DNA-dependent ATPase B of FM3A cells

Its separation from DNA polymerase α

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One form of DNA-dependent ATPase (DNA-dependent ATPase B) has been purified from FM3A cells. In this report, we describe the association of DNA polymerase α activity with DNA-dependent ATPase B through a series of purification steps and the final separation of the two enzymes by glycerol gradient centrifugation operated at a low salt concentration.

DNA-dependent ATPase B *FM3A cells* *DNA polymerase α* *Separation* *Glycerol gradient centrifugation* *Low salt condition*

1. INTRODUCTION

We have demonstrated the existence of three forms of DNA-dependent ATPase in calf thymus, which are designated A1, A2 and B [1]. We have sought analogous enzymes in FM3A cells which are a promising source for the isolation of DNA replication enzymes since a method has been developed, which enables us to obtain a large amount of S-phase accumulated cells grown in mouse [2]; several temperature-sensitive mutants have also been isolated from the cells [3–5]. Preliminary study has revealed that three forms of DNA-dependent ATPase are also present in FM3A cells. In the course of purification of each ATPase, we have encountered the difficulty to remove the contaminating DNA polymerase α activity from the preparation of DNA-dependent ATPase B.

This report describes the association of DNA polymerase α activity with DNA-dependent ATPase B through a series of purification steps and the final separation of the two enzymes by glycerol gradient centrifugation operated at a low salt concentration.

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2. MATERIALS AND METHODS

FM3A cells were grown, synchronized to S-phase by FUdR and harvested as in [2], and stored frozen at −80°C until use. DNA-dependent ATPase and DNA polymerase α were assayed as in [1] and [2], respectively. DNA-dependent ATPase B from FM3A cells was prepared by the modified methods in [1] as follows. Frozen stock of 1×10^{11} cells were thawed, suspended in buffer 1 (20 mM KP_i buffer (pH 7.5), 0.1 mM Na_3EDTA, 1 mM 2-mercaptoethanol and 0.25 mM phenylmethyl sulfonyl fluoride) and homogenized by sonication. The sonicate (750 ml) was made 0.3 M KCl by the addition of 3.3 M KCl in buffer 1, extracted and clarified by centrifugation (Hitachi RP42, 40000 rev./min, 1 h). The extract was applied to DEAE-cellulose column (1000 ml) equilibrated with 0.3 M KCl in buffer 1. The flow-through fraction (DNA-free extract, 910 ml) was precipitated by 25–80% ammonium sulfate fractionation, dissolved in and dialyzed against 50 mM KCl in

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buffer 2 (buffer 1 plus 20% ethylene glycol). The dialyzate was subjected to 2nd DEAE-cellulose column chromatography at 50 mM KCl in buffer 2. As was the case with calf thymus [1], the flow-through fraction contained DNA-dependent ATPase A activity. DNA-dependent ATPase B was eluted with 0.25 M KCl in buffer 2 together with DNA polymerase α activity. This preparation of DNA-dependent ATPase was further purified as in section 3.

3. RESULTS AND DISCUSSION

To establish in vitro DNA replication system and to evaluate the role of an enzyme in DNA replication, it is necessary to have an enzyme preparation which is free from the known DNA replication enzymes and proteins such as DNA polymerases and DNA binding proteins. Among these enzymes and proteins, DNA polymerase α was carefully monitored during the series of purification steps of DNA-dependent ATPase B because it had been observed that the DNA-dependent ATPase from mouse myeloma was associated with DNA polymerase α during early steps of purification [6]. Although DNA-dependent ATPase from mouse myeloma was reported to be separated from DNA polymerase α by phosphocellulose column chromatography, the DNA-dependent ATPase B fraction from FM3A cells prepared as in section 2 was not well separated from DNA polymerase α by phosphocellulose column chromatography (fig. 1). The elution pattern was not improved even when the volume of the elution buffer or column bed size was changed (not shown). This poor resolution between DNA polymerase α and DNA-dependent ATPase B might be due to the heterogeneity of DNA polymerase α of FM3A cells as shown in [2].

The phosphocellulose fraction was further purified by hydroxyapatite column chromatography (fig. 2). DNA polymerase α activity contaminated through the previous steps was

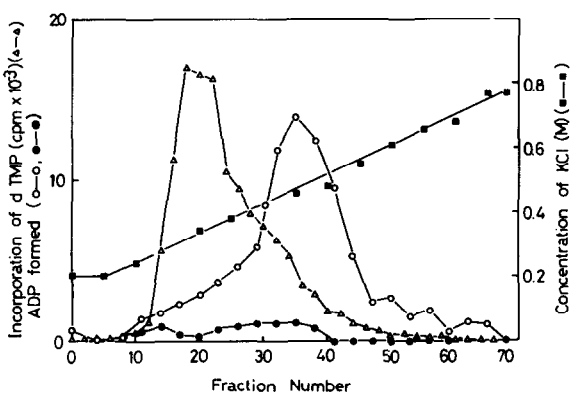

Fig. 1. First phosphocellulose column chromatography. DEAE-cellulose fraction of DNA-dependent ATPase B prepared as in section 2 was adjusted to 0.2 M KCl and applied onto phosphocellulose column (100 ml) and eluted with 1000 ml of a linear gradient of KCl from 0.2–0.8 M in buffer 2. ATPase activity in the presence (○—○) or absence (●—●) of heat-denatured DNA. DNA polymerase α activity (▲—▲). Concentration of KCl (■—■).

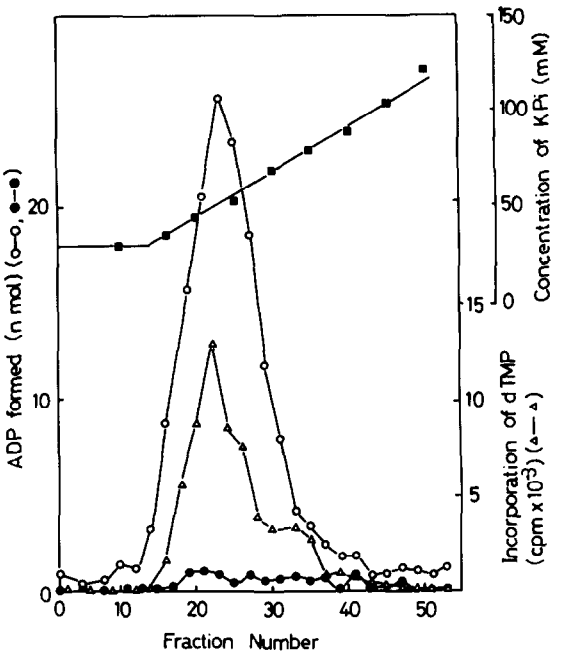

Fig. 2. Hydroxyapatite column chromatography. Phosphocellulose fraction (fractions 29–42 of fig. 1, 210 ml) was adjusted to 0.5 M KCl in buffer 2 and applied onto hydroxyapatite column (25 ml). After the column was washed with the above buffer and buffer 2 containing 30 mM KP_i and 0.5 M KCl, 250 ml of a linear gradient from 30–150 mM KP_i in buffer 2 containing 0.5 M KCl was applied. ATPase activity in the presence (○—○) or absence (●—●) of heat-denatured DNA. DNA polymerase α activity (▲—▲). Concentration of KP_i (■—■).

co-purified again with the ATPase activity on this purification step in the presence of 0.5 M KCl. Alteration of the [KCl] in the elution buffer had no influence on the elution pattern.

A further attempt to separate the two enzymes by the use of two successive chromatographies on native DNA–cellulose and second phosphocellulose columns was unsuccessful. DNA polymerase γ detected until the hydroxyapatite column step was completely removed away during native DNA-cellulose column chromatography.

The final and the only method which proved effective for the separation of the two enzymes was glycerol gradient centrifugation in the presence of low concentration of salt (fig. 3). DNA-dependent ATPase B activity sedimented at 5.5 S, whereas DNA polymerase α sedimented at 10 S, suggesting that the latter was aggregated under low salt condition, since DNA polymerase α activity sedimented more slowly in the presence of 0.5 M KCl resulting in a rather broad peak and incomplete separation from the ATPase whose sedimentation was unaffected by changing the salt concentration.

The above results not necessarily prove specific interaction between DNA-dependent ATPase and DNA polymerase α, but rather they must be taken as a warning in evaluation of the purity of DNA polymerase α from various cells and tissues. Some of preparations of DNA polymerase α cannot strictly rule out the possible contamination of DNA-dependent ATPase activities which might be co-purified through conventional procedure as we have used in this study. In addition, the findings that DNA polymerase α possesses a novel 'primase' activity [7,8], may have to take into consideration a possibility of the participation of DNA-dependent ATPase, because proteins which have DNA-dependent ATPase activity are clearly demonstrated to play an important role in the priming step of prokaryotic DNA replication systems [9].

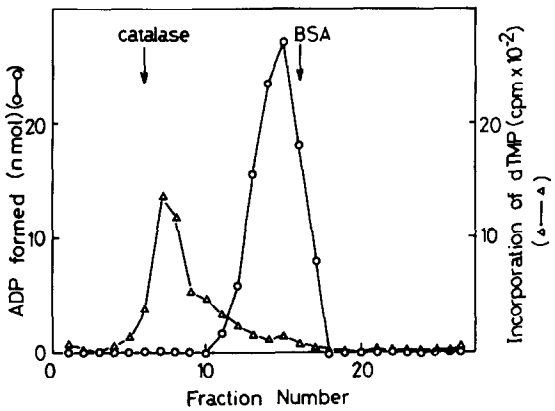

Fig. 3. Glycerol gradient centrifugation. A DNA-dependent ATPase B fraction purified through native DNA-cellulose column and second phosphocellulose column chromatography after hydroxyapatite column chromatography (fig. 2) was layered on glycerol gradient from 20–40% in buffer 1 containing 40 mM KCl. Centrifugation was performed for 40 h at 52000 rev./min in a Hitachi RPS65-T rotor at 4°C. Fraction was collected from the bottom. Marker proteins used were catalase (11 S) and bovine serum albumin (4.4 S). ATPase activity (○—○). DNA polymerase α activity (Δ—Δ).

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