TWO SPECIES OF RNA POLYMERASE II RELEASED FROM RAT LIVER CHROMATIN

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1. Introduction

In eukaryote nucleus, RNA polymerase exists in two forms; 'free' and 'engaged' or 'bound' forms [1]. The 'free' enzyme appears to diffuse out of the nuclei during homogenization of tissues and nuclei in isotonic sucrose [2,3]. On the contrary, the 'engaged' enzyme is bound to the chromatin in a tight transcription complex [4]. This 'bound' enzyme is complexed with the chromatin so tightly that it is usually solubilized by sonication in high ionic strength media [4]. However, since the sonication procedure has been shown to cause a drastic change in chromatin structure [5,6], the RNA polymerase solubilized by sonication may not retain such a intact entity as exists in vivo. To avoid this problem in solubilization of RNA polymerase, we employed the disruption of nuclei with micrococcal nuclease. The implication is that, upon nucleolytic cleavage of chromatin, the 'bound' RNA polymerase may be released in an intact structure and further expected to retain the factors that might otherwise be lost by sonication in high ionic strength media.

The present communication provides evidence that, in the micrococcal nuclease digest of rat liver chromatin, there exist two species of RNA polymerase II, which are distinguished from each other notably by their different transcriptional ability for chromatin template.

2. Materials and methods

2.1. *Preparation of nuclei*

Rat liver nuclei were prepared by repeated washing in isotonic sucrose as described in [7]. In this nuclear preparation, there was little 'free' RNA polymerase as reported in [3,8]. Unwashed nuclei were prepared from rat liver by the hypertonic-sucrose method of [9]. Rat liver chromatin was prepared according to method of [10] with a modification as in [11].

2.2. *Nuclease digestion*

Nuclei were suspended to give A_{260} of 40, in medium A containing 10 mM Tris–HCl (pH 7.5), 80 mM NaCl, 0.2 mM dithiothreitol, 0.1 mM phenylmethyl-sulfonyl fluoride, 12.5% glycerol and 0.1 mM $CaCl_2$. The nuclear suspension was digested with 250 units/ml of micrococcal nuclease (Worthington, PFCP) for 2.5 min at 37°C. The digestion was terminated by cooling on ice and addition of ethyleneglycol bis(β-aminoethyl ether) *N,N,N′,N′*-tetraacetic acid (EGTA) at a final concentration of 0.5 mM. After centrifugation at 3000 × *g* for 10 min at 4°C, the supernatant was assayed for the release of material absorbing at 260 nm and for acid-solubility as in [12].

2.3. *Sucrose density gradient centrifugation*

1.5 ml aliquots of the nuclease digest were layered over 7.5% to 22.5% sucrose gradients (28.5 ml) in a medium containing 10 mM Tris–HCl (pH 7.5), 80 mM NaCl, 0.2 mM dithiothreitol, 0.5 mM EDTA and 12.5% glycerol, and centrifuged for 20 h at 25 000 rev./min in Hitachi RPS-25 tubes at 4°C. The gradients were fractionated from the bottom into 20 fractions with simultaneous recording of A_{260}.

2.4. *Assay for RNA polymerase activity*

0.75 ml of each fraction was mixed with 1.5 ml of saturated $(NH_4)_2SO_4$ (adjusted to pH 7.9 with NH_4OH). After standing for 30 min at 0°C, the sus-

pension was centrifuged at 20 000 × *g* for 15 min at 4°C. The supernatant was drained by inverting the centrifuge tube for 30 min at 4°C, and the precipitate was dissolved in 0.4 ml of a medium which contained 50 mM Tris–HCl (pH 7.9), 20% glycerol, 5 mM $MgCl_2$, 0.1 mM EDTA and 0.2 mM dithiothreitol. 0.2 ml of the resulting solution was assayed for RNA polymerase activity in a mixture containing the following in a final volume of 250 μl; 50 mM Tris–HCl (pH 7.9), 1 mM dithiothreitol, 0.2 mM each of ATP, GTP and CTP, 4 mM $MgCl_2$, 2 mM $MnCl_2$, 50 mM $(NH_4)_2SO_4$ and 0.5 μCi of [5,6-^{3}H]UTP (1 Ci/mmol, New England Nuclear). As an exogenous template, native calf thymus DNA (40 μg/ml) or rat liver chromatin (150 μg DNA/ml) was used. After incubation at 30°C for 30 min, the mixtures were cooled to 0°C and 3 ml of 10% trichloroacetic acid containing 1% sodium pyrophosphate was added. The acid-insoluble material was collected on Whatman GF/F filters, washed, dried and the radioactivity counted in a toluene-based scintillator. RNA polymerase II activity was calculated from the difference between [^{3}H]UMP incorporation in the presence and absence of α-amanitin (0.5 μg/ml).

3. Results and discussion

Figure 1A shows the distribution of RNA polymerase II activity on sucrose density gradient that was released from the chromatin by digestion of rat liver nuclei. Employing native calf thymus DNA as a template, two peaks of activity were recognized. The peak just ahead of the monomeric nucleosome (peak 2) corresponds to the position of purified RNA polymerase II, as judged by its sedimentation coefficient (15–16 S) [13]. Besides this peak, there was another peak of activity (peak 1) that sedimented faster in the sucrose gradient. When a preparation of chromatin having low endogenous RNA polymerase activity [11] was used as an exogenous template, peak 1 was found to transcribe the chromatin-template much more effectively than peak 2. These results strongly indicate that there exist two forms of chromatin-bound RNA polymerase II in the cell nucleus which, upon digestion with micrococcal nuclease, are released as two distinct species (peak 1 and peak 2) separated from each other by sucrose

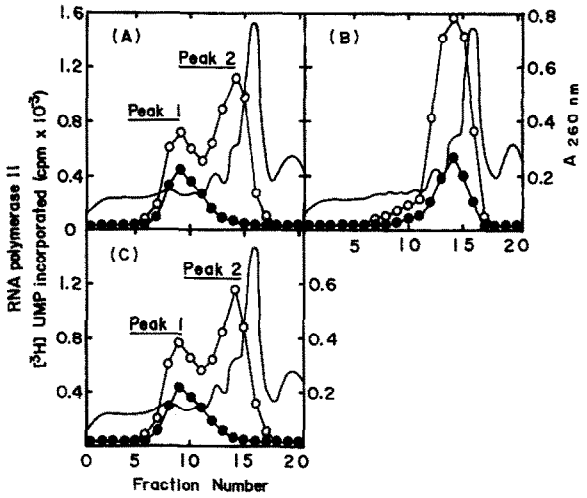

Fig.1. Distribution of RNA polymerase II activity in micrococcal nuclease digest of rat liver nuclei on sucrose density gradient. The micrococcal nuclease digest (4.5 ml) of rat liver nuclei was prepared as described in section 2 to give the acid-solubility of 6.0% and divided into three equal portions (1.5 ml). The first portion was kept aside for control (A). The second (B) and the third (C) were made 500 mM with 5 M NaCl. Then, mixture (C) was dialyzed against a large volume of a buffer containing 10 mM Tris–HCl (pH 7.5), 80 mM NaCl, 0.2 mM dithiothreitol, 0.5 mM EDTA and 12.5% glycerol, at 4°C for 2 h. All preparations (A, B and C) were fractionated on sucrose density gradients. Each fraction obtained was assayed for RNA polymerase II activity, using calf thymus DNA (○——○) or rat liver chromatin (●——●) as an exogenous template. In the latter case, endogenous activity (49 cpm) in the chromatin-template was subtracted as the blank value. In (A), with DNA-template in the presence of α-amanitin (0.5 μg/ml), the [^{3}H]UMP incorporations of peak 1 (fraction 9) and peak 2 (fraction 14) were 20 and 246 cpm, respectively.

density gradient centrifugation. It is probable that these two species of RNA polymerase II molecules might have different physiological functions. In fact, in a separate experiment employing DEAE–Sephadex chromatography, we could demonstrate that peak 1 but not peak 2 contained a factor which enhanced the transcription of chromatin-template by purified RNA polymerase II (unpublished data). Peak 1 as well as peak 2 were found also in the nuclear digest of unwashed nuclei, indicating that peak 1 polymerase was not generated during the repeated and lengthy washing of the nuclei.

When the micrococcal nuclease digest was brought to an NaCl concentration of 500 mM prior to sucrose density gradient centrifugation, peak 1 activity disappeared, and the enzyme activity for chromatin-transcription was recovered at the position of peak 2 together with a concomitant increase in the enzyme activity for the DNA-template (fig.1B). Furthermore, when the sample exposed to the high salt concentration was dialyzed back to a lower ionic strength (80 mM NaCl), the enzyme activity of peak 1 resumed its original position as seen in fig.1C. From these results, we assume that peak 1 is a complex which, at 500 mM NaCl, could be dissociated into some component and the enzyme carrying the factor required for the chromatin-transcription. As to the component, we have not enough information at the moment, but the following finding indicates that a certain kind of RNA molecule is comprised in peak 1. Namely, when the micrococcal nuclease digest of nuclei was subjected to a combined RNase A and T_1 digestion (50 μg/ml each) at 37°C for 10 min prior to sucrose density sedimentation, peak 1 disappeared completely, and the activity for chromatin-transcription was recovered in the peak 2 region as in the case of the high ionic strength experiment (fig.1B). As relatively high concentrations of RNase A and T_1 were required to dissociate peak 1, it seemed that the RNA molecule was protected from the action of RNase in the peak 1 complex.

To examine whether the release of two species of RNA polymerase II was peculiar to micrococcal nuclease, a different type of nuclease was employed. When DNase I was used to solubilize RNA polymerase, both peak 1 and peak 2 were also observed.

Recent studies have demonstrated that micrococcal nuclease preferentially excises nucleosomes from the actively transcribed chromatin regions [14,15], and that, by a mild micrococcal nuclease digestion, the major activity of template-engaged RNA polymerase II is easily recovered with 'polynucleosomes', aggregates of 6 to 30 covalently linked nucleosomes [16]. Hence, it might be interesting to examine the localization of the above mentioned two species of RNA polymerase II molecules. When the nuclei were digested under mild conditions and the resulting supernatant and pellet (fraction S and P) further treated with DNase I, most of peak 2 activity was found in fraction S, whereas peak 1 activity was exclusively in fraction P (fig.2A,B). These results,

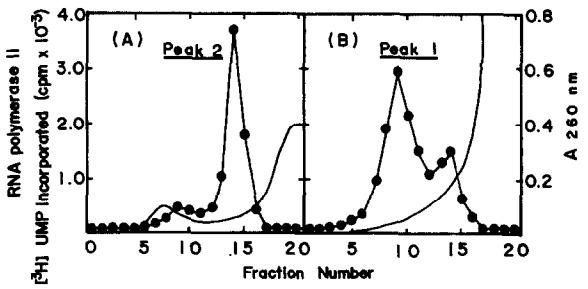

Fig.2. Differential solubilization of two species of chromatin-bound RNA polymerase II. Rat liver nuclei suspended in 4 ml of medium A was incubated at 30°C for 1.5 min with 1.5 units/ml of micrococcal nuclease. Digestion was stopped by the addition of 2 mM EGTA (final concentration). The suspension was centrifuged at 1000 × g for 10 min to separate pellet (fraction P) and supernatant (fraction S). Under these conditions, 15% of the material absorbing at 260 nm was liberated in the supernatant. The pellet was then suspended in 2 ml of medium A. Aliquots (2 ml) of fraction P and S were made 0.2 mM with 1 M $MgCl_2$, and incubated at 37°C for 2.5 min with 0.5 $\mu g/(A_{260}$ of fraction P and S) of DNase I (Sigma, DN-CL). The reaction was terminated by the addition of 1 mM EDTA (final concentration). After centrifugation at 3000 × g for 10 min, the supernatants were fractionated on sucrose density gradients, and assayed for RNA polymerase II activity using DNA as a template. (A) fraction S; (B) fraction P.

together with the facts reported by others [14–16], may suggest that peak 2 comprises a species of RNA polymerase II responsible for transcription in the cell nucleus. Although the functional significance of RNA polymerase II in peak 1 is not clear at the present time, it would be worth emphasizing that the enzyme is associated with a factor responsible for chromatin-transcription and probably with a certain kind of RNA molecule.

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