LOOSELY BOUND CHROMATIN COMPONENTS ARE RESPONSIBLE FOR DIFFERENT ELECTROPHORETIC MOBILITY OF THE CHROMATIN COMPLEXED WITH RNA POLYMERASE II

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1. Introduction

Eukaryotic chromatin is organized in a repeating subunit structure, the nucleosome, which is basically composed of histones (H_1, H_{2A}, H_{2B}, H_3 and H_4) and DNA [1]. In the transcriptionally active chromatin, nucleosome-like structures are also present, although they differ from those of the bulk chromatin [2]. The altered structure appears to originate from covalent modifications and depletion of particular nucleosomal constituents (e.g., acetylation of histones, lack of H_1 histone), and from the association of other components (e.g., high-mobility-group proteins) [3].

The transcriptionally active chromatin has been most successfully characterized by hybridization of the isolated DNA with cDNA of poly(A)-containing mRNA [4]. The engaged RNA polymerase molecules are bound to the chromatin in a tight transcription complex as revealed by biochemical studies [5], and the active chromatin, which carries the bound RNA polymerases in the characteristic transcription unit, is distinguished by electron microscopy [6,7].

Here we show that the chromatin carrying the bound RNA polymerase II molecule has a different electrophoretic mobility from the bulk chromatin.

2. Materials and methods

2.1. *Digestion and fractionation of nuclei*

Washed rat liver nuclei, retaining little free RNA polymerase activity, were prepared as in [8]. The nuclei were suspended to give A_{260} of 75 in medium A [10 mM Tris–HCl (pH 7.5), 0.2 mM dithiothreitol (DTT), 0.2 mM phenylmethylsulfonyl fluoride (PMSF), 12.5% glycerol containing 3 mM $MgCl_2$ and 0.1 mM $CaCl_2$. The suspension was warmed to 30°C for 30 min and digested with micrococcal nuclease (300 U/ml; Worthington) for the time indicated in the text. The digestion was terminated by cooling on ice and addition of ethyleneglycol bis(β-aminoethyl ether) *N,N,N',N'*-tetraacetic acid to 0.5 mM final conc. After centrifugation for 10 min at 10 000 × *g*, the resulting first supernatant fraction (S_1) was saved, and the pelleted nuclei were lysed by suspension in medium A containing 0.5 mM EDTA. The suspension was centrifuged at 15 000 × *g* for 10 min to yield the second supernatant fraction (S_2).

For extraction of the loosely bound chromatin components, the S_2 fraction was made 0.35 M with 5 M NaCl, and the chromatin was pelleted through a 15% sucrose pad containing 0.35 M NaCl in medium A by centrifugation at 100 000 × *g* for 15 h in Hitachi RP 65 rotor.

2.2. *Fractionation of the nuclear digest*

2.2.1. Column electrophoresis

Electrophoresis on a Sephadex G-10 column (2.5 × 60 cm) was carried out using the Uniphor column electrophoresis system (LKB, Sweden). The sample (3–5 ml) was loaded onto the column equilibrated with 10 mM Tris–acetate (pH 8.0), 10 mM sodium acetate, 0.5 mM EDTA, 0.2 mM DTT, 0.2 mM PMSF and 12.5% glycerol (column buffer). The lower anode and upper cathode reservoirs, which contained the same buffer, were separated from the column by a semipermeable membrane (LKB, cot.

no. 7790-24). Electrophoresis was carried out at 10 mA for 17 h with cooling of the whole system at 2°C. After electrophoresis, the sample was collected from the column with the column buffer, monitoring A_{260}.

2.2.2. Sucrose density gradient centrifugation

The sample (1.5–2.5 ml) was layered, in a Hitachi RPS 27-2 tube, on a 7.5–22.5% linear sucrose gradient (30 ml) with a 60% sucrose cushion (3 ml) in medium A containing 10 mM NaCl and 0.5 mM EDTA.

2.3. *Assay for RNA polymerase II activity dependent on an exogenous DNA template*

The activity was measured after recovering the enzyme by ammonium sulphate precipitation as in [9]. Under this condition, chromatin-bound RNA polymerase II activity, which was measured as in section 2.4., was hardly detected.

2.4. *Assay for chromatin-bound RNA polymerase II activity*

The enzyme activity was also measured without ammonium sulphate precipitation. The assay condition was the same as above, except that the exogenous DNA template was omitted and the ammonium sulphate was raised to 200 mM which permitted only the elongation of RNA chains [10,11]. RNA polymerase II activity detected under this condition was considered to originate from the enzyme complexed with chromatin, hence to represent the chromatin carrying the bound enzyme.

3. Results and discussion

We have reported that the digestion of rat liver nuclei with micrococcal nuclease liberated the engaged RNA polymerase II in two forms that could be distinguished from each other in several respects: one form behaved like a free enzyme, the other like an enzyme complexed with some RNA and mono- or dinucleosome enriched with non-histone proteins [9,12]. In these experiments, the nuclei were digested to a rather extensive degree, and the two forms could be separated by various procedures. The enzymes of both forms, however, were detected as free enzyme that could utilize the exogenous DNA template, when they were recovered by ammonium sulphate precipitation [9,12]. Here, we attempted to solubilize the engaged RNA polymerase II in rather large chromatin fragments. This aim was attained by mild micrococcal nuclease digestion of the nuclei, especially in a medium containing 3 mM $MgCl_2$.

Rat liver nuclei were digested at 30°C with micrococcal nuclease in medium A containing 0.1 mM $CaCl_2$ and 3 mM $MgCl_2$, and the nuclear digest was fractionated into the first (S_1) and the second (S_2) supernatants, as in section 2. As seen in the time course of digestion (fig.1A), the chromatin released into fraction S_2 increased gradually with a concomitant linear increase of acid-soluble material. Electrophoretic analysis of the DNA in fraction S_2 revealed a series of DNA fragments that were multiples of a unit of ~200 basepairs in length*. The DNA in fraction S_1, however, revealed a single band corresponding to monomer length, which increased in size as a

* The DNA size of fraction S_2 (7.5 min digestion) was 200–6000 basepairs

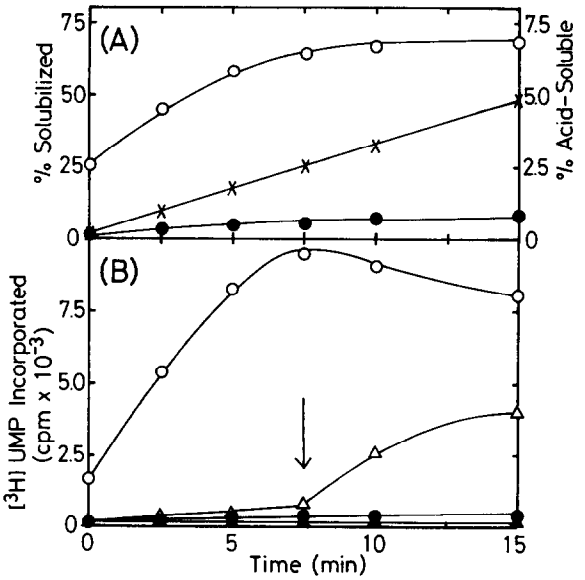

Fig.1. Time course of micrococcal nuclease digestion. Nuclei from rat liver were digested with 300 U micrococcal nuclease/ml. At the indicated times, aliquots of the nuclear suspension were withdrawn and fractionated as in section 2. (A) Digestion was monitored by the $\%A_{260}$ released into fraction S_1 (•) or S_2 (○); and by the $\%A_{260}$ rendered acid-soluble (×). (B) Each fraction was assayed for RNA polymerase II activity dependent on the exogenous DNA template: S_1 (▲), S_2 (△); and for chromatin-bound RNA polymerase II activity: S_1 (•), S_2 (○). The arrow indicates the digestion time (7.5 min) used for the experiment shown in fig.2–4.

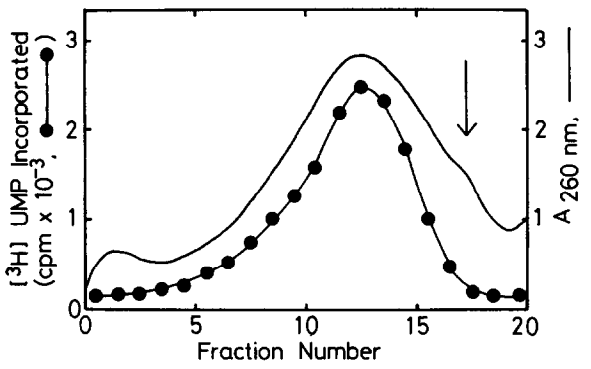

Fig.2. Distribution on a sucrose density gradient of the chromatin-bound RNA polymerase II activity in fraction S_2. Fraction S_2 (2.5 ml, 7.5 min digestion) was centrifuged at 18 000 rev./min for 17 h at 4°C, as in section 2. The gradient was fractionated from the bottom into 20 fractions with simultaneous recording of A_{260}. Each fraction was assayed for chromatin-bound RNA polymerase II activity. The arrow indicates the position of nucleosome monomer.

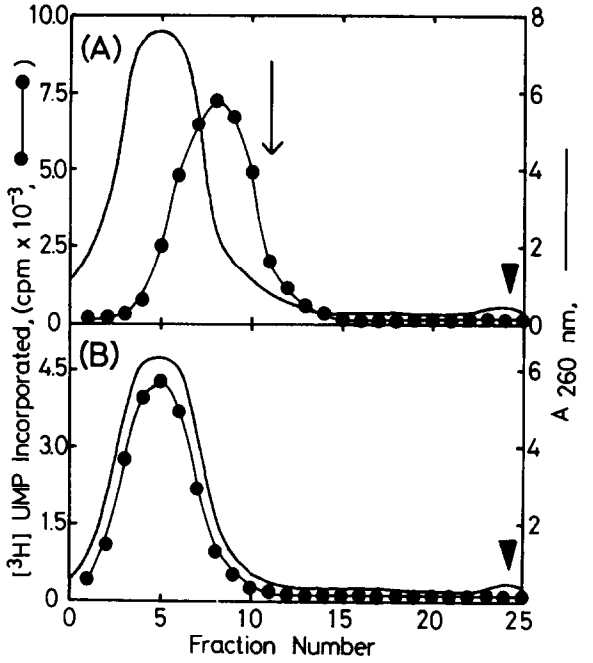

Fig.3. Sephadex G-10 column electrophoresis of fraction S_2: bulk and RNA polymerase II-containing chromatins. Fraction S_2 (40 ml) prepared as shown in fig.1, was concentrated to 10 ml ultrafiltration using an Amicon XM-300 membrane and divided into two portions. One portion was used as control (A). The other (B) was treated with 0.35 M NaCl and centrifuged through a 15% sucrose pad as in section 2. The pelleted chromatin was thoroughly resuspended in 5 ml column buffer. Both preparations (A,B) were electrophoresed on a Sephadex G-10 column and fractions were assayed for chromatin-bound RNA polymerase II activity. The arrow indicates the migration position of purified RNA polymerase II and the arrow heads the origin of electrophoresis.

function of digestion time. The electrophoretic profile of the protein from fraction S_1 revealed the presence of the core histones and a set of proteins characteristic of the ribonucleoprotein (RNP) particles [13], and the depletion of H_1 histone (not shown).

When assayed for RNA polymerase II activity (fig.1B), fraction S_1 did not exhibit any significant activity toward both the exogenous DNA and the endogenous chromatin templates. In contrast, fraction S_2 contained predominantly the chromatin-bound enzyme activity, which increased up to a point where ~2.5% of the total DNA was rendered acid-soluble (7.5 min). On the other hand, up to 7.5 min digestion, free enzyme was only slightly detected in fraction S_2. Thereafter, the enzyme abruptly increased with a concomitant decrease in the bound enzyme. These results indicate that, in the presence of Mg^{2+} (3 mM), the engaged RNA polymerase II is preferentially liberated in the chromatin-bound state by mild micrococcal nuclease digestion.

When fraction S_2 was analyzed on a sucrose density gradient (fig.2), the chromatin-bound RNA polymerase II activity* was distributed together with the bulk chromatin fragment. It is clear that chromatin fragments both with and without RNA polymerase II molecules are heterogenous in size, and have very similar hydrodynamic properties. However, they could be separated from each other by electrophoresis on a Sephadex G-10 column. As seen in fig.3A, the chromatin fragment complexed with RNA polymerase II migrated more slowly than bulk chromatin, but faster than free RNA polymerase II. In addition, the chromatin fragment with or without RNA polymerase II molecules migrated in a well-confined peak; indicating that each chromatin fragment had a characteristic electric surface charge/unit mass.

The electrophoresis of fraction S_2 was further performed after extraction of loosely-bound chromatin components with 0.35 M NaCl. It was found that, in contrast to the unextracted sample whose RNA polymerase II activity migrated more slowly than the

* The redigestion of fraction S_2 with DNase I gave exclusively the peak 2 enzyme (designated in [9]) which seemed to originate from the active chromatin [12]

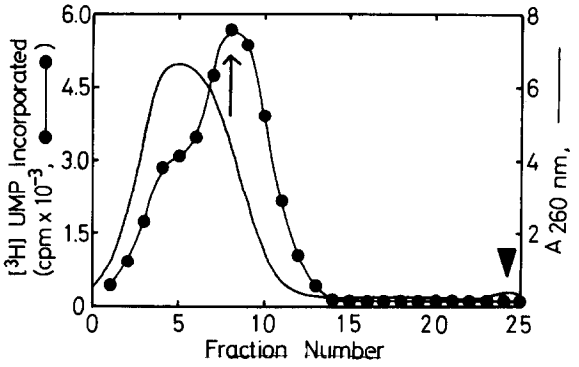

Fig.4. Reconstitution of the RNA polymerase II-containing chromatin. Fraction S_2 (20 ml) was concentrated to 5 ml and centrifuged through a 15% sucrose pad as in section 2. The pelleted chromatin representing 95% of recovered A_{260}, was resuspended in 2.5 ml 0.35 M NaCl in medium A. The supernatant loosely-bound fraction which contained little chromatin-bound RNA polymerase II activity, was concentrated to 2.5 ml by ultrafiltration using an Amicon PM-10 membrane. The salt-washed chromatin was mixed with the loosely-bound fraction, and dialyzed overnight against column buffer. The sample was electrophoresed and fractions were assayed for chromatin-bound RNA polymerase II activity. The incompleteness of reconstitution seemed to originate from inactivation and/or degradation of some component(s) prior to the dialysis, since when the fraction S_2 made 0.35 M NaCl was treated similarly without the separation of the loosely-bound fraction, all of the enzyme activity migrated at its original position (indicated by arrow).

bulk chromatin fragment (fig.3A), the enzyme activity of the salt-treated sample co-migrated with the bulk chromatin (fig.3B). Furthermore, when the salt-washed chromatin was reconstituted with the loosely bound fraction, most of the chromatin-bound RNA polymerase II activity resumed its original position (fig.4). However, the bulk chromatin did not exhibit detectable changes in its electrophoretic mobility after the salt treatment. These results indicate that, the components which can be removed by 0.35 M NaCl extraction are responsible for the altered electrophoretic mobility of the RNA polymerase II-containing chromatin.

These findings are interpreted to mean that the loosely bound chromatin components are the constituents of the chromatin which carries the bound RNA polymerase II molecules, and suggest that they take part in the maintenance of the altered chromatin structure which is possibly required for the enzyme to be engaged in RNA synthesis. In this respect, it should be emphasized that 0.35 M NaCl-soluble non-histone chromatin proteins have been reported to be involved in gene regulation or in the active chromatin structure [14,15]. It is possible to suppose that the nascent RNP fibrils attached to the transcribing RNA polymerase II molecules are the effective component that change the electrophoretic mobility. However, this is unlikely since the RNP particles themselves migrate approximately to the same position as bulk chromatin on electrophoresis (not shown).

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