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Fractionation of Liver Chromatin

(DNase II/differential precipitation/RNA polymerase/rat liver)

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ABSTRACT Rat liver chromatin has been fractionated into two fractions on the basis of precipitability in standard saline after mild treatment with DNase II. The major portion of liver chromatin contains little nonhistone protein and is enriched in histones, while a minor portion of such chromatin, with which RNA polymerase is associated, is highly enriched in proteins other than histone and impoverished in histones. Although the relative concentrations of the several histone species present in two chromatin fractions are identical, binding of histones to DNA in the minor fractions appears to be modified, presumably by the presence of proteins other than histones.

The chromatin isolated from interphase nuclei can serve as a template for DNA-dependent RNA synthesis (1, 2). The template activity of isolated chromatin is, however, restricted to only certain portions of chromosomal DNA (2). It has been further shown that the template restriction in isolated chromatins is tissue-specific and that the regulatory state in the cell is well preserved in isolated chromatins (3–6).

In a series of chromatins isolated from different tissues (8) and from different stages of development (9, 10), a greater percentage of nonhistone proteins are present in more template-active chromatins, suggesting that chromosomal nonhistone proteins are in some way associated with active portions of the genome, whereas the template activity correlates inversely with the histone content. In the present experiments, rat liver chromatin has been fractionated into portions rich in nonhistone proteins, and histone-rich portions. It is the chromatin fraction rich in nonhistone proteins that contains endogenous RNA polymerase activity and, therefore, may represent template-active portions of liver chromatin.

MATERIALS AND METHODS

Preparation of Chromatin. Rat liver chromatin was prepared as described (2), except that 0.05 M Tris buffer (pH 8) was replaced by 0.01 M Tris buffer (pH 8) throughout the procedure. The gelatinous, purified chromatin sediment obtained after centrifugation through dense sucrose was washed once with Tris buffer (0.01 M, pH 8) by centrifugation at 30,000 × *g* for 20 min and resuspended in the same buffer. In some experiments, the chromatin thus obtained was sheared with a Virtis homogenizer at 30 V for 90 sec and centrifuged at 30,000 × *g* for 20 min. The supernatant, in which the majority of chromatin was recovered, will be referred to as sheared chromatin.

Fractionation of Chromatin. Rat liver chromatin was incubated with 25 μg/ml of crude DNase II (Worthington) in 0.01 M Tris buffer (pH 8) containing 0.1 mM EDTA at 37°C for various lengths of time. The concentration of chromatin was equivalent to 250–400 μg of DNA per ml. After incubation, the mixture was centrifuged at 30,000 × *g* for 20 min. To the supernatant, 0.11 volume of ten-times concentrated standard saline–citrate (SSC, 0.15 M NaCl–0.015 M sodium citrate) was added dropwise with stirring, and the stirring was continued for 15–30 min. This solution was then centrifuged at 30,000 × *g* for 20 min. The supernatant (SSC-soluble fraction) was centrifuged in a Spinco no. 40 rotor at 80,000–100,000 × *g* for 10–16 hr with a cushion of 1 ml of 1.7 M sucrose containing 0.01 M Tris buffer (pH 8) at the bottom of the tube. After centrifugation, the sucrose layer, including the pellet, was resuspended in 0.01 M Tris buffer (pH 8) and dialyzed against the same buffer. The sediment (SSC-precipitable fraction) was washed once with SSC, resuspended in 0.01 M Tris buffer (pH 8), and sedimented by centrifugation in a Spinco no. 40 rotor as described above.

To obtain deproteinized DNA, each chromatin fraction was mixed with an equal volume of 6 M CsCl and centrifuged in a Spinco SW-39 rotor at 39,000 rpm for 48 hr. The DNA sediment was resuspended in dilute saline–citrate (0.015 M NaCl–0.0015 M sodium citrate) and dialyzed against this solution overnight.

Analyses of Chromatin Fractions. DNA was determined directly from the UV absorbance of chromatin fractions, after correction for turbidity as described (11). Histones and nonhistone proteins were fractionated according to Marushige and Bonner (2) and determined by the method of Lowry *et al.* (12) with bovine-serum albumin as standard. Electrophoreses of histones in polyacrylamide gels were described (11).

The sedimentation coefficient of DNA was determined by band sedimentation in self-forming D_2O gradients (13) in the Spinco analytical centrifuge equipped with the photoelectric scanner and recorder. Molecular weight of DNA was calculated from its sedimentation coefficient, according to Studier (14). Calculations were performed on the IBM 7094 and the IBM 360/50 computers.

Chromatin was melted with the Beckman DK-2 ratio-recording spectrophotometer equipped with a temperature-controlled cuvette holder in 0.25 mM sodium EDTA, titrated

Abbreviation: SSC, standard saline–citrate (0.15 M NaCl–0.015 M Na_3 citrate).

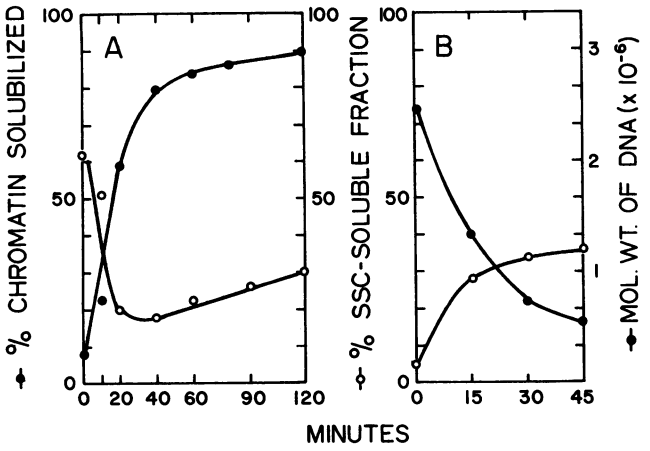

FIG. 1. Action of DNase II on rat liver chromatin. Incubation was at 37°C with 25 μg/ml of DNase II. (*A*) rat liver chromatins solublized by DNase treatment were determined after centrifugation at 30,000 × *g* for 20 min (*solid circles*). Solubilized chromatins were treated with SSC and the percentage of SSC-soluble fractions was determined after centrifugation at 30,000 × *g* for 20 min (*open circles*). (*B*) sheared chromatins from rat liver incubated for various lengths of time were sedimented at 80,000 × *g* for 16 hr and resuspended in Tris buffer (0.01 M, pH 8). DNA was prepared from aliquots and analyzed by sedimentation (*closed circles*). Aliquots were treated with SSC and the percentage of SSC-soluble fractions was determined after centrifugation at 30,000 × *g* for 20 min (*open circles*).

with NaOH to pH 8. The increase of A_{257} was recorded every 2°C, and the slope of the melting curve at each temperature was obtained essentially as described by Li and Bonner (15). Meltings were done over a range of DNA concentrations of 0.375–0.488 A_{257} in a 1 cm-path length at 22°C, but results were normalized to a DNA concentration of 0.500 A_{257}.

RNA polymerase activities of chromatin fractions were assayed by incubation in a reaction mixture containing 20 mM Tris buffer (pH 8), 2 mM $MgCl_2$, 3 mM $MnCl_2$, 0.1 mM dithiothreitol, 0.5 mM (each) of ATP, GTP, and CTP, and 0.1 mM [^{3}H]UTP (100 Ci/mol) at 37°C for 10 min. After incubation, the reaction was stopped by the addition of 10% trichloroacetic acid. The acid-insoluble material was collected by filtration through a membrane filter, washed four times with 5 ml of 10% trichloroacetic acid, and the radioactivity was determined in the Beckman LS-250 liquid scintillation counter.

RESULTS

Since template-active portions and repressed portions in the genome of higher organisms are likely to be intimately mixed, their separation should require fragmentation of chromatins into small pieces. We have used a mild nuclease digestion as the means to obtain small fragments of chromatins. Among readily available deoxyribonucleases, DNase II (Worthington) has been chosen for the following reasons: the reaction requires no divalent metal ions that would cause aggregation of chromatin and chromosomal proteins, and the optimum pH of this enzyme is 4.8; therefore, a very mild degradation may be obtained at neutral pH. In fact, under the conditions used in the present experiments, only about 5% of pure, deproteinized DNA became acid-soluble when incubated with enzyme for 90 min. Finally, this enzyme has been reported to cleave both DNA strands simultaneously, at or near the same site (16).

Rat liver chromatins obtained by the present method are viscous, high molecular weight aggregates, essentially completely sedimentable at 15,000–30,000 × *g* for 20 min. The size of DNA prepared from such chromatin by the procedure of Marmur (17) is generally found to be 25–26 S. Although little or no chromosomal DNA becomes acid soluble by the DNase treatment used in the present experiments, the action of the DNase can be clearly seen by the solubilization of the chromatin. As shown in Fig. 1*A* (*closed circles*), the percentage of chromatin no longer sedimentable at 30,000 × *g* increases sharply with incubation time up to 40 min; about 90% of the chromatin is solubilized after 90 min. The majority of the solubilized chromatin can, however, still be sedimented by high-speed centrifugation (e.g., 100,000 × *g* for 16 hr). When previously sheared chromatin from rat liver is incubated, the action of DNase II can best be shown by the decrease in molecular weight of its DNA, as shown in Fig. 1*B* (*closed circles*). The size of DNA, originally 14 S, decreases to 8.6 S after incubation for 45 min.

One of the characteristic properties of isolated chromatins is their precipitability in physiological saline (0.15 M NaCl). Such precipitation has been shown to be due to ionic neutralization of negative charges on the chromatin (18). Since chromatin precipitates at physiological ionic strength, it seems probable that in interphase nuclei the majority of chromatin is in a highly aggregated state, which would seem quite unfavorable for transcription. The template-active portion of chromatin, which constitutes only a small fraction of the total genome (8), could, however, possess such a chemical nature that it remains soluble at physiological ionic strength. We have, therefore, attempted to fractionate the chromatin fragments obtained by DNase treatment on the basis of their solubility in standard saline–citrate.

TABLE 1. *Chemical composition of chromatin fractions*

Expt.	Incubation* (min)	Fraction†	Composition relative to DNA: Histone protein	Composition relative to DNA: Nonhistone protein
1	40	S	0.74	1.41
	60	S	0.82	1.31
	120	S	0.84	0.94
	40	P	1.22	0.15
	60	P	1.19	0.16
	120	P	1.18	0.13
2	60	S	0.91	1.36
	60–60‡	P	1.15	0.10
3§	0	Total	1.01	0.52
	20	S	0.86	0.64
	20	P	1.13	0.11

* Incubations were at 37°C with 25 μg/ml of DNase II.

† S and P represent SSC-soluble fractions and SSC-precipitable fractions, respectively (see *Methods*).

‡ SSC-precipitable fractions obtained after 60 min of incubation were again incubated with DNase II for 60 min and precipitated with SSC.

§ Sheared chromatins were used in these experiments.

Rat liver chromatins solubilized with DNase II as shown in Fig. 1A (*closed circles*) were treated with SSC. The precipitate was sedimented by centrifugation at 30,000 × *g* for 20 min. As shown in Fig. 1A (*open circles*), the percentage of the SSC-soluble fraction is high at early stages of the DNase treatment, decreases to about 20% at 40 min, and then increases gradually with increase incubation time. The enrichment of SSC-soluble fractions in the solubilized chromatins at the early stages of incubation suggests that those portions of the chromatin that are not precipitable with SSC are solubilized more rapidly, presumably due to a higher susceptibility to the enzyme. Consistent with this view is the observation that when sheared chromatin is treated with DNase, the percentage of SSC-soluble fractions increases sharply during the early portion of the incubation, as shown in Fig. 1B (*open circles*).

The chemical compositions of the SSC-soluble and SSC-precipitable fractions obtained from rat liver chromatins subjected to various DNase treatments are presented in Table 1. The composition of whole chromatin solubilized by mechanical shearing is shown in Expt. 3 of this table. It is clear that the SSC-soluble fractions are highly enriched in nonhistone proteins and impoverished in histones, while the SSC-precipitable fractions are enriched in histones and contain little nonhistone proteins. In addition, there is a significant decrease of nonhistone-protein content of the SSC-soluble fraction between 60 and 120 min of incubation (Expt. 1). This, together with the observation that the percentage of SSC-soluble fraction increases gradually during prolonged incubation with DNase (Fig. 1A) suggests that the SSC-soluble fraction becomes increasingly contaminated with the SSC-precipitable fraction. It seems likely that during the prolonged incubation the size of chromatin fragments becomes smaller and, therefore, sedimentation of aggregates formed in SSC becomes increasingly difficult under the centrifugal conditions used. The low nonhistone-protein content of the SSC-soluble fraction obtained with sheared chromatin (Expt. 3) is probably also due to contamination by the SSC-precipitable fraction. In fact, the percentage of the SSC-soluble fraction after 20 min of incubation of sheared chromatin is as high as that of unsheared chromatin treated with DNase for 120 min (Fig. 1A and B). No appreciable change is observed in the composition of the SSC-precipitable fraction obtained from chromatin treated with DNase II for 40–120 min (Expt. 1). A further purification of the SSC-precipitable fraction by a second DNase treatment, followed by SSC fractionation, does not produce any substantial change in its chemical composition (Expt. 2).

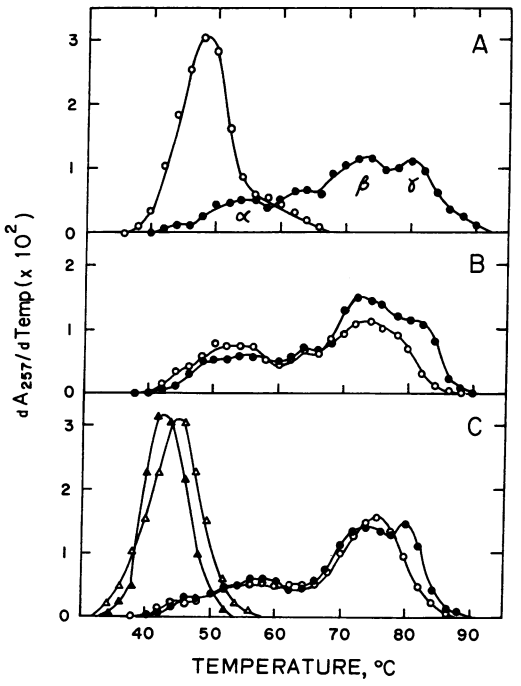

FIG. 2. Derivative melting profiles of chromatin fractions. (*A*) (control), sheared chromatin (*solid circles*) and rat liver DNA (*open circles*); (*B*) SSC-soluble (*open circles*) and SSC-precipitable fractions (*solid circles*) obtained from chromatins treated with 25 μg/ml of DNase II for 40 min; (*C*) SSC-soluble fractions (*open circles*) from chromatin treated with 25 μg/ml of DNase II for 60 min and their DNA (*open triangles*), and SSC-precipitable fractions (*solid circles*) from chromatin treated with 40 μg/ml of DNase II for 60 min and their DNA (*solid triangles*).

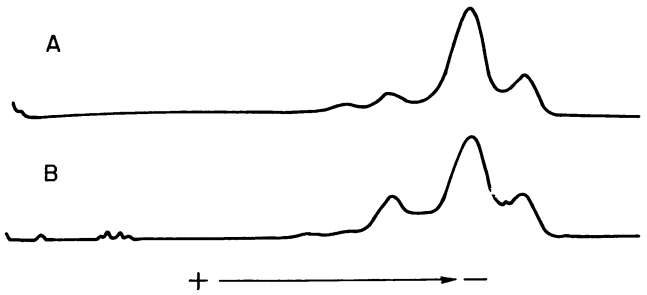

FIG. 3. Electrophoresis of histones of chromatin fractions. About 20 μg of histones were applied to a gel and electrophoresed for 90 min at 4–5 mA per tube. The gels were stained with Amido schwartz and scanned with the Canalco model E microdensitometer. (*A*) histones of SSC-soluble fractions; (*B*) histones of SSC-precipitable fractions.

Heat denaturation profiles of SSC-soluble and SSC-precipitable fractions of liver chromatins are analyzed in Fig. 2. The DNA isolated from these chromatin fractions exhibits hyperchromicity identical to that of DNA of untreated rat liver chromatin, indicating that there are no long stretches of single-strand breaks in the DNA of these chromatin fractions. However, their lowered melting temperature (compare Fig. 2A with C) suggests that their DNA does contain some nicks. As seen in Fig. 2A, melting of rat liver chromatin, in a good agreement with observations with pea-bud chromatin (15), consists of three major melting components (designated as α, β, and γ in Fig. 2A). Data of Fig. 2B and C clearly show that melting-component γ is nearly absent, if not completely abolished, in the SSC-soluble fractions of chromatin. Li and Bonner (15) have shown that melting bands at 66 and 81°C, which appear to correspond to β and γ in this experiment, are due to stabilization of DNA by histones, while the melting band at 52°C, corresponding to α in this experiment, is due to binding of nonhistone proteins and to coupling of melting at boundaries and gaps between various melting components. As shown in Fig. 3, the SSC-soluble and SSC-precipitable fractions of chromatin contain the same histone fractions at the same relative concentrations. The lack or diminution of melting-component γ in the SSC-soluble fractions of chromatin, therefore, suggests that in such portions of chromatin the binding of histones to DNA is modified, presumably by the presence of nonhistone proteins.

TABLE 2. *RNA polymerase activities of chromatin fractions*

Chromatin fractions	Reaction mixture	pmol of UMP incorporated per ml per 10 min at 37°C
SSC-soluble	Complete	3.73
	−ATP, −GTP, −CTP	0.00
	$-Mn^{++}$, $-Mg^{++}$	0.00
SSC-precipitable	Complete	0.00

Reaction mixtures (1 ml) contained 300 μg of DNA as SSC-soluble or SSC-precipitable fractions obtained from chromatins treated with DNase II for 60 min. In this experiment, 5 μg/ml of purified DNase II (Worthington) was used. Chromatins were prepared and fractionated in the presence of 0.1 mM dithiothreitol.

The question as to whether a chromatin fraction is derived from a template-active portion or from a repressed portion of the genome may be answered by assay of the endogenous RNA polymerase activity of each. As can be seen in Table 2, the SSC-soluble fractions of chromatin support the incorporation of [^{3}H]UTP into the 10% trichloroacetic acid-precipitable fraction, and such incorporation is completely abolished by the omission of divalent metal ions or nucleoside triphosphates. No RNA polymerase activity is detectable in SSC-precipitable fractions of chromatin. The SSC-soluble fraction is, therefore, presumably the template-active one.

DISCUSSION

In native chromatins (2), as well as in reconstituted nucleohistones (19, 20), the binding of histones to DNA has been shown to restrict the ability of DNA to support RNA synthesis. That histones lack an ability to recognize specific DNA base sequences implies that they bind indiscriminately to any part of the genome and can, therefore, cause premature cessation of transcription. This view leads to the idea that template-active genes should be free from inhibitory effects of histones over the entire genomal length. One possibility is, therefore, that free DNA (or DNA with which only nonhistone proteins are complexed) constitutes the template-active genes. A melting band whose melting temperature is identical to that of free DNA has been observed in pea-bud chromatins (15). It has not, however, been shown that this melting band represents a melting of DNA of genomal length.

Another possibility is that in template-active genes, the binding of histones to DNA is modified in such a way that histones do not obstruct the execution of transcription. In the present experiments, the SSC-soluble fraction of chromatin, which is highly enriched in nonhistone proteins (Table 1), contains endogenous RNA polymerase (Table 2) and is, therefore, likely to represent the template-active portions of liver chromatin. In this chromatin fraction, the amounts of histones are reduced relative to DNA (Table 1), and their binding to DNA appears to be different than that by which histones are bound in the remaining portion of liver chromatin (Fig. 2) (this latter class being precipitable in SSC). These results are consistent with the second possibility above, that histone binding is modified in template-active chromatin.

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