DNase I SENSITIVE CHROMATIN IS ENRICHED IN THE ACETYLATED SPECIES OF HISTONE H4

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1. Introduction

DNase I recognizes and selectively digests transcriptionally-competent chromatin regions [1–5]. The DNase I sensitivity of these regions may be due to packaging or to an altered nucleosome conformation at transcriptionally-competent loci.

We have designed a fractionation procedure which allows the isolation of the DNase I sensitive chromatin fraction. The fraction is enriched in the acetylated species of histone H4. Furthermore, the fraction contains low levels of histone H1 and high levels of the non-histone chromosomal proteins, including the HMG proteins.

2. Materials and methods

2.1. *Digestion of nuclei*

Trout testis nuclei were prepared as in [6], except that the nuclei were treated with the protease inhibitor, phenylmethylsulfonyl fluoride (0.1 mM). Washed nuclei (20 A_{260}/ml) were incubated with 1 μg/ml DNase I (Sigma) at 15°C in Buffer A (10 mM Tris–HCl (pH 7.4), 3 mM $MgCl_2$ and 10 mM NaCl) for 10 min. The reaction was terminated by placing the solution on ice. When these conditions are used, 5% of the total A_{260} is released in the supernatant. For micrococcal nuclease digestions, nuclei (40 A_{260}/ml) in buffer B (40 mM Tris–HCl (pH 7.4), 25 mM KCl, 1 mM $MgCl_2$, 0.25 M sucrose and 1 mM $CaCl_2$) were incubated with 100 A_{260} units/ml micrococcal nuclease (Sigma) at 25°C for 4 min. The reaction was terminated by making the solution 1 mM in EGTA (pH 7.0) and placing it on ice. The nuclei were collected by centrifugation at 3000 × *g* for 10 min and the supernatant, SO, placed on ice.

2.2. *Stepwise extractions*

The digested nuclei were resuspended in buffer C (50 mM Tris–HCl (pH 7.5), 25 mM KCl, 2 mM $MgCl_2$ and 0.25 M sucrose) containing 0.1 M NaCl and incubated for 20 min at 0°C. The nuclear suspension was centrifuged at 3000 × *g* for 10 min and the supernatant, SS1, removed. The above steps (resuspension of the nuclei, incubation and centrifugation) were sequentially repeated using buffer C containing 0.2 M, 0.4 M and finally 0.6 M NaCl. The supernatants were obtained after each centrifugation, yielding SS2, SS4 and SS6, respectively. Alternatively, digested nuclei were extracted with 0.2 M NaCl in buffer C yielding the supernatant SS2T after centrifugation.

2.3. *Fractionation of the salt extracted products*

Salt-extracted fractions were applied to either a Bio-Gel A-5 m column (90 × 1.5 cm) or a Bio-Gel A-0.5 m (40 × 1.5 cm) column equilibrated with 10 mM Tris–HCl (pH 7.5), 0.7 mM EDTA and 0.4 M NaCl at 4°C as in [7].

2.4. *Preparation of histones*

Supernatants and pooled column fractions were dialyzed overnight at 4°C against 10 mM NH_4HCO_3 and lyophilized. Lyophilized samples and nuclei were extracted with 0.4 N H_2SO_4 (30 min on ice) and insoluble material removed by centrifugation (3000 × *g* for 10 min). The acid extracts were dialyzed overnight at 4°C against 0.1 N acetic acid, lyophilized and redissolved in distilled water.

2.5. *Acrylamide gel electrophoresis*

15% Polyacrylamide–SDS slab gels were run as in [8]. Acid/urea gels were run as in [9]. 10% Denaturing DNA polyacrylamide–SDS slab gels were prepared as in [10] except that 0.1% SDS was added to the gel and to the electrophoresis buffer.

3. Results and discussion

The fractionation procedure is a modification of that in [11]. The nuclei were digested with DNase I (1 μg/ml for 10 min at 15°C), then sequentially extracted with increasing concentrations of NaCl.

The % of total A_{260} material released in each fraction is presented in table 1. The values that were obtained for each salt-extracted nuclear fraction did not vary appreciably with the extent of digestion over 1.5–30% solubilization of the DNA. The results suggest that each fraction represents a distinct chromatin subpopulation.

The DNA fragment size associated with each fraction was examined on 10% denaturing polyacrylamide gels (fig.1). Fractions SS1, SS2, SS4 and SS6 presented the characteristic 10 base DNA repeat pattern. However, fractions SS4 and SS6 had a greater level of DNA fragments which were larger than ~140 bases in length, as compared to fractions SS1 and SS2. Thus, the fraction of chromatin which is most sensitive to DNase I digestion is eluted at the lowest NaCl concentrations.

Gel exclusion chromatography of the various fractions on Bio-Gel A-5 m or Bio-Gel A-0.5 m columns (not shown) suggested that the average size of the nucleosome species increased with each successively higher NaCl concentration. When 5% of the total DNA was solubilized in SO, SS1 and SS2 contained predominantly mononucleosomes, while SS4 contained mainly dinucleosomes and polynucleosomes.

The proteins associated with each fraction were analyzed on a 15% polyacrylamide-SDS gel (fig.2). Each fraction contained the core histones, H2A, H2B, H3 and H4. The majority of the core histones

Table 1
DNA content of salt-extracted chromatin fractions

Fraction	Total A_{260} in each fraction (%)
SS1	3.1 ± 0.7
SS2	2.6 ± 0.5
SS4	31.5 ± 3.0
SS6	23.3 ± 3.3

The A_{260} absorbing material released in the salt-extracted nucleosome fractions after DNase I digestion of trout testis nuclei was measured by adding 100 μl of the fraction to 0.9 ml 0.6 M NaCl in buffer C and reading the A_{260}

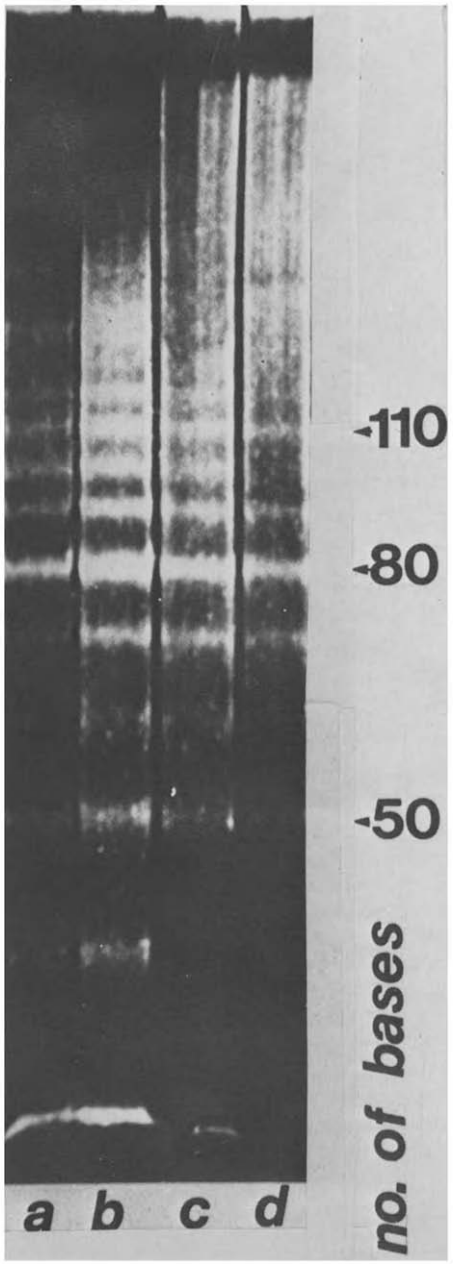

Fig.1. Polyacrylamide gel electrophoresis of single-stranded DNA fragments generated by DNase I digestion of trout testis nuclei and released by stepwise increases in NaCl concentrations. The salt-extracted fractions were dialyzed overnight against 10 mM NH_4HCO_3 and samples corresponding to 0.5 A_{260} were lyophilized. The samples were redissolved in sample buffer containing SDS and applied to the gel. The gel was stained with ethidium bromide and photographed under ultraviolet light. (a–d) Fractions successively released by buffer C containing 0.1, 0.2, 0.4 and 0.6 M NaCl (SS1–SS6), respectively.

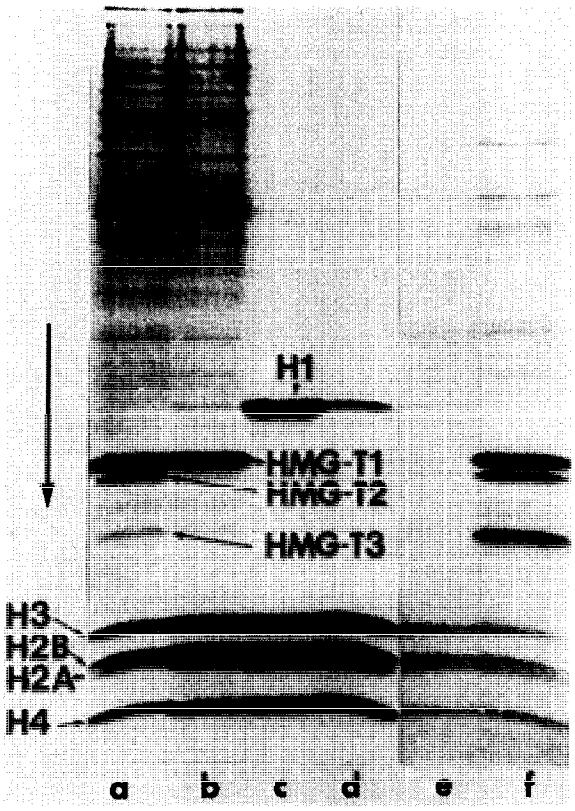

Fig.2. 15% Polyacrylamide–SDS gel electrophoretic separation of proteins released from DNase I digested trout testis nuclei by successively higher NaCl concentrations. The salt-extracted fractions were dialyzed overnight against 10 mM NH_4HCO_3 and samples corresponding to 0.6 A_{260} were lyophilized. The samples were redissolved in sample buffer containing SDS and applied to the gel. The gel was stained with Coomassie blue. (a–d) Fractions released from DNase I digested nuclei by buffer C containing 0.1, 0.2, 0.4 and 0.6 M NaCl (SS1–SS6), respectively; (e) fraction released by digestion of nuclei with DNase I (SO (DNase I)); (f) fraction released by digestion of nuclei with micrococcal nuclease (SO (micrococcal)).

were associated with nucleosomes, i.e., little or no free histone was present (not shown). Fractions SS1 and SS2 contained low levels of H1. In addition, these fractions contained high levels of nonhistone chromosomal proteins, including HMG-T1 (equivalent to HMG-T [12]), HMG-T2 and HMG-T3 (now known to be a proteolytic product). The nomenclature assigned to these proteins will be described elsewhere (B. S. Bhullar, E. P. M., in preparation). Fractions SS4 and SS6 contained large amounts of H1, this histone being highest in SS4.

Fraction SO (DNase I) (fig.2(e)) consisting of material solubilized by DNase I digestion of nuclei contained low levels of both non-histone chromosomal proteins and core histones. However, fraction SO (micrococcal) (fig.2(f)) consisting of analogous material solubilized from micrococcal nuclease digested nuclei had large amounts of the HMG proteins along with low levels of the core histones. These core histones are probably associated with nucleosomes that have been solubilized, as the SO (micrococcal) fraction contained DNA fragments ~146 and 170 base pairs long (not shown).

The levels of acetylated histone species associated with the various salt-extracted fractions were examined on acid/urea gels. In addition, the levels of acetylated histone species associated with undigested nuclei (C) and with SS1 derived from a micrococcal digest were also analyzed. Histones from fractions SS4 and SS6 were analyzed directly on the gels. With SS1 and SS2, it was found necessary to remove the non-histone chromosomal proteins (NHCP) associated with these fractions before electrophoresis, as the NHCPs interfered with the resolution of the acetylated histone species.

To remove the NHCPs, fraction SS2T was further fractionated on a Bio-Gel A-0.5 m column equilibrated with 0.4 M NaCl, 10 mM Tris–HCl (pH 7.5) and 0.7 mM EDTA. Histones associated with mono-nucleosomes were extracted and then resolved on the gels.

Table 2
Proportion of acetylated species of histone H4 in salt-extracted nucleosome fractions released from either micrococcal nuclease or DNase I digested nuclei

Fraction	Ratio of H4 $\frac{1A_1 + 2A_2 + 3A_3}{A_0 + A_1 + A_2 + A_3}$ [a]
SS1 (micrococcal)	1.47 ± .07
SS2T (monomer)	1.32
SS4	1.08 ± .03
SS6	0.94 ± .03
Control (undigested nuclei)	0.97

[a] Determined from the A_{550} peak heights of the scanned H4 species

Histones, prepared from either undigested nuclei or salt-extracted nucleosome fractions following micrococcal nuclease or DNase I digestion as in section 2, were separated on acid–urea gels. The gels were stained with Coomassie blue and scanned at 550 nm using a Gilford spectrophotometer

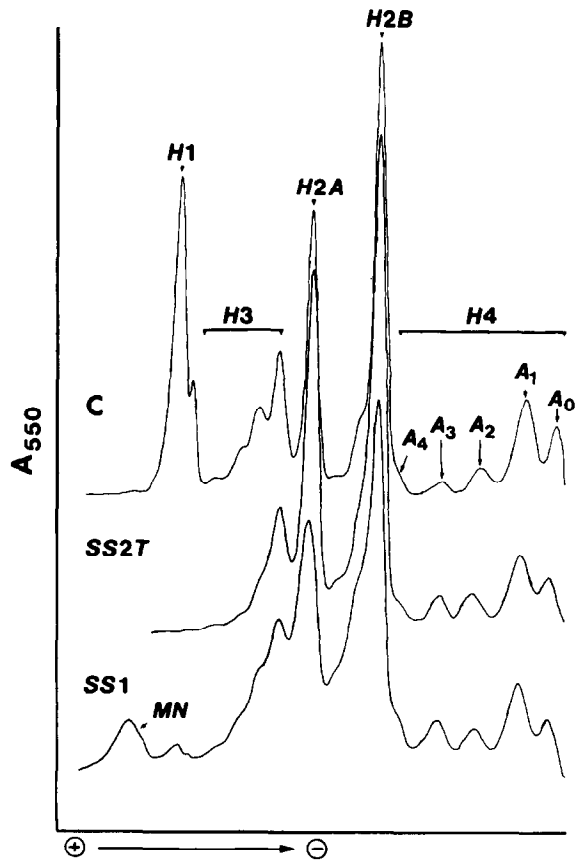

Fig.3. Gel scans of histones analyzed on acid–urea gels. Histones were acid-extracted from either undigested nuclei (C), nucleosomes that had been released from micrococcal nuclease digested nuclei by buffer C containing 0.1 M NaCl (SS1 micrococcal)) or mononucleosomes (SS2T (DNase I)) that were prepared by extracting DNase I digested nuclei with buffer C containing 0.2 M NaCl, and fractionating the solubilized material on a Bio-Gel A-0.5 m column as in section 2. Gels were stained with Coomassie blue and scanned in a Gilford spectrophotometer at 550 nm.

Abbreviations: MN, micrococcal nuclease. A_0, unacetylated H4; A_1, A_2, A_3 and A_4, mono-, di-, tri- and tetra-acetylated H4, respectively

Gel scans of the histone species associated with undigested chromatin (control), SS1 (from a micrococcal nuclease digest) and SS2T (from a DNase I digest) are shown in fig.2. As previous experiments (submitted) have demonstrated that highly acetylated histone H4 was associated with nucleosomes present the SS1 fraction obtained from micrococcal ase-digested nuclei, the histones associated with the SS1 (micrococcal) fraction were compared to those associated with the SS2T (monomer) obtained from DNase I digested nuclei. Both SS1 (micrococcal) and SS2T (monomer) contained higher levels of the acetylated H4 species.

Table 2 shows the proportion of acetylated H4 species associated with each fraction. Both SS2T (monomer) and SS1 (micrococcal) have the highest levels of acetylated H4 species while SS4, SS6 and undigested chromatin contain lower levels. SS6 and undigested chromatin contain the lowest levels of acetylated H4. Although the level of acetylated H4 species associated with SS1 (micrococcal) is greater than that of SS2T (monomer) (DNase I), the levels of acetylated H4 species associated with SS2T (micrococcal) and with SS2T (monomer) (DNase I) are similar (not shown).

It should be noted that developing trout testis has a very low histone deacetylase activity, as seen by the slow turnover of labelled histone acetyl groups [13]. In order to detect comparable highly acetylated chromatin fractions in cultured mammalian cells, it will probably be necessary to include sodium butyrate in the isolation buffers to inhibit the histone deacetylases [14,15].

In summary, the fractionation procedure described here allows the isolation of a DNase I sensitive chromatin fraction. This fraction is enriched in NHCP which includes the HMG proteins. Furthermore, the nucleosomes associated with this fraction contain higher levels of the acetylated histone H4 species.

A possible role for histone acetylation may be to unfold the chromatin fiber [16–18]. That the N-terminal parts of the core histones interact with the internucleosomal DNA has been suggested [18]. In this way, chromatin may be maintained in a compact state. Acetylation at the N-terminal parts of the core histones would weaken or prevent the interactions, and the chromatin fiber would unfold. The extended form of chromatin is probably a required structural feature of transcriptionally competent chromatin [19].

Our results suggest that the level of the acetylated H4 species is directly correlated with the DNase I susceptibility of chromatin. This is consistent with the observations [17,20] that the highly acetylated chromatin of butyrate-treated cells is more sensitive to DNase I. High levels of the acetylated H4 species may thus allow chromatin to adopt an extended structure and maintain a DNase I sensitive, transcriptionally competent structure.

Acknowledgement

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