presence and absence of 100 μg/mL of α-amanitin, a concentration sufficient to inhibit completely RNA polymerase II (Schultz & Hall, 1976). The fraction of α-amanitin sensitive activity (polymerase II) is minimal (less than 10%) in the absence of added salt and increases to 35% of the total activity assayed at 0.3 M ammonium sulfate. The absolute amount of endogenous polymerase II activity is stimulated more than twofold by the addition of salt and is optimal at 0.3 M ammonium sulfate. For yeast nuclei (Figure 1), the α-amanitin resistant activities (due to the combined endogenous polymerases I and III) are greatest in the absence of added salt. Therefore, assays were conducted at 0.02 M ammonium sulfate in order to enhance the amount of RNA polymerase III activity measured.

α-Amanitin Sensitivity of Endogenous RNA Polymerase Activity in Isolated Nuclei. When the α-amanitin sensitivity of nuclear RNA synthesis is measured in the presence of 0.02 M ammonium sulfate, the results shown in Figure 2 are obtained. About 10% of the nuclear activity is inhibited by a concentration of α-amanitin of 50 μg/mL, which is sufficient to completely inhibit the isolated RNA polymerase II (Schultz & Hall, 1976). At the higher concentrations of α-amanitin required to inhibit RNA polymerase I (50% inhibited at 600 μg/mL; Schultz & Hall, 1976), there is an additional 30%

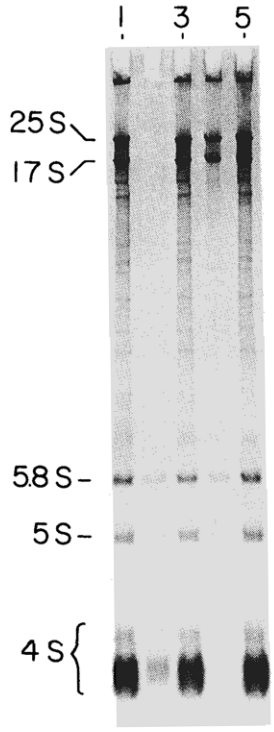

FIGURE 3: Slab gel electropherograms of RNA extracted from isolated nuclei. This figure is a photograph of a section of the polyacrylamide slab gel from the experiment of Figure 4. The gel was stained with Stains-All and photographed using a green filter. The direction of electrophoresis was from top to bottom. The positions of migration of ribosomal 25S and 17S RNAs and 5.8S, 5S, and 4S RNAs are indicated on the figure. Slots 1, 3, and 5 show the stain pattern for RNA extracted from isolated nuclei incubated in the presence of the following concentrations of α-amanitin: slot 1, 50 μg/mL; slot 3, 2400 μg/mL; and slot 5, no α-amanitin. This pattern of stainable bands is very reproducible and has been seen for the RNA from three different preparations of nuclei. Slot 4 shows the stain pattern for unlabeled yeast total RNA prepared as described in Materials and Methods. Slot 2 shows the pattern for a mixture of 5.8S, 5S, and 4S RNAs prepared by direct phenol extraction of whole yeast cells (Holley et al., 1961). The absence of intermediate-sized discrete bands in slot 2 indicates that it is unlikely that those discrete bands are the result of specific aggregates of low molecular weight RNA species. Additional results (not shown) indicate that those intermediate-sized discrete bands are not present in the total yeast transfer RNA (Boehringer Mannheim GmbH) used as carrier.

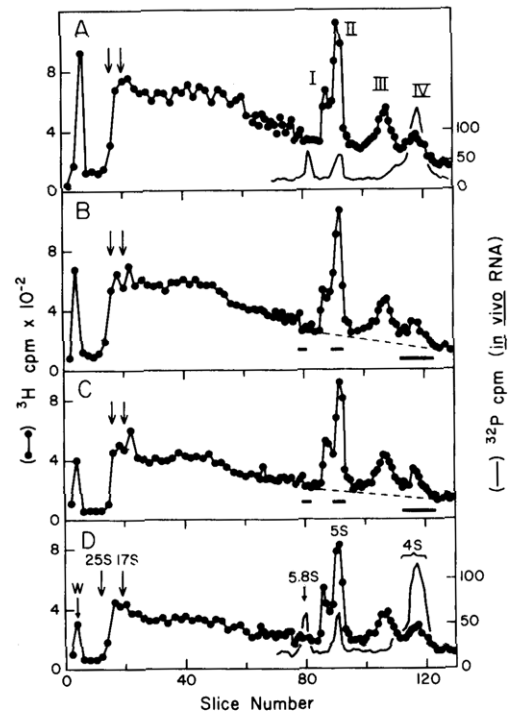

FIGURE 4: Polyacrylamide gel electrophoresis of RNAs synthesized in vitro by isolated yeast nuclei in the presence of increasing concentrations of α-amanitin. Aliquots (20 μL) of a suspension of isolated yeast nuclei were incubated for RNA synthesis in a final reaction volume of 100 μL as described in Materials and Methods, except that the [^{3}H]UTP specific activity was increased to 20 Ci/mmol and ammonium sulfate was present at 0.02 M. Final concentrations of α-amanitin present in the incubations were as follows: (A) no α-amanitin; (B) 50 μg/mL; (C) 480 μg/mL; and (D) 2.4 mg/mL. At the conclusion of the incubation the RNA was extracted and analyzed by electrophoresis in polyacrylamide gel slabs as described in Materials and Methods. A small amount (1500 cpm) of an electrophoretically purified RNA mixture containing ^{32}P-labeled ribosomal 5.8S RNA, 5S RNA, and tRNA sythesized in vivo was included as an internal marker with each in vitro sample. Strips of the slab gel were cut into 1.1-mm slices and solubilized, and radioactivity was measured as described in Materials and Methods: (—) in vivo marker [^{32}P]RNAs; (●—●) [^{3}H]RNAs synthesized in vitro by nuclei. Slices 1–70 contained no ^{32}P radioactivity above background (18 cpm). In panels B and C, horizontal bars indicate the positions of the ^{32}P-labeled 5.8S RNA, 5S RNA, and 4S RNA markers. The vertical arrows at the left end of each panel correspond to the position of 25S and 17S ribosomal RNA markers. The peak of ^{3}H radioactivity at the extreme left end of each panel (identified with a W in panel D) represents material which did not enter the gel. All values were corrected for spillover of ^{32}P into the ^{3}H channel (2%), but no backgrounds were subtracted. The dotted line (- - -) in panels B and C represents the background used for calculations of the areas under each of the low molecular weight RNA peaks as described in the text.

inhibition of RNA synthesis, although no clear inflection point is seen. About 60% of the endogenous polymerase activity is not inhibited by the highest concentrations of α-amanitin tested (2.4 mg/mL). Because the isolated polymerase I is inhibited 85% by that concentration of drug while polymerase III is inhibited less than 30% (Schultz & Hall, 1976; Valenzuela et al., 1976), it appears that the major part of the endogenous activity in nuclei assayed in the presence of 0.02 M ammonium sulfate is due to RNA polymerase III. It is unlikely that the failure to observe more inhibition by α-amanitin is due to a nonspecific binding of the drug by nuclear proteins and a reduced effective concentration, since about a 5% greater inhibition at each drug concentration than that shown in Figure 2 was obtained when the experiment was repeated with a fourfold greater concentration of nuclei in the incubation mixture. The degree of inhibition seen at each drug concentration was very similar (±5%) for four different preparations of yeast nuclei and three different batches of α-amanitin. The main variation between nuclear preparations was in the amount of endogenous polymerase II activity.

Analysis of RNA Products Synthesized in the Presence of α-Amanitin. RNA synthesized in vitro by isolated yeast nuclei was examined by polyacrylamide slab gel electrophoresis. Figure 3 shows a photograph of one such gel following staining with Stains-All. The presence of stainable quantities of the ribosomal 25S RNA, 17S RNA, 5.8S RNA, and 5S RNA species is presumably due to the ribosomes which are seen to be studding the outer nuclear envelope in electron micrographs of the isolated nuclei. The most interesting result to note, however, is the presence of a large number of additional discrete RNA bands (Figure 3).

The distribution of radioactivity within the gel slab was determined as described in Materials and Methods. The results for four representative RNA samples are shown in Figure 4. Isolated yeast nuclei synthesize four discrete classes of low molecular weight RNA (less than 5.8 S). In Figure 4 these are labeled as peaks I–IV, respectively, in order of decreasing molecular weight. In addition, a large amount of RNA heterogeneous in size in the range from 7 S to greater than 25 S

TABLE I: α-Amanitin Sensitivity of RNA Synthesis in Isolated Yeast Nuclei.[a]

		% of synthesis by control				
		α-Amanitin concn (μg/mL)				
RNA species	Control	50	200	480	1200	2400
Total RNA	100	88	82	78	71	63
Heterodisperse	100	90	80	79	65	60
Peak I	100	107	92	135	124	115
Peak II	100	102	101	99	101	102
Peak III	100	100	103	107	112	104
Peak IV	100	99	95	105	108	108
Coeff of variation		±10.5%	±6.9%	±11.2%	±9.1%	±10.2%

[a] These results are the composite of two independent, but related experiments and include data from the experiment presented in Figure 4. The values for total RNA are calculated from the total ^3H cpm incorporated into RNA in the incubations used to synthesize RNA for analysis on polyacrylamide gels. The average control value is 268 500 cpm incorporated. The values for the amount of heterodisperse RNA and RNA species in peaks I, II, III, and IV synthesized in the presence of different concentrations of α-amanitin are calculated as described in the text, and are expressed relative to the control incubation without α-amanitin. The coefficient of variation at each drug concentration was calculated as the sum of the coefficient of variation of the data for the total ^3H cpm incorporated in the control incubation and the coefficient of variation for the total ^3H cpm incorporated in a particular reaction with the indicated concentration of α-amanitin. For the small RNA synthesis results, these values are a minimum estimate of the variability in the data as they do not include any estimate of the uncertainty involved in determining the background levels under peaks I–IV (background A described in Materials and Methods). The mean coefficient of variation for the entire data set is ±9.6%. The average control values for radioactivity in RNA corresponding to heterodisperse RNA and peaks I, II, III, and IV, respectively, are 182 700, 3760, 11 900, 9150, and 5150 cpm.

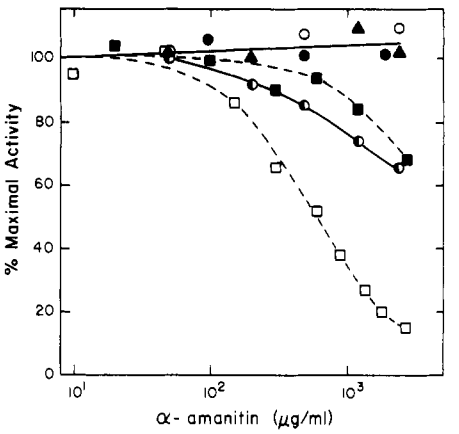

FIGURE 5: Effect of α-amanitin concentration on the synthesis of RNA by isolated yeast nuclei. The data are taken from the experiment presented in Table I and several related experiments. The percent radioactivity remaining in each class of RNA as a function of α-amanitin concentration was calculated as described in the text. For the low molecular weight RNAs, the results obtained by summing up the total radioactivity present in peaks I, II, III, and IV (after background subtractions) are presented for three separate experiments (●, ○, ▲). At each α-amanitin concentration, the results are expressed as a percentage of the amount of radioactivity present in the low molecular weight RNAs in the absence of the drug. For the heterodisperse RNAs, the plotted results are calculated from the data presented in Table I (◐). In order to examine the effects of α-amanitin on the synthesis of only those heterodisperse RNAs made by the endogenous RNA polymerase I and III activities, the results are normalized as follows: for each α-amanitin concentration, the amount of radioactivity incorporated into heterodisperse RNA is expressed as a percentage of the radioactivity incorporated into that class of RNA in the presence of sufficient α-amanitin (50 μg/mL) (◐) to inhibit RNA polymerase II, but not polymerases I and III (Schultz & Hall, 1976). The 100% values (with appropriate background subtraction) are 40 300 cpm (●), 28 400 cpm (▲), 30 900 cpm (○), and 160 100 cpm (◐). The inhibition curves for the partially purified RNA polymerase I (□- - -□) and RNA polymerase III (■- - -■) are replotted from Figure 4 of Schultz & Hall (1976).

(referred to as heterodisperse RNA) is synthesized in vitro (Figure 4). In order to facilitate the identification of these in vitro RNAs, they were coelectrophoresed with a mixture of ^{32}P-labeled in vivo 5.8S RNA, 5S RNA, and 4S RNA. The RNA species in peak II comigrates exactly with the in vivo ribosomal 5S RNA marker, suggesting that peak II represents 5S RNA. Peak IV contains a more heterogeneous size class of RNAs which coelectrophorese with the marker 4S RNA. In addition, there are two classes of RNA synthesized in vitro (peaks I and III) which show electrophoretic mobilities similar to the yeast tRNA precursors (pre-4S RNA) observed by Blatt & Feldmann (1973). In preliminary experiments in which nuclei were incubated with [^3H]UTP for 4 min and then chased with excess unlabeled UTP for various lengths of time, there was a decrease with time of the amount of radioactivity present in peaks I and III and a concomitant increase in the amount of radioactivity present in the 4S region of the gel while the 5S region was not detectably affected (J. Bennetzen, personal communication). This further suggests that the RNA species in peaks I and III represent tRNA precursors.

In order to define which RNA polymerase(s) function in the synthesis of these discrete classes of low molecular weight RNA, the effect of α-amanitin on the synthesis of the small RNA peaks and the heterodisperse RNA class was examined. The results of one such experiment are presented in Figure 4. To quantitate the effect of α-amanitin on the synthesis of each class of RNA, the data from the experiment of Figure 4 and several related experiments were analyzed as described in Materials and Methods. In Table I, the resulting values for the amount of each class of RNA synthesized in the presence of various concentrations of α-amanitin are expressed as a percentage of the radioactivity incorporated into that class of RNA in the absence of the drug. The synthesis of the heterodisperse RNA is inhibited about 10% by concentrations sufficient to completely inhibit RNA polymerase II (50 μg/mL) and inhibited 40% by concentrations of α-amanitin (2.4 mg/mL) sufficient to inhibit greater than 85% of the isolated RNA polymerase I activity (Schultz & Hall, 1976). On the other hand, the synthesis of all four classes of low molecular weight RNA (peaks I–IV) exhibits no significant degree of inhibition by α-amanitin concentrations as high as 2.4 mg/mL (see Table I).

In Figure 5, the α-amanitin sensitivities observed for the in vitro synthesis of the combined low molecular weight RNA fraction and for synthesis of heterodisperse RNA by isolated nuclei are compared with those of the partially purified RNA polymerases I and III. As discussed below, since polymerase III is the only known RNA polymerase in yeast nuclei which is substantially resistant to α-amanitin concentrations exceeding 2 mg/mL (Schultz & Hall, 1976; Valenzuela et al., 1976), the qualitative similarity of the α-amanitin inhibition patterns for polymerase III and for small RNA synthesis suggests that yeast 5S RNA and 4S RNA precursor transcripts are synthesized in vivo by RNA polymerase III.

The data presented in Figure 5 show that the synthesis of the heterodisperse RNA exhibits a sensitivity intermediate between those observed for the isolated RNA polymerases I and III. About 65% of the control value (at 50 μg/mL) is still synthesized in the presence of 2.4 mg/mL of the drug, a con-

centration at which nearly all of the residual synthesis is expected to be due to polymerase III activity. As may be seen in panel D of Figure 4, this heterodisperse RNA synthesized in the presence of high concentrations of α-amanitin (2.4 mg/mL) includes a mixture of RNA species ranging in size from 5.8 to 25 S. A substantial fraction of the radioactivity is still present in the 17S region (Figure 4D). It is unlikely that the radioactivity in the high molecular weight region is the result of aggregation of the low molecular weight RNAs, as there is no detectable ^{32}P radioactivity in that region as would be expected if the ^{32}P-labeled marker RNAs are aggregating with the in vitro synthesized low molecular weight RNAs. As discussed below, these results suggest that the endogenous RNA polymerase III activity in yeast nuclei might synthesize some intermediate and high molecular weight RNAs in addition to the low molecular weight 5S RNA, 4S RNA, and 4S RNA precursors.

Discussion

The observation that the yeast RNA polymerases show a differential sensitivity toward inhibition by α-amanitin (Schultz & Hall, 1976) suggested that an approach similar to that of Weinmann & Roeder (1974) might be employed to determine the transcriptional functions of these enzymes. In order to determine which of the RNA polymerase(s) are responsible for the synthesis of 4S and 5S RNA, an in vitro nuclear transcription system was developed which actively synthesizes these small RNAs. The isolated nuclei also synthesize a large amount of RNA heterogeneous in size in the range from 7 S to greater than 25 S (termed heterodisperse RNA).

The effects of α-amanitin on the synthesis of 5S RNA, pre-4S RNA, 4S RNA, and the heterodisperse RNA class have been examined. In interpreting these results, I will make the assumption that α-amanitin inhibits with equal effectiveness both the activity of a particular purified RNA polymerase on exogenous DNA and the activity of the corresponding endogenous enzyme in isolated nuclei. Two lines of evidence support this assumption. First, in the case of the vertebrate nuclear transcription systems, it has been clearly demonstrated that the endogenous RNA polymerase activities in nuclei show the same sensitivities to α-amanitin as do the corresponding purified class I, class II, and class III enzymes (reviewed in Roeder, 1976). Second, for the case of isolated yeast chromatin, Wintersberger et al. (1973) find that the endogenous RNA polymerase II activity is inhibited 50% at about 1.5 μg/mL α-amanitin and is totally inhibited by α-amanitin concentrations of 20 μg/mL, a result very similar to that observed for the isolated yeast RNA polymerase II (Schultz & Hall, 1976).

Using the data in this paper in conjunction with published results for the in vitro α-amanitin sensitivities of the three yeast RNA polymerases (Schultz & Hall, 1976; Valenzuela et al., 1976), a comparison can be made between the α-amanitin sensitivity of yeast polymerase III activity in vitro and the α-amanitin sensitivity of the synthesis of 5S RNA and pre-4S RNA in isolated yeast nuclei. At α-amanitin concentrations up to 600 μg/mL, neither the in vitro polymerase III activity nor the endogenous nuclear activity responsible for the synthesis of the RNA species in peaks I–IV (Figures 4 and 5) is significantly inhibited. In contrast, there is significant inhibition of both yeast RNA polymerase I and II activities by 600 μg/mL α-amanitin (Schultz & Hall, 1976; Valenzuela et al., 1976; Huet et al., 1975). Therefore, polymerases I and II appear not to be responsible for the synthesis of these small RNA species in yeast nuclei.

A more definitive basis for identifying yeast RNA polymerase III with 5S RNA and pre-4S RNA transcription might be sought by precise comparison, throughout the entire range of α-amanitin concentrations, of the inhibition curves for RNA polymerase III in vitro and small RNA synthesis in nuclei. Such a comparison cannot be made with high precision because, for both processes, the maximum observed inhibition is small, evidence for any inhibition is equivocal, and very high drug concentrations are required. In particular, there is some uncertainty regarding the degree of sensitivity of the isolated polymerase III to very high concentrations of α-amanitin (>1 mg/mL). In different studies, the sensitivity of yeast polymerase III to an α-amanitin concentration of 2 mg/mL has varied from less than 5% (Valenzuela et al., 1976) to 15% (L. Schultz, unpublished results) to 25% inhibition (Schultz & Hall, 1976). In contrast, the inhibition curves for the isolated RNA polymerases I and II are very reproducible even at high α-amanitin concentrations (L. Schultz, unpublished results; Schultz & Hall, 1976; Valenzuela et al., 1976). This suggests that the variable partial inhibition of polymerase III might be the result of nonspecific effects at very high drug concentrations (>1 mg/mL). Thus, the possibility must be considered that polymerase III is actually substantially more resistant to the specific inhibitory effects of α-amanitin than is apparent from the results presented in Figure 5. The observation that small RNA synthesis is not significantly inhibited suggests that such nonspecific effects, if they exist, may be less apparent in the nuclear transcription system possibly as a consequence of the differences in biochemical composition between isolated nuclei and the in vitro transcription system employing isolated polymerase III and purified DNA.

A further important consideration in interpreting these results concerns the apparent α-amanitin resistance of small RNA synthesis in isolated yeast nuclei. As indicated in Table I, there is a mean coefficient of variation of $\pm$9.6% for the small RNA synthesis results. This value is a minimum estimate of the variability as it does not take into consideration the uncertainties involved in determining the background levels under peaks I–IV in Figure 4. Hence, although no inhibition is apparent in the data presented in Table I and Figure 5, these results do not exclude the possibility that small RNA synthesis might show a slight (5–10%) sensitivity to inhibition by high concentrations of α-amanitin.

Therefore, although there is not a perfect quantitative agreement between the α-amanitin inhibition curves for the isolated polymerase III and that for small RNA synthesis in nuclei, the differences are probably not significant in view of the experimental variability in the small RNA synthesis data and the equivocal nature of the polymerase III inhibition results. On the other hand, the inhibition curves for the isolated polymerases I and II are clearly very different from that observed for small RNA synthesis in isolated nuclei. As the in vitro inhibition results for polymerases I and II are very reproducible (Schultz & Hall, 1976; Valenzuela et al., 1976), it is very unlikely that they are responsible for the synthesis of small RNAs in yeast nuclei. Hence, since RNA polymerase III is the only known DNA-dependent RNA polymerase in yeast nuclei which is resistant to 600 μg/mL α-amanitin and substantially resistant to 2.0 mg/mL of the drug (Schultz & Hall, 1976; Valenzuela et al., 1976), the qualitative similarity of the α-amanitin inhibition patterns for polymerase III and for small RNA synthesis suggests that yeast RNA polymerase III is responsible for the synthesis of 5S RNA and pre-4S RNA transcripts.

While it is possible that an uncharacterized fourth class of RNA polymerase activity in yeast nuclei might be responsible for the synthesis of the low molecular weight RNAs, this

possibility seems very unlikely. Since small RNA synthesis in both normal whole cells (Shulman et al., 1977) and isolated nuclei (Figure 4) accounts for greater than 10% of the measured transcription, it seems likely that such a fourth class of RNA polymerase, if it exists, would have been detected in studies characterizing and purifying the isolated yeast RNA polymerases. However, only three classes of yeast nuclear RNA polymerases have been detected in a number of different studies involving a variety of different enzyme solubilization and purification methods (Adman et al., 1972; Brogt & Planta, 1972; Ponta et al., 1972; Tipper, 1973; Sajdel-Sulkowska & Halvorson, 1975; Schultz & Hall, 1976; Valenzuela et al., 1976). Consequently, it seems very improbable that an α-amanitin resistant RNA polymerase activity other than polymerase III is responsible for the synthesis of low molecular weight RNAs in yeast nuclei. Thus, yeast RNA polymerase III appears to be functionally analogous to the vertebrate class III RNA polymerase, the enzyme which synthesizes 5S RNA and pre-4S RNA (Weinmann & Roeder, 1974; Weinmann et al., 1974a,b; Weil & Blatti, 1976).

There are two general implications of these results. First, they suggest that the function for synthesis of low molecular weight RNAs is more closely associated with the catalytic and chromatographic properties of a class III RNA polymerase than with the degree of α-amanitin sensitivity. Second, the results suggest that RNA polymerase III transcribes the 5S genes in yeast, as it does in vertebrates, despite the very great differences in 5S gene organization (see introductory statement). If, as suggested by the in vitro transcription experiments of Van Keulen et al. (1975) and Holland et al. (1977), yeast RNA polymerase I synthesizes the large ribosomal RNA precursor, then the transcriptional functions of both RNA polymerases I and III are conserved throughout evolution despite major changes in the chromosomal organization of the genes which each enzyme transcribes.

The effect of α-amanitin on the synthesis of heterodisperse RNA by isolated yeast nuclei has also been examined. The following discussion will be concerned with that fraction of the heterodisperse RNA made by RNA polymerases other than polymerase II; hence, the amount of RNA synthesized at each α-amanitin concentration will be compared to that made at 50 μg/mL α-amanitin. The synthesis of the heterodisperse RNA has a sensitivity to high concentrations of α-amanitin which is intermediate between those observed for the isolated RNA polymerases I and III. If we assume that heterodisperse RNA consists of a mixture of molecules some of which are made by RNA polymerase I and others by RNA polymerase III, it is possible to determine what proportion of the transcripts should be made by each polymerase activity in order to most closely approximate the α-amanitin inhibition curve observed for heterodisperse RNA synthesis. The best fit to the data is obtained with values of 60% polymerase III transcripts and 40% polymerase I transcripts. These results suggest that yeast RNA polymerase III might also be responsible for the synthesis of some intermediate and high molecular weight RNA species.

The particular transcriptional functions that have been clearly demonstrated for the class III RNA polymerases (see introductory statement) might lead one to conclude that these enzymes are specialized for the synthesis of small RNA molecules. However, in addition to the results presented above, there are some other indications that the class III polymerases might have additional functions. Studies of RNA synthesis in isolated nuclei from yeast (De Kloet & Beltz, 1975), *Dictyostelium* (Jacobson et al., 1974), and HeLa cells (Zylber & Penman, 1971) indicate that poly(A)-containing and/or heterogeneous nuclear RNA are synthesized by two distinct enzymes, one sensitive and the other resistant to low concentrations of α-amanitin. Therefore, it remains to be determined both for yeast and for other eukaryotes whether RNA polymerase III synthesizes exclusively low molecular weight RNAs or may also synthesize some intermediate and high molecular weight RNA species, such as the nuclear precursors for special classes of messenger RNA.

Acknowledgments

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References

Adman, R., Schultz, L. D., & Hall, B. D. (1972) *Proc. Natl. Acad. Sci. U.S.A. 69,* 1702.

Andrew, C., Hopper, A. K., & Hall, B. D. (1976) *Mol. Gen. Genet. 144,* 29.

Bhargava, M. M., & Halvorson, H. O. (1971) *J. Cell Biol. 49,* 423.

Birnstiel, M. L., Chipchase, M., & Speirs, J. (1971) *Prog. Nucleic Acid Res. Mol. Biol. 11,* 351.

Blatt, B., & Feldmann, H. (1973) *FEBS Lett. 37,* 129.

Brogt, Th. M., & Planta, R. J. (1972) *FEBS Lett. 20,* 47.

Buhler, J.-M., Sentenac, A., & Fromageot, P. (1974) *J. Biol. Chem. 249,* 5963.

Cabib, E. (1971) *Methods Enzymol. 22,* 120.

Chambon, P. (1974) *Enzymes, 3rd Ed. 10,* 261.

Dahlberg, A. E., Dingman, C. W., & Peacock, A. C. (1969) *J. Mol. Biol. 41,* 139.

Darnell, J. E., Jr. (1968) *Bacteriol. Rev. 32,* 262.

De Kloet, S. R., & Beltz, W. R. (1975) *Arch. Biochem. Biophys. 167,* 322.

Dingman, C. W., & Peacock, A. C. (1968) *Biochemistry 7,* 659.

Edstrom, J.-E., & Lambert, B. (1975) *Prog. Biophys. Mol. Biol. 30,* 57.

Hagen, F. S., & Young, E. T. (1974) *Biochemistry 13,* 3394.

Hartwell, L. H., Culotti, J., & Reid, B. (1970) *Proc. Natl. Acad. Sci. U.S.A. 66,* 352.

Holland, M. J., Hager, G. L., & Rutter, W. J. (1977) *Biochemistry 16,* 16.

Hollenberg, C. P. (1973) *Biochemistry 12,* 5320.

Holley, R. W., Apgar, J., Doctor, B. P., Farrow, J., Marini, M. A., & Merrill, S. H. (1961) *J. Biol. Chem. 236,* 200.

Huet, J., Buhler, J.-M., Sentenac, A., & Fromageot, P. (1975) *Proc. Natl. Acad. Sci. U.S.A. 72,* 3034.

Jacobson, A., Firtel, R. A., & Lodish, H. F. (1974) *J. Mol. Biol. 82,* 213.

Lohr, D., & Van Holde, K. E. (1975) *Science 188,* 165.

Peacock, A. C., & Dingman, C. W. (1968) *Biochemistry 7,* 668.

Ponta, H., Ponta, U., & Wintersberger, E. (1972) *Eur. J. Biochem. 29,* 110.

Raymond, S., & Nakamichi, M. (1962) *Anal. Biochem. 3,* 23.

Reeder, R. H., & Roeder, R. G. (1972) *J. Mol. Biol. 67,* 433.

Roeder, R. G. (1974) *J. Biol. Chem. 249,* 241.

Roeder, R. G. (1976), in *RNA Polymerase* (Losick, R., & Chamberlin, M., Eds.) p 285, Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.

Roeder, R. G., & Rutter, W. J. (1970) *Proc. Natl. Acad. Sci.*

U.S.A. 65, 675.
Rubin, G. M., & Sulston, J. E. (1973) *J. Mol. Biol. 79,* 521.
Sajdel-Sulkowska, E., & Halvorson, H. O. (1975) *J. Cell Biol. 67,* 377a.
Schultz, L. D. (1976), Ph.D. Dissertation, University of Washington, Seattle, Wash.
Schultz, L. D., & Hall, B. D. (1976) *Proc. Natl. Acad. Sci. U.S.A. 73,* 1029.
Schwartz, L. B., Sklar, V. E. F., Jaehning, J. A., Weinmann, R., & Roeder, R. G. (1974) *J. Biol. Chem. 249,* 5889.
Shulman, R. W., Sripati, C. E., & Warner, J. R. (1977) *J. Biol. Chem. 252,* 1344.
Sklar, V. E. F., & Roeder, R. G. (1975) *Fed. Proc., Fed. Am. Soc. Exp. Biol. 34,* A2448.
Sklar, V. E. F., & Roeder, R. G. (1977) *Cell 10,* 405.
Tipper, D. J. (1973) *J. Bacteriol. 116,* 245.
Udem, S. A., & Warner, J. R. (1972) *J. Mol. Biol. 65,* 227.
Udvardy, A., & Seifart, K. H. (1976) *Eur. J. Biochem. 62,* 353.
Valenzuela, P., Hager, G. L., Weinberg, F., & Rutter, W. J. (1976) *Proc. Natl. Acad. Sci. U.S.A. 73,* 1024.
Van Keulen, H., Planta, R. J., & Retel, J. (1975) *Biochim. Biophys. Acta 395,* 179.
Wallace, H., & Birnstiel, M. L. (1966) *Biochim. Biophys. Acta 114,* 296.
Weil, P. A., & Blatti, S. P. (1976) *Biochemistry 15,* 1500.
Weinmann, R., & Roeder, R. G. (1974) *Proc. Natl. Acad. Sci. U.S.A. 71,* 1790.
Weinmann, R., Brendler, T. G., Raskas, H. J., & Roeder, R. G. (1976) *Cell 7,* 557.
Weinmann, R., Raskas, H. J., & Roeder, R. G. (1974a) *Proc. Natl. Acad. Sci. U.S.A. 71,* 3426.
Weinmann, R., Raskas, H. J., & Roeder, R. G. (1974b) *Cold Spring Harbor Symp. Quant. Biol. 39,* 495.
Wintersberger, U., Smith, P., & Letnansky, K. (1973) *Eur. J. Biochem. 33,* 123.
Zylber, E. A., & Penman, S. (1971) *Proc. Natl. Acad. Sci. U.S.A. 68,* 2861.

CORRECTIONS

Repair of Nitrous Acid Damage to DNA in *Escherichia coli,* by Ricardo Da Roza,* Errol C. Friedberg,* Bruce K. Duncan, and Huber R. Warner, Volume 16, Number 22, November 1, 1977, pages 4934–4939.

On page 4938, column 2, third line from the bottom, the reference Ljungquist and Lindahl (1977) is cited, but it is not listed under References. The complete reference is: Ljungquist, S., and Lindahl, T. (1977), *Nucleic Acids Res. 4,* 2871.

Rabbit Liver Transglutaminase: Physical, Chemical, and Catalytic Properties, by Takashi Abe, Soo Il Chung, Richard P. DiAugustine, and J. E. Folk,* Volume 16, Number 25, December 13, 1977, pages 5495–5501.

On page 5495, column 2 of the Abstract, the sentence starting on line 6 should read: This finding is consistent with an earlier report (Tyler, H. M., and Laki, K. (1967) *Biochemistry 6,* 3259) that a transamidase isolated from rabbit liver is different in specificity from guinea pig liver transglutaminase.

On page 5496, column 1, the paragraph starting on line 19 should read: There is a report that a transamidase partially purified from rabbit liver displays little, or no, activity for hydroxylamine incorporation into Z-L-glutaminylglycine. It is apparent from the findings presented here that, indeed, this is a primary difference in catalytic specificity between the transglutaminases from livers of rabbit and guinea pig.

Conversion of Skeletal Muscle Glycogen Synthase to Multiple Glucose 6-Phosphate Dependent Forms by Cyclic Adenosine Monophosphate Dependent and Independent Protein Kinases, by Joan Heller Brown, Barbara Thompson, and Steven E. Mayer,* Volume 16, Number 25, December 13, 1977, pages 5501–5508.

On page 5503, column 2, paragraph 3, the sentence starting on line 8 should read: The protein was again precipitated with 15% Cl_3AcOH, and both the supernatant and precipitated protein were *ashed* . . . , not washed as printed.

Methylation of Glucagon, Characterization of the Sulfonium Derivative, and Regeneration of the Native Covalent Structure, by T. Michael Rothgeb, Barry N. Jones, Daniel F. Hayes, and Ruth S. Gurd,* Volume 16, Number 26, December 27, 1977, pages 5813–5818.

On page 5814, column 1, line 6 of paragraph 1 should read: second buffer of 200 mM ammonium acetate, instead of 20 mM ammonium acetate as printed.