Structure of active chromatin: higher-order folding of transcriptionally active chromatin in control and hypothyroid rat liver

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Investigations have been carried out into the salt-induced higher-order folding in the transcriptionally active chromatin region of rat liver nuclei by nuclease digestion, sedimentation and CD. The sensitivity of active chromatin in nuclei to micrococcal nuclease was suppressed by raising the ionic strength from 25 to 90 mM, indicating the occurrence of salt-induced condensation. The rate of sedimentation of fractionated inactive chromatin fragments of both moderate (~ 3.5 kbp) and large (~ 8.8 kbp) size increased maximally to the same extent, while that of active chromatin fragments was dependent on their size. The rate of sedimentation of moderately sized active chromatin fragments (~ 5.5 kbp) showed a maximal 15% increase at 90 mM ionic strength. In contrast, a large increase (at least 60%) in the sedimentation rate of large active chromatin fragments (~ 21 kbp) was observed at

65 mM ionic strength. A reasonable degree of higher-order folding was observed in large active chromatin fragments even at 25 mM ionic strength. On considering the percentage increase in sedimentation rate as a measure of the higher-order folding of chromatin, a different type of higher-order folding was observed in active chromatin fragments. Although the percentage increase in sedimentation decreased from 40 to 24% with an increase in the size of active chromatin from ~ 3 to ~ 9 kbp, a further increase in size up to 16 kbp brought the percentage increase back to 40%. CD studies agreed with the conclusions drawn from sedimentation studies. Active chromatin from hypothyroid rats showed similar folding behaviour, but the order of folding was slightly lower than for control active chromatin, at all sizes.

INTRODUCTION

In the eukaryotic genome, DNA is folded at a primary level by wrapping around histone octamers at regular intervals, forming a 10 nm thick polynucleosomal filament. On raising the ionic strength to a near physiological level, this filament undergoes histone-H1-mediated folding into 30 nm fibres [1]. This is referred to as 'higher-order folding' and is a characteristic feature of transcriptionally incompetent chromatin in interphase nuclei. The globular domain of histone H1 is located between the entering and leaving ends of nucleosomal DNA. Its C-terminus with its highly basic extended tail brings about the higher-order folding [2]. Transcriptionally active chromatin has been shown to possess a polynucleosomal structure, but with an unknown type of alteration in the nucleosomal structure [3]. Active chromatin is also known to lack nucleosomal structure at some specific sites in the 5' upstream regulatory regions of genes [4]. Very specific models for the condensed fibre have been proposed [5,6] which are essentially refinements of the Finch and Klug solenoid model. On the other hand, it is presumed that zig-zag arrays of nucleosomes exist in solutions of low ionic strength [7–9]. Further compaction of the zig-zag leads to a helical structure with distinctly different properties from the solenoid model. In the zig-zag model the nucleosomes in the beaded chain are not consecutive, and the linker DNA is not coiled but remains extended. A number of non-symmetrical models have also been proposed. However, no single model has been proved to be the correct structure. Using low-temperature preparation techniques, it has been observed that the fibre has continuously variable structure [10].

Extensive investigations have been carried out to discover whether active genes are devoid of higher-order folding. This idea gained support from early studies showing that histone H1

is absent from active chromatin [11–14]. However, later studies presented evidence in favour of the presence of H1 in active chromatin [1,15,16]. This controversial situation has been attributed to the rapid rearrangement and exchange of H1 from active chromatin fragments during its isolation at the moderate salt concentrations used in the above studies [17]. It has been shown by cross-linking studies that H1 is located in *Drosophila* heat-shock genes [18], other genes of puffed regions [19], active Balbiani ring genes in chironomus polytene chromosomes [20], potentially active genes in chicken erythrocytes [21] and transcriptionally active genes in *Tetrahymena thermophila* [22]. However, a partial depletion of linker histones has been reported, on the basis of a slightly decreased efficiency of cross-linking of H1 to DNA [21,22]. Alternatively, the lower level of cross-linking of H1 to DNA may not be due to its partial depletion, but to a change in the mode of binding to the altered active nucleosomal structure [18].

Potentially active β -globin gene chromatin fragments from chicken erythrocytes showed a 50% decrease in higher-order folding compared with inactive chromatin [23]. This fragment contains a part of 5' upstream and downstream regions containing DNase I-hypersensitive sites. Sedimentation studies with shorter fragments, containing only the coding region, did not reveal any differences in higher-order folding compared with bulk chromatin fragments [24]. Only a small difference in the degree of higher-order folding of active chromatin as compared with inactive chromatin was observed in micrococcal nuclease (MNase)-digested chromatin from chicken oviduct [25]. Electron microscopic studies have indicated that the fully extended chromatin fibre of active Balbiani ring genes is rapidly folded into 30 nm fibres between consecutive RNA polymerases [26]. The property of inactive chromatin whereby MNase-cleaved fragments are held together is absent from active chromatin [27].

Abbreviation used: MNase, micrococcal nuclease.

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Previous experiments have often utilized cells that were relatively inactive. The folding of chromatin in such cells may not be typical of eukaryotic cells in general [28]. The above studies on higher-order folding have also involved the use of total chromatin at a salt concentration at which the possibility of rearrangement and exchange of H1 from active chromatin (low-affinity sites) to inactive chromatin (high-affinity sites) cannot be ruled out. Hence we planned to study in detail the higher-order folding of active chromatin isolated from rat liver nuclei under low-ionic-strength conditions (with minimum perturbation) using nuclease digestion, sedimentation and CD. In addition, studies have been extended to monitor the effects of hypothyroidism on the higher-order folding of active chromatin. Hypothyroidism in the rat was chosen for these studies because the effect of this condition on transcription is confined to only 10 % of transcriptionally active genes [29,30], while its action on growth retardation is linked to the entire genome, consisting of total active and inactive chromatin. Thus any changes observed in the folding of total active chromatin on induction of hypothyroidism will be mainly associated with growth of the tissue.

MATERIALS AND METHODS

All operations were carried out at 0–4 °C, unless otherwise indicated.

The isolation of rat liver nuclei and transcriptionally active chromatin, measurement of the kinetics of nuclease digestion of nuclei, DNA extraction and its electrophoretic analysis, electrophoresis of nuclear proteins and nucleic acid estimations were carried out as described in the accompanying paper [30a]. The induction of hypothyroidism in rats was carried out as described in the preceding paper [30b].

Isolation of a transcriptionally inactive chromatin fraction

It has been observed in our laboratory that, when nuclei are digested with endonucleases at 4 °C, no active chromatin fragments are extracted into the medium. However, a similar digestion with MNase at 4 °C yields chromatin fragments that are extracted efficiently. This latter supernatant, expected to be depleted in active chromatin, was utilized for further studies. This observation enabled us to isolate chromatin fragments depleted in active chromatin. Nuclei were digested with MNase (1 unit/mg of DNA) at 4 °C for 90 min in buffer A (10 mM Tris/HCl, pH 7.8, 10 mM NaCl, 1 mM PMSF), so that the bulk chromatin was digested to oligo- and poly-nucleosomal fragments. The resulting solution was centrifuged at 12000 *g* for 10 min and the supernatant was collected. EDTA was added to a final concentration of 2.5 mM to inhibit nucleases. This preparation was termed 'inactive chromatin'.

Fractionation of chromatin fragments

Active and inactive chromatin (10–15 A_{260} /ml) isolated by the above procedure were passed through a Sepharose 4B column (30 cm × 1 cm) equilibrated with low-salt buffer (buffer A containing 2.5 mM EDTA). Fractions of 1 ml were collected and their absorbance measured at 260 nm. The fractions at the void volume peak were used for various studies. Other fractions of the column profile were discarded.

Alteration of the ionic strength of chromatin solutions

Boiled RNase was added to the peak fraction obtained from the above step. This solution was slowly dialysed against buffers of the required ionic strength at 4 °C for 12 h. Dialysates were

centrifuged at 12000 *g* for 10 min to remove any aggregated material. Supernatants were collected and the absorbance at 260, 280 and 320 nm was measured. These chromatin samples were used for various studies within a few hours of dialysis against the respective ionic strength buffers.

Sedimentation studies

Chromatin samples (200 μ l; 3 A_{260} /ml), obtained after dialysis as described above against buffers of appropriate ionic strength, were loaded on 5–25 % isokinetic sucrose gradients in the same buffer. The tubes were centrifuged at 4 °C using an SW 50.1 swing-out rotor in a Beckman L8-55M ultracentrifuge at 85000 *g* for 2 h 15 min. Gradients were fractionated on an ISCO gradient analyser, and 0.3 ml fractions were collected while monitoring the absorbance at 254 nm. DNA extracted from these fractions was analysed by agarose gel electrophoresis. Peak fractions were also analysed by SDS/PAGE to confirm the intactness of proteins.

CD studies

RNA-free chromatin samples dialysed against buffers of different ionic strengths, as described above, were taken at a concentration of 1 A_{260} /ml for CD measurements. A 1 cm path-length cell was used, and CD spectra were recorded between 250 and 320 nm at 25 °C on a JASCO J500A spectropolarimeter. Although the samples were not visibly turbid, we measured their absorbance at 320 nm. Molar ellipticities were calculated using the formula:

$$[\theta]_{\text{m.r.w.}} = 100 \times \theta_{\text{obs}}/cl$$

where θ_{obs} is the observed ellipticity in degrees, c is molar concentration and l is the path length. We used a molar absorption coefficient for DNA of 6600 $\text{M}^{-1} \cdot \text{cm}^{-1}$. Molar ellipticity is expressed in degrees $\cdot \text{cm}^2 \cdot \text{dmol}^{-1}$.

RESULTS

Nuclease-sensitivity studies

In earlier studies, nuclease digestions were carried out in the presence bivalent cations at concentrations which maintain the chromatin in a higher-order folded form. Thus the effect of nuclease-sensitivity on the higher-order folding of chromatin could not be checked. In contrast, we carried out the endogenous digestion of nuclei in the absence of bivalent cations. By raising the univalent salt concentration from 25 to 90 mM, we were able to estimate the extent of suppression of the nuclease-sensitivity of active chromatin showing higher-order folding. This active chromatin is a preferentially digested minor chromatin fraction representing active genes (see the accompanying paper [30a]). Therefore we carried out the digestions with nuclei from control and hypothyroid rats at ionic strengths of 25 and 90 mM and measured the time of appearance of the preferentially digested minor chromatin fraction at different salt concentrations (Figure 1). In control nuclei, a faint DNA smear appeared within 2 min of digestion at 25 mM ionic strength (Figure 1A). A similar minor DNA smear appeared between 5 and 10 min of digestion on raising the ionic strength to 90 mM (Figure 1B). The rise in ionic strength from 25 to 90 mM led to a suppression of nuclease-sensitivity of control active chromatin of between 2.5- and 5-fold. In the case of nuclei from hypothyroid rats, a similar extent of digestion was observed after 4 and 16 min at 25 and 90 mM respectively, indicating an approx. 4-fold suppression of nuclease-sensitivity by the increase in ionic strength (Figures 1C and 1D).

Figure 1 Influence of ionic strength on the nuclease-sensitivity of chromatin from control and hypothyroid rat liver

Nuclei from control (**A, B**) and hypothyroid (**C, D**) animals were incubated with MNase (0.36 unit/mg of DNA for control and 0.12 unit/mg of DNA for hypothyroid) at 37 °C in 10 mM Tris/HCl buffer containing 1 mM PMSF and 10 mM NaCl (**A, C**) or 90 mM NaCl (**B, D**) for the times indicated. DNA (50 µg) from these digests was electrophoresed in a 0.7% agarose gel and stained with ethidium bromide.

These observations indicate the presence of higher-order folding in active chromatin, and suggest that hypothyroidism has little effect on this folding.

Isolation of transcriptionally inactive chromatin

For sedimentation studies, it was necessary to isolate chromatin that was free of active chromatin. This was achieved by incubation of nuclei in the absence and presence of MNase at 4 °C. Figure 2 shows the agarose gel electrophoresis pattern of nuclei under these conditions. When nuclei were incubated in the absence of MNase at 4 °C, chromatin was preferentially digested by endonucleases present in the nuclei (lighter smear below bulk DNA band; Figure 2, lane b). Preferentially digested chromatin which belongs to active gene class (see the accompanying paper [30a]) was not extracted (lanes c and d). This may be due to its association with the nuclear matrix; the latter is destroyed on incubation at 37 °C, and thus the active chromatin is extracted [30a]. On the other hand, incubation of nuclei in the presence of MNase at 4 °C generated chromatin of oligo- and polynucleosomal size (lane e) which was extracted efficiently (lanes f and g). In our experiments, the bulk chromatin represented 65% of the total chromatin. This provides evidence that active chromatin is associated with the nuclear matrix at 4 °C and is not released into the supernatant.

Figure 2 Isolation of inactive chromatin

Nuclei were incubated at 4 °C with and without MNase (1 unit/mg of DNA) in 10 mM Tris/HCl, pH 7.8, containing 10 mM NaCl and 1 mM PMSF. Lane a, nuclei at zero time (100 µg of DNA); lane b, nuclei after 120 min of incubation in the absence of MNase (100 µg of DNA); lanes c and d, DNA was extracted from the supernatant after 120 min of incubation in the absence of MNase from 300 and 600 µg of nuclei respectively; lane e, nuclei were digested with MNase for 90 min; lanes f and g, DNA was extracted from the supernatant, after 90 min of incubation with MNase, from 45 and 90 µg of nuclei respectively.

Fractionation of chromatin

Figure 3(A) shows the elution profile of chromatin fractions on a Sepharose 4B column. The fraction at the void volume (10 ml) peak was analysed by agarose gel electrophoresis (Figure 3B) and SDS/PAGE (Figure 3C). Chromatin fragments of sizes above octanucleosome were eluted in the void volume, while fragments of lower size, as well as ribonucleoprotein particles, were eluted later (results not shown). Thus the chromatin fragments of greater size, free of ribonucleoprotein particles, were obtained. Figure 3(B), lane d clearly indicates that the chromatin fraction of the void volume was free from RNA and short oligonucleosomes of less than octanucleosomal size. SDS/PAGE of this chromatin indicated that only a small amount of non-histone protein remained associated with it.

Inactive chromatin was fractionated in a similar manner before being utilized in other studies.

Sedimentation studies

The effect of ionic strength on the rate of sedimentation of moderately sized active and inactive chromatin fragments is shown in Figure 4(A). Bell-shaped profiles are observed in both cases. The sizes of the fractions at the peak of the sedimentation profile at different ionic strengths are compared. On raising the ionic strength to 65 and 90 mM, the rate of sedimentation of inactive chromatin (~3.5 kbp size) was increased by 15% and 40% respectively. This indicates complete folding at 90 mM ionic strength. However, in the case of active chromatin, no clear profile was observed with a distinct peak at 65 mM ionic strength. However, at 90 mM a clear peak was observed. The fraction at the peak (~5.5 kbp) showed a slightly higher rate of sedimentation (15%) than at 25 mM ionic strength.

A larger inactive chromatin fraction (~8.8 kbp) did not show an altered sedimentation rate compared with smaller fragments at any ionic strength (Figure 4B, right-hand panel). As with moderately sized inactive chromatin, similar increases of 15% and 40% in the sedimentation rate were observed at 65 mM and 90 mM ionic strength respectively. With active chromatin fragments of larger size (~21 kbp), the rate of sedimentation was

Figure 3 Fractionation of chromatin on Sepharose 4B

(A) An active chromatin fraction obtained as the supernatant from nuclei after a brief digestion (1.5 ml of 36 A_{260}) was applied on a Sepharose 4B column (1 cm $\times$ 50 cm) and 0.5 ml fractions were collected. (B) Agarose-gel electrophoresis of DNA at various stages. Samples of 50 μ g of DNA were loaded in each lane. The samples were not treated with RNase. The gel was stained with ethidium bromide and photographed under UV light. DNA was from: lane a, nuclei; lane b, digested nuclei; lane c, supernatant recovered from digested nuclei; lane d, chromatin fraction from void volume. (C) SDS/PAGE of proteins. Proteins from active chromatin were extracted with 0.25 M HCl. The gel was stained with Coomassie Blue. Lanes: M, marker proteins; a, unfractionated active chromatin; b, active chromatin after fractionation. Positions of histones are indicated.

increased by 60% on increasing the ionic strength to 65 mM (Figure 4B, left-hand panel). The rate of sedimentation of active chromatin did not show any further increase even at 90 mM ionic strength. Figure 4(C) shows the sizes of the DNA used for sedimentation studies and the peak fractions at different ionic strengths.

The folding behaviour of inactive chromatin is independent of its size. Active chromatin fragments of moderate size show a lower level of folding, while those of greater size show a higher degree of salt-induced folding.

Sedimentation studies with active chromatin from hypothyroid rats

Similar sedimentation studies were performed with active chromatin from hypothyroid rat liver (Figure 5). The sizes of the DNAs of active chromatin from control and hypothyroid rat livers that were loaded on the gradient are shown in Figure 5(C). Sedimentation profiles of active chromatin from control and hypothyroid rats at various ionic strengths are shown in Figures 5(A) and 5(B) respectively. At 90 mM ionic strength the sedimentation profiles of active chromatin from both control and hypothyroid rats were shifted towards the bottom of the gradient, indicating the presence of salt-induced higher-order folding.

To investigate the relationship between the order of folding and the size of the chromatin fragments, various fractions of the sedimentation study were analysed by agarose-gel electrophoresis (Figure 6). From the electrophoretic mobilities of DNA size markers, a plot was obtained of distance migrated against log molecular size (Figure 7A). From this standard graph, the sizes of the DNAs in the chromatin of the sucrose-density-gradient fractions were calculated. Since this sucrose gradient is an isokinetic gradient, the fraction number will be directly proportional to the sedimentation rate of the chromatin. A linear plot was obtained for the control active chromatin at 25 mM ionic strength between 2.5 and 17 kbp (Figure 7B), but the line bends gradually towards the right above $\sim$ 17 kbp. On raising the ionic strength from 25 mM to 90 mM the plot was shifted

more to the right, indicating higher-order folding. In addition, the bending started at a lower size ($\sim$ 10 kbp) at 90 mM ionic strength.

To measure higher-order chromatin folding, a graph was drawn of percentage change in sedimentation rate against DNA size (Figure 7C). The curve was biphasic for chromatin fragments of 3–17 kbp at 25 mM ionic strength. From 3 to 9 kbp the percentage change in sedimentation rate decreased gradually from 42% to 22%, but above this size it again increased gradually, to 40% at $\sim$ 17 kbp. In the case of inactive chromatin, the percentage change in sedimentation rate was 40% for chromatin fragments of 3.5 and 8.8 kbp under similar conditions (Figure 4). Inactive chromatin fragments of greater size should also show the same percentage change in sedimentation rate, as indicated by the shape of the sedimentation profile (Figures 4A and 4B). This also supports the result of Figure 4(B) showing that the active chromatin fragment of $\sim$ 21 kbp undergoes a 60% increase in sedimentation rate on increasing the ionic strength from 25 to 90 mM. Thus the maximum difference in higher-order folding between active and inactive chromatin fractions is reflected only in fragments in the moderate size range ($\sim$ 9 kbp). This difference decreases with chromatin fragments of smaller or greater size. Active chromatin from hypothyroid rats also showed a linear response, but the curves were shifted to the left at both 25 and 90 mM ionic strength, indicating that these active chromatin fragments have a slightly lower level of folding in comparison with those from control animals (Figure 7B). When the ionic strength was raised to 90 mM, the percentage increase in sedimentation rate had a minimum value of 22% at $\sim$ 9 kbp. If the size of the chromatin was either greater or less than 9 kbp, the percentage increase in sedimentation rate increased to 32% (Figure 7C). As the studies were mainly concerned with changes in the higher-order folding of active chromatin caused by hypothyroidism, no attempts were made to analyse the sizes of the DNA fragments in the gradient fractions after sedimentation of inactive chromatin at various ionic strengths.

Figure 4 Effect of ionic strength on the sedimentation of active and inactive chromatin

Experimental details were as described in the Materials and methods section. For (A) and (B), the left panels show active chromatin and the right panels show inactive chromatin. Sedimentation profiles of fractionated chromatin are shown: (A) moderately sized fragments (0.6 of A_{260}); (B) large-sized fragments (0.6 of A_{260}), at an ionic strength of 25 mM (solid line), 65 mM (dashed line) and 90 mM (dotted line). (C) DNA from chromatin fractions used for sedimentation: lane a, nuclei (50 μ g); lane b, digested nuclei (50 μ g); lane c, unfractionated active chromatin (50 μ g); lane d, MNase-digested nuclei for isolation of inactive chromatin (50 μ g); lane e, unfractionated inactive chromatin; lanes f, g and h, moderately sized active chromatin fragments dialysed against 25, 65 and 90 mM ionic strength buffers respectively; lanes i, j and k, fractionated large inactive chromatin fragments dialysed against 25, 65 and 90 mM ionic strength buffers respectively; lanes l, m and n, peak fractions from sedimentation profiles of moderately sized active chromatin fragments at 25, 65 and 90 mM ionic strength respectively; lanes o, p and q, large inactive chromatin fragments at 25, 65 and 90 mM ionic strength respectively; lane r, peak fraction from sedimentation profile of large fractionated active chromatin fragments at 25 mM ionic strength; lanes s, t and u, peak fractions from the sedimentation profile of inactive chromatin fragments of moderate size at 25, 90 and 65 mM ionic strength respectively. Lanes M contain DNA size markers.

The increase in the rate of sedimentation was due to increased folding of the chromatin fragments, and not to aggregation. This was concluded on the basis of the following observations: (i) the DNA bands in successive fractions of the sucrose gradients showed an upward shift, and (ii) the broadness of the DNA bands (Figure 6) was as expected on the basis of diffusion of DNA in the sucrose gradient. The right half of the curve (Figure 7C) is questionable because of poor resolution of DNA in the large size range. Possible contamination of large-sized active chromatin fragments with inactive gene sequences cannot be ruled out. However, the left half of the curve appears to be reliable.

CD studies

Next we carried out studies on the salt-induced higher-order

folding of chromatin using CD. CD spectra of active and inactive chromatin fragments were recorded at 25, 65 and 90 mM ionic strength. Figure 8 shows the sizes of the DNA from control and hypothyroid rat liver used for CD studies. Moderately sized active chromatin fragments (peak at ~ 5.5 kbp) had a CD spectrum (Figure 9A) similar to that of inactive chromatin at 25 mM ionic strength (Figure 9B). The molar ellipticity at 273 nm was identical for active and inactive chromatin. However, a 20% decrease in ellipticity at 273 nm was observed for the active chromatin when the ionic strength was increased from 25 to 90 mM, and the CD spectrum showed a dip above 290 nm. A larger decrease of 44% in ellipticity at 273 nm was observed with inactive chromatin, but the CD spectrum did not show the dip at 290 nm.

Larger active chromatin fragments (average size ~ 21 kbp) (Figure 9C) showed a molar ellipticity at 273 and 282 nm of 60%

Figure 5 Effect of ionic strength on the sedimentation of active chromatin from control and hypothyroid rat liver

Sedimentation analyses of active chromatin isolated from control and hypothyroid rat liver were performed as described in the Materials and methods section. Sedimentation profiles are shown of (A) control rat liver active chromatin ($1 A_{260}$) and (B) hypothyroid rat liver active chromatin ($1 A_{260}$) at an ionic strength of 25 mM (solid line) and 90 mM (dashed line). (C) DNA was extracted from samples utilized for sedimentation studies. DNA from control liver nuclei: lane a, nuclei at zero time; lane b, nuclei digested for 5 min; lane c, supernatant from nuclei digested for 5 min; lanes d and e, active chromatin at 25 mM and 90 mM ionic strength respectively. DNA from hypothyroid liver nuclei: lane f, nuclei at zero time; lane g, nuclei digested for 5 min; lane h, supernatant from nuclei digested for 5 min; lanes i and j, active chromatin at 25 mM and 90 mM ionic strength respectively. Samples of 50 μ g of DNA were loaded in each lane.

of that of inactive chromatin of similar size at 25 mM ionic strength. The dip above 290 nm was again present (Figure 9C); this is a characteristic feature of folded active chromatin. This spectrum suggests that large active chromatin fragments display some folding even at 25 mM ionic strength, as observed in the sedimentation studies (Figure 7B). An increase in ionic strength to 65 mM caused a greater decrease in the molar ellipticity at 273 nm of active chromatin (50%) compared with inactive chromatin (30%). No further decrease in ellipticity was found at 90 mM ionic strength for active chromatin, whereas the ellipticity of inactive chromatin fell by a further 44%. Active chromatin from hypothyroid animals, containing large fragments (peak at ~ 20 kbp), had a CD spectrum (Figure 9D) similar to that of control active chromatin of a similar size (Figures 9C and 9D), with a higher ellipticity value at 273 nm. The percentage decrease in molar ellipticity on raising the ionic strength from 25 to 90 mM was lower (36%) than for the control (50%). A dip above 290 nm was again observed.

DISCUSSION

It was reported previously that the increase in the sedimentation rate of chromatin fragments at ionic strengths up to 25 mM is due to the linear compaction of neighbouring nucleosomes, with no increase in fibre thickness [31,32]. Further increases in

Figure 6 DNA size analysis of gradient fractions from the sedimentation profiles of active chromatin from control and hypothyroid rat liver

DNA was extracted from the gradient fractions indicated. The sedimentation of active chromatin from hypothyroid (A, B) and control (C, D) rats was carried out at an ionic strength of 25 mM (A, C) and 90 mM (B, D). Lanes M contain molecular size markers.

sedimentation rate at higher ionic strength are due solely to higher-order folding, and such folding approaches near completeness at around 90 mM. To determine the higher-order folding in transcriptionally active and inactive chromatin, we have studied the differences in nuclease-sensitivity, rate of sedimentation and CD on raising the ionic strength above 25 mM.

With nuclei from both hypothyroid and control rats, the sensitivity to MNase of active as well as bulk chromatin in nuclei was decreased by 2.5–5-fold on raising the ionic strength (Figure 1). However, the differential digestion pattern of chromatin persisted even at 90 mM ionic strength. This indicates that, although its nuclease-sensitivity is decreased by higher-order folding, active chromatin is still more sensitive than inactive chromatin at higher ionic strength.

The percentage increase in the rate of sedimentation of chromatin fragments with ionic strength was used as a measure of higher-order folding. From a plot of the percentage increase in sedimentation rate against DNA size (Figure 7C), the following observations were made. (i) Control active chromatin fragments of moderate size (~ 9 kbp) had only half of the higher-order folding found in inactive chromatin. (ii) In contrast with inactive chromatin, active chromatin fragments smaller or larger than 9 kbp showed increased levels of higher-order folding. This is in agreement with the substantially lower levels of folding of potentially active β -globin gene chromatin fragments of moderate size (~ 6.3 kbp) [23]. Normal levels of folding in shorter β -globin gene fragments [24] and a slightly lower level of folding of active ovalbumin gene fragments of large size (~ 9.7 – 15.9 kbp) [25]

Figure 7 Relationship between chromatin size and electrophoretic mobility

(A) Distances migrated by the different DNA fragments of the DNA marker in the agarose gel of Figure 6. The negatives of Figure 6 were scanned and the weight average sizes of the DNA in the indicated gradient fractions were estimated. (B) Relationship between fraction number and size of DNA in that fraction. ○, Control active chromatin at 25 mM ionic strength; ●, control active chromatin at 90 mM; △, hypothyroid active chromatin at 25 mM; ▲, hypothyroid active chromatin at 90 mM. (C) The sedimentation distance of a chromatin fragment of a particular DNA size was determined at 25 and 90 mM ionic strength from (B). The percentage increase in sedimentation rate (% Δs) for that chromatin size was calculated using the formula:

$$\% \Delta s = [(d_{90} - d_{25}) / d_{25}] \times 100$$

where d_{90} and d_{25} are the sedimentation distances at 90 and 25 mM ionic strength respectively. This was repeated for each DNA size indicated in (B). Values for control inactive chromatin were calculated from the DNA gel of Figure 4. ●, Control active chromatin; ▲, hypothyroid active chromatin; ■, control inactive chromatin.

Figure 8 Protein and DNA size analysis of samples used for CD

(A) SDS/PAGE of proteins. Lanes: a, unfractionated active chromatin; b, fractionated active chromatin; c, unfractionated inactive chromatin; d, fractionated active chromatin after dialysis; e, fractionated inactive chromatin after dialysis; M, molecular mass markers. Positions of histones are indicated. (B) Agarose gel electrophoresis of DNA from large active (lanes a–c) and inactive (lanes d–f) chromatin fragments dialysed against 25 mM (a, d), 65 mM (b, e) and 90 mM (c, f) ionic strength buffers. Lane M contains DNA size markers. (C) Agarose gel electrophoresis of DNA used for CD. Sources of DNA were as follows. Control rat liver: lane a, nuclei at zero time; lane b, nuclei digested for 5 min; lane c, supernatant from nuclei digested for 5 min; lanes d and e, moderately sized active chromatin fragments at 25 and 90 mM ionic strength respectively. Hypothyroid rat liver: lane f, nuclei at zero time; lane g, nuclei digested for 5 min; lane h, supernatant from nuclei digested for 5 min; lanes i and j, large active chromatin fragments at 25 and 90 mM ionic strength respectively.

were observed on comparison with inactive gene fragments of similar sizes. However, the dependence of folding on the size of the chromatin fragment was not investigated in these studies. The folding of control active chromatin fragments of large size approached a near plateau level at 65 mM, in contrast with only a partial folding of inactive chromatin fragments at this ionic strength. Fisher and Felsenfeld [25] reported that the folding of active ovalbumin gene fragments of larger size (~ 9.7 – 15.9 kbp) was optimal at 50 mM ionic strength, whereas an ionic strength of 100 mM was required for complete folding of inactive β -globin gene fragments of similar size.

In the CD studies (Figure 9) a 44% decrease in molar ellipticity at 273 nm was observed on higher-order folding of inactive

chromatin, which indicates that this was mediated by bending of the DNA. A similar fall in molar ellipticity at 273 nm was observed for active chromatin, which was also due to higher-order folding. The decrease in molar ellipticity was greater for large than for moderately sized active chromatin fragments. Large active chromatin fragments from hypothyroid animals showed a smaller decrease in ellipticity than active chromatin fragments of a similar size range from controls. Thus the CD observations support the conclusions drawn from the sedimentation studies. The results of these two studies indicate that the higher-order folding of active chromatin is also due to bending of the DNA. At present, the biochemical significance of a lower level of folding in active chromatin from hypothyroid

Figure 9 CD spectra of fractionated chromatin at different ionic strengths

CD spectra of chromatin fractionated on Sepharose-4B at 25 °C are shown. A concentration of 1 A_{260} /ml was used for recording the spectra, with a 10 mm path-length cell. Samples used were active (A) and inactive (B) chromatin of moderate size from control rat liver, and active chromatin of large size from control rat liver (C) and hypothyroid rat liver (D). Ionic strength: solid line, 25 mM; dotted line, 65 mM; dashed line, 90 mM.

rats is not clear. A dip was always observed above 290 nm in CD spectra for active chromatin only. This suggests that active chromatin adopts a different type of secondary structure of DNA. At present an adequate explanation cannot be given as to why such a dip appears above 290 nm on higher-order folding. However, a dip between 270 and 300 nm has been observed for Z-DNA [33].

If it is assumed that the low level of higher-order folding is due to the partial depletion of histone H1 in active chromatin, then such H1 depletion must be by approx. 50%. However, such a conclusion is not supported by our studies on the stoichiometric analysis of histones in active and bulk chromatin (see the accompanying paper [30a]). Secondary folding in the H1-reconstituted chromatin has been observed to be semi-co-operative [34], not co-operative [34], even with H1 stoichiometry. Hence it seems that the partial depletion of histone H1 in active chromatin, as suggested by Kamakaka and Thomas [21], may not be the cause of a lower level of higher-order folding. An

altered mode of interaction of histone H1 with the nucleosomes may be a more likely reason for a decrease in higher-order folding.

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REFERENCES

- 1 Van Holde, K. E. (1988) in *Chromatin* (Rich, A., ed.), pp. 317–354, Springer-Verlag, New York
- 2 Allan, J., Hartman, P. G., Crane-Robinson, C. and Aviles, F. X. (1980) *Nature* (London) **288**, 675–679
- 3 Weintraub, H. and Groudine, M. (1976) *Science* **193**, 848–856
- 4 Yaniv, M. and Cereghini, S. (1986) *CRC Crit. Rev. Biochem.* **21**, 1–26
- 5 Butler, P. J. G. (1984) *EMBO J.* **3**, 2599–2604
- 6 McGhee, J. D., Rav, D. C., Charney, E. and Felsenfeld, G. (1980) *Cell* **22**, 87–96
- 7 Staynov, D. Z. (1981) *Int. J. Biol. Macromol.* **5**, 310–311
- 8 Woodcock, C. L. F., Frado, L. L. Y. and Rattner, J. B. (1984) *J. Cell Biol.* **99**, 42–52
- 9 Worcel, A., Strogatz, S. and Riley, D. (1981) *Proc. Natl. Acad. Sci. U.S.A.* **78**, 1461–1465
- 10 Horwitz, R. A., Agard, D. A., Sedat, J. W. and Woodcock, C. L. (1994) *J. Cell Biol.* **125**, 1–10
- 11 Ridsdale, J. A. and Davie, J. R. (1987) *Nucleic Acids Res.* **15**, 1081–1096
- 12 Rocha, E., Davie, J. R., Van Holde, K. E. and Weintraub, H. (1984) *J. Biol. Chem.* **259**, 8558–8563
- 13 Rose, S. M. and Garrard, W. T. (1984) *J. Biol. Chem.* **259**, 8534–8544
- 14 Xu, M., Barnard, M. B., Rose, S. M., Cockerill, P. N., Huang, S. Y. and Garrard, W. T. (1986) *J. Biol. Chem.* **261**, 3838–3845
- 15 Berent, S. L. and Sevall, J. S. (1984) *Biochemistry* **23**, 2977–2983
- 16 Nielson, P. E. (1981) *FEBS Lett.* **135**, 173–176
- 17 Caron, F. and Thomas, J. O. (1981) *J. Mol. Biol.* **146**, 513–537
- 18 Nacheva, G. A., Guschin, D. Y., Preobrazhenskaya, O. V., Karpov, V. L., Ebralidse, K. K. and Mirzabekov, A. D. (1989) *Cell* **58**, 27–36
- 19 Hill, R. J., Watt, F., Wilson, C. M., Fifi, T., Underwood, P. A., Tribbick, G., Geysen, H. M. and Thomas, J. O. (1989) *Chromosoma* **89**, 411–421
- 20 Ericsson, C., Grossbach, U., Björkroth, B. and Daneholt, B. (1990) *Cell* **60**, 73–83
- 21 Kamakaka, R. T. and Thomas, J. O. (1990) *EMBO J.* **9**, 3997–4006
- 22 Dedon, P. C., Saults, J. A., Allis, C. D. and Gorovsky, M. A. (1991) *Mol. Cell. Biol.* **11**, 1729–1732
- 23 Kimura, T., Mills, F. C., Allan, J. and Gould, H. V. (1983) *Nature* (London) **306**, 709–712
- 24 Caplan, A., Kimura, T., Gould, H. and Allan, J. (1987) *J. Mol. Biol.* **173**, 57–70
- 25 Fisher, E. and Felsenfeld, G. (1986) *Biochemistry* **25**, 8010–8016
- 26 Grossbach, E. R., Björkroth, B. and Daneholt, B. (1990) *Cell* **60**, 78–83
- 27 Weintraub, H. (1984) *Cell* **38**, 17–27
- 28 van Holde, K. E. and Zlatanova, J. (1995) *J. Biol. Chem.* **270**, 8373–8376
- 29 Liaw, C., Seelig, S., Mariash, C. N., Oppenheimer, J. H. and Toule, H. C. (1983) *Biochemistry* **22**, 213–221
- 30 Seelig, S., Jump, D. B., Towle, H. C., Liaw, C., Mariash, C. N., Schwartz, H. L. and Oppenheimer, J. H. (1982) *Endocrinology* **110**, 671–673
- 30a Tikoo, K., Gupta, S., Hamid, Q. A., Shah, V., Catterjee, B. and Ali, Z. (1997) *Biochem. J.* **321**, 273–279
- 30b Tikoo, K. and Ali, Z. (1997) *Biochem. J.* **321**, 281–287
- 31 Ausio, J., Borochoy, N., Seger, D. and Eisenberg, H. (1984) *J. Mol. Biol.* **177**, 373–398
- 32 Butler, P. J. G. and Thomas, J. O. (1980) *J. Mol. Biol.* **140**, 505–529
- 33 Riazance, J. H., Baase, W. A., Johnson, W. C., Hall, K., Cruz, P. and Tinoco, I. (1985) *Nucleic Acids Res.* **13**, 4983–4989
- 34 Sun, J. M., Ali, Z., Lurz, R. and Ruiz-Carrillo, A. (1990) *EMBO J.* **9**, 1651–1658