FURTHER CHARACTERIZATION OF A RIBONUCLEOTIDE-POLYMERIZING ENZYME FROM HISTOPLASMA CAPSULATUM. II. POSSIBLE ROLE IN CELLULAR METABOLISM

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SUMMARY - An enzyme, ribonucleotide polymerase, isolated from the yeast phase of a fungus, Histoplasma capsulatum has been found to stimulate the incorporation of dTMP in the reaction catalysed by DNA polymerase from H. capsulatum and E. coli. The stimulation is dependent on the amount of ribonucleotide polymerase added. The data indicate that protein-protein interaction is responsible for the increase in DNA synthesis. It is suggested that ribonucleotide polymerase may be involved in supplying short RNA primers for DNA polymerase.

INTRODUCTION

We have recently described a novel ribonucleotide-polymerizing enzyme from the yeast phase of a dimorphic pathogenic fungus, Histoplasma capsulatum (1). This enzyme, which we have called ribonucleotide polymerase, is not dependent on DNA template, and produces short oligonucleotides (5-6 residues per average chain). The enzyme contains a very tightly bound RNA primer (1-3 residues). In this communication we describe some additional properties of ribonucleotide polymerase and show that the enzyme stimulates the incorporation of nucleotides into DNA in the reaction catalysed by DNA polymerase from H. capsulatum and E. coli.

MATERIALS AND METHODS

Unlabeled ribonucleoside triphosphates and calf thymus DNA were purchased from Sigma Chemical Co. A 20:1 complex of poly(dA)·p(dT)$_{10}$, deoxyribonucleoside triphosphates, and DNA polymerase (E. coli B) were obtained from P-L Biochemicals. ^{3}H-labeled UTP and dTTP were from New England Nuclear and Schwarz/Mann, respectively.

RNA polymerase, ribonucleotide polymerase, and fraction II of histin (an RNA polymerase inhibitor) were prepared and assayed as described previously (1,2). Glycerol gradient centrifugations were also performed as before (1). Activated calf thymus DNA was prepared as described by Uyemura and Lehman (3). DNA polymerase activity was assayed in a final volume of 125µl. The reaction mixture contained the following: 1.25µmol of Tris-HCl (pH 7.85); 0.75µmol of

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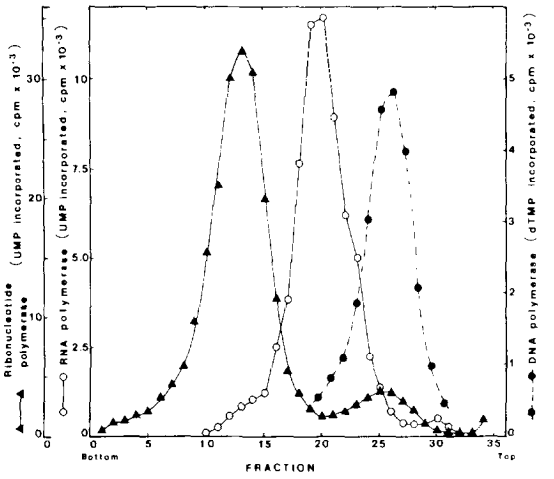


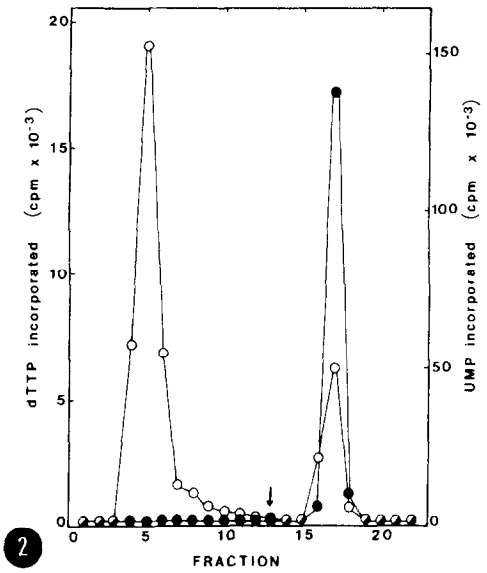
Fig. 1. Glycerol gradient centrifugation of ribonucleotide polymerase, RNA polymerase, and DNA polymerase from the yeast phase of H. capsulatum. A sample of crude extract (19mg of protein) was applied to a 36-ml glycerol gradient (15-30%, v/v), and centrifuged and fractionated as described (1). Each fraction was assayed for the three activities (30µl/assay). Native calf thymus DNA was used in RNA polymerase assays. Activated DNA was used in DNA polymerase assays.

MgCl ; 2.0µmol of NaCl; 0.025µmol each of dATP, dCTP, and dGTP; 0.0075µmol of unlabeled dTTP; and 2µCi of [^{3}H]dTTP (specific radioactivity 15Ci/mmol). The native, activated, or heat-denatured calf thymus DNA (10µg/assay) was used as a template, as indicated. The volumes were adjusted with water, and the reaction started by enzyme addition. After 60 min at 23°C the reactions were terminated by pipetting 100µl aliquots on DEAE-cellulose discs (Whatman DE81). The discs were processed and radioactivity measured as described (1).

DEAE-cellulose chromatography of DNA polymerase and ribonucleotide polymerase was performed as follows. The resin was equilibrated with 0.05M ammonium sulfate in a buffer containing 0.05M Tris-HCl (pH 7.85), 25% (v/v) glycerol, 0.5mMEDTA, and 0.5mM dithiothreitol. A sample of crude extract was applied to the column at the rate of 1 column volume per hour. The column was washed with 2 volumes of the equilibrating buffer, and then with 2 volumes of 0.40M ammonium sulfate in the same buffer. The active fractions were adjusted to 50% glycerol and stored at -25°C.

RESULTS

Glycerol gradient centrifugation and DEAE-cellulose chromatography - The heavy species of ribonucleotide polymerase can be easily and reproducibly separated from both RNA polymerase and DNA polymerase of the yeast phase of Histoplasma capsulatum (Fig. 1). However, the light form of ribonucleotide polymerase and DNA polymerase of the fungus sedimented at the same position in



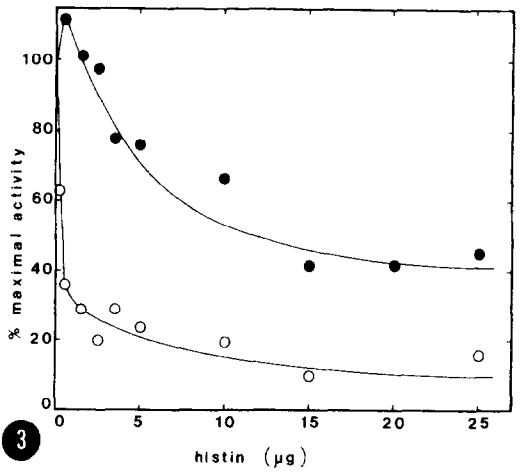


Fig. 2. DEAE-cellulose chromatography of DNA polymerase and ribonucleotide polymerase from the yeast phase of Histoplasma capsulatum. A sample of crude extract (32mg of protein) was applied to a 10-ml DEAE-cellulose column (0.8 x 5cm) and the enzyme activities eluted as described in MATERIALS AND METHODS. Fractions (2ml) were collected and 30µl samples assayed for DNA polymerase (O———O) and ribonucleotide polymerase (●———●) activity. The arrow indicates buffer change from 0.05M to 0.40M ammonium sulfate.

Fig. 3. Inhibition of RNA polymerase and ribonucleotide polymerase by histin. The enzymes (30µl samples of each) were assayed with increasing amounts of histin. The maximal activity (100%) for RNA polymerase (O———O) was 3,297cpm; the maximal activity (100%) for the heavy form of ribonucleotide polymerase (●———●) was 12,710cpm.

the gradient. In order to obtain a DNA polymerase fraction free of ribonucleotide polymerase activity, a DEAE-cellulose column was used. Fig. 2 shows that DNA polymerase of H. capsulatum can be separated into two fractions, one which was not retained on the column, and the other which required high salt concentration for elution. The early fraction was completely free of ribonucleotide polymerase. This fraction, and E. coli DNA polymerase were used in the subsequent experiments.

Effect of histin on polymerization of nucleotides - In contrast to extracts from the yeast phase, those prepared from the mycelial form of H.

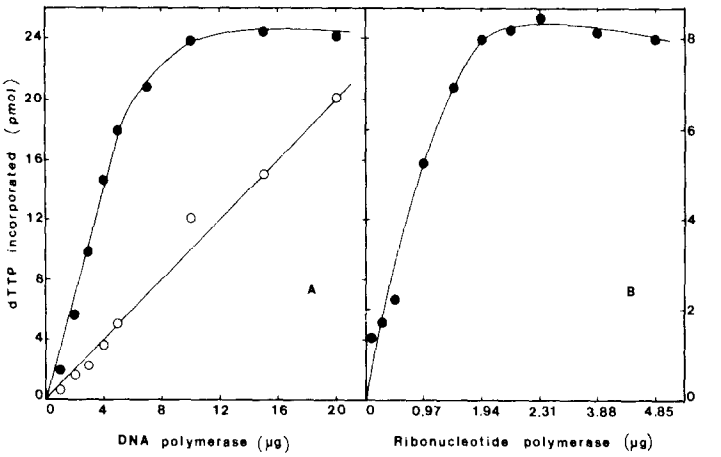


Fig. 4. Stimulation of DNA synthesis by ribonucleotide polymerase. DNA polymerase from E. coli was assayed in the absence and the presence of the heavy species of ribonucleotide polymerase obtained from the glycerol gradient shown in Fig. 1. Panel A - DNA polymerase alone (O——O) or with constant amount (0.97µg) of ribonucleotide polymerase (●——●); Panel B - constant amount of DNA polymerase (2µg) assayed with the increasing amounts of ribonucleotide polymerase. Heat-denatured calf thymus DNA was used in all assays.

capsulatum contained only traces of RNA polymerase (4) or ribonucleotide polymerase (1) activity. The absence of RNA polymerase was correlated with the presence of a potent inhibitor called histin (2,4). As shown in Fig. 3, histin exerted a significant, although not complete, inhibitory effect on ribonucleotide polymerase. On the other hand, neither E. coli DNA polymerase, nor H. capsulatum DNA polymerase were affected by histin (data not shown). Thus, the inhibitor is specific in that it acts only when ribonucleotide polymerization is involved.

Stimulation of DNA synthesis by ribonucleotide polymerase - Because DNA synthesis involves the participation of short RNA primers, and because ribonucleotide polymerase produces short oligonucleotide chains, it was of interest to see whether this enzyme would influence the activity of DNA polymerase. As shown in Fig. 4, the addition of ribonucleotide polymerase to the E. coli DNA polymerase reaction resulted in a considerable increase (up to 5-fold) in the incorporation of deoxyribonucleotides into DNA. The increase was independent

of the template concentration (not shown) and was not due to non-specific stimulation by protein or RNA because the addition of the equivalent amounts of yeast RNA and bovine serum albumin brought about a 50% drop in DNA synthesis. The same 2.5-5-fold stimulation of activity was observed with H. capsulatum DNA polymerase using both native and denatured calf thymus DNA but not poly(dA)·p(dT)$_{10}$. Ribonucleotide polymerase alone did not catalyse DNA synthesis in the presence of either $MgCl_2$ or $MnCl_2$, nor was any incorporation seen when DNA template was omitted from the assay. Addition of histin had no effect on DNA polymerase activity or on the stimulation by ribonucleotide polymerase.

DISCUSSION

We have previously (1) characterized ribonucleotide polymerase with respect to catalytic properties, molecular weight, and the critical kinetic parameters of the assay. The present results show that ribonucleotide polymerase can stimulate activity of DNA polymerase from both E. coli and H. capsulatum. The stimulation was independent of template concentration but depended on the amounts of DNA polymerase and ribonucleotide polymerase present in the reaction (Fig. 4). The observed effect of varying the DNA polymerase concentration and keeping ribonucleotide polymerase constant (or vice versa) indicated that the two enzymes interacted directly to reach a plateau at which they would become saturated, so that no further increase in activity could be seen.

Our previous experiments (1) showed that ribonucleotide polymerase contained a small, very tightly bound RNA fragment. Therefore it is possible that the observed increase in DNA synthesis was due to the introduction of primers for DNA polymerase. This would help to explain why histin had no effect on the stimulation of synthesis. However, yeast RNA alone or in combination with bovine serum albumin did not stimulate DNA polymerase activity suggesting that the priming effect would have to depend on the func-

tion or conformational state of ribonucleotide polymerase.

The participation of RNA in biosynthesis of DNA is a well-documented phenomenon (5-7). In some cases a specific RNA polymerase is known to be involved in the production of RNA primers needed for the initiation of DNA replication (8,9). A recent report by Plevani and Chang (10) suggested that in Saccharomyces cerevisiae all known forms of RNA polymerase are so utilized. Except for this report, there is paucity of information concerning the enzyme(s) involved in the formation of RNA primers in eucaryotic organisms. It is tempting to hypothesize that ribonucleotide polymerase might be such an enzyme in H. capsulatum. We are at present attempting to purify and stabilize DNA polymerases of the fungus and to isolate intact DNA to use them in a homologous system coupled with ribonucleotide polymerase. Until this is achieved, and the reaction product is analysed, the proof for the proposed role of the enzyme will be only circumstantial.

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