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FINESTRUCTURE AND TEMPLATE ACTIVITIES OF DNA-HISTONE COMPLEXES RECONSTITUTED IN THE PRESENCE AND ABSENCE OF UREA

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Abstract. Several DNA-histone complexes were reconstituted in the presence and absence of urea. The fiber size of DNA-histone H1 complex was about 20 Å in width with knobs 100 to 250 Å in diameter interspersed at an average interval of about 1,100 Å. H1 was associated with DNA segments corresponding to a DNA size of fewer than 100 base pairs. DNA-histones H2A, H2B, H3 and H4 complex consisted of globular subunits 100 to 150 Å in diameter alternating with thin strands, like beads on a string. DNA-whole histones complex was 200 to 250 Å in width and had a condensed configuration. The nuclease digestion pattern of the complexes containing histones H2A, H2B, H3 and H4 was regular, similar to that of chromatin, and was disrupted by urea. The complex containing H1 was inactive for *in vitro* RNA synthesis by *Escherichia coli* RNA polymerase, whereas the other complexes were active. The complexes reconstituted in the absence of urea had template activities slightly less than in the presence of urea.

Key words: finestructure, template activity, DNA-histone complex, reconstitution, electron microscopy, nuclease digestion.

Electron microscopy and biochemical studies have shown that chromatin fibers of eukaryotic cells consist of globular subunits alternating with thin strands, like beads on a string (1–4). However, the details of the organization of these chromosomal substructures are not clear at present.

Stein *et al.* have reported that the components of chromosomal proteins of reconstituted rat liver chromatin are identical to those of native rat liver chromatin (5). Chromatin reconstituted after its dissociation into DNA and proteins mediates the transcription of tissue-specific RNA (6–9) and specific messenger RNA (10, 11). This suggests that chromosomal components reassociate with DNA in a highly specific manner during reconstitution.

The structure and function of chromatin, especially the role of individual histones, are studied by the interaction of DNA with purified histone fractions (12, 13). Histones complexed with DNA are thought to control the transcription of DNA. Numerous investigators have reported that lysine-rich histones H1 and H2B are the best repressors of DNA template activity (14–16) and that

arginine-rich histones H3 and H4 are the most efficient repressors (17, 18). Furthermore, conformational changes of the chromatin fibers affect on the *in vitro* DNA-dependent RNA synthesis (19).

In the present comunication, DNA-histone complexes were prepared by salt-step gradient dialysis in the presence and absence of urea, and were examined by electron microscopy and biochemical methods. We report on the conformational and functional differences of the DNA-histone complexes resulting from DNA-histone interaction.

MATERIALS AND METHODS

Preparation of DNA and histones. DNA and histones were prepared from quick-frozen calf thymus according to the methods of Marmur (20) and of Johns (21). Chromatin was prepared by the method described by Bonner *et al.* (22). DNA was isolated from this chromatin by the method of Marmur (20). A whole histone fraction was extracted from this chromatin with 0.4 N H_2SO_4. The chromatin was dialyzed against 0.7 mM sodium phosphate buffer (pH 7.5). The dialyzed material was extracted overnight in the presence of 0.4 N H_2SO_4 with vigorous stirring and was centrifuged at 70,000×g for 20 min. Four volumes of cold (−20°C) ethanol were added to the supernatant which was kept at −20°C for 24 h. The white precipitate was collected by centrifugation at 2,000×g for 5 min, washed twice with acetone, and dried under vacuum conditions. The other histone fractions (H1 only, or H2A, H2B, H3 and H4 fraction) were prepared by the method of Johns (21).

Complex formation and purification. Reconstitution of DNA-histone complexes was performed by a salt-step gradient dialysis method in the presence (salt-urea method) and in the absence (salt method) of urea. DNA and histones were each dissolved in a medium consisting of 2.0 M NaCl, 5.0 M urea, 10 mM $NaHSO_3$ and 10 mM Tris-HCl (pH 7.5) at a concentration of 200 μg/ml. The DNA and histones were mixed at a histone/DNA ratio (w/w) of 1.0.

1) *Salt-urea method.* The DNA-histone mixtures were dialyzed against 200 volumes of 5.0 M urea, 10 mM $NaHSO_3$ and 10 mM Tris-HCl (pH 7.5) for 8 h in a decreasing NaCl gradient (2.0, 1.5, 1.0, 0.8, 0.6, 0.4, 0.3, 0.15, and to 0 M).

2) *Salt method.* The DNA-histone mixtures were dialyzed against 200 volumes of 10 mM $NaHSO_3$ and 10 mM Tris-HCl (pH 7.5) for 8 h in a decreasing NaCl-urea gradient: 2.0 M NaCl-5.0 M urea; 1.5 M NaCl-2.5 M urea; 1.0 M NaCl-1.0 M urea; 0.8 M NaCl-0.5 M urea; 0.6 M NaCl-0.1 M urea; 0.4 M NaCl; 0.3 M NaCl; 0.15 M NaCl; and to 0 M. The DNA-histone complexes thus formed were purified by passage through Bio-Gel A15m (100 to 200 mesh) columns (1 cm×50 cm). Free histones were removed from the DNA-histone complexes. The columns were washed with 20 to 25 times the void volume with 10 mM $NaHSO_3$, 10 mM Tris-HCl (pH 7.5) and 5.0 M urea or with 10 mM $NaHSO_3$ and 10 mM Tris-HCl (pH 7.5). The excluded fractions were dialyzed for 12 h against 500 volumes of 1.0 mM Tris-HCl (pH 7.5). These procedures were performed at 0 to 4°C.

Nuclease digestion and gel electrophoresis. DNA-histone complexes (100 μg/ml) were digested for 10 min at 37°C with 20 μg/ml of staphylococcal nuclease (Worthington; 6,000 units/mg) in 0.34 M sucrose-buffer A as described (2). Digestion was terminated by the addition of EDTA, SDS and NaCl (2). Each digestion product was extracted twice with equal volumes of a mixture of chloroform and isoamyl alcohol (24 : 1, v/v). The aqueous phase was dialyzed overnight against 20 mM Tris-HCl (pH 7.4) and 1 mM EDTA at 2 to 4°C. DNA samples were subjected to electrophoresis on 2.0% agarose slab gel (15.0 cm × 17.0 cm × 0.2 cm) for 2.5 h at 4V/cm-gel at room temperature. The gel was stained with ethidium bromide (0.5 μg/ml). Gel electrophoretic analysis of the histones extracted from reconstituted DNA-histone complexes was performed on 15% gels containing urea as described by Panyim and Chalkley (23) for 3 h at 1.5 mA/tube. The gels (0.6 cm in diameter; 10 cm in length) were stained for 3 h with 0.1% amido black in 20% methanol and 7% acetic acid.

Analytical determination of DNA-histone complex. The DNA content of complexes was estimated by absorbance at 260 nm using $E\ \frac{1\,mg/ml}{260\,nm} = 20$. The histone content of complexes was determined by the procedure of Lowry *et al.* (24). The template activities of DNA-histone complexes and DNA for RNA synthesis were estimated by measuring the incorporation of nucleoside triphosphates into acid-insoluble material in 10 min at 37°C in 0.2 ml of incubation mixture containing 40 mM Tris-HCl buffer (pH 7.9 at 4°C); 50 mM KCl; 8 mM $MgCl_2$; 2 mM $MnCl_2$; 1 mM dithiothreitol (DTT); 1 unit of *E. coli* RNA polymerase; 0.4 mM each of ATP, CTP and GTP; 0.04 mM ^{3}H-UTP (1 μCi); and 1 μg of DNA or DNA-histone complex. The reaction was terminated by adding 3 ml of cold 7% trichloroacetic acid (TCA)-0.1 M sodium pyrophosphate (SPP) solution. The reaction mixture was kept overnight in an ice-bath. The precipitate was collected on Whatmann "GF/C" filters, washed with 30 ml of 7% TCA-0.1 M SPP and dried. The precipitates were counted in 10 ml of toluene scintillation liquid (4.00 mg/ml PPO-0.25 mg/ml POPOP) with a scintillation counter.

Electron microscopy of DNA-histone complex. Samples of the DNA-histone complex for fractions 8 to 10 in Fig. 1 were prepared by rotary shadowing and negative staining. The DNA concentration in the final complex solution was 10 to 20 μg/ml. This solution was placed directly on carbon-coated collodion grids. The grids were dipped into 90% ethanol, dried on filter papers and shadowed with platinum : palladium (80 : 20) at an angle of about 7°. The negatively contrasted specimens were prepared by directly staining the grids with 1% uranyl acetate. The grids prepared by each method were observed with a electron microscope. The distance between knobs of complex fibers was measured with a map ruler.

RESULTS

Preparation of DNA-histone complexes. Three types of DNA-histone complexes were reconstituted from calf thymus histones and calf thymus DNA. Complex A consisted of histone H1 and DNA; Complex B consisted of histones

H2A, H2B, H3, H4 and DNA; and Complex C consisted of histones H1, H2A, H2B, H3, H4 and DNA. The three DNA-histone mixtures in the salt-urea and salt methods had only slight differences in exclusion chromatographic patterns in salt gradient dialysis against 0.6 M NaCl (Fig. 1a). In salt gradient dialysis

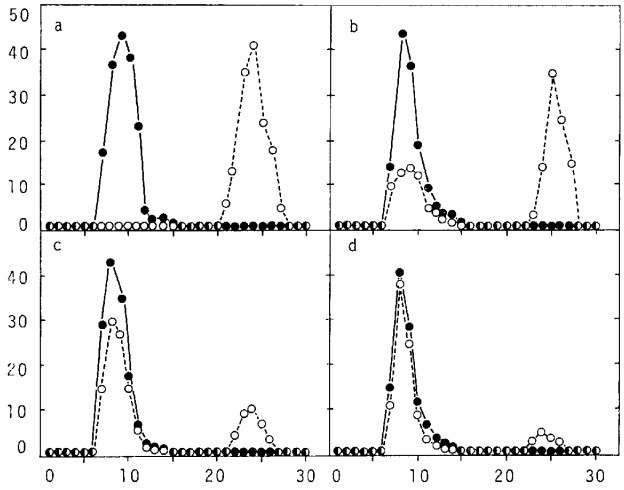

Fig. 1. Exclusion patterns of total DNA and histone contents in DNA-histone mixture and complexes reconstituted by the salt-urea method. (a) DNA-histone H1 mixture prepared by dialysis at an ionic strength of 0.6 M NaCl. (b) Complex A (DNA-histone H1). (c) Complex B (DNA-histones H2A, H2B, H3 and H4). (d) Complex C (DNA-whole histones). Abscissa: fraction number; ordinate: DNA and histone contents of the complexes. ●—●, weight of DNA (μg/ml); ○···○, weight of histone (μg/ml); and ◐, points of overlap of ● and ○.

against 0.15 M NaCl, Complexes A and C became turbid earlier than Complex B. Histone H1 may be responsible for this difference. At 5 h after dialysis against 0.15 M NaCl, all complexes became gelatinous.

The Bio-Gel filtration profiles of the reconstituted DNA-histone complexes in the presence of urea are shown in Figs. 1b, 1c and 1d. The profiles in the absence of urea were also similar but are not shown. The DNA-histone complexes were eluted at void volume, and the free histones were eluted within fractions 20 to 30. The purified complexes in fractions 8 to 10 were pooled, and the weight ratio of histone to DNA for Complexes A, B and C was determined (Table 1).

Gel electrophoretic patterns of the histone fractions associated with DNA are shown in Fig. 2. The digestion fragments from Complex A gave a single band on agarose gels corresponding to a DNA size of fewer than 100 base pairs (Figs. 3a and 3b), but the size was not determined accurately. Both of the

TABLE 1. TEMPLATE ACTIVITIES OF RECONSTITUTED DNA-HISTONE COMPLEXES FOR *in vitro* RNA SYNTHESIS

Template	Histone/DNA ratio (w/w)	[^{3}H]UMP incorporation (cpm)	Template activity relative to DNA
Control DNA	0	21,815	1.00
Complex A[a]	0.35	3,992	0.18
	(0.40)[b]	(3,272)	(0.15)
Complex B[c]	0.71	9,140	0.42
	(0.81)	(8,944)	(0.41)
Complex C[d]	0.89	2,901	0.13
	(0.95)	(3,054)	(0.14)

a DNA-histone H1 complex. *b* The values with and without brackets indicate the values of transcription with complexes prepared by the salt and salt-urea methods, respectively, as described in the text. *c* DNA-histones H2A, H2B, H3 and H4 complex. *d* DNA-whole histones complex.

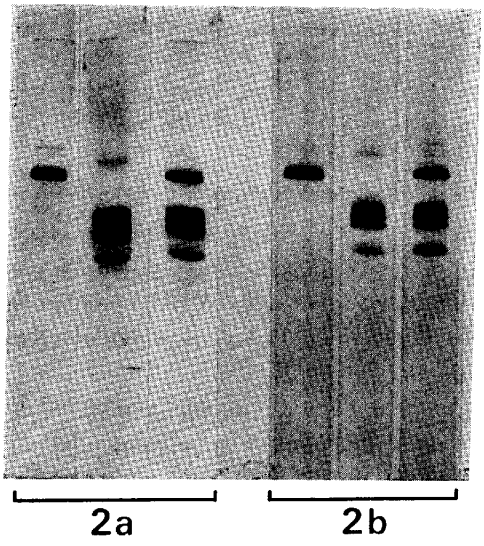

Fig. 2. Gel electrophoretic patterns of histone fractions associated with DNA in the absence (2a) and presence (2b) of urea. Histone fractions extracted from the complexes were subjected to electrophoresis. (Left to right (a), (b)) Histone fraction extracted from DNA-histone H1 complex, DNA-histones H2A, H2B, H3 and H4 complex and DNA-whole histones complex.

DNA fragments from Complexes B and C had regular patterns of bands as chromatin (Figs. 3c, 3d, 3e and 3f). However, these repeated structures were partially disrupted in the presence of urea during complex formation (Figs. 3c and 3e).

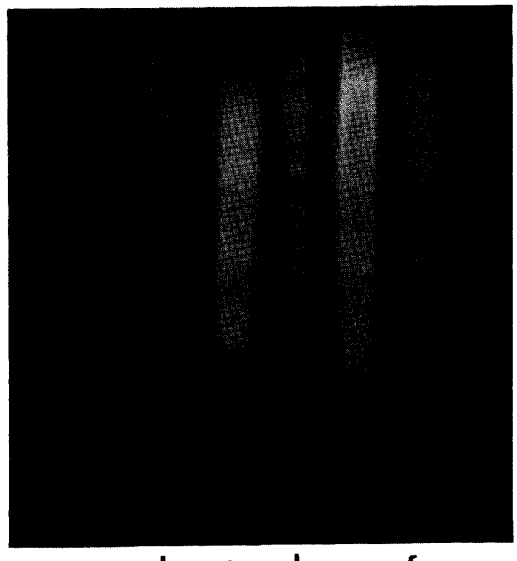

Fig. 3, Nuclease digestion patterns of several DNA-histone complexes reconstituted in the presence (a, c and e) and absence (b, d and f). DNA-histone H1 (a and b); DNA-histones H2A, H2B, H3 and H4 complex (c and d); and DNA-whole histones complex (e and f). Migration is from top to bottom.

Electron microscope observations. Complex A had fiber structures consisting of knobby regions alternating with thinner regions. The smallest knobs were about 100 Å in diameter and the largest about 250 Å. The distance between knobs was about 1,100 Å for an average interval. The thinner thread-like fibers were 20 to 30 Å in width. The thinner fibers that linked those knobs appeared as histone-free DNA regions.

Complex B consisted of globular subunits 100 to 150 Å in diameter alternating with thin strands, like beads on a string (Figs. 4b, 4e). Although a repeated structure similar to chromatin was observed at intervals of about 70 to 100 Å (Fig. 4e), the globular structure disrupted as the concentration of urea was increased during reconstitution (Fig. 4b). These conformational changes were also confirmed by a method involving limited digestion as described above (Figs. 3c, 3d, 3e, 3f).

Complex C consisted of thicker fibers with partially condensed regions (Figs. 4c, 4f). The thicker fibers were about 200 to 250 Å in width; that is, about corresponded to ten to twelve times the width of naked DNA. Condensed regions were seen in different areas of the same sample. The repeated structure was confirmed in this complex with nuclease digestion also (Figs. 3e, 3f). The three types of structures described here were also observed in samples prepared by rotary shadowing.

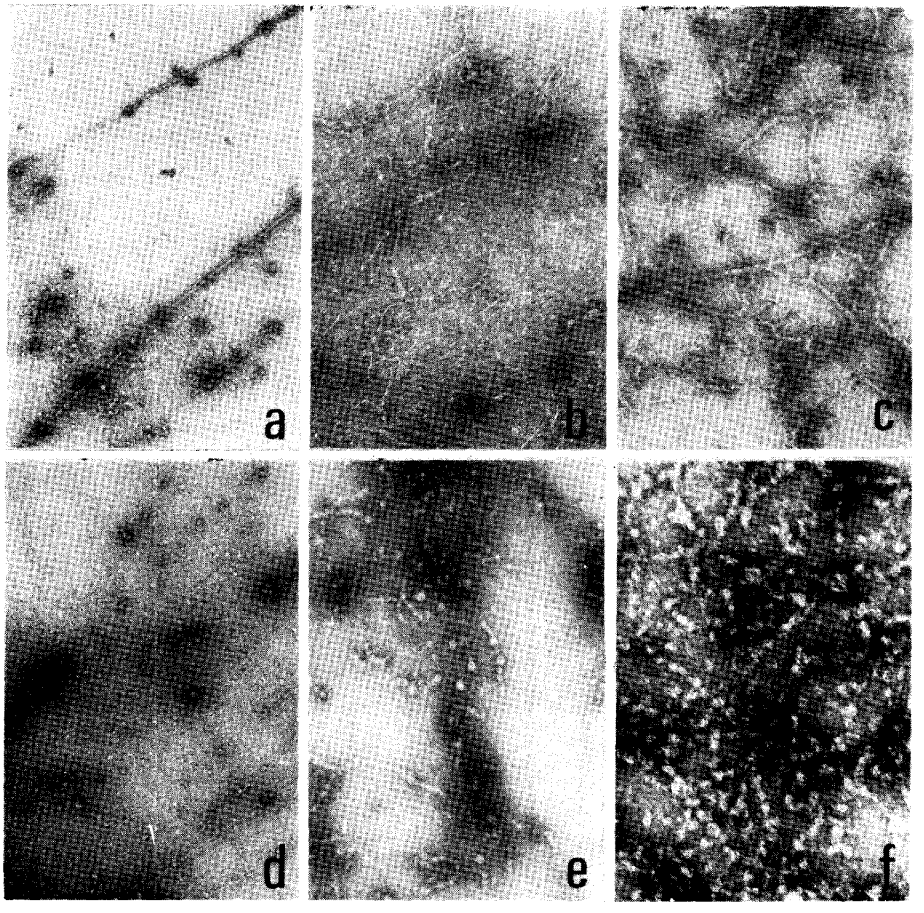

Fig. 4. Electron micrograph of calf thymus DNA-histone complexes reconstituted in the presence (a, b and c) and absence (d, e and f) of urea. (a and d) DNA-histone H1. (b and e) DNA-histones H2A, H2B, H3 and H4. (b) Globular subunits are seen at intervals of about 70 to 100 Å. (e) Subunit structures (diameter 100 to 250 Å) are seen at intervals of about 70 to 100Å. (c and f) DNA-whole histones. ×46,000.

Template activity of DNA-histone complex. The last two columns of Table 1 show the template activities of reconstituted complexes and the ratios of template activity of the complexes to that of naked DNA used as control. Complex B had an activity 42% of the control DNA whereas Complexes A and C had 18% and 13%, respectively. Complexes reconstituted in the absence of urea had template activities slightly less than those of the complexes reconstituted in the presence of urea.

DISCUSSION

Complex A was characterized by fiber structures with knobs along the fibers (Figs. 4a, 4d). The knobs of these fibers were interpreted as arising from the interaction between the H1 histones complexed with DNA and may have folding structures of DNA. Digestion of chromatin fibers with deoxyribonuclease (DNase) has revealed that the blocking effect of histones on DNA accessibility to DNase is severely limited by histone proteins complexed with DNA (25–27) and that the lysine-rich histones H1 and H2B block accessibility more effectively than the arginine-rich histones H3 and H4 (26). This blocking effect of histone H1 may be related to conformational changes of complex fibers, such as the knob structure described here. It has been reported that the DNA-H1 complex exhibited a biphasic melting process (28–30). The first-step (about 45°C) of transition was due to the histone H1-free DNA regions and the second-step (about 75°C) to the DNA regions complexed with histone H1 (30, 31). Therefore, according to the fiber size, the histone-to-DNA ratio (0.35–0.40, wt/wt) and the melting profiles, the thinner regions of fibers described above may be histone H1-free DNA regions.

Complex B was characterized by a repeated structure similar to chromatin (Figs. 3c, 3d, 4b, 4e). The disruption of repeating structures by urea probably arises from perturbation of the specific interaction among the histones complexed with DNA fibers. The configurational changes are consistent with those of previous studies of urea denaturation of chromatin periodic structure (32, 33).

Complex C was characterized by thicker fibers and partially condensed configurations (Figs. 4c, 4f) and was composed of the repeated structure similar to chromatin (Figs. 3e, 3f). These features partially resemble those of chromatin reported by others (34, 35, 36). Electron microscopic studies have shown that chromatin fibers are relatively homogeneous in width, although the diameter of the unit fiber has been variously estimated as 30 to 40 Å (37), 100 Å (34) and 230 Å (35). Nonuniform condensed protuberances were often observed in this complex. This irregularity has been reported in native chromatin (34, 35, 36). These condensed regions were interpreted as localized folding rather than higher localized histone concentrations. Magnetic resonance studies have suggested that histone H1 is necessary for the condensation of chromatin (38). It is speculated that nonhistone proteins are concerned with the fluidity of various higher order structures of chromatin to accompany the cell cycle or RNA synthesis. It appears that the intact chromatin fibers are partially heterogeneous and that the heterogeneity is closely related to histone H1 and nonhistone proteins. Of course, other factors may be at least partially responsible for these configurational changes, such as charge effects (especially at low ionic strength) or an increase in histone aggregations, or an increase in the extent of complex fiber aggrega-

tion.

Complexes A and C inhibited RNA synthesis more efficiently than the Complex B (Table 1). This is consistent with the results reported by others (14–16, 39) and strongly suggests that histone H1 inhibits *in vitro* RNA synthesis more efficiently than other histones.

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