Ribonucleic Acid Polymerases of the Yeast Phase of 
*Histoplasma capsulatum*

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Ribonucleic acid (RNA) polymerases of *Histoplasma capsulatum* (yeast phase) were fractionated by phosphocellulose chromatography and partially characterized. Three distinct, active fractions were seen. The major RNA polymerase species was inhibited strongly by α-amantin, whereas the other two were resistant. When either slightly purified (HSE) extract or the major active component was assayed at 37 C, the incorporation of tritiated uridine monophosphate into RNA stopped after 10 to 15 min. In contrast, the synthesis continued for at least 1 h at 23 C. The other two RNA polymerase species exhibited higher rates of incorporation when tested at 37 C, and continued to synthesize RNA even after 60 min. However, by that time the levels of incorporation at 23 C were higher than at 37 C for all three enzymes. The temperature sensitivity was not affected by changing substrate concentration or employing either native or denatured calf thymus deoxyribonucleic acid as a template. These results are compared with the data obtained with RNA polymerases from different fungi and other organisms. A possible involvement of RNA polymerase(s) in morphological differentiation of *H. capsulatum* is discussed.

*Histoplasma capsulatum* is a medically important dimorphic fungus. The multicellular mycelial form converts to a unicellular yeastlike form when the fungus infects and multiplies in a susceptible host. The conversion of mycelium to yeast can also be triggered in vitro in the laboratory by a temperature shift from 23 to 37 C. Almost immediately after the shift, there is a transient period of cessation of net ribonucleic acid (RNA) synthesis which lasts for 4 h (S. Cheung et al., manuscript in preparation). RNA synthesis then resumes at a normal rate as the conversion proceeds. This suggested to us that RNA polymerases might be directly involved in, or affected by, the conversion process.

In this paper we present data on RNA polymerases isolated from yeastlike cells of *H. capsulatum*. We have found that the enzymes in vitro show differential responses to incubation at 23 and 37 C, and that the predominant species of RNA polymerases is rapidly but reversibly inactivated at elevated temperature.

**MATERIALS AND METHODS**

**Organism.** *H. capsulatum* (Down’s strain, mating type [−]) from the permanent stock culture collection of this laboratory was grown in the yeast phase at 37 C in liquid YEPD medium (1% yeast extract, 2% peptone, 2% glucose) in a New Brunswick rotary shaker. Flasks were inoculated at a density of 10⁶ cells/ml, and growth continued for about 60 h to yield mid-exponential-phase cells. Mid-exponential-phase cultures at 60 h yielded about 6 to 8 g of cells per liter (wet weight). Cells were harvested by filtration on membrane filters (0.45 μm; Millipore Corp.), washed with sterile water, and processed without delay.

**Chemicals.** Unlabeled ribonucleotides, calf thymus deoxyribonucleic acid (DNA, type V), and phenylmethylsulfonyl fluoride (PMSF) were purchased from Sigma Chemical Co. [H]-labeled uridine triphosphate ([H]UTP; 13 Ci/mmol) was purchased from Schwarz BioResearch. α-Amanitin was obtained from Henley Co., N.Y. Rifampin was obtained from Dow Chemical Co., Zionsville, Ind., and the rifamycin derivative AP/013 (3-formyl rifamycin SV-O-n-octyloxime) was from Gruppo LePetit, Italy. DE-81 filters and phosphocellulose (P1) were purchased from Whatman. Sephadex G25 and diethylaminoethyl (DEAE)-Sephadex were from Pharmacia, Sweden.

**Extraction of RNA polymerase.** The extraction procedure for RNA polymerase was similar to that employed by Adman et al. (1), except for the following modifications. The suspension obtained after shaking cells with glass beads was centrifuged once at 3,000 × g for 10 min to remove glass beads and debris. The sonic oscillation step was omitted. The resulting supernatant fluid was treated with (NH₄)₂SO₄ (0.36 g/ml), and the suspension stirred for 30 min at 4 C and then centrifuged for 25 min at 48,000 × g. The pellet was dissolved in a minimal volume of buffer.
containing 0.05 M tris-(hydroxymethyl)aminomethane (Tris)-hydrochloride (pH 7.9), 25% glycerol, 0.5 mM ethylenediaminetetraacetic acid, 0.5 mM diithiothreitol, and 5 mM MgCl₂ (TGMD buffer). FMSF was added to a final concentration of 0.2 mg/ml. The solution was then centrifuged at 159,000 x g for 1 h. The resulting supernatant fluid was passed through a Sephadex G25 column equilibrated with 0.025 M ammonium sulfate in TGMD buffer in order to lower the concentration of ammonium sulfate. The eluate was subjected to sonic oscillation for 30 s at setting 3 of a Branson Sonifier (micropipet attachment). The solution was centrifuged for 1 h at 177,000 x g, and the supernatant fluid (high-speed enzyme[HSE]) was either stored at -65 C or subjected immediately to column chromatography.

**P-cellulose chromatography.** Phosphocellulose (P-cellulose) was washed according to the instructions of the manufacturer and equilibrated with 0.05 M ammonium sulfate in TGED (no MgCl₂) buffer. A sample of HSE was applied to the column at the rate of about 0.5 column volume per h. The column was washed with equilibrating buffer, and the activity was eluted with a linear gradient of 6 column volumes of 0.05 to 0.40 M ammonium sulfate in TGED buffer. The concentration of (NH₄)₂SO₄, was measured with a Radiometer (Copenhagen) conductivity meter. The active fractions were pooled and stored at -65 C.

**RNA polymerase assay.** The standard incubation mixture of 0.125 ml contained the following (final concentrations): 0.04 M Tris-hydrochloride (pH 7.9), 1.28 mM MnCl₂, 0.4 mM each of adenosine triphosphate, cytosine triphosphate, and guanosine triphosphate, 80 μg of native calf thymus DNA per ml, and 2 μCi of tritiated UTP (1.28 μM). Reaction was linear in respect to UTP concentration up to at least 17.28 μM, and up to 40 μg of protein (HSE) or 50 μlitters of RNA polymerase fractions (when corrected for ammonium sulfate concentration [see text]). Time of incubation was 60 min unless indicated otherwise. The filters were washed by the method of Lindell et al. (8), dried, and counted in a Nuclear-Chicago liquid scintillation counter (Mark I). RNA polymerase activity was expressed as counts of [³H]uridine monophosphate ([³H]UMP) incorporated into polynucleotides. One picomole of UMP was equivalent to 4,200 counts/min. Protein was determined by the method of Lowry et al. (9), or by absorbance at 280 nm.

**RESULTS**

**Column chromatography.** Although it is a nearly standard procedure to use an anion exchanger, such as DEAE-Sephadex or DEAE-cellulose, as the first step in purification of RNA polymerases (1, 8, 11, 13), we could not obtain satisfactory and reproducible chromatographic patterns with DEAE-Sephadex columns; both the number of activity peaks and their relative heights were variable. Instead, good reproducibility was obtained when equivalent extracts were fractionated on a cation exchanger, P-cellulose. A typical chromatographic pattern obtained with P-cellulose column is shown in Fig. 1. Three well-separated fractions with activity (PC I, PC II, and PC III) were seen, the first one strongly inhibited by the fungal toxin α-amanitin (80% inhibition at 5 μg/ml). On this basis, PC I may correspond to the RNA polymerase II or B of other organisms, as eluted from DEAE-Sephadex (7, 8). The other two (α-amanitin insensitive) peaks (PC II and PC III) may correspond to DEAE-Sephadex polymerases I and III or to the RNA polymerase I fractions (1, 4, 5, 12). The relative abundance of each component was PC I, 69.4%; PC II, 13.9%; and PC III, 16.6%.

These values were not corrected for salt effects which are described below. None of the enzymes was sensitive to rifampin (32 μg/ml), and all were inhibited 50 to 60% by 32 μg of the rifamycin derivative AF/013 per ml.

**Ammonium sulfate dependence of enzyme activity.** Eukaryotic RNA polymerases have, in general, different salt optima (1, 11, 13). These optima were determined for HSE and for the three activities (Fig. 2) by using preparations dialyzed for 4.5 h against 0.05 M (NH₄)₂SO₄ in TGED buffer. Ammonium sulfate concentration in dialyzed fractions was 0.07 M. Optimal salt concentrations for PC I and PC II were determined to be about 0.05 M and 0.017 to 0.045 M, respectively; PC II was rapidly inactivated above 0.045 M salt concentration. PC III was most active at the lowest attainable salt concentrations (0.017 M ammonium sulfate).

![Fig. 1. Phosphocellulose chromatography of high-speed enzyme (HSE) from yeast-like cells of H. capsulatum. HSE (63 mg of protein) was applied to a column (1.9 by 7 cm) equilibrated with 0.05 M ammonium sulfate in TGED buffer, and the enzyme activity was eluted with a 120-ml linear gradient of 0.05 to 0.40 M (NH₄)₂SO₄ in TGED buffer. Fractions were collected into tubes containing 80 μlitters of bovine serum albumin (20 mg/ml), and 50-μlitter samples were assayed for activity for 60 min at 23 C in the absence (●) or the presence (O) of α-amanitin (5 μg/ml). Total volume of each fraction was 1.6 ml. Activity was expressed as counts of UMP incorporated per minute. After assaying, the fractions were pooled as follows: PC I, fractions 32 to 39; PC II, fractions 52 to 55; and PC III, fractions 67 to 71.](http://jb.asm.org)
Since the enzyme I subfractions (i.e., Iₐ and Iₖ) have been shown to have identical salt optima (4, 5, 12), the data suggest that PC II and PC III are different enzyme species. On the basis of these results, however, we cannot be certain that PC II and PC III correspond to DEAE-Sephadex polymerases I and III or to polymerase I subfractions.

The method used for assay of the column fractions (50-µliter samples diluted into final volume of 125 µliters) resulted in assay concentrations of ammonium sulfate which were optimal for PC I and nearly optimal for PC II. However it became apparent that the activity for PC III was markedly underestimated because of the inhibitory effect of (NH₄)₂SO₄.

When corrections for ammonium sulfate inhibition were made, the relative amounts of the three activities were: PC I, 47.7%; PC II, 16.6%; and PC III, 35.7%. It seemed very likely, on the basis of abundance and the P-cellulose positions of the enzyme elution, that PC III was equivalent to polymerase I of DEAE-Sephadex chromatography profile. (In support of this, when DEAE-Sephadex enzymes are chromatographed on P-cellulose, the order of elutions is: polymerase II, III, and I [R.G. Roeder, personal communication].)

**Effect of temperature on RNA polymerase activity.** Because temperature shifts play such an important role in the life cycle of H. capsulatum and because a change from 23 to 37°C causes a temporary halt in net RNA synthesis, the three enzyme activities were tested at both 23 and 37°C. The initial experiments with HSE preparation (Fig. 3) showed that the activity at 23°C was much higher and that the incorporation of labeled UMP continued for a much longer period of time than at 37°C. When the three fractions were tested (Fig. 3), it appeared that qualitatively PC I behaved like HSE and that RNA production virtually stopped after about 10 to 15 min at 37°C. Instead, at 23°C, RNA synthesis continued for at least 60 min.

In contrast to the results with RNA polymerase PC I, polymerases PC II and PC III at 37°C had higher reaction rates (20 and 40 min, respectively) than at 23°C, and continued to synthesize RNA at the elevated temperature even after 60 min. However, by that time the levels of incorporation at 23°C were higher than at 37°C for all three enzymes.

The sensitivity of PC I to elevated temperature was not a result of enzyme denaturation at 37°C. PC I was preincubated for 1 h at either 23 or 37°C and subsequently assayed for activity at both temperatures (Fig. 4). Preincubation at 23 or 37°C for 1 h had no effect on the subsequent activity of the enzyme. In addition, when the assay temperature was shifted from 37 to 23°C, the reaction resumed immediately at a rate almost equal to that of the 23°C control. Similarly, HSE was not affected by preincubation at 37°C for up to 1 h (data not shown).

In most of the experiments described here, HSE was assayed at 0.08 M ammonium sulfate. This tended to optimize the relative activity of α-amanitin-sensitive enzyme (PC I) since PC II and PC III are about three times less active at this salt concentration (Fig. 2). Therefore, it is not surprising that HSE and PC I responded in
a similar way to temperature (Fig. 3). However, when the sensitivity of HSE to α-amanitin was tested at 23 and 37°C, it appeared that at 23°C the inhibition remained essentially constant (~72%) throughout the incubation period, but at 37°C it decreased slowly to about 42% (Fig. 5). This decrease was presumably due to the fact that PC II and PC III are relatively stable at 37°C and are not inhibited by α-amanitin. In support of this, the actual incorporation of radioactivity in the presence of α-amanitin (16 μg/ml) is slightly higher at 37°C than at 23°C for up to 30 min (data not shown).

One possible explanation of the observed selective inactivation of PC I at 37°C could be that it was contaminated with a nuclease which destroyed the RNA product at a higher temperature, or with a factor conferring temperature sensitivity on the enzyme. To test these possibilities, PC I and PC III were mixed and assayed at 23 and 37°C in the presence and absence of α-amanitin (16 μg/ml) (Fig. 6).

In the presence of the inhibitor, virtually all activity that remained was due to PC III (Fig. 6, panel B). As one would expect, activity at 37°C was thus as high or higher than at 23°C. The difference between inhibited and uninhibited samples represents the activity of PC I (Fig. 6, panel C). As expected, the incorporation of UMP is higher at 23 than at 37°C. Thus, no specific factor or nucleolytic activity is associated with the PC I preparation. Moreover, incubation of PC I with radioactive ribosomal RNA also indicated that no nuclease was present in the preparation since no degradation occurred (data not shown).

A possibility that PC I was selectively af-
fected at elevated temperature because of limiting concentrations of UTP in the assay (1.28 μM) is unlikely because increasing the UTP concentration 13.5 or 63.5 times had no effect on the temperature-related behavior of the enzyme at 37 C (although the incorporation at both 23 and 37 C increased 12-fold and 18-fold with respective increases in the UTP concentrations [data not shown]). The same temperature sensitivity of PC I was observed with denatured calf thymus DNA instead of native DNA as a template (not shown).

DISCUSSION

RNA polymerase fractions from H. capsulatum. RNA polymerase activity from yeasts like cells of H. capsulatum could be reproducibly resolved into three components by chromatography on P-cellulose. One of these components, PC I, was sensitive to α-amanitin and presumably was equivalent to RNA polymerase II or B as eluted from DEAE-Sephadex (7, 8). The other two components probably corresponded to DEAE-Sephadex polymerases I and III. It is important to point out that PC I was much less sensitive to α-amanitin than is sea urchin or mammalian polymerase II (7, 8), but was similar to the enzyme from the yeast Saccharomyces cerevisiae (1). Amounts as high as 16 μg of α-amanitin per ml were needed to inhibit PC I 90 to 95%.

Unlike RNA polymerases from other fungi such as Allomyces arbuscula (3) or S. cerevisiae (1), which yield multiple enzyme species on DEAE-cellulose or DEAE-Sephadex columns, RNA polymerase from H. capsulatum could not be fractionated on DEAE-Sephadex. It is not clear why no reproducible and satisfactory patterns were obtained, but it may be due to some peculiarity in the chemical composition of the extracts.

Temperature sensitivity of eukaryotic RNA polymerases. We have found only a few reports on effects of temperature on RNA polymerase activity in subcellular systems. Marzluff et al. (10) have shown that, in nuclei isolated from mouse myeloma cells, RNA synthesis at 25 C continued for up to 1 h, but at 37 C ceased after 10 to 15 min. These results were comparable to the results reported here with the crude HSE preparation. However, in our studies, the apparent cessation of RNA synthesis probably only reflected the fact that PC I, the predominant species, was affected by temperature (Fig. 3). Also, we emphasize that the relatively high salt concentrations used to optimize incorporation in HSE were far from optimal for PC II and PC III. These two activities thus contributed little to RNA synthesis in the standard assay system, and it seems plausible that a similar situation may hold in the experiments with unfractionated nuclei from mouse myeloma cells (10).

Shields and Tata (14) have reported that RNA polymerases A and B of rat liver and yeast nuclei exhibit differential stability upon heating to 45 C. In their experiments, RNA polymerase B or II (α-amanitin sensitive) was more heat resistant than polymerase A or I. We have not tried to preheat the enzyme to 45 C, but preincubation of PC I at 37 C for 1 h did not affect the activity as subsequently assayed at 23 C (Fig. 4). The fact that preincubation at 37 C did not have an effect on activity means that either the enzyme renatured very rapidly when transferred to permissive temperature or it did not denature at all. Possibly at 37 C the capacity of enzyme to form an initiation complex or to participate in chain elongation or termination diminished as the time of assay was extended.

Role of RNA polymerases in development. When a culture of H. capsulatum growing at 23 C was shifted to 37 C, RNA synthesis was brought to a temporary halt and resumed only after several hours (S. Cheung et al., manuscript in preparation). Our experiments showed that only one species of RNA polymerase was temperature sensitive; on this basis, one would expect that, at the higher temperature, RNA synthesis in vivo would continue, albeit at somewhat slower rate. A number of hypotheses can be put forward to explain this apparent contradiction. For example, it could be that internal pools of RNA precursors were temporarily depleted during the transition period, or that the permeability of cell membrane changed in such a way that nutrients from the medium could not be taken up by the cells. A more interesting possibility may be that, in the mycelial phase (23 C), the functions of all three RNA polymerases, or at least of the two predominant ones (PC I and PC III), are temperature sensitive, and that some time at 37 C must elapse before these enzymes are converted to temperature resistant forms or new ones are made. If this were the case, it would mean that RNA polymerases in H. capsulatum are under a specific control system, a suggestion consistent with the finding that in stationary cells the α-amanitin sensitive activity is markedly reduced (data to be published).

No changes in RNA polymerase have been observed in the life cycles of other dimorphic...
organisms such as *A. arbuscula* (3) or *C. crescentus* (2) or with developmental stages of the cellular slime mold *Dictyostelium discoideum* (11). On the other hand, development of *Rhizopus stolonifer* (6) seems to involve a change in RNA polymerase content and properties. The question of the possible involvement of RNA polymerases in the life cycle of *H. capsulatum* may be resolved by the study of these enzymes from mycelium and from the transitional form of this organism.

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**LITERATURE CITED**