LOCATION OF ENDOGENOUS RNA POLYMERASE B IN A SUB-FRACTION OF RAT LIVER CHROMATIN

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1. Introduction

Sheared chromatin can be fractionated in two main components, differing by their aggregation properties [1–3]. It has been suggested that these fractions represent eu- and heterochromatin, largely on the basis of their template activity in vitro. However, shearing seems to alter the chromatin native characteristics [4,5]. On the other hand, regulation of transcription of eukaryotic DNA is not easily explained by the degree of chromatin accessibility or compaction [6–8]. Firstly, DNA is not necessarily repressed by histone binding [9], and some repressed DNA sequences are accessible to DNase or polylysine [6,7]. Secondly, fl histone, the role of which is especially important in modulating accessibility of DNA [6] and the conformation of chromatin in a compact or loose structure [10,11], seems to control the level of DNA replication, rather than transcription [10–12]. Thirdly, condensation of chromatin is a function of the content of repetitive DNA sequences, perhaps because of interactions between proteins specifically bound to the same DNA sequences: therefore, for some authors, heterochromatin is a minor fraction which comprises satellite DNA [13]. The extended euchromatic fraction, containing the bulk of DNA, does thus comprise repressed as well as derepressed sequences. One can therefore ask whether the 'extended fraction' obtained by shearing chromatin really contains the derepressed DNA sequences. Its greater template capacity for exogenous RNA polymerase in vitro could be attributed to the loss of fl histone [14,15], which could result from the shearing process, since it is known that fl histone can be rapidly displaced from its binding sites on DNA [6,16–18].

We report here that the loose chromatin fraction released by shearing is enriched in endogenous RNA polymerase B. This means that this fraction might be active for transcription in situ.

2. Material and methods

Rat liver chromatin was extracted and purified as in [6]. Its concentration was always expressed as μg of DNA.

Chromatin fractionation was achieved by shearing at 1250 psi in a French pressure cell, followed by elution at 1 mg/ml in a 100–200 mesh BioGel A50m column (50 × 1.5 cm) equilibrated in 0.1 M $MgCl_2$, 0.15 M KCl, 5 mM $NaHSO_3$, 0.1 mM dithiothreitol and 10 mM Tris–HCl (pH 8); 1-ml fractions were collected at a rate of 30 ml/h.

Binding of *O*-[^{3}H]methyl-demethyl-γ-amanitin (0.05 μCi/μg) to chromatin was performed before or after shearing, for 1 h at 0°C in 2 mM $MnCl_2$, 1 mM dithiothreitol, 0.2 M $(NH_4)_2SO_4$ and 50 mM Tris (pH 8). Chromatin was then precipitated by a two-fold dilution in 5 mM $MgCl_2$, 10 mM Tris (pH 8), and resuspended in the elution buffer for BioGel A50m chromatography.

Endogenous chromatin RNA polymerase activity was assayed by an 1 h-incubation in 0.2 M $(NH_4)_2SO_4$, 2 mM $MnCl_2$, 5 mM dithiothreitol, 0.1 mM ATP, 0.1 mM GTP, 0.5 μM [^{3}H] UTP (40 Ci/mM), 0.5 μM [^{3}H] CTP (20 Ci/mM) and 40 mM Tris (pH 8). When incubation was performed before fractionation,

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chromatin was precipitated after incubation by a twofold dilution in 5 mM $MgCl_2$, 10 mM Tris (pH 8), washed twice in this medium and resuspended in the elution buffer.

Nucleotide incorporation was measured by precipitation of RNA in 10% cold trichloroacetic acid with 100 μg of yeast RNA as carrier. Acid-insoluble material was collected and washed on GF/C (Whatman) filters.

3. Results

Rat liver chromatin is able to bind *O*-[^{3}H]methyl-demethyl-γ-amanitin, before as well as after shearing (fig.1). When chromatin is fractionated by elution on a BioGel A50m column, amanitin seems to be associated with the excluded minor fraction, which represents 10–20% of total DNA. This suggests that the first DNA fraction is enriched in RNA polymerase B-associated sequences, since the binding of amanitin to this polymerase is very specific and stoichiometric [19]. The amount of RNA polymerase B was calculated to be about 3 to $6 \cdot 10^{10}$ molecules per μg of DNA. This is in agreement with the results of Cochet-Meilhac et al. [19], who estimate that rat liver nuclei contain $7.5 \cdot 10^{10}$ molecules of RNA polymerase B per μg of DNA, since it is known [20] that as much as 50% of total nuclear RNA polymerase may exist in the form of a free enzyme, not associated with chromatin. Therefore it seems that amanitin saturates chromatin RNA polymerase, and that most of active RNA polymerase B is conserved in chromatin during the isolation procedure.

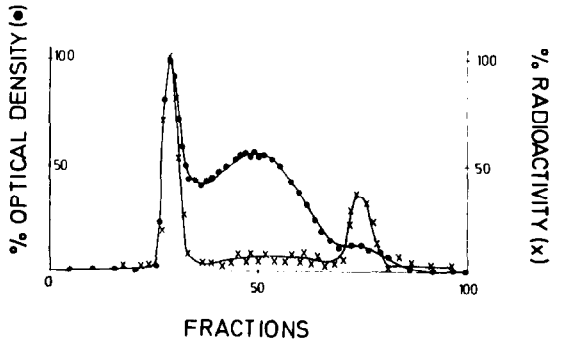


Fig.1. Binding of *O*-[^{3}H]methyl-demethyl-γ-amanitin to chromatin. Chromatin (250 μg) was incubated with an excess (0.1 μg) of [^{3}H]amanitin as described in Methods, and the bulk of non-reacted amanitin was eliminated by chromatin centrifugation (10 000 *g*; 10 min). Chromatin was then resuspended in 0.1 M $MgCl_2$, 0.15 M KCl, 5 mM $NaHSO_3$, 0.1 mM dithiothreitol and 10 mM Tris (pH 8), and eluted on a BioGel A50m column (50 × 1.5 cm); fractions of 1 ml were collected. Results are expressed as percentages of the OD_{260} nm (•) or of the radioactivity (×), taking as 100% the maximal OD or radioactivity of the first peak. Maximal OD was 0.31; 600 cpm were associated with the first peak. The background values (30 cpm/fraction) were substracted. Identical results were obtained by labelling chromatin before or after shearing.

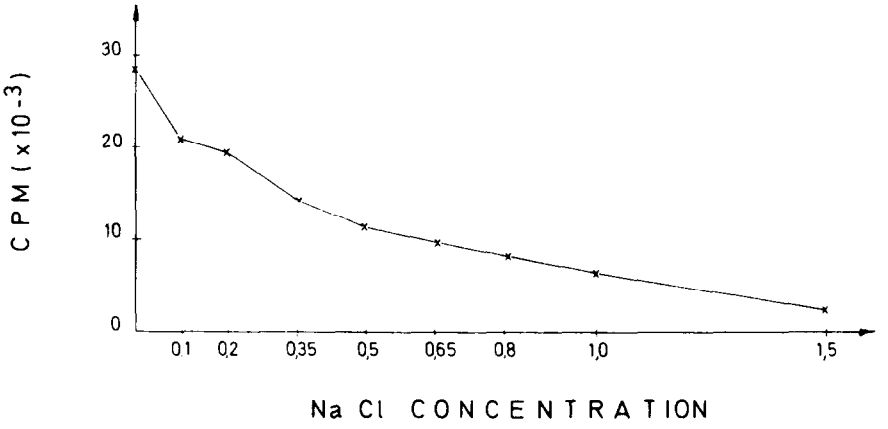
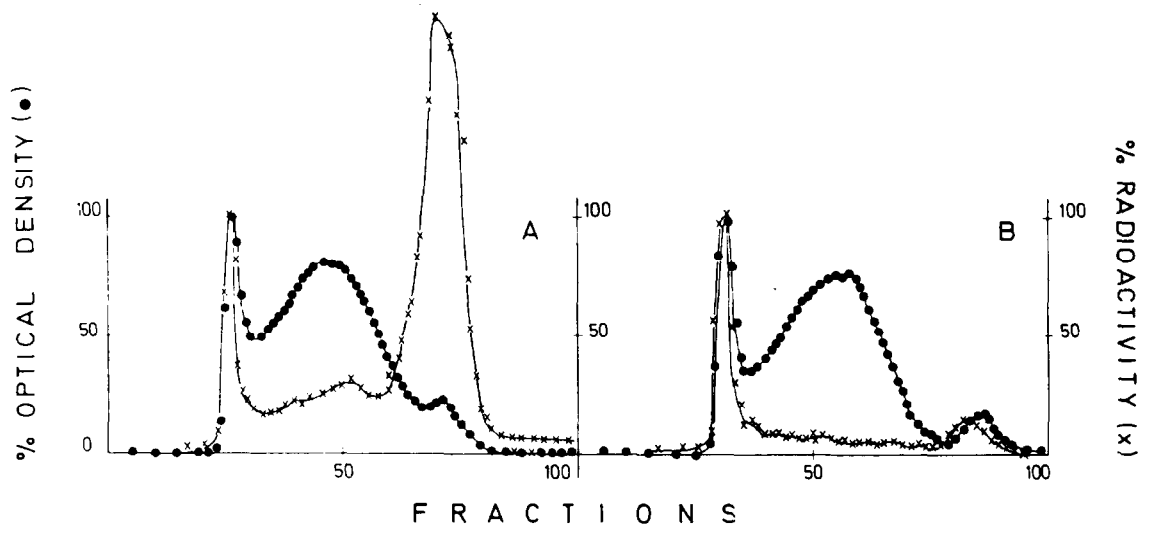


Fig.2. Release of endogenous RNA polymerase from chromatin as a function of ionic strength. Chromatin samples were extracted for 1 h at 4°C by different NaCl concentrations, then centrifuged for 40 h at 49 000 rev/min in a SW 50.1 (Spinco) rotor on a cushion of 1 ml 1.8 M sucrose, 10 mM Tris (pH 8). Chromatin pellets were washed and resuspended in 5% glycerol, 5 mM $NaHSO_3$, 1 mM dithiothreitol and 10 mM Tris (pH 8). 25 μg of each sample were incubated for 30 min at 37°C in 0.2 M $(NH_4)_2SO_4$, as described in Methods for the assay of endogenous RNA polymerase activity.

While only slight association is found in the second, included, peak, some radioactivity is found in the last fractions (fig.1). This might mean that some RNA polymerase is liberated from chromatin during fractionation in 0.1 M $MgCl_2$ and 0.15 M KCl, and appear as labelled molecules at the last part of the chromatin eluate [1]. That RNA polymerase can actually be dissociated from chromatin as a function of ionic strength is shown in fig.2.

Residual RNA polymerase associated with chromatin is thus probably firmly bound to DNA, as when it is actively engaged in transcription [20]. It should be noted here that the affinity of amanitin for RNA polymerase B is increased at high ionic strengths [19]. Binding of amanitin to chromatin seems not to be due to some non-specific adsorption, since the binding curve, as a function of amanitin concentration, is linear up to a plateau; moreover, after preincubation of chromatin with unlabelled amanitin, no radioactivity was found in the first peak of the chromatin eluate.

Another way to test the presence of endogenous RNA polymerase is to measure its activity in high salt in vitro (fig.3); it is known [21] that this activity is essentially due to elongation of pre-existing RNA chains. By using this method, it is apparent that the first chromatin fraction contains a much greater concentration of RNA polymerase activity per μg of DNA, whether the assay is done before (fig.3A) or after shearing (fig.3B), or after fractionation (fig.3C). The second peak contains a maximum of 33% of the first fraction activity, at each of the DNA concentrations



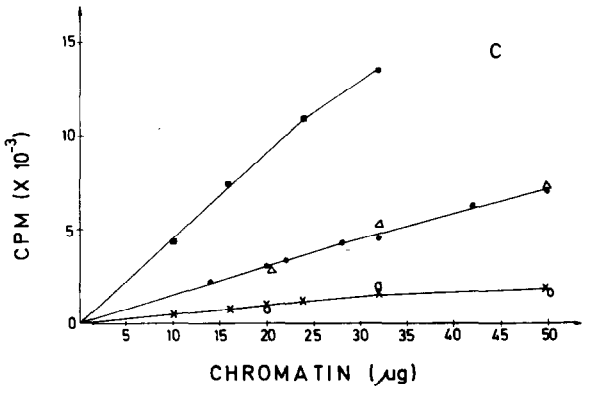


Fig.3. Endogenous RNA polymerase activity of fractionated chromatin. (A) Chromatin (250 μg) was incubated for 1 h at 37°C in 0.2 M $(NH_4)_2SO_4$ before shearing, as described in Methods for the assay of endogenous RNA polymerase activity. Results are expressed as percentages of the OD_{260} nm (•) or of the radioactivity (×), taking as 100% the maximal OD or radioactivity of the first peak. Maximal OD was 0.33; 34 050 cpm were associated with the first peak. (B) Same legend as for (A), except that chromatin was sheared before incubation. Maximal OD was 0.33; 13 510 cpm were associated with the first peak. (C) Different amounts of total sheared chromatin (■), or of the first (•) and the second peak (top fractions) (×), were incubated for 1 h at 37°C under the conditions described in Methods for endogenous RNA polymerase assay. In (△) and (○), the incubation medium for the first and the second fraction, respectively, contained 1 mg/ml of heparin.

tested. The third peak of radioactivity observed in (A) could be attributed to the liberation, from chromatin, of sheared RNA fragments adsorbed to chromatin during fractionation. In all cases, the activity was inhibited by 50–80% in the presence of 1 μg α-amanitin per 50 μg of chromatin. Since heparin does not stimulate RNA synthesis in high salt (fig.3C), it is concluded that a maximal elongation rate was attained, and that no initiation by released RNA polymerase was possible; an interference due to RNase activity is excluded in those conditions, since heparin, which is a powerful inhibitor of RNase [21], does not affect the results.

It was important to estimate the length of the DNA pieces in the two fractions, since a difference in RNA chain elongation could be due to a difference in template size. It is indeed obvious that chromatin endogenous RNA polymerase activity is decreased by a lowering of DNA size (fig.4). However, the DNA in the two fractions is of about the same length, as seen in table 1.

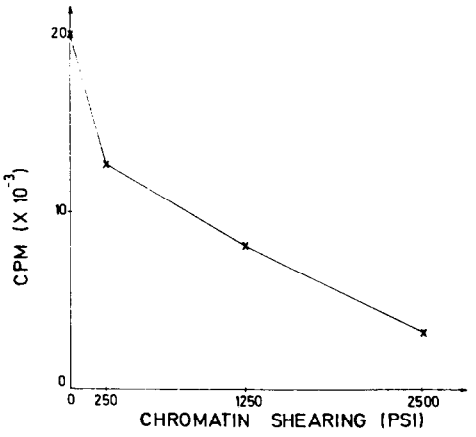


Fig.4. Inhibition of endogenous RNA polymerase activity as a function of chromatin DNA shearing. Samples (15 μg) of sheared chromatin preparations were incubated for 1 h at 37°C in the conditions described in Methods for endogenous RNA polymerase assay.

4. Discussion

Chromatin sheared and fractionated in 0.1 M $MgCl_2$ and 0.15 M KCl has probably lost its physiological constitutive properties. In particular, we have shown [6] that fl histone is released from DNA in 0.1 M $MgCl_2$, the salt concentration needed to solubilize chromatin. Moreover, labelled probe DNA mixed with chromatin becomes partially protected against DNase I after shearing chromatin (unpublished results): this means that during shearing some fl histone molecules can be displaced from their original binding sites, and can cover exogenous naked DNA [6]. On the other hand, new initiation sites for exogenous RNA polymerase appear after shearing [6], and endogenous RNA polymerase is partially lost during elution in high salt. The fractions isolated under those conditions are thus not useful for further studies on chromatin function. However, it is possible to conclude that the first fraction represents a set of DNA sequences particularly well transcribed in vivo, since it contains a much greater concentration of endogenous RNA polymerase than the bulk of chromatin. This fraction is also enriched in non-histone acidic

Table 1
Chromatin DNA length as a function of shearing and fractionation

Chromatin	S_{20}	Molecular weight (daltons)	minimal length (base pairs)
Native	11.6	710 000	2370
Sheared	7.3	226 000	750
First peak	8.2	298 000	1000
Second peak (top fractions)	7.6	250 000	830

DNA was extracted from different chromatin preparations, and centrifuged (0.5 OD_{260} nm/ml) at 48 000 rev/min in 0.9 M NaCl, 0.1 M NaOH for 3 h in a Beckmann analytical ultracentrifuge (AN-F rotor; scanning every 16 min). The mol. wt of DNA (single-stranded) was calculated from sedimentation data according to Studier's relation [22].

proteins (ratio to DNA= 1.6 for the first fraction, and 0.4 for the second), while the histone content is about the same for the two fractions. These observations are compatible with the idea that non-histone proteins de-repress or modulate the level of transcription inhibition exerted by the histones. The recent discovery that histones form non-specific, self-assemblages in subunits on DNA [23–27] leads to the suggestion that the organ-specific transcription of particular DNA sequences could be due to local modifications of a general chromatin architecture ([7]; and see, for example, J. Paul's model in [28]). After shearing, those sequences no longer aggregate and can be isolated as separate entities.

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