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SEPARATION OF A STIMULATORY FACTOR OF RNA POLYMERASE II FROM PROTEIN KINASE ACTIVITY OF EHRLICH ASCITES TUMOR CELLS

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1. Introduction

The regulation of eukaryotic gene expression is one of the most exciting problems in modern biology. Since Roeder and Rutter reported the multiple forms of eukaryotic RNA polymerase [1], extensive studies have been done on the purification of these enzymes. Along with the purification of the enzymes, there have been many papers reporting the factors stimulating these enzymes, especially RNA polymerase II [2–6]. These factors are expected to play important roles in the regulation of eukaryotic transcription. In order to elucidate the function of these factors, we purified one of these factors, named S-II, from Ehrlich ascites tumor cells [7]. This factor is a basic protein having molecular weight of 38 000 and stimulates only RNA polymerase II and distinctly insensitive to RNA polymerase I. The stimulation cannot be detected when poly [d(A–T) is used as template under the conditions where more than ten-fold stimulation is obtained with homologous DNA as template.

Recently, Dahmus showed that a stimulatory factor of RNA polymerase II from Novikoff hepatoma cells, named HLF_2, contained protein kinase activity [8]. He proposed that phosphorylation of RNA polymerase II by this factor was required for the subsequent stimulation of RNA synthesis.

In this paper we report results showing that S-II could not be copurified with protein kinase and that protein kinase activity was not essential for the stimulation of RNA polymerase II.

2. Methods

2.1. *Assay of the stimulation of RNA synthesis*

The reaction mixture contained in a total volume of 0.25 ml, 10 μmol of Tris–HCl, pH 7.9, 0.75 μmol of $MnCl_2$, 1.15 μmol $MgCl_2$, 12.5 μmol $(NH_4)_2SO_4$, 0.017 μmol EDTA, 1 μmol β-mercaptoethanol, 0.0625 μmol each CTP, ATP and GTP, 0.00625 μmol UTP, 0.5 μCi [^{3}H]UTP (20 Ci/mmol), 5 μg Ehrlich ascites tumor DNA, and 10 units partially purified RNA polymerase II [9]. To this reaction mixture were added 10–50 μl of test fractions and the incorporation of UMP into acid-insoluble fraction was compared with that in reaction mixture without the test fraction after incubation for 60 min at 37°C. One stimulation unit was defined as the amount which enhanced the activity of 10 units of RNA polymerase II to 11 units under these conditions. This procedure is essentially the same as reported before [7].

2.2. *Assay of protein kinase*

The reaction mixture contained in 0.2 ml total volume, 10 μmol of Tris–HCl, pH 7.9, 10 μmol $MgCl_2$, 15 nmol EGTA, 5 nmol [γ-^{32}P]ATP (50–60 cpm/pmol) and 500 μg of casein (α-casein, Sigma Biochem. Co.) or 120 μg of histone (calf thymus whole histone type-II, Sigma Biochem. Co.). When histone was used as substrate, 1 nmol cyclic AMP was added to the reaction mixture. After shaking for 10 min at 37°C, the reaction was terminated by adding 2 ml 7.5% trichloroacetic acid and acid-insoluble radioactivity was counted. One unit of protein kinase

activity was defined as the amount of enzyme that catalyses the transfer of 1 nmol phosphate from ATP in 1 min to substrate under the conditions described above.

3. Results

Recently we purified a stimulatory factor of RNA polymerase II from Ehrlich ascites tumor cells [7]. The purification procedure involved the following steps: disruption of cells in a buffer containing 0.25 M ammonium sulfate and sonication, ammonium sulfate precipitation, chromatography on DEAE-cellulose, ammonium sulfate fractionation, chromatographies on phosphocellulose followed by CM-cellulose and gel-filtration through Bio-Gel P-60.

According to Dahmus' hypothesis, protein kinase activity associated with stimulatory factor is essential for the stimulation of RNA polymerase II. Since Dahmus' stimulatory factor HLF_2 and our factor S-II can be purified from the DEAE-cellulose flowthrough-fraction by nearly the same procedures, we carefully checked the protein kinase activity of S-II fraction at each step of purification. Stimulatory activity and protein kinase activity of each fraction eluted from a column of phosphocellulose were assayed. Two stimulatory factors, S-I and S-II, can be resolved at this step as reported previously [7]. As shown in fig.1, both peaks of S-I and S-II did not coincide with any peak of protein kinase activity which phosphorylate casein or histone.

The stimulatory factor S-II eluted from phosphocellulose was further fractionated on a column of CM-cellulose. Again, the peak of stimulatory factor S-II did not coincide with that of protein kinase activity which phosphorylate casein, as shown in fig.2. The protein kinase activity phosphorylating histone was not detected on this chromatogram.

Therefore, it may be possible to conclude that

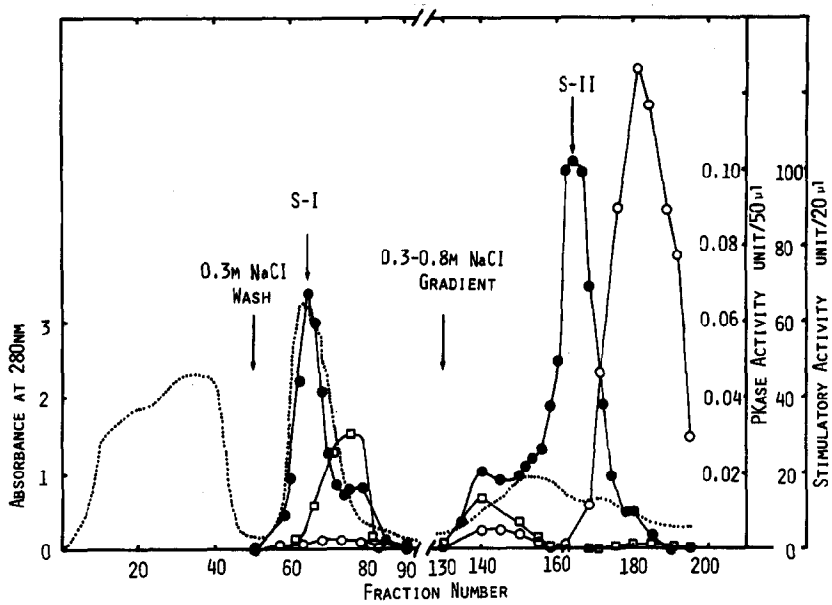

Fig.1. Phosphocellulose column chromatography of stimulatory factors and protein kinase. The precise procedures to isolate stimulatory factor S-II was described elsewhere [7]. The material not adsorbed on DEAE-cellulose was fractionated with ammonium sulfate. Taking protein fraction precipitating between 50–85% saturation of ammonium sulfate, it was dialyzed against a buffer consisting of 0.01 M Tris–HCl, pH 7.9 and 5 mM β-mercaptoethanol. About 1600 mg of protein was applied to a column of phosphocellulose (4 × 7 cm) which had been equilibrated with the same buffer. After washing the column with the buffer containing 0.3 M NaCl, the column was developed with a linear gradient of 600 ml from 0.3–0.8 M NaCl in the buffer. Stimulatory activity and protein kinase activity in each fraction were assayed. (●——●) Stimulatory activity, (○——○) protein kinase activity phosphorylating casein, (□——□) protein kinase activity phosphorylating histone, (----------) absorbance at 280 nm.

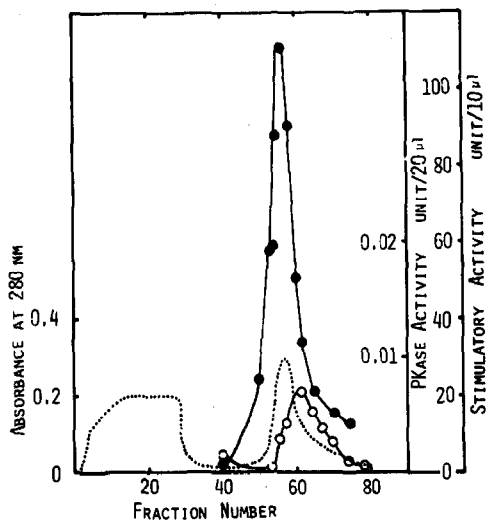

Fig.2. CM-cellulose chromatography of stimulatory factor S-II. S-II fraction eluted from phosphocellulose was dialyzed extensively and then applied to a column of CM-cellulose (1.6 × 3.5 cm). Adsorbed activity was eluted with a linear gradient of 200 ml from 0–0.2 M NaCl in the buffer described in the legend to fig.1. Stimulatory activity and protein kinase activity in each fraction were assayed. (●——●) Stimulatory activity, (○——○) protein kinase activity phosphorylating casein, (----------) absorbance at 280 nm.

stimulatory factor S-II did not contain protein kinase activity. The specific activity of stimulatory factor S-II and that of protein kinase activity at each purification step is shown in table 1. The specific activity of S-II increased about 13 fold over DEAE-cellulose flowthrough-fraction, whereas that of protein kinase activity decreased to 0.06-fold. This result clearly indicates that S-II could not be copurified with protein kinase activity.

The ATP analogue, adenylimidodiphosphate (AMP-PNP) is an effective inhibitor of enzymes which cleave β–γ linkage. When ATP and GTP in the reaction mixture of RNA synthesis were replaced with AMP–PNP and GMP–PNP respectively, the incorporation of [^{3}H]UMP into RNA fraction was reduced to about 50% that of original system. Under these conditions, the effect of S-II on RNA synthesis was examined. If protein kinase is essential for the stimulation of RNA synthesis, no stimulation of RNA

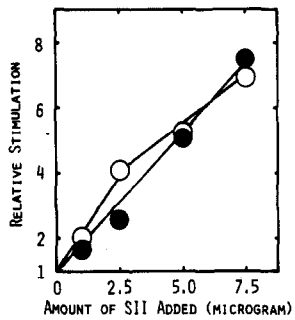

Fig.3. The effect of AMP–PNP and GMP–PNP on the stimulation of RNA polymerase II by S-II. The assay was as described in Methods. Each reaction contained 10 units of RNA polymerase II and various amounts of S-II. (○——○) Stimulation in the regular system, (●——●) stimulation in the system where ATP and GTP were replaced with AMP–PNP and GMP–PNP, respectively.

Table 1
Summary of the purification of stimulatory activity and protein kinase activity in S-II fraction

Step	Protein (mg)	Stimulatory activity			Protein kinase activity[a]		
		Total activity (units)	Specific activity	Recovery (%)	Total activity (units)	Specific activity	Recovery (%)
DEAE-cellulose flowthrough	2800	2 350 000	839 (1)	100	1 518 000	542 (1)	100
Phosphocellulose S-II fraction[b]	22.5	175 000	7780 (9.3)	7.4	7000	311 (0.57)	0.5
CM-cellulose S-II fraction	4.8	51 800	10 800 (13)	2.2	144	31 (0.06)	0.01

[a] Casein was used as phosphate acceptor for the assay of protein kinase
[b] At this step stimulatory factor S-I and S-II were resolved

synthesis should be observed, since there is no effective phosphate donor in the reaction mixture. However, as shown in fig.3, significant stimulation of RNA synthesis by S-II was observed under the condition where ATP and GTP were replaced with AMP–PNP and GMP–PNP, respectively.

The increase of relative stimulation was proportional to the amount of S-II added and this increase was comparable to that in the control system. Therefore, it can be concluded that protein kinase activity was not required for the stimulation of RNA synthesis by the stimulatory factor S-II.

4. Discussion

The detailed mechanism of the stimulation of RNA synthesis by stimulatory factors obtained from various sources has remained unknown. The phosphorylation of RNA polymerase II by these factors is certainly a possibility, since it is known that phosphorylation of RNA polymerase II by protein kinase results in an apparent stimulation of RNA synthesis [10]. Recently Dahmus reported evidence that protein kinase activity was essential for the stimulation of RNA polymerase II by a stimulatory factor HLF_2 isolated from Novikoff hepatoma cells. This factor seems to have similar characteristics to the stimulatory factor S-II which we isolated from Ehrlich ascites tumor cells, since both factors are basic protein and purified from DEAE-cellulose flowthrough-fraction of the crude cell extracts. However, in the case of S-II, the protein kinase activity was not co-purified with the stimulatory activity and the S-II fraction recovered from a column of CM-cellulose contained very low activity of protein kinase. Moreover, the stimulation of RNA synthesis by S-II was detected under the conditions where protein kinase was inactive. Therefore, we concluded that the stimulation of RNA synthesis by S-II did not require the protein kinase activity as was observed with HLF_2. The mechanism of the stimulation of RNA synthesis by S-II is not clear at the moment. However, a possibility that a nonspecific stimulation by nucleases contaminating in S-II fraction has been excluded as reported previously [7].

Acknowledgement

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