Effect of Elevated Temperature on Growth and Ribonucleic Acid Synthesis in *Cryptococcus albidus*

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When a growing culture of *Cryptococcus albidus* was shifted from 27 to 37 °C, growth ceased abruptly and the cells died rapidly (over 90 % after 4 h). The temperature shift resulted in a rapid cessation of [³H]uridine incorporation (as perchloric acid-insoluble material). DEAE-Sephadex chromatography of crude extract from *C. albidus* cells revealed the presence of two major enzyme fractions with RNA polymerase activity. The fractions were insensitive to α -amanitin, and both synthesized RNA efficiently at 23 °C but not at 37 °C. Neither proteolytic digestion of the enzymes nor ribonuclease degradation of the RNA products could account for the observed decline in RNA synthesis at the elevated temperature.

INTRODUCTION

The yeast *Cryptococcus albidus* is classified as a non-pathogenic member of the genus *Cryptococcus*, partly on the basis of nutritional requirements and partly on the basis of its inability to grow at 37 °C (Emmons *et al.*, 1977). A number of reports have described the effects of the elevated temperature on whole cells of various cryptococci (Dabbagh *et al.*, 1974; Tang & Howard, 1973), but macromolecular synthesis in cell-free systems has not been investigated. In this communication we present evidence that RNA synthesis in *Cryptococcus albidus* strain CR172108 is severely restricted by a temperature shift from 23 to 37 °C. Both crude extract and individual RNA polymerases exhibit the same pattern of inhibition. The cessation of RNA synthesis *in vitro* is paralleled by the cessation of growth and uridine incorporation *in vivo*.

METHODS

Organism. *Cryptococcus albidus* (strain CR172108) was obtained from Dr Geoffrey Land (Wadley Institute, Dallas, Texas, U.S.A.). The media used for the cultivation of the fungus were described previously (Dabbagh *et al.*, 1974). Growth was measured in a Spectronic 20 (Bausch and Lomb) colorimeter at 420 nm, and by viable cell count on Sabouraud agar plates as previously described (Dabbagh *et al.*, 1974).

Chemicals. Unlabelled ribonucleoside triphosphates and calf thymus DNA (type I) were purchased from Sigma; [³H]UTP (25 Ci mmol⁻¹) was from New England Nuclear Corporation; [³H]uridine (25 Ci mmol⁻¹) was from Schwarz/Mann, Orangeburg, New Jersey, U.S.A.; Sephadex G-25 and DEAE-Sephadex A25 were from Pharmacia; and α -amanitin was from Boehringer.

Extraction and assay for RNA polymerase. Cells were grown in 10 l complex medium (Dabbagh *et al.*, 1974) for 33 h at 27 °C, harvested by centrifugation and washed with distilled water (total yield 40 g wet weight). The soluble fraction (high-speed enzyme, HSE) was obtained as described previously (Boguslawski *et al.*, 1974). RNA polymerase activity was assayed at either 23 or 37 °C under otherwise standard conditions (Boguslawski *et al.*, 1977) using [³H]UTP as labelled RNA precursor.

DEAE-Sephadex chromatography. DEAE-Sephadex A25 was washed and equilibrated with the standard

Fig. 1. Growth of *C. albidus* at 27 °C (○) and 37 °C (●). (a) Cultures of *C. albidus* were grown on minimal medium (Dabbagh *et al.*, 1974) in triple-baffled flasks with side arms (Bellco) at 27 °C. At the indicated time (arrow), one culture was transferred to a waterbath shaker at 37 °C and incubation was continued. (b) Decline in cell viability upon incubation at 37 °C (the initial cell count was 8.5×10^5 cells ml⁻¹).

buffer as described by Roeder & Rutter (1969), except that $MgCl_2$ was omitted. A sample of HSE was applied to the column at a rate of about 0.5 column vol. h⁻¹. The column was washed with the same buffer and the activity was eluted with a linear gradient of 7 column vol. of 0.05 to 0.42 M-ammonium sulphate in the standard buffer. The active fractions were pooled and stored at -70 °C.

Ribonuclease activity. Degradation of RNA was monitored using ³H-labelled total yeast RNA. The RNA was prepared by growing *Saccharomyces cerevisiae* in the presence of [³H]uridine and extracting RNA with phenol. Radioactive RNA was mixed with the fractions tested for ribonuclease activity and incubated at the appropriate temperature. At specified times, samples (100 µl) of the mixtures were removed and pipetted on to DEAE-cellulose filter discs (DE81, Whatman). The discs were then processed as described by Roeder (1974) for the determination of radioactivity. Control experiments with ribonuclease A (Sigma) showed over 99% degradation of the RNA in 60 min at 23 °C.

Other procedures. The incorporation of [³H]uridine into whole cells was followed as previously described (Dabbagh *et al.*, 1974). The concentration of $(NH_4)_2SO_4$ in column fractions was measured with a Radiometer (Copenhagen, Denmark) conductivity meter CDM 2e. Protein was determined by the method of Lowry *et al.* (1951).

RESULTS

Growth of C. albidus at 27 and 37 °C and incorporation of [³H]uridine

In partial agreement with the findings of other investigators (Dabbagh *et al.*, 1974; Tang & Howard, 1973), our data (Fig. 1a) show that a shift from the permissive (27 °C) to the non-permissive (37 °C) environment resulted in an abrupt cessation of growth. The cessation of growth was followed by rapid cell death (Fig. 1b), so that 90% of the cells died within 4 h after the shift. No substantial morphological change or cell lysis was observed at 37 °C, but there was an initial slight increase in turbidity.

The inhibition of growth at the elevated temperature was not due to a decrease in protein synthesis as measured by incorporation of [¹⁴C]isoleucine (data not shown). However, when [³H]uridine incorporation into perchloric acid (PCA)-insoluble material was tested (Fig. 2), it appeared that the shift to 37 °C resulted in the cessation of incorporation of radioactivity. This could be partially explained by an increase in ribonucleolytic activity at 37 °C and/or by lower permeability of the cells to uridine. Alternatively, it was possible that RNA synthesis was affected by the temperature rise. Therefore a cell-free extract (high-speed enzyme, HSE) was prepared and RNA polymerase activity was tested at 23 and 37 °C.

Fig. 2

Fig. 2. Incorporation of [³H]uridine into whole cells at 27 °C (○) and 37 °C (●). Cells were grown on minimal medium (Dabbagh *et al.*, 1974) at 27 °C to an absorbance of 0.19 at 420 nm. At that time (time zero on the graph), 50 μ Ci of [³H]uridine and 12 μ mol of unlabelled uridine carrier were added to a 50 ml culture and incorporation was allowed to proceed for 1.5 h. A portion of the culture was then transferred (arrow) to 37 °C and incubation continued for 2.5 h. Samples (1 ml) were taken and radioactivity was determined as described in Methods.

Fig. 3

Fig. 3. Effect of temperature on RNA polymerase activity. (a) High-speed enzyme (HSE) (52.5 μ g protein) was assayed for 60 min as described in Methods at 23 °C (○) and 37 °C (●). (b) HSE was preincubated at 37 °C and at various times samples (52.5 μ g protein) were taken and assayed for polymerase activity at 23 °C (▲) for 60 min. In all assays, the (NH₄)₂SO₄ concentration was 0.046 M.

RNA synthesis *in vitro*

The incorporation of [³H]UMP into RNA continued for at least 60 min at 23 °C (Fig. 3a). In contrast, the incorporation at 37 °C was halted in less than 30 min. However, the preincubation of HSE at 37 °C for up to 60 min and subsequent assay at 23 °C did not decrease [³H]UMP incorporation (Fig. 3b). Thus, the enzyme itself is not permanently damaged at the elevated temperature.

It was of interest to know whether the temperature sensitivity was a property of one or more RNA polymerase forms; therefore, a sample of HSE was fractionated on a DEAE-Sephadex column (Fig. 4). Two well-separated fractions with activity were seen, one eluting at an ammonium sulphate concentration of about 0.10 M (enzyme I), and the other at about 0.18 to 0.23 M (enzyme II). On the basis of their positions of elution, these fractions probably correspond to the known eukaryotic RNA polymerases I and II (Jacob, 1973). Strikingly, however, neither of the enzymes showed any sensitivity to the fungal toxin α -amanitin, even at a toxin concentration of 80 μ g ml⁻¹. This is consistent with the observation that growth of *C. albidus* is not inhibited by α -amanitin, nor is incorporation of [³H]uridine into RNA affected by the drug (data not shown).

The enzymes I and II were tested for activity at 23 and 37 °C. Both RNA polymerases behaved exactly like the HSE preparation (Fig. 3). It was possible, however, that the observed apparent cessation of RNA synthesis at 37 °C was due to contaminating ribonucleolytic activity present in the enzyme fractions. To test this possibility, the two enzymes were mixed with [³H]uridine-labelled yeast RNA and incubated at 23 and 37 °C for 60 min. Samples of the mixtures were taken at various times and the amount of RNA remaining was determined. At the same time, standard RNA polymerase assays were performed with enzymes I and II to determine the extent of RNA synthesis. The results (Fig. 5) show the

Fig. 4

Fig. 5

Fig. 4. DEAE-Sephadex chromatography of high-speed enzyme (HSE). HSE (49 mg protein) was applied to a column (0.95 × 7 cm) equilibrated with the standard buffer and the column was washed and developed with a 140 ml gradient of 0.05 to 0.42 M-(NH₄)₂SO₄ in the same buffer (---). Fractions (1 ml) were collected and 1 mg bovine serum albumin was added to each. Samples (50 μl) were assayed for RNA polymerase activity (○) for 60 min at 23 °C under standard conditions.

Fig. 5. RNA polymerase activity and ribonucleolytic degradation catalysed by enzyme fractions I and II. RNA polymerase activity in enzyme fractions I (△) and II (▲) was assayed at 0.05 M-(NH₄)₂SO₄ at 23 and 37 °C under otherwise standard conditions. Incorporation of [³H]UMP into RNA was measured at the indicated times. The ratios of incorporation at 37 °C to incorporation at 23 °C were calculated and the values for the 5 min points were taken as 1.0. Ribonucleolytic activity was determined for the two enzyme fractions as described in Methods. The added ³H-labelled RNA (time zero) for enzyme I (○) represented 7500 c.p.m. (23 °C) and 8000 c.p.m. (37 °C). The ratio (8000/7500) was taken as 1.0. The added RNA for enzyme II (●) represented 7670 c.p.m. (23 °C) and 6730 c.p.m. (37 °C). The ratio (6730/7670) was taken as 1.0. The incubations were carried out in the complete RNA polymerase assay systems except that [³H]UTP was replaced by the equivalent amount of unlabelled UTP.

relative decline in the amount of added ³H-labelled RNA and the relative decline in the RNA polymerase activity in the course of the experiment. The data are presented as normalized ratios of polymerase activity or RNA degradation at 37 °C to the corresponding activity at 23 °C. In this manner a correlation is obtained between RNA polymerase and ribonuclease activities observed at both 23 and 37 °C. It is apparent that both enzymes were equally sensitive to elevated temperature and that the slight contaminating ribonuclease activity could not be responsible for the observed cessation of RNA synthesis at 37 °C. Because saturating amounts of DNA template were used in the assays, a deoxyribonuclease activity could also be discounted. Identical results were obtained with the HSE preparation (not shown).

DISCUSSION

Our results show that growth and uptake of an RNA precursor by *C. albidus* was halted when cells were shifted from 27 to 37 °C. An adequate explanation for this phenomenon was provided by the observed inhibition of RNA synthesis *in vitro* at the non-permissive temperature. This contrasts, however, with an earlier report (Tang & Howard, 1973) in which only little effect on RNA synthesis could be seen. Perhaps this discrepancy is due to the difference in viabilities of the strains used. Incubation of our strain for 4 h at 37 °C resulted in the death of over 90 % of the cells as measured by viable cell count. In contrast, Tang & Howard (1973) showed that with their strain even 18 h incubation left about 90 % of the cells alive. This was determined by the eosin dye exclusion technique which may account in part for the disparity of the results. Further complication is added by the report of Dabbagh *et al.* (1974) who found that cells of various species of saprophytic cryptococci, including *C. albidus*, lysed soon after the temperature shift. Neither we nor Tang & Howard (1973) observed cell lysis within the 37 °C incubation period used.

In agreement with the *in vivo* data, RNA synthesis in the crude preparation (HSE) and in fractionated RNA polymerases from our strain of *C. albidus* was restricted at 37 °C. Our results demonstrate that this restriction was not due to increased RNA degradation. A possibility that proteolytic enzymes may damage the enzyme during incubation at 37 °C could also be discounted because full activity was restored as soon as the temperature was lowered to 23 °C. This phenomenon is similar to that described previously for RNA polymerase PC I from *Histoplasma capsulatum* (Boguslawski *et al.*, 1974). The molecular basis for the temperature effect is at present unknown but it is possible that the enzyme's ability for chain elongation or reinitiation decreased with prolonged incubation at 37 °C. It remains to be seen whether other cryptococci, especially *C. neoformans* (capable of growth at 37 °C), possess a temperature-insensitive RNA polymerase.

The two enzyme fractions obtained from DEAE-Sephadex seem to correspond to eukaryotic RNA polymerases I and II; however, neither of them was inhibited by α -amanitin. No sensitivity to the toxin could be detected in HSE or any other subfraction of the total cell homogenate (data not shown). An identical extraction procedure applied to *H. capsulatum* (yeast form) or to *S. cerevisiae* cells yielded RNA polymerase preparations which were inhibited by about 70 % by α -amanitin (Boguslawski *et al.*, 1974, and unpublished data). Thus, it seems unlikely that the α -amanitin-sensitive RNA polymerase was selectively lost during the course of extraction. Furthermore, although rare, the lack of sensitivity to the toxin is not unique among eukaryotes and has been demonstrated previously in *Rhizopus stolonifer* spores (Gong & Van Etten, 1972), and in the mycelial form of *H. capsulatum* (Boguslawski *et al.*, 1975; McMillian *et al.*, 1977).

In summary, our observations show that RNA synthesis in *C. albidus* is severely restricted at 37 °C. This is sufficient to account for the cessation of growth, but other factors may be involved in the rapid cell death. Although our results are in conflict with the data of others (Dabbagh *et al.*, 1974; Tang & Howard, 1973), this may be due to differences between strains. The available strains of *C. albidus* are not well characterized genetically, and variations are possible. It is only recently that the existence of the perfect state of *C. neoformans* has been firmly established (Kwon-Chung, 1975). If, as suggested by Van der Walt (1967), *C. albidus* also produces the sexual state, the genetic analysis and comparison of the strains will become feasible.

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REFERENCES

- BOGUSLAWSKI, G., SCHLESSINGER, D., MEDOFF, G. & KOBAYASHI, G. (1974). Ribonucleic acid polymerases of the yeast phase of *Histoplasma capsulatum*. *Journal of Bacteriology* **118**, 480–485.
- BOGUSLAWSKI, G., MEDOFF, G., SCHLESSINGER, D. & KOBAYASHI, G. S. (1975). Histin, an RNA polymerase inhibitor isolated from *Histoplasma capsulatum*. *Biochemical and Biophysical Research Communications* **64**, 625–632.
- BOGUSLAWSKI, G., ZEHRING, W. A., MEYER, R. R. & PARR, J. S. (1977). Characterization of a novel ribonucleotide-polymerizing enzyme from a fungus, *Histoplasma capsulatum*. *Journal of Biological Chemistry* **252**, 4337–4346.
- DABBAGH, R., CONANT, N. F., NIELSEN, H. S. & BURNS, R. O. (1974). Effect of temperature on saprophytic cryptococci: temperature-induced lysis and protoplast formation. *Journal of General Microbiology* **85**, 177–189.
- EMMONS, C. W., BINFORD, C. H., UTZ, J. P. & KWON-CHUNG, K. J. (1977). *Medical Mycology*, 3rd edn, p. 226. Philadelphia: Lea and Febiger.
- GONG, C.-S. & VAN ETEN, J. L. (1972). Changes in soluble ribonucleic acid polymerases associated with the germination of *Rhizopus stolonifer* spores. *Biochimica et biophysica acta* **272**, 44–52.
- JACOB, S. T. (1973). Mammalian RNA polymerases. *Progress in Nucleic Acid Research and Molecular Biology* **13**, 93–126.
- KWON-CHUNG, K. J. (1975). Description of a new

- genus, *Filobasidiella*, the perfect stage of *Cryptococcus neoformans*. *Mycologia* **67**, 1197–1200.
- LOWRY, O. H., ROSEBROUGH, N. J., FARR, A. L. & RANDALL, R. J. (1951). Protein measurement with the Folin phenol reagent. *Journal of Biological Chemistry* **193**, 265–275.
- MCMILLIAN, R., KUMAR, B. V., MEDOFF, G. & KOBAYASHI, G. S. (1977). *Abstracts of the 77th Annual Meeting, American Society for Microbiology, May, 1977*, abstract F60, p. 128.
- ROEDER, R. G. (1974). Multiple forms of deoxyribonucleic acid-dependent ribonucleic acid polymerase in *Xenopus laevis*. *Journal of Biological Chemistry* **249**, 241–248.
- ROEDER, R. G. & RUTTER, W. J. (1969). Multiple forms of DNA-dependent RNA polymerase in eukaryotic organisms. *Nature, London* **224**, 234–237.
- TANG, S. L. & HOWARD, D. H. (1973). Metabolism, macromolecular synthesis, and nuclear behavior of *Cryptococcus albidus* at 37 C. *Journal of Bacteriology* **115**, 574–581.
- VAN DER WALT, J. P. (1967). Sexually active strains of *Candida albicans* and *Cryptococcus albidus*. *Antonie van Leeuwenhoek* **33**, 246–256.