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DNA-BINDING PROPERTY OF FREE AND CHROMATIN-BOUND RNA POLYMERASE II

Analysis by DNA–Sephadex G-25 chromatography

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1. Introduction

At present, the biological determinants that localize RNA polymerase molecules in the cell nucleus are not fully elucidated. Since it seemed possible that the enzyme's DNA-binding property might be one of the determinants, we examined DNA-binding activity of the extracted RNA polymerase II by affinity chromatography of native DNA that was covalently coupled to Sephadex G-25 [1,2].

We found that:

(1) The free RNA polymerase II of the nuclear soluble fraction revealed a very low-binding activity as compared with the enzyme extracted from the chromatin-bound fraction;

(2) The nuclear soluble RNA polymerases once purified by DEAE-Sephadex A-25 chromatography, acquired DNA-binding activities;

(3) The weak binding of the nuclear soluble enzymes was due to association of heat-stable protein(s).

2. Materials and methods

2.1. *Isolation of nuclei and fractionation of nuclear RNA polymerase II*

Livers of male Sprague-Dawley rats were minced with scissors in 2 vol. 2.1 M sucrose/3 mM $MgCl_2$, and homogenized with 10 strokes in a Potter-type homogenizer. The homogenate was filtered through 4 layers of gauze, diluted with 8 vol. same medium, and centrifuged at 28 000 rev./min for 60 min in a Hitachi RP-30 rotor. The enzymes of the nuclear soluble fraction was extracted from the pelleted nuclei as in [3], and concentrated by ultrafiltration with an Amicon XM-50 membrane. After the same extraction was repeated twice more, the nuclear pellet was homogenized by hand with a Potter-type homogenizer in 0.5 M NaCl/buffer A (10 mM Tris–HCl (pH 7.9)/ 12.5% (v/v) glycerol/0.5 mM EDTA/0.1 mM dithiothreitol), and centrifuged. The supernatant 'loosely chromatin-bound fraction' was concentrated as above. After washing the chromatin pellet in the 0.5 M NaCl-containing medium once more, the enzyme which remained bound to the chromatin was solubilized by nuclease digestion (section 2.2). This fraction was designated as the residual chromatin fraction.

2.2. *Partial purification of RNA polymerase II for DNA-affinity chromatography*

The nucleic acids in the enzyme fractions were removed with nucleases under a modified condition of [4]. The nuclear fractions starting from 40 g livers were digested with 500 μg DNase I (Sigma, bovine pancreas, 2155 Kunits/mg) and 250 μg RNase A (Sigma, bovine pancreas, type I-A) in 10 ml 10 mM $MgCl_2$/0.5 M NaCl/buffer A. For digestion of the residual chromatin fraction, DNase I was added at 100 μg/ml. After incubation at 30°C for 5 min, each digest was precipitated with 2 vol. saturated $(NH_4)_2SO_4$ (pH 7.9). The precipitates collected by centrifugation were dissolved in 4 ml 0.5 M NaCl/buffer A, applied to a column of Bio-Gel A-5m (1.4 × 95 cm) and eluted with the same buffer at 6 ml/h (2.2 ml/fraction). The enzymes in the region of 1.3–2.0 V_e/V_o (ratio of the elution volume to void volume) were pooled, and precipitated with 2 vol. saturated $(NH_4)_2SO_4$ (pH 7.9). The collected precipitates were dissolved in buffer A

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and dialyzed extensively against 5 mM NaCl/buffer A. When necessary, the enzymes were further purified by DEAE-Sephadex column chromatography as in [5], followed by concentration and dialysis as in a similar manner for the Bio-gel enzymes. These samples were employed for the following DNA–affinity chromatography.

2.2. *DNA–Affinity chromatography*

DNA-binding property was studied by use of DNA–Sephadex columns. Calf thymus double-stranded DNA (Sigma, highly polymerized) was coupled to Sephadex G-25 (fine) as in [1,2]. The samples (1.0–1.5 ml) were applied to affinity columns (1.4 X 6 cm), which were pre-equilibrated with 5 mM NaCl/buffer A, and eluted with a linear NaCl gradient (5 mM–1.0 M) at 3 ml/h. The chromatography was performed at a DNA–ligand concentration enough for the applied proteins as in [1,2]. The DNA content of affinity resin was determined by A_{260} which was released by extensive digestion with DNase I, assuming that 1 mg/ml has an A_{260} of 30. The salt concentration of each fraction was determined by conductimetry. Protein content was estimated from A_{260} and A_{280} according to the equation in [6]. RNA polymerase II assay was done as in [7].

3. Results and discussion

3.1. *DNA–affinity column chromatography of the partially purified RNA polymerase II*

The RNA polymerase II of rat liver nuclei was fractionated into 3 populations; the nuclear soluble, loosely chromatin-bound (which was extracted with 0.5 M NaCl), and tightly chromatin-bound (which was solubilized from the residual chromatin by digestion with DNase I in 0.5 M NaCl) fractions. Since enzymes are present in the nucleus interacting with chromatin or DNA with different affinities, it seemed interesting to examine whether there was any difference in their binding affinity for DNA. For this purpose, the 3 enzyme preparations were subjected to DNA–affinity column chromatography. The enzyme preparations were pre-treated with DNase I and RNase A to remove nucleic acids that would otherwise compete with DNA in the affinity column, and further purified on a Bio-Gel A-5m column in the presence of 0.5 M NaCl. The enzymes were then applied to the columns of DNA–Sephadex at 5 mM NaCl in buffer A, and eluted with 5 mM–1.0 M NaCl. The main enzyme activity in the nuclear soluble fraction was recovered at the flow through fractions with an accompanying small activity eluting at ~30 mM NaCl (fig.1a). In contrast to this, the enzyme that was solubilized from the chromatin with 0.5 M NaCl was much adsorbed to the DNA column, and eluted as a broad peak around 100 mM NaCl (fig.1b). Furthermore, the enzyme of the residual chromatin fraction exhibited a prominent DNA-binding peak at 100 mM NaCl with a trailing shoulder at 200 mM NaCl (fig.1c). The percent enzyme activity eluting above 50 mM NaCl was 74%, 52% and 13% with the residual chro-

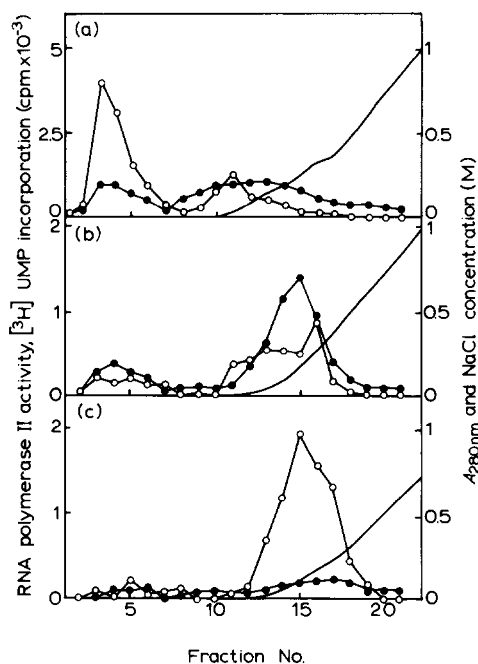

Fig.1. DNA-affinity chromatography of partially purified RNA polymerase II. The nuclear soluble (a), 0.5 M NaCl soluble (b) and residual chromatin (c) fractions were purified partially on a column of Bio-gel A-5m and applied to parallel columns of DNA–Sephadex G-25 as in section 2. The adsorbed proteins were eluted with a linear NaCl gradient from 5 mM–1.0 M. The chromatographic conditions were at a ligand DNA/applied protein (w/w) ratio of 3.2 for the nuclear soluble fraction, 4.0 for the 0.5 M NaCl soluble chromatin fraction and 5.8 for the residual chromatin fraction, respectively. Each column fraction was assayed for RNA polymerase II activity (○), and monitored for the optical absorbance at 280 nm (●) and NaCl concentration (——). Note that most of the nuclear soluble enzymes were not adsorbed on the column. In the case of these nuclear soluble enzymes, the elution profile was very similar to (a) even on a column that contained 5- or 10-fold more DNA.

matin, 0.5 M NaCl soluble and nuclear soluble fractions, respectively. These results indicate that there is a parallel relationship between the enzymes' extractability from the nucleus and their DNA-binding property: the chromatin-bound enzyme possesses high DNA-binding activities, whereas the nuclear soluble enzyme a very low activity.

3.2. *Occurrence of a DNA-binding inhibitor in the nuclear soluble fraction*

As revealed in section 3.1, free RNA polymerase II in the nuclear soluble fraction did not bind to DNA significantly. The result was rather unexpected because RNA polymerase has been characterized as a typical DNA-binding protein [8–10]. The different DNA-binding activities of the 3 enzyme fractions observed above suggest that the molecular entities of the isolated RNA polymerases are different according to its localization in the cell nucleus. In fact, as shown below, the low binding activity of the nuclear soluble fraction was found attributable to a dissociable substance which inhibits DNA-binding of the enzyme: when the enzymes were purified on a column of DEAE-Sephadex A-25, they became to exhibit a significant DNA-binding activity (fig.2a). Moreover, when each DEAE-Sephadex A-25 fraction was subsequently tested for the inhibitor activity by adding back to the enzyme fraction, the pass-through fractions (50 mM $(NH_4)_2SO_4$) were found to contain the DNA-binding repressing activity (fig.2b). The results indicate that the enzyme in the nuclear soluble fraction was composed of a RNA polymerase, which itself had a DNA-binding activity, and a dissociable substance that acted as an inhibitor against DNA-binding.

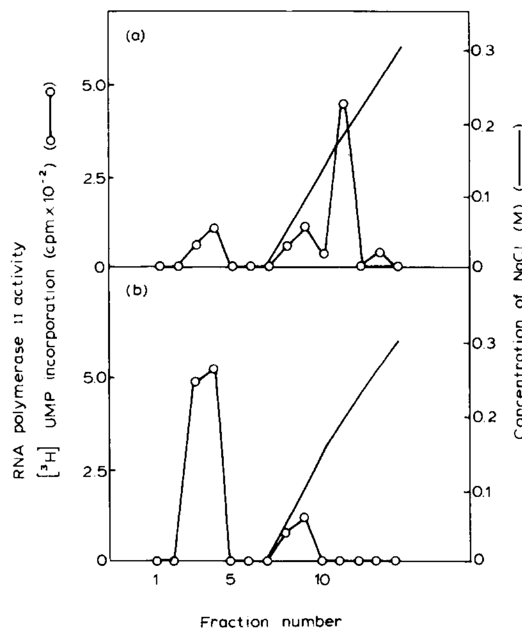

Fig.2. Occurrence of a DNA-binding inhibitor in the nuclear soluble fraction. The nuclear soluble enzymes purified partially by a column of Bio-gel A-5m were further purified by DEAE-Sephadex A-25 chromatography: (a) DEAE-Sephadex RNA polymerase II (0.31 mg protein) was then analyzed on a column of DNA–Sephadex (1.4 × 7 cm, 5.0 mg DNA) under the elution condition in section 2; (b) purified enzyme (0.31 mg protein) and the DEAE-Sephadex A-25 pass-through fraction (0.43 mg protein) were mixed, stood for 30 min at 0°C, then applied to a DNA column (1.4 × 7 cm, 5.0 mg DNA).

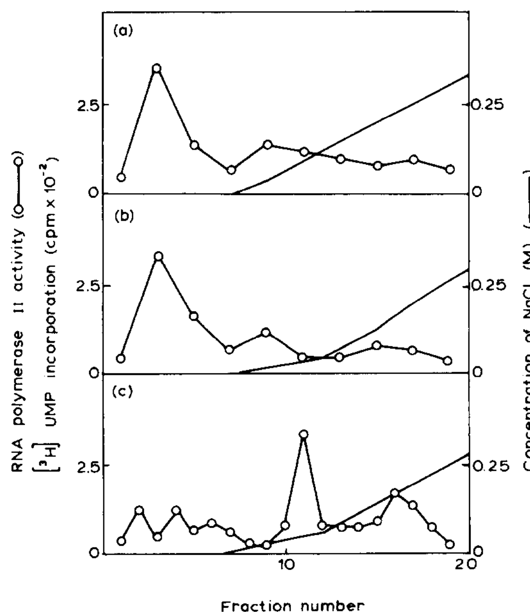

Fig.3. Characterization of the DNA-binding inhibitor. The nuclear soluble fraction was partially purified on a column of Bio-gel as in section 2 and fractionated by DEAE-Sephadex chromatography into the inhibitor and enzyme fractions. The inhibitor which was recovered at the flow-through, was heat-treated at 86°C for 5 min, or incubated with trypsin (100 μg/ml) at 30°C for 15 min followed by addition of soya bean trypsin inhibitor (100 μg/ml). The preparations (0.43 mg protein) were then mixed with the enzyme (0.31 mg protein) that was purified by DEAE-Sephadex, dialyzed against buffer A–5 mM NaCl, and analyzed on DNA–affinity columns as shown in fig.1. For collection of the effluents of the trypsin-treated sample, 20 μg trypsin inhibitor was included in each collection tube. The inhibitor received: (a) no treatment; (b) heat-exposure; (c) trypsin digestion.

Some properties of the inhibitor were studied. After treatment at 85°C for 5 min, the inhibitor still retained 95% of the original activity, indicating its stability against heat (fig.3a,b). When the inhibitor was digested with hydrolyzing enzymes (DNase I, RNase A and trypsin), only trypsin destroyed its activity (fig.3c). In addition, the inhibitor was characteristically found in the nuclear soluble fraction, and not detectable in the chromatin fraction. Furthermore, even when the chromatography was carried out on a column that contained as much as 5–10-fold more DNA, the elution of the enzyme (nuclear soluble fraction from the Bio-Gel column) was very similar to that obtained under a standard condition (fig.1a). This result supports the notion that the inhibitor would exist in association with the enzymes, and not in a free form that had a DNA-binding activity, which would occupy the DNA hence reduce the available DNA on the column for the enzyme.

We do not know at present the significance of the DNA-binding inhibition by the heat-stable protein(s). However, the inhibitor may be involved in the regulation of the number of the enzyme which is used for the nuclear RNA synthesis. The DNA-binding demonstrated in the present chromatography was not so strong (<0.5 M NaCl) as expected for those which are engaged in RNA chain synthesis, in fact, the engaged enzyme in the transcriptional complex does not dissociate from DNA template even at 2 M NaCl [11,12]. Since the binding observed in this study occurred: (i) at <500 mM NaCl; (ii) at 0–4°C; and (iii) with no ribonucleotide triphosphates; it seems not to be the binding involved in the initiation or elongation of RNA synthesis. Rather the observed binding is likely to occur in the pre-initiation step such as promoter-site recognition by the enzyme [13].

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