CHARACTERIZATION OF THREE PROTEINS STIMULATING RNA POLYMERASE II

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Received 16 April 1981; revised version received 26 May 1981

1. Introduction

We have purified three proteins (S-II, S-II′, S-I(b)) that specifically stimulate the activity of RNA polymerase II of Ehrlich ascites tumor cells [1,2]. Structural studies showed that S-II′ was a phosphorylated form of S-II [3] and that radioiodinated S-I(b) and S-II′ gave identical peptide maps, whereas the M_r of S-I(b) (24 000) was significantly different from that of S-II′ (41 000) [2]. These proteins were found to share common antigenicity and to be located entirely in the nucleoplasm, not in the nucleoli [4]. Antibodies raised against S-II selectively inhibited α-amanitin-sensitive RNA synthesis in isolated nuclei, indicating that these proteins are essential for transcription by RNA polymerase II in vivo [5]. Moreover, when S-II was added exogenously to isolated nuclei from spleen cells of anemic mice, it significantly enhanced the synthesis of globin mRNA as well as α-amanitin-sensitive RNA synthesis [6].

This paper describes studies on the optimum conditions for stimulation of RNA polymerase II using purified stimulatory proteins and enzyme. Under these conditions, RNA polymerase II and a stimulatory protein are suggested to interact in a molar ratio of 1:1, and it was possible to obtain significant stimulation with a few nanograms of purified stimulatory proteins. These proteins did not seem to participate in the initiation of RNA synthesis from the promoter region of the adenovirus major late gene, under these conditions.

2. Materials and methods

RNA polymerase II was purified to homogeneity from Ehrlich ascites tumor cells by the following procedures: chromatography on DEAE-cellulose, phosphocellulose, DEAE-Sephadex, sucrose density gradient centrifugation and finally a 2nd DEAE-Sephadex chromatography (in preparation). The final preparation gave a single band by non-denaturing gel electrophoresis and it separated into 11 polypeptides on SDS–polyacrylamide gel. Stimulating proteins S-II and S-II′ were purified as in [1] and S-I(b) was purified as in [2]. The preparations each gave a single band by SDS–polyacrylamide gel electrophoresis.

3. Results

We established the optimum conditions for stimulation of RNA polymerase II using the 3 purified stimulatory proteins and purified RNA polymerase II from Ehrlich ascites tumor cells. These conditions are summarized in the legend to fig.1. Under the conditions in [1], stimulation was ≤5–6-fold, whereas under these conditions it was >20-fold. The main difference between the two conditions was the salt concentration. The effect of $(NH_4)_2SO_4$ on the stimulation of RNA synthesis under the new conditions is shown in fig.1(A). The activity of RNA polymerase II alone increased gradually with increase in the concentration of $(NH_4)_2SO_4$. However, RNA synthesis in the presence of a stimulatory protein was maximum when $(NH_4)_2SO_4$ in the reaction mixture was 0–5 mM. The stimulation decreased markedly at higher concentrations of $(NH_4)_2SO_4$ and almost no stimulation was detected at 50 mM. The effect of $(NH_4)_2SO_4$ on stimulation of RNA synthesis was essentially the same with all 3 stimulatory proteins. Thus, it was concluded that $(NH_4)_2SO_4$ was not necessary to measure the overall stimulatory effect of these proteins.

The stimulatory effects of the 3 proteins were also

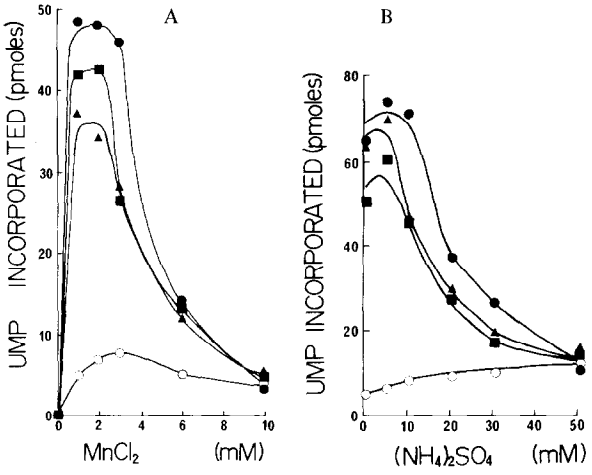

Fig.1. Effects of $(NH_4)_2SO_4$ and $MnCl_2$ on the activity of the stimulatory proteins of RNA polymerase II: 0.4 μg RNA polymerase II was assayed in the absence (○) or presence of S-II (10 ng (●)), S-II′ (10 ng (■)) and S-I(b) (6 ng (▲)), respectively, with increasing amounts of (A) $(NH_4)_2SO_4$ and (B) $MnCl_2$. The assay medium contained, in a total volume of 250 μl, 15 mM of Tris–HCl (pH 7.9), 2 mM β-mercaptoethanol, 2 mM $MnCl_2$, 200 μM each of ATP, GTP and CTP, 20 μM of UTP, 1 μCi [^{3}H]UTP (20 Ci/mmol), 1.25 μg purified Ehrlich ascites tumor DNA, 13% (v/v) glycerol, 0.04% (v/v) Triton X-100 and purified RNA polymerase II. The medium was incubated for 30 min at 37°C in the presence or absence of stimulatory proteins and incorporation of UMP into the acid-insoluble fraction was measured.

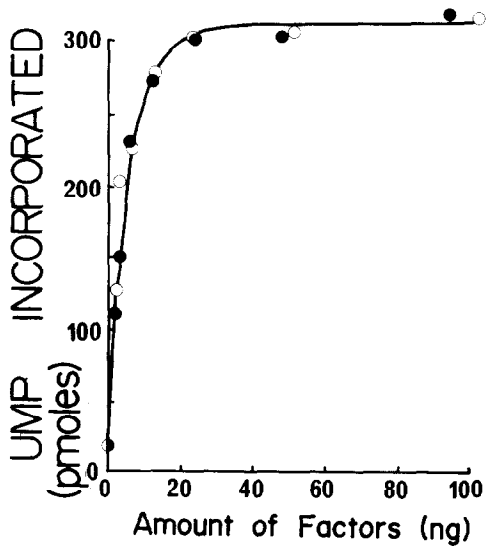

Fig.2. Dose–responses of S-I(b) (●) and S-II (○): 1 μg RNA polymerase II was assayed with increasing amounts of S-I(b) and S-II.

greatly affected [$MnCl_2$] in the reaction mixture. As shown in fig.1(B), $MnCl_2$ was essential for the expression of stimulatory activity, having a very narrow optimum concentration range of 1–2 mM; higher [$MnCl_2$] decreased the stimulatory activity markedly. Similar results on the effect of $MnCl_2$ were obtained with all 3 stimulatory proteins. It was found that $MnCl_2$ could not be replaced by $MgCl_2$ and that $MgCl_2$ was not essential for either RNA polymerase II itself or stimulation of RNA synthesis (not shown). Addition of 0.04% (v/v) of Triton X-100 was effective for stabilizing the activity of RNA polymerase II. The 3 stimulatory proteins had qualitative similar stimulatory effects on overall RNA synthesis.

The dose-dependencies of the effects of S-II and S-I(b) were tested under the optimal conditions using 1 μg RNA polymerase II. As shown in fig.2, a few nanograms of S-II or S-I(b) markedly enhanced the activity of RNA polymerase II, which reached a plateau with ~50–100 ng. The same result was obtained with S-II′. Since the M_r of RNA polymerase II was calculated to be 480 000, it is likely that the stimulatory activity reached a plateau when the molar ratio of RNA polymerase II to stimulatory protein(s) was ~1:1. Thus, probably these proteins interact with RNA polymerase II stoichiometrically, not catalytically.

The next question was what kind of RNA is synthesized in the presence of these proteins. To answer this question we employed a truncated DNA of adenovirus major late gene as template. It is known that this DNA contains only one initiation site for RNA polymerase II, and that when it is used as template for RNA synthesis in a HeLa cell lysate in which faithful transcription occurs [7], the major product is RNA 536 nucleotides long which starts from the correct initiation site [8]. If the stimulatory proteins are essential to allow RNA polymerase II to distinguish the correct initiation site and if the enhanced RNA synthesis starts from this site, the product should give a discrete band of RNA of 536 nucleotides on acrylamide gel. To examine this point, RNA synthesis was performed using purified RNA polymerase II in the presence or absence of stimulatory proteins, and the resulting RNA was extracted and analyzed. Incorporation of [α-^{32}P]UMP into the acid-insoluble fraction was enhanced ~10-fold in the presence of each stimulatory protein. An autoradiography of the RNA is shown in fig.3. No discrete band was detected when RNA synthesized by purified RNA polymerase II with

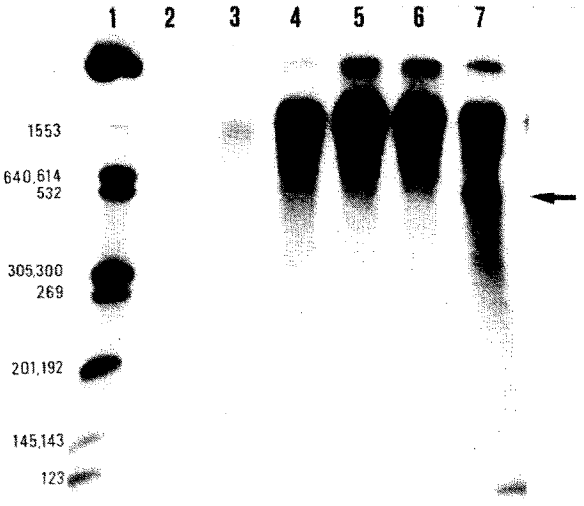

Fig.3. Analysis of RNA synthesized in vitro. RNA synthesis was performed in the presence of each stimulatory protein in reaction mixtures containing 0.5 μCi [α-^{32}P]UTP and 0.25 μg *Sma*I digest of pBR313 DNA containing *Sma*I-F of adenovirus major late gene as in [8]. RNA was extracted and electrophoresed on 5% (w/v) acrylamide–7 M urea gel as in [11], then autoradiographed: (1) terminally labeled φX 174 *Hha*I fragments (used as size markers); (2) background (without template DNA); (3) RNA synthesized by RNA polymerase II alone; (4–6) RNA synthesized in the presence of S-I(b), S-II and S-II', respectively; (7) RNA synthesized in HeLa cell lysate [7] with the same template. The arrow indicates the position of RNA having 536 nucleotides.

or without stimulatory proteins was analyzed. The RNA produced gave a smear on autoradiography and only its intensity increased on addition of a stimulatory protein. Thus random RNA synthesis occurred under these conditions and the stimulatory proteins enhanced this RNA synthesis. No significant difference was detected in the autoradiographic patterns of the products with the 3 stimulatory proteins. As a control, RNA synthesized in a HeLa cell lysate is shown in lane 7. In this case a discrete band of 536 nucleotides is clearly visible.

4. Discussion

There are several reports on the multiplicity of stimulatory proteins of RNA polymerase II [9]. This multiplicity is partly due to modification of a single protein. Then the question arose of whether there was any functional difference in the modified proteins. We found that the 3 purified stimulatory proteins, which are immunologically cross-reactive, respond in the same way to changes in $[MnCl_2]$ and $[(NH_4)_2SO_4]$. The stimulatory proteins were very susceptible to the presence of these two components in the reaction mixture, and their optimum conditions for stimulation of RNA synthesis were the same. Thus, we could not detect any qualitative difference between these proteins as far as overall stimulation of RNA synthesis in vitro was concerned.

In addition to RNA polymerase II, some protein factor(s) is (are) essential for the initiation of RNA synthesis from the correct initiation site [8,10]. We found that the 3 stimulatory proteins did not induce correct initiation in vitro with truncated adenovirus DNA containing the late promotor region as template. However, we could not exclude the possibility that these stimulatory proteins may be some of several essential proteins involved in initiation of RNA synthesis from the promoter region. Since these proteins were shown to participate in meaningful RNA synthesis in isolated nuclei [5,6], we assume that they are modulators of the rate of RNA synthesis in vivo by interacting with RNA polymerase II. The rate of RNA synthesis may differ for various genes and thus its control should be important in the regulation of eukaryotic gene expression.

Acknowledgements

We are grateful to Dr Y. Suzuki for providing truncated adenovirus major late gene containing the promoter region and HeLa cell lysate for faithful transcription. This study was supported by a grant-in-aid for Scientific Research from the Ministry of Education, Science and Culture of Japan.

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