polymerase along the template in the direction of transcription, possibly driven by conformational changes in the protein induced by nucleotide binding.

Acknowledgments

The excellent technical assistance of Judy Pascale-Judd is gratefully acknowledged.

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Comparison of the Ribonucleic Acid Polymerases from Both Phases of *Histoplasma capsulatum*†

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ABSTRACT: The three ribonucleic acid (RNA) polymerases (ribonucleoside triphosphate RNA nucleotidyltransferases, EC 2.7.7.6) of the two phases (yeast and mycelial) of the dimorphic fungus *Histoplasma capsulatum* have been purified and characterized. The corresponding enzymes from the two phases differ in sensitivity to α-amanitin, ion and salt requirements, temperature sensitivity, and subunit structure. This is the first case in which such qualitative differences in RNA polymerases have been demonstrated in two growth states of the same organism.

Histoplasma capsulatum is the etiologic agent of histoplasmosis, an infection worldwide in occurrence and endemic in the Mississippi and Ohio Valley areas of the United States (Goodwin & Des Prez, 1973). The mycelial phase of the dimorphic fungus is found in soil, and the yeast phase is the parasitic form found in infected tissues. In cultures, the mycelial phase grows at 25 °C and the yeast phase grows at 37 °C. The reversible conversion from one phase to the other is easily accomplished by shifting the temperature of incubation of the culture between 25 and 37 °C.

Because we found that the transition from the mycelial to the yeast phase was accompanied by marked changes in RNA synthesis (Cheung et al., 1974), studies of the ribonucleic acid (RNA) polymerases (ribonucleoside triphosphate ribonucleotidyltransferases, EC 2.7.7.6) of both phases of *H. capsulatum* were pursued. Yeast cells of *H. capsulatum* were found to contain three distinct species of RNA polymerases, but mycelial cells contained only one, with very low activity (Boguslawski et al., 1975). We felt it was unlikely that mycelial cells did not contain a full complement of RNA polymerases, and therefore it was probable that the major portion of RNA polymerase activity was masked in mycelial extracts. This notion is confirmed by the work reported here in which further purification of polymerase activity has exposed three enzymes in each phase.

Even with further purification, it is clear that each of the purified polymerases from one phase differs from the corresponding enzyme in the other. The purification procedure and characterization of all six polymerases (three from each phase) with respect to subunit composition, α-amanitin sensitivity, and enzymatic properties are reported here.

Materials and Methods

Biochemicals. The following materials were purchased from Sigma Chemical Co., St. Louis, MO: calf thymus deoxyribonucleic acid, DNA type I, unlabeled nucleoside triphosphates, phenylmethanesulfonyl fluoride, α-amanitin, and diethylaminoethyl (DEAE)-Sephadex (A25). [^{3}H]UTP (13 Ci/mmol) and ammonium sulfate (enzyme grade) were purchased from Schwarz/Mann, Orangeburg, NJ. Polyacrylamide gel reagents were obtained from Eastman Organic, Rochester, NY, and crystalline bovine serum albumin (Pentex) was purchased from Miles Laboratories, Inc., Elkhart, IN. Yeast extract was obtained from Difco Laboratories, Detroit, MI. Polymin P was obtained from Boehringer Mannheim, Indianapolis, IN. All other chemicals used were purchased from commercial sources and were of the highest analytical grade.

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Organism. H. capsulatum [Down's strain, mating type (−) from the permanent stock culture collection of this laboratory] was used throughout this study; the fungi were grown in liquid GYE medium (2% glucose and 1% yeast extract) as previously described (Boguslawski et al., 1975). Incubation was carried out at 37 °C for the yeast phase and at 25 °C with constant aeration for the mycelial phase.

Salt and Protein Measurements. Ammonium sulfate concentrations were measured with a Radiometer conductivity meter (type CDM 2e) following a 10-fold dilution of the sample with water. Protein concentrations were measured by the spectrophotometric method of Groves et al. (1968). α-Amanitin concentration was estimated from the extinction coefficient as described by Gebert et al. (1967).

Preparation of Ion-Exchange Resins. DEAE-Sephadex (A25), DEAE-cellulose, and phosphocellulose were processed for chromatography according to the directions of the manufacturer.

RNA Polymerase Assay. The standard assay mixture of 0.125 mL contained the following: 40 mM Tris-HCl (pH 7.9); 1.28 mM $MnCl_2$; 0.4 mM each of ATP, CTP, and GTP; 2 μCi of [^{3}H]UTP (1.28 μM); 10 μg of native calf thymus DNA; 100 μg of bovine serum albumin. The reactions were initiated by the addition of the RNA polymerases, except when the effect of temperature was studied. In those experiments, the reaction was initiated by the reaction mixture. After a 60-min incubation at 25 °C, the reactions were terminated by spotting 0.1 mL of the assay mixture on Whatman DE-81 filter disks. The disks were washed by the method of Lindell et al. (1970), dried, and counted in a liquid scintillation counter.

For measurement of the effects of metal ions and α-amanitin, specified concentrations of these agents were added to the reaction mixture before the RNA polymerase reaction was initiated.

One unit of RNA polymerase activity was defined as the amount of enzyme required for the incorporation of 1 pmol of [^{3}H]UMP under the above conditions. One picomole was equal to 2600 cpm as determined on a Beckman scintillation counter.

RNA Polymerase Purification. (*a*) *Preparation of Crude Extract.* Yeast cells or mycelia were suspended in 2 volumes of buffer A containing 0.05 M Tris-HCl (pH 7.9), 25% glycerol, 5 mM $MgCl_2$, 0.5 mM dithiothreitol (DTT), and 1.7 mM phenylmethanesulfonyl fluoride. The cells were then mixed with twice their weight of glass beads (acid washed) and homogenized for 2 min in a Braun homogenizer (Model MSK) cooled with CO_2. The cells were further disrupted by subjecting the cell suspension to four 30-s bursts with a Sonifier cell disrupter (Model 185M, Ultrasonic, Inc.) at maximum power using a microtip probe. The sonicate was centrifuged for 10 min at 12000*g* in a Sorvall SS-34 rotor, and the resultant supernatant was further clarified by centrifugation at 100000*g* in a Beckman Type 50.1 rotor.

(*b*) *Polymin P Precipitation.* The protein concentration of the high-speed supernatant was adjusted to 6–8 mg/mL with buffer A, and 10 μL/mL of a 10% solution of Polymin P was added to the high-speed supernatants. The resultant mixture was left in the cold for 20 min, and the precipitate was collected by centrifugation at 5000*g* in a Sorvall SS-34 centrifuge.

(*c*) *Extraction of the Enzymes.* The Polymin P precipitate obtained above was suspended in 0.2 M $(NH_4)_2SO_4$ in buffer B (buffer A without Mg^{2+}) for 10 min in the cold. The suspension was centrifuged at 5000*g* in a Sorvall SS-34 rotor. The extraction was repeated a second time, and the supernatants from both extractions were combined and centrifuged at 12000*g* in a Sorvall SS-34 rotor to remove any fine precipitates. The protein fraction containing the RNA polymerases from the supernatant was then precipitated with 40% (w/v) $(NH_4)_2SO_4$. The precipitate was collected by centrifugation, suspended in a small volume of buffer B, and desalted by using a Sephadex G-25 column equilibrated with buffer B.

(*d*) *DEAE-Sephadex Chromatography.* The Polymin P fraction prepared above was applied to a DEAE-Sephadex column previously equilibrated with buffer B. The bound protein was eluted with a 300-mL linear gradient of 0.025–0.4 M $(NH_4)_2SO_4$ in buffer B. Fractions of 5 mL were collected at a flow rate of 15 mL/h and assayed for RNA polymerase activity. The enzyme activities are named in the order of their elution as I, II, and III according to the procedure of Roeder & Rutter (1969) and Blatti et al. (1971).

(*e*) *Heparin–Sepharose Chromatography.* DEAE-Sephadex fractions containing greater than 90% of the activity for each of the three RNA polymerase peaks (see Figure 1) were pooled and concentrated by $(NH_4)_2SO_4$ precipitation followed by extensive dialysis against buffer B. RNA polymerases I and III were charged to heparin–Sepharose columns prepared as described earlier (Iverius, 1971). Enzymes were eluted with a linear gradient from 0.025 to 1.0 M $(NH_4)_2SO_4$ in buffer B. This affinity column is applicable only to the enzymes I and III. Enzymes II had a very high affinity for the column and could not be eluted even at 2 M salt concentrations.

(*f*) *Glycerol Gradient Centrifugation.* Fractions containing more than 90% of the total activity from heparin–Sepharose for enzymes I and III and from DEAE-Sephadex for enzyme II were pooled and concentrated by $(NH_4)_2SO_4$. All precipitated enzymes were suspended in a small volume of buffer C (buffer B with 10% glycerol) containing 0.1 M $(NH_4)_2SO_4$. Samples of 0.5–1.0 mL of each of the concentrated RNA polymerase activities were layered on 11-mL linear gradients of 15–30% glycerol in buffer B containing 0.1 M $(NH_4)_2SO_4$ and centrifuged for 14.5 h at 100000*g* in a Beckman SW40 rotor. Fractions of 0.5 mL were collected and assayed for RNA polymerase activity.

(*g*) *Second DEAE-Sephadex Chromatography.* As the heparin–Sepharose chromatography of enzyme II was not possible, this enzyme was purified by chromatography of the active fractions from the glycerol density gradient on a second DEAE-Sephadex column. This was performed as described earlier for the Polymin P purified crude supernatant, except that the column size was approximately one-fourth that of the original column. The activity obtained from this second chromatography was further purified on a glycerol density gradient as described above.

(*h*) *Polyacrylamide Gel Electrophoresis.* Purified yeast and mycelial RNA polymerases from the glycerol gradient fractions were subjected to electrophoresis under both denaturing and nondenaturing conditions. Electrophoresis under nondenaturing conditions was performed according to the method of Sklar & Roeder (1975), using 6 or 4.5% polyacrylamide gels at a current of 3 mA/gel. The gels were stained overnight with 0.05% Coomassie brilliant blue in a solution of 50% methanol and 10% acetic acid and destained by diffusion in 10% acetic acid and 30% methanol. The assay for RNA polymerase activity after nondenaturing polyacrylamide gel electrophoresis was performed by cutting unstained gels and incubating 0.5-mm thick slices in the standard assay mixture described above for 90 min at 23 °C.

Electrophoresis under denaturing conditions was performed according to Laemmli (1970) by using 9% polyacrylamide gels.

Table I: Summary of Purification of RNA Polymerases from *H. capsulatum*[a]

fraction	enzyme	vol (mL)		protein (mg)		total act.		sp act.		purifn (x-fold)	
		Y	M	Y	M	Y	M	Y	M	Y	M
crude supernatant	total	81	70	875	749	4170	4170	4.7	5.1	1	1
Polymin P ppt eluted with 0.2 M $(NH_4)_2SO_4$	total	60	60	228	252	151, 506	52509	664	208		
DEAE-Sephadex	I	4.5	3.5	5.5	8.4	396	139	72	17	15	3
	II	3.0	2.0	6.9	15	707	1162	102	78	21	15
	III	2.0	2.0	3.5	2.6	4526	4626	1293	1779	275	349
heparin-Sepharose	I	5.0	5.0	3.8	3.0	566	354	151	154	32	30
	III	6.0	6.0	0.48	1.75	2927	14435	6098	8249	1297	1617
glycerol density gradient	II	2.5	3.0	4.2	6.8	705	1135	168	167	36	38
DEAE-Sephadex	II	2.0	2.0	0.24	0.16	83	84	345	523	73	107
glycerol density gradient	I	2.0	2.0	0.89	0.65	247	148	278	228	59	45
	II	2.0	2.0	0.069	0.07	28	55	403	797	85	156
	III	2.0	2.0	0.116	0.29	185	451	1592	1556	339	305

[a] Y = yeast phase; M = mycelial phase.

Samples were precipitated with 5% trichloroacetic acid and centrifuged for 20 min at 8000*g* in a Sorvall SS-34 rotor. After the supernatant was removed, the precipitate was washed with acetone. Residual acetone was removed by heating the samples for 2 min at 50 °C. Samples were then dissolved in sample buffer (62 mM Tris-HCl, pH 6.8, 1% β-mercaptoethanol, 2% $NaDodSO_4$,[1] and 10% glycerol), and electrophoresis was performed at a constant voltage of 100 V. The gels were stained by the procedure described above.

Molecular weights of the subunits of the RNA polymerases were estimated by using *Escherichia coli* β-galactosidase and alkaline phosphatase, catalase, bovine serum albumin, ovalbumin, lactic dehydrogenase, soybean trypsin inhibitor, and chymotrypsin as the marker proteins. Molecular weights were estimated from the migration of these known proteins by using a programmed calculator. The relationship between the molecular weights and the migration was determined as described by Shapiro et al. (1967).

Results

Purification Procedure for Yeast and Mycelial RNA Polymerases. An outline of the procedure for the purification of the RNA polymerases and the total protein, specific activities, and levels of purification at each stage is presented in Table I. The first step in the purification, involving Polymin P, was essential to permit reproducible patterns of elution of the individual RNA polymerase species in the subsequent DEAE-Sephadex step. Our previous publication had used the traditional procedure of polymerase purification of DEAE-Sephadex chromatography of crude samples. This had resulted in three peaks for yeast but only one of low activity for mycelia. Three mycelial enzyme activities could be obtained only if phosphocellulose chromatography or the Polymin P step was carried out before DEAE-Sephadex chromatography. We believe that either of these steps removed material present in the mycelial phase which inhibited RNA polymerase activity (Boguslawski et al., 1975; Kumar et al., 1977).

The enzymes were designated I, II, and III in order of their elution from DEAE-Sephadex. Each RNA polymerase was further purified by affinity chromatography and glycerol density gradient centrifugation, and these steps resulted in increases in the specific activities of the enzymes.

The complete purification procedure yielded yeast RNA polymerases I, II, and III with an overall purification with respect to activity per milligram of protein of 59, 85, and 339, respectively. The final purification levels of mycelial RNA polymerases I, II, and III were 45-, 156-, and 305-fold, respectively. The molecular weights of RNA polymerases I, II, and III for yeast phase were 645 500, 557 000, and 613 000, and the molecular weights of mycelial enzymes I, II, and III were 565 500, 542 000, and 670 000. All of the polymerases were very labile after the last step of purification. Polymerases I of the yeast and mycelial phases, which were present in the largest quantities, were the least stable of the enzymes.

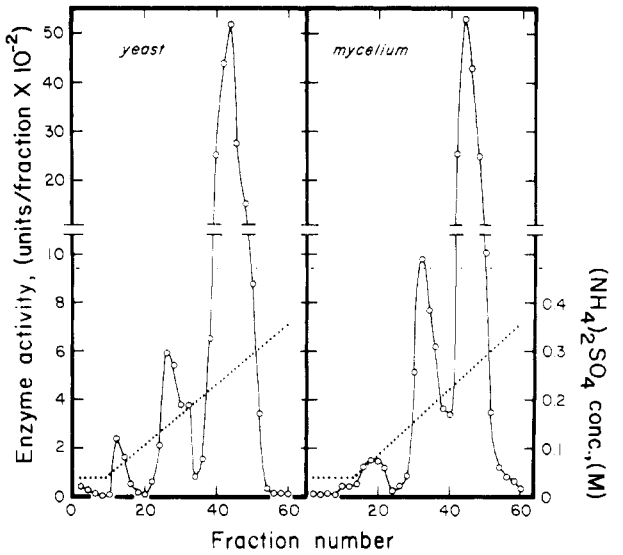

FIGURE 1: DEAE-Sephadex chromatography of the RNA polymerases. High-speed supernatants of the crude extracts of yeast and mycelial cells were absorbed to DEAE-Sephadex columns (2.5 × 24 cm) after partial purification by Polymin P precipitation. The enzymes were eluted with 300 mL of a linear gradient of 0.025–0.4 M $(NH_4)_2SO_4$ in buffer B. 5-mL fractions were collected, and alternate fractions were assayed for RNA polymerase activity.

DEAE-Sephadex Chromatography of Yeast and Mycelial RNA Polymerases. The elution patterns of the yeast and mycelial phase enzymes from a DEAE-Sephadex column are shown in Figure 1. The peak activities of the individual enzymes varied, depending on the extent of cell breakage. However, the overall separation and elution patterns for the mycelial and yeast enzymes were reproducible. Yeast and mycelial RNA polymerases II and III eluted from DEAE-Sephadex at similar ammonium sulfate concentrations. However, RNA polymerase I from yeast eluted from the column at a lower salt concentration than RNA polymerase I from mycelia. When the individual RNA polymerases were rechromatographed separately on 1 × 5 cm DEAE-Sephadex columns, the elution characteristics remained the same.

[1] Abbreviation used: $NaDodSO_4$, sodium dodecyl sulfate.

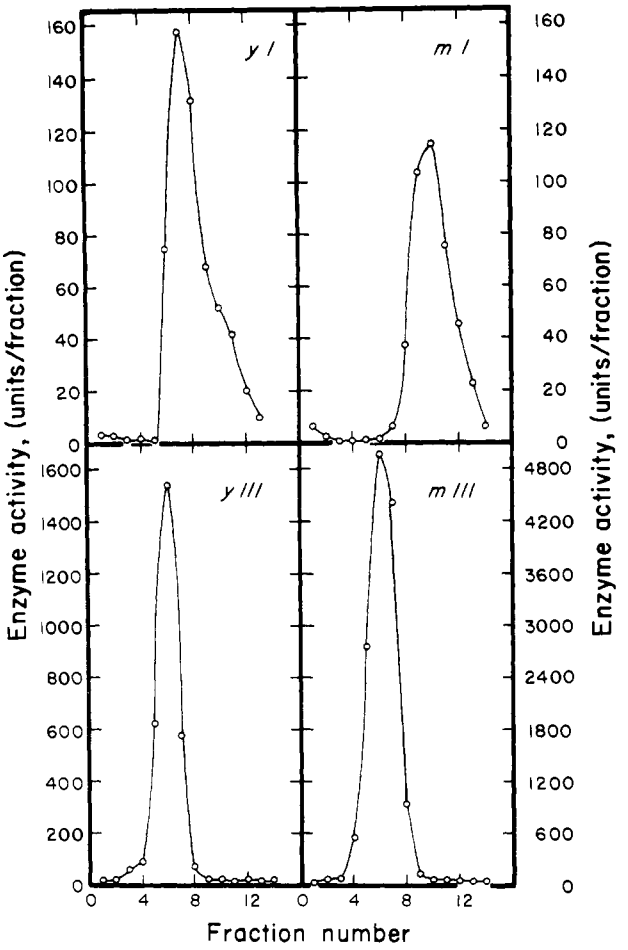

FIGURE 2: Heparin–Sepharose affinity chromatography of RNA polymerases I and III from yeast and mycelial phases. Fractions of the enzymes pooled from DEAE-Sephadex columns were concentrated by $(NH_4)_2SO_4$ precipitation, desalted by dialysis against buffer B, and absorbed to heparin–Sepharose columns (0.5 × 4 cm) previously equilibrated with buffer B containing 0.025 M $(NH_4)_2SO_4$. Enzyme activity was eluted with a linear gradient of 0.025–1 M $(NH_4)_2SO_4$ in buffer B. y represents yeast phase, and m represents mycelial phase.

Heparin–Sepharose Affinity Chromatography. The elution profiles of enzymes I and III from heparin–Sepharose are given in Figure 2. Both of the enzymes from both phases were eluted at ~0.25 M salt concentration. This step purified enzymes I and III of any contaminating II, as 100% of enzyme II bound very strongly to the resin and could not be eluted from the column even at 2 M salt concentration. Because of their positions of elution from the DEAE-Sephadex column, cross-contamination of I and III was very unlikely.

Glycerol Density Gradient Centrifugation. Figure 3 shows the patterns of sedimentation of RNA polymerases through 15–30% glycerol density gradients. Enzymes from the heparin–Sepharose chromatography (Figure 2) or the second DEAE-Sephadex chromatography for enzyme II were layered on a 15–30% glycerol density gradient as described under Materials and Methods. All the enzymes sedimented around a density of 1.065 g/cm^3.

Nondenaturing Polyacrylamide Gel Electrophoresis. Figure 4 shows the polyacrylamide gels after electrophoresis of the purified enzymes under nondenaturing conditions. Glycerol gradient fractions having the highest activity for each of the mycelial and yeast RNA polymerases were subjected to electrophoresis under native conditions as described under Materials and Methods. Each of the purified enzymes showed only one band which contained the polymerase activity.

$NaDodSO_4$–Polyacrylamide Gel Electrophoresis. Structural analysis of the RNA polymerases was done by using $NaDodSO_4$–polyacrylamide gel electrophoresis. The composition of each enzyme under denaturing conditions is given in Figure 5.

To simplify the analysis, we have assumed that yeast RNA polymerases I, II, and III correspond to mycelial enzymes I, II, and III, respectively, as has been done by previous workers. Such a comparison was justified in our case not only because the method of purification of the enzymes was identical for both phases of the organism but also because of the close similarities in their enzymatic properties described below.

Each enzyme was composed of nine subunits. A direct comparison of the subunits of corresponding enzymes showed the following differences.

Enzyme I of the yeast phase had one subunit of 115 000 molecular weight that was absent in the corresponding mycelial enzyme. Mycelial RNA polymerase I had a subunit of molecular weight 35 000 that was absent in the yeast RNA polymerase I. Apart from these differences, both enzymes were composed of subunits of the following molecular weights: 185 000, 135 000, 55 000, 49 500, 46 000, 25 500, 19 000, and 15 000.

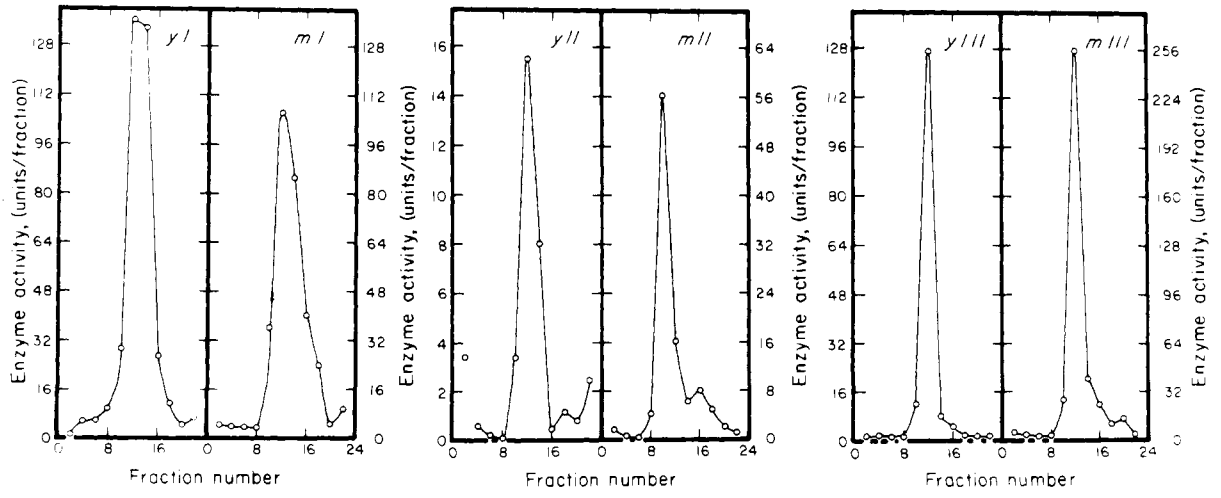

FIGURE 3: Glycerol density gradient centrifugation of the RNA polymerases. Active peak fractions from the DEAE-Sephadex chromatography of the RNA polymerases II and those from heparin–Sepharose for I and III were individually pooled and concentrated by salt precipitation. They were then centrifuged for 14 h at 100000*g* in an SW 40 rotor on a 15–30% gradient of glycerol. 0.5-mL fractions were collected, and 25-μL alternate fractions were assayed for RNA polymerase activity.

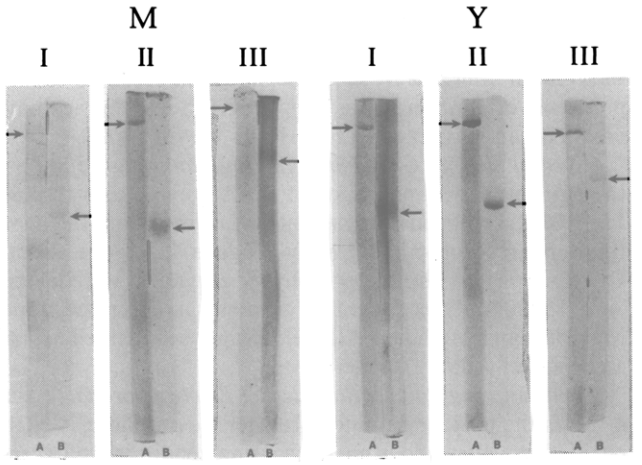

FIGURE 4: Native polyacrylamide gel electrophoresis of the RNA polymerases. Active fractions from the glycerol density gradient were subjected to electrophoresis under native conditions on 6 and 4.5% gels. 3–5 μg of protein was applied per gel. Electrophoresis was done under a constant current of 3 mA/gel. The gels were stained with Coomassie blue and destained as described under Materials and Methods. The concentration of acrylamide was 6% for (A) and 4.5% for (B). The arrows indicate the positions of the protein bands.

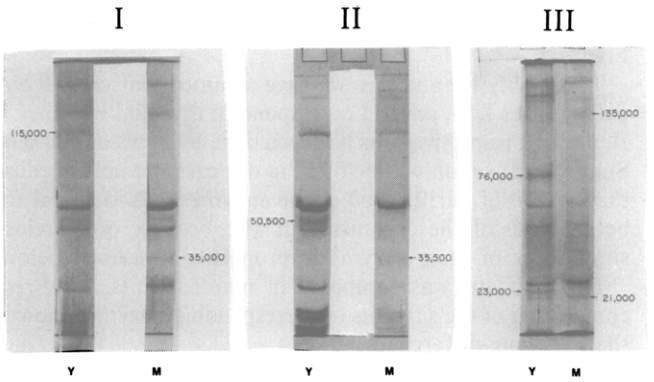

FIGURE 5: $NaDodSO_4$–polyacrylamide gels of the RNA polymerases. 2–5 μg of the RNA polymerases from yeast and mycelial phase cells was subjected to electrophoresis under denaturing conditions on 9% gels. Electrophoresis was done by using Tris–glycine buffer containing 0.1% $NaDodSO_4$ at a constant voltage of 100 V. Yeast and mycelial enzymes are designated by Y and M. Protein bands specific for each phase are shown by arrows followed by their molecular weights.

Enzyme II of the yeast phase had one subunit with a molecular weight of 50 500 that was absent in the mycelial phase enzyme. Mycelial polymerase II had one subunit of molecular weight 35 500 that was absent in the yeast phase enzyme. The rest of the subunit composition of the enzymes was identical and was composed of subunits of the following molecular weights: 185 000, 135 000, 60 000, 46 000, 25 500, 21 000, 18 500, and 15 500.

Enzyme III of the yeast phase had two subunits of molecular weights 76 000 and 23 000 that were absent in the mycelial phase. The mycelial phase enzyme had two subunits of molecular weights 135 000 and 21 000 that were absent in the yeast phase. The remainder of the subunits of the two enzymes were identical and had the following molecular weights: 185 000, 155 000, 51 000, 46 000, 36 000, 25 500, and 15 500.

Enzymatic Properties. (*a*) *α-Amanitin Sensitivity.* Figure 6 shows the sensitivities of the different RNA polymerases to the fungal toxin α-amanitin. Of the three enzymes from the yeast phase cells, enzyme III showed the most sensitivity to the drug. One microgram of α-amanitin per mL of reaction mixture inhibited the enzyme to 50% of its activity. Enzyme II required ~1000 μg/mL drug for 50% inhibition. Enzyme

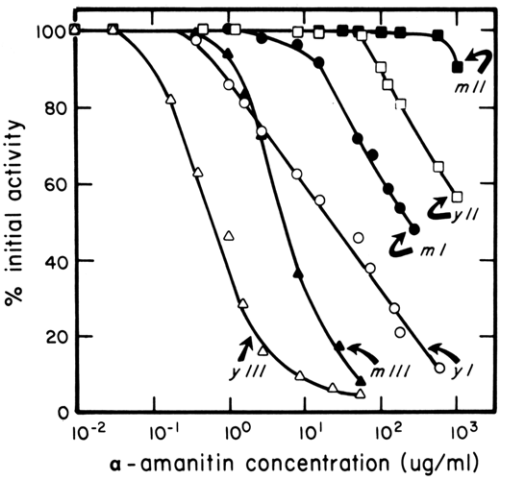

FIGURE 6: α-Amanitin sensitivity of RNA polymerases. Equal quantities (2–4 units) of yeast and mycelial phase enzymes were assayed in the presence of specified concentrations of α-amanitin at room temperature for 60 min. The percentage of the initial activity is plotted against α-amanitin concentration for each enzyme. Yeast and mycelial phase enzymes are designated by y and m, respectively.

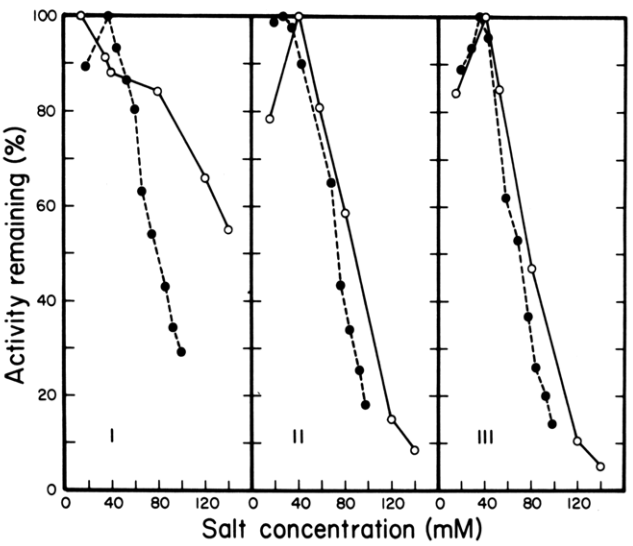

FIGURE 7: Effect of salt concentrations on the RNA polymerase activity. Enzymes from yeast (○) and mycelia (●) were incubated in the presence of specified concentrations of ammonium sulfate. The highest activity obtained at any particular salt concentration was taken as 100%, and other activities were computed as a percentage of the highest activity.

I showed intermediate sensitivity, requiring ~16 μg/mL drug for 50% inhibition.

The mycelial phase RNA polymerases were all 6–10-fold less sensitive to α-amanitin than were the corresponding yeast phase enzymes. This difference in sensitivity to α-amanitin was present at every stage of purification. Mycelial enzyme III was the most sensitive and required 6 μg of α-amanitin for 50% inhibition. Mycelial enzyme I was intermediate and required ~120 μg/mL for 50% inhibition, and mycelial enzyme II was only slightly inhibited by 1 mg/mL α-amanitin.

(*b*) *Effect of Salt.* Figure 7 shows the effects of various concentrations of $(NH_4)_2SO_4$ on the activities of the enzymes. Yeast phase enzyme I showed an activation curve slightly different from that of the corresponding mycelial enzyme. The salt activation curves of enzymes II and III were almost superimposable.

(*c*) *Effect of Metal Ions.* Figure 8A shows the effect of Mg^{2+} on the activities of the RNA polymerases. There were

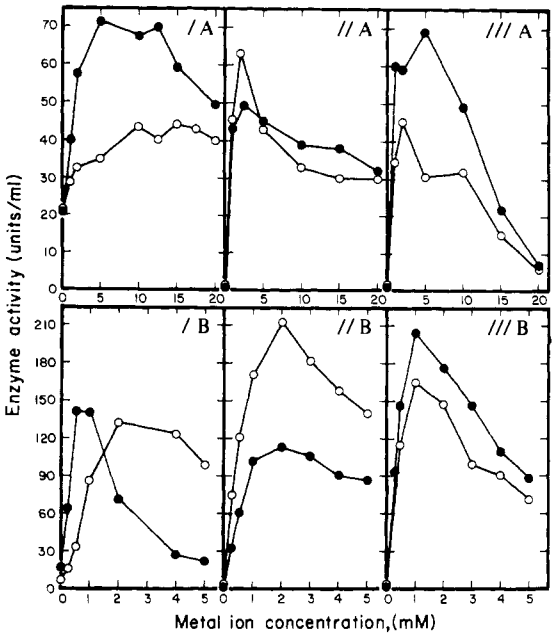

FIGURE 8: Effect of metal ions on the activity of RNA polymerases. RNA polymerases were assayed at specified concentrations of magnesium (top panel, A) and manganese (bottom panel, B) at room temperature for 60 min. Equal amounts of yeast phase (O) and mycelial phase (●) enzymes were used in the assay.

no differences in the effects of Mg^{2+} on the activities of the enzymes from each phase.

Mn^{2+} ion exhibited some differential effects on the activities of the RNA polymerases (Figure 8B). A significant difference was present in the case of enzyme I. Mycelial phase enzyme I showed a broad optimum between 0.6 and 1.4 mM Mn^{2+}. In contrast, yeast phase I exhibited an optimum between 2.5 and 4.0 mM Mn^{2+}. Enzymes II and III showed similar patterns of activity.

(*d*) *Effect of Temperature.* Figure 9 shows the effect of temperature on the enzyme activities. All the enzymes from both phases incorporated [^{3}H]UMP at a constant rate for at least 60 min when incubated at 25 °C. On the other hand, at 37 °C all of the enzymes incorporated [^{3}H]UMP linearly for only ~20 min. Thus, 25 °C appears to be the preferred temperature for the in vitro activities of all the RNA polymerases.

Figure 10 shows the activities of the enzymes from yeast and mycelial phases at 25 °C after a preincubation for specified periods of time at 37 °C. Yeast phase enzyme I showed a constant increase in its activity at 25 °C with increasing periods of preincubation at 37 °C, whereas the mycelial phase polymerase I lost its activity at 25 °C in a linear fashion as the time of preincubation at 37 °C increased. Yeast phase enzyme II behaved similarly to enzyme I for the first few minutes of preincubation at 37 °C. Thereafter, the enzyme lost 35% of its activity in 20 min of preincubation. Mycelial enzyme II gradually lost activity for the first 20 min of preincubation and then plateaued at 60% of initial activity. After 40 min at 37 °C, enzymes from the two phases had similar activities. Both yeast and mycelial enzymes III were inactivated by preincubation at 37 °C, although the loss of activity for the yeast phase enzyme was appreciably less than that of the mycelial phase enzyme at any given time.

Discussion

The purification procedure reported here has resulted in three species of RNA polymerases from both the yeast and mycelial phases of *H. capsulatum*. We believe that the six

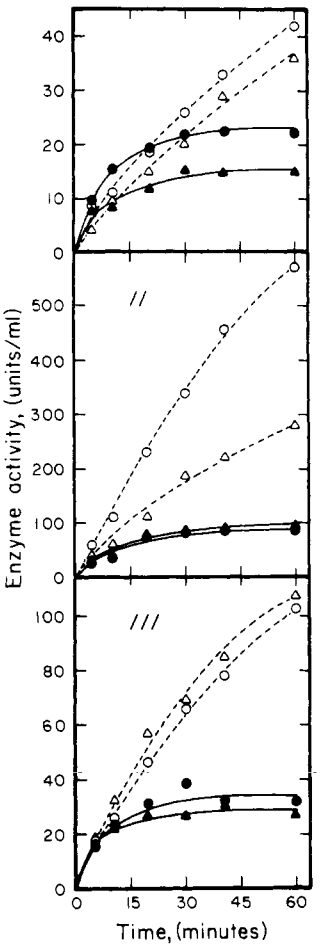

FIGURE 9: Comparison of RNA polymerase activity at 37 and 25 °C. RNA polymerases I, II, and III from the yeast and mycelial phases were assayed at 37 and 25 °C for 60 min. For comparison, the same amounts of each enzyme from each phase were used at both of the temperatures. Closed symbols (● and ▲) represent the activities at 37 °C, and opened symbols (O and △) represent the activities at 25 °C. Yeast enzymes are designated by O and ●. Mycelial phase enzymes are designated by △ and ▲.

enzymes obtained from our procedure have been purified to electrophoretic homogeneity because (1) specific activity fell with further purification after the last step despite a decrease in protein concentration, (2) one band was seen on nondenaturing gels, and the molecular weights of each enzyme and the number of subunits on $NaDodSO_4$ gels were consistent with what has been seen with other RNA polymerases, and (3) fractions from either side of the peaks of RNA polymerase activity after glycerol gradient centrifugation had the same specific activities and showed the same mobilities on gel electrophoresis.

This work extends earlier experiments from our laboratory which showed that partially purified extracts from the yeast phase had three RNA polymerases whereas the mycelial phase had only one of very low activity (Boguslawski et al., 1975). The main differences between the purification procedures was the addition, in the present method, of the Polymin P precipitation step before DEAE-Sephadex chromatography. This early step in the isolation of the mycelial RNA polymerases was necessary for the subsequent resolution into three separate mycelial polymerase activities on DEAE-Sephadex. DEAE-cellulose chromatography followed by phosphocellulose chromatography could be substituted for Polymin P precipitation, and this also led to the elution of three enzymes from DEAE-Sephadex columns (data not shown). We do not know

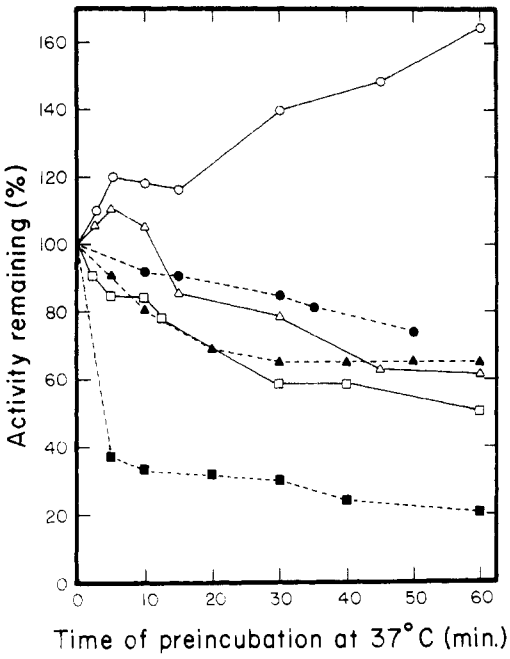

FIGURE 10: Effect of preincubation at 37 °C on the activity of RNA polymerases at 25 °C. RNA polymerases from yeast and mycelial phases were incubated at 37 °C for the specified times and assayed at 25 °C. Activity obtained at each time of preincubation was expressed as a percentage of nonpreincubated activity. ○, △, and □ are enzymes I, II, and III from the yeast phase, and ●, ▲, and ■ are enzymes I, II, and III from the mycelial phase.

why the individual RNA polymerases of the mycelial phase could not be resolved on DEAE-Sephadex if these prior steps were omitted, but it is probable that an interfering substance or inhibitor present in the mycelial phase and not in the yeast was removed. The increase in total recovery of enzyme activity after the Polymin P step supports this possibility, as do the previous reports of inhibitors of RNA polymerase activity in the mycelial phase (Boguslawski et al., 1975; Kumar et al., 1977).

The recovery of three RNA polymerases from each phase of *H. capsulatum* is in agreement with similar work on other eucaryotes. A number of the properties of these polymerases are comparable to those from other eucaryotes. For example, the number of protein subunits observed in disassociating electrophoretic gels (9 to 10), the elution profiles from column chromatography, and the salt and metal ion requirements of the enzymes were similar to those of enzymes from other eucaryotes (Chambon, 1975). Therefore, we think it is unlikely that any of the protein bands observed in the dissociating gels result from degradation during extraction. The similarities between the RNA polymerases of *H. capsulatum* and those of other eucaryotes make the differences between the enzymes of the two phases, described below, all the more unexpected.

The RNA polymerases from both phases were also similar to those of other eucaryotes in regard to the number of enzymes that are responsive to α-amanitin; one of the enzymes was very sensitive, one was intermediate, and one was resistant. However, the pattern of sensitivity differs from that described for *Saccharomyces cerevisiae* (Schultz & Hall, 1976) or other eucaryotes (Roeder, 1976). In both phases of *H. capsulatum*, RNA polymerase II was most resistant to α-amanitin and polymerase III was most sensitive, whereas in higher eucaryotes RNA polymerase II was most sensitive and polymerase I was most resistant. In *S. cerevisiae*, enzyme III was most resistant and enzyme II was most sensitive (Schultz & Hall, 1976).

Because the pattern of sensitivity to α-amanitin is so different from that in other organisms, this frequently used criterion to classify RNA polymerases is not available to us. Thus, we have had to depend on the order of elution from a DEAE-Sephadex column to identify the enzymes. Immunological or other criteria will have to be used to determine which RNA polymerase from each phase of *H. capsulatum* truly corresponds to the polymerases in other eucaryotes. However, our purpose here was to purify and compare enzymes from one phase with the corresponding ones in the other. Fortunately, in the case of *H. capsulatum*, the corresponding enzymes according to elution from DEAE-Sephadex were also most equivalent when the criterion of α-amanitin sensitivity and other enzymatic and structural properties were compared.

The amount of α-amanitin required for 50% inhibition of the *H. capsulatum* sensitive enzyme (enzyme III) was similar to what has been observed for the sensitive enzyme of *S. cerevisiae* (Schultz & Hall, 1976) and about 100-fold higher than what has been observed for the α-amanitin-sensitive enzyme (enzyme II) from vertebrates (Roeder, 1976). The intermediate-sensitive enzyme of the yeast phase of *H. capsulatum* (enzyme I) required 16 μg/mL α-amanitin, which was in the range of the intermediate-sensitive enzymes of vertebrates (Virgil et al., 1976). The same enzyme in the mycelial phase required 120 μg/mL α-amanitin, which was similar to the case for the intermediate enzyme of *S. cerevisiae*.

The resistance to α-amanitin was more extreme in the three enzymes isolated from the mycelial phase of *H. capsulatum*. The mycelial enzymes were 6–10-fold more resistant to α-amanitin at every level of purification than the yeast phase enyzmes. The differences in α-amanitin sensitivity, ion and salt requirements, temperature sensitivity, and subunit structure represent the first case in which such differences in specific properties of corresponding RNA polymerases have been noted in two phases of the same organism. Quantitative changes have been found in the levels of the RNA polymerases during embryonic development of *Xenopus laevis* (Roeder, 1974; Grippo et al., 1976) and *Artemia salina* (Renart & Sebastian, 1976) and in resting and growing fibroblasts (Mauck, 1977). Similar changes in the levels of activity of the enzymes have also been reported in *Mucor rouxii* during the change from yeast to mycelial growth (Young & Whiteley, 1975) and in *Dictyostelium discoideum* (Pong & Loomis, 1973) and *Allomyces arbuscula* (Cain & Nester, 1973) during the differentiation process of these organisms. However, none of those systems have shown the kind of structural or functional differences in the enzymes from different phases that *H. capsulatum* exhibits.

The significance of the differences in the RNA polymerases of the two phases of *H. capsulatum* is unknown. Marked changes occur in RNA synthesis (Cheung et al., 1974) during the transition of the organism from the yeast phase to the mycelial phase, and the different properties of the RNA polymerases may affect the transition by controlling this process.

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Characterization of Glycine-Rich Proteins from the Ribonucleoproteins Containing Heterogeneous Nuclear Ribonucleic Acid†

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ABSTRACT: The salt-soluble 28 000–38 000-dalton proteins were isolated from ribonucleoproteins containing heterogeneous nuclear RNA and partially purified. They were glycine-rich proteins (22–26 mol/100) and contained a small amount of N^G-dimethylarginine. Their N-terminal amino acid was blocked. Their p*I* was basic, extending from 6.95 to 9.20. Some 40 different polypeptides were demonstrated by combining molecular weight and p*I* determinations. Comparison of peptidic maps and of peptide size after trypsin and thermolysin digestion indicated the presence of only four proteins. The pattern of distribution of p*I* showing series of discrete major and minor bands common to two or three polypeptides of different apparent molecular weight was also compatible with the existence of four proteins and in addition supported the idea that the multiplicity of polypeptides was due to extensive posttranslational modifications.

The nuclear ribonucleoproteins containing heterogeneous nuclear RNA (hnRNP)[1] are made of two classes of constituents, 30–50S monoparticles and other complexes much more heterogeneous in size (Stévenin et al., 1977). In contrast to heterogeneous complexes, the monoparticles accumulated at 30–50S upon a mild ribonuclease treatment of the hnRNP (Samarina et al., 1968; Stévenin et al., 1970, 1977), they were easily dissociated by salt treatment (Gallinaro et al., 1975), and they were relatively poor in phosphoproteins (Stévenin et al., 1977; Gallinaro et al., 1975; Fuchs & Jacob, 1979). Approximately 20 major proteins could be detected in monoparticles by monodimensional gel electrophoresis (Stévenin et al., 1979). Some of them were acidic (p*I* of 5–7) and their molecular weight extended from 40 000 to 110 000. Another group was made of basic proteins (p*I* of 7–9), clustered between 28 000 and 38 000 daltons. They represented approximately 25% of the total hnRNP proteins. We started a study of these proteins in rat brain, our aim being, at long term, to determine whether the assembly of these basic hnRNP proteins may govern the structure of hnRNP as other basic proteins do in the assembly of chromatin or ribosomes.

A large-scale method had been devised previously, allowing the clear-cut separation of heterogeneous complexes from monoparticle proteins after salt dissociation (Fuchs & Jacob, 1979). The 28 000–38 000-dalton proteins could then be separated from the higher molecular weight proteins by gel chromatography. In the present work, we further fractionated the mixture of basic proteins by combining gel and carboxymethylcellulose chromatographies. The amino acid composition and the p*I* of individual proteins or groups of proteins were determined, and peptidic maps were established. The results support the idea of the existence of only four poly-

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[1] Abbreviations used: RNP, ribonucleoproteins; hnRNP, ribonucleoproteins containing heterogeneous nuclear RNA.

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