Modulation of the higher-order folding of chromatin by deletion of histone H3 and H4 terminal domains

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The 'tails' of histones H3 and H4 were removed by light *in situ* trypsin digestion of the nuclei. The alterations in the higher-order folding of chromatin resulting from this treatment were monitored by ethidium bromide titration. We found that DNA-intercalation of ethidium bromide under these conditions exhibited a complex concentration effect that was dependent on the extent of chromatin folding. This most likely reflects the structural transitions of chromatin during its folding as a result of the changes in the nucleosome linker twist [Woodcock, Grigoryev, Horowitz and Whitaker (1993) Proc. Natl. Acad. Sci. U.S.A. **90**, 9021–9025]. These results strongly suggest that the H3 and H4 terminal domains play a very important role in chromatin folding. We discuss the molecular basis of this phenomenon and propose a novel generalized model for the higher-order folding of chromatin.

INTRODUCTION

The organization of eukaryotic chromatin consists of a complex hierarchy of folding levels that need to be sequentially uncoiled during the process of gene activation [1,2]. The basic unit of this chromatin organization is the nucleosome core particle in which 146 bp of DNA are wrapped around a histone octamer in approx. 1.8 left-handed superhelical turns. The histone octamer contains two copies of each of the histones H2A, H2B, H3 and H4 [3,4]. Histone H1 binds to the region of the entry and exit of DNA from the nucleosome core covering roughly an additional 20 bp of DNA. The location of histone H1 and its ability to determine the exiting and entering angles of the DNA from the nucleosome result in a zig-zag-like organization of chromatin (Figure 1A), which is abolished by H1 depletion [5]. At the next level of organization, the nucleosome chain folds into a higher-order superstructure to form a fibre of 30–40 nm diameter. This folding is very important because the architecture of the fibre ultimately defines the organization of the substrate in which the genetic processes take place. Attempts made in the past to ascertain the internal organization of the chromatin fibre have resulted in a plethora of different models (reviewed in [6]). However, none of these models can completely account for all the experimental data available. Indeed, increasingly more evidence has shown that a highly regular 'textbook' model of chromatin higher-order structure may not be acceptable any longer [7,8] and that emphasis should be placed on a nucleofilament folding through rotation between adjacent nucleosomes [9]. This paper represents a further attempt to contribute to this evidence.

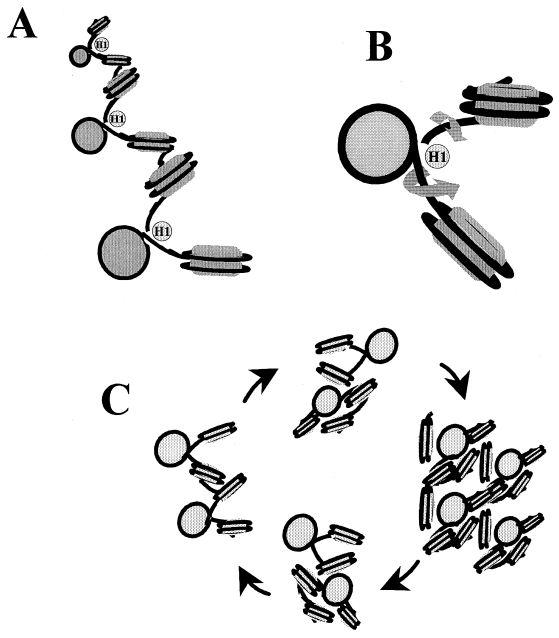

Figure 1 A schematic representation of the folding of the nucleosome fibre caused by continuous 'unwinding' of the internucleosomal linkers

Histone H1 sets the entry–exit angles of the DNA from the nucleosome resulting in a zig-zag conformation of the extended nucleosome filament (**A**). Changes in the nucleosome linker twist (**B**) result in structural transitions of chromatin through a chromatin folding 'cycle' (**C**). See also Figure 2 in [27].

In this article we evaluated the contribution of histone H3 and H4 'tails' to the folding of chromatin into its higher-order structure.

MATERIALS AND METHODS

Isolation and trypsin treatment of HeLa S3 nuclei

Human HeLa S3 cells were grown in RPMI 1640 medium supplemented with 10 % (v/v) fetal calf serum at 5 % CO_2 and 36.6 °C. Cells were collected at a density of $(0.1–0.3) \times 10^6$ cells/ml by centrifugation (1000 ***g***, 15 min) and lysed in 50 mM triethanolamine (TEA) /HCl, pH 8.0, containing 10 mM NaCl, 4 mM $MgCl_2$, 5 mM 2-mercaptoethanol and 0.125 % detergent Nonidet P40 (NP-40) buffer at 4 °C. Nuclei were pelleted in an Eppendorf microfuge (2000 ***g***, 10 min, 4 °C) and washed once with the same buffer. Nuclei were next resuspended in 10 mM TEA/HCl, pH 8.0, 5 mM NaCl, 1 mM 2-mercaptoethanol buffer

Abbreviations used: TEA, triethanolamine; NP-40, Nonidet P40.

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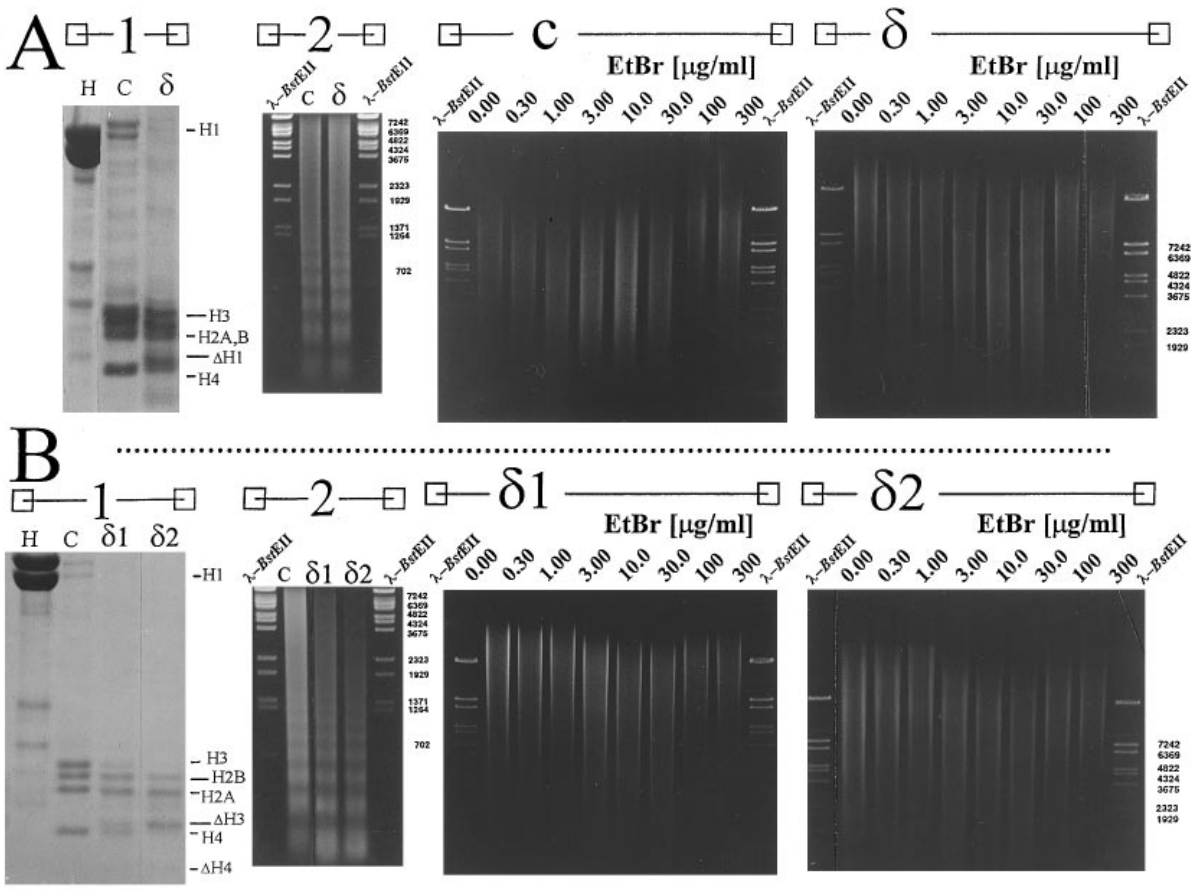

Figure 2 Micrococcal nuclease digestion of trypsinized HeLa S3 nuclei subjected to progressive ethidium bromide intercalation

HeLa S3 cell nuclei were isolated in low-salt buffer (described in the Materials and methods section) and digested with 0 ('c'), 1 ('δ'), 5 ('δ1') and 10 ('δ2') μg/ml of trypsin. The histone composition and the nucleosome ladder gel corresponding to the different trypsin-digested nuclei are shown in lanes 1 and 2 respectively. Histones were extracted from nuclei using 0.5 M HCl [12] and analysed in SDS/13%-polyacrylamide gels [13]: lane H, 0.4 M NaCl protein extract obtained from chicken erythrocyte nuclei [34]. To analyse the nucleosomal repeat of trypsinized nuclei, nuclei were digested with 200–300 units/ml of micrococcal nuclease, DNA was isolated and analysed in 1.5% agarose gels (**A**2, **B**2) (see the Materials and methods section). To analyse the effects of trypsin on the pattern of higher-order chromatin folding, HeLa nuclei, after trypsin treatment, were incubated with various amounts of ethidium bromide for 1 h and fixed by glutaraldehyde. Fixed nuclei were digested with 10 units/ml of micrococcal nuclease and then DNA was released by extensive treatment with proteinase K. After isolation, DNA was analysed in either 0.5% (**A**, 'c' and 'δ') or 0.25% (**B**, 'δ1' and 'δ2') agarose gels. The marker used for the DNA gels is a *Bst*EII restriction digest of the λ phage DNA. The number of base pairs corresponding to the different fragments is shown down the right hand side of these gels.

and the trypsin was added at the desired concentrations (1–10 μg/ml). Trypsin digestion (5 min, room temperature) was stopped by addition of 1 vol. of the same buffer containing 2 mM PMSF. The trypsinized nuclei were centrifuged as above and were next resuspended (1×10^6 nuclei/ml) in 10 mM TEA/HCl, pH 8.0, 5 mM NaCl, 1 mM $MgCl_2$, 1 mM 2-mercaptoethanol, 0.5 mM PMSF buffer containing ethidium bromide at the desired concentrations. These nuclear suspensions were incubated on ice for 60 min and then fixed by the addition of 0.25% glutaraldehyde overnight. The fixed nuclei were centrifuged as above and were next resuspended in 50 mM TEA/HCl, pH 8.0, 100 mM NaCl, 4 mM $MgCl_2$, 1 mM $CaCl_2$, 5 mM 2-mercaptoethanol, washed twice with the same buffer, and finally resuspended at 1×10^6 nuclei/ml for nuclease digestion.

Micrococcal nuclease analysis of ethidium bromide-treated nuclei

Micrococcal nuclease digestion (15 min, room temperature) was terminated by addition of 1 vol. of 10 mM NaCl, 1% SDS solution and extracted with 1 vol. of phenol/chloroform (1:1, v/v) mixture. The organic phase was precipitated with 4 vols. of ethanol at -20 °C for 2–3 h. The precipitate was resuspended (0.1–0.2 ml/10^6 fixed nuclei) in 10 mM EDTA, 100 mM NaCl, 1% SDS buffer, containing 0.1 mg/ml proteinase K. The proteinase digestion (37 °C, 12 h) was extracted with 1 vol. of phenol/chloroform. The DNA from the aqueous phase was ethanol-precipitated and dissolved in the gel-loading buffer [1 mM EDTA, 0.01% Bromophenol Blue, 10% (v/v) glycerol] containing 0.01 mg/ml of RNase A.

Analysis of protein composition of ethidium bromide-treated HeLa nuclei

Histones were extracted from nuclei using 0.5 M HCl [10]. Proteins were analysed in SDS/13%-polyacrylamide gels [11].

Analysis of the DNA sizes

The DNA gels such as those shown in Figure 2 were photographed under UV light and the images were digitized and

computer analysed. This analysis was conducted with the help of an Easy Image Plus Gel Documentation System (Herolab. Syst.) with the appropriate software. The average DNA sizes were taken to be those corresponding to the weight-average values of the DNA size distribution.

RESULTS

Experimental background

In a previous paper [12] we have described the complex association between chromatin higher-order folding and the changes in the twist of the internucleosomal DNA regions. The analysis was carried out in living human erythroleukaemia K562 cells using *in situ* chloroquine intercalation to change the helical twist of nucleosomal linker DNA. In the present study we have employed a similar experimental approach to examine the folding pattern of higher-order chromatin structure. We have taken advantage of the intercalative DNA binding of ethidium bromide to examine the differences in the higher-order folding of chromatin lacking histone H3 and H4 'tails' in comparison with native chromatin. Ethidium bromide is a well-characterized aromatic dye which, upon binding, unwinds the DNA duplex by about 26 ° per intercalated ethidium molecule [13]. Ethidium bromide intercalation has been shown to have a strong preference for linker, but not nucleosomal DNA [14–16] and under the conditions used in our study its binding does not lead to any significant reorganization of chromosomal proteins [14]. The intercalator-dependent modulation of chromatin higher-order folding at the dinucleosome level has been well characterized by electron microscopy [14]. Direct measurements of the free energy of ethidium intercalation by fluorescent spectroscopy reveals a competition between chromatin folding and ethidium intercalation [14]. These effects were attributed to ethidium-induced changes in the twist of linker DNA rather than to any other possible effects [14,17]. Therefore, it is possible to analyse the intercalator-mediated reorganization of chromatin higher-order structure to assess the chromatin folding (its position in the chromatin 'folding cycle', i.e. see Figure 1C and also the Discussion). Micrococcal nuclease seems to be an adequate probe for chromatin conformation in this case because the DNA of the nucleosome core is highly protected from nuclease digestion. Therefore, the relative sensitivity of chromatin to the nuclease (affecting mainly the DNA linker regions) reflects structural differences mainly in the organization of chromatin fibre [12,18].

Removal of H3/H4 histone 'tails' by mild trypsin treatment of isolated HeLa nuclei

The analysis was carried out on intact nuclei isolated from human HeLa S3 cells. Nuclei were isolated by mild lysis of the cells with the non-ionic detergent NP-40 at a hypotonical salt concentration to minimize the rearrangement of chromatin structure. Histone H3 and H4 'tails' were removed by light trypsin digestion of isolated nuclei. Figure 2(A1) shows the state of the histones in the nuclei following trypsin treatment. At the lowest trypsin concentrations used by us (1–2 μg/ml) essentially no intact histone H1 remains while all four core histones still remain mainly undegraded (Figure 2A1, lanes c and δ). A set of shorter peptide fragments was observed on the gel together with a peptide corresponding to the globular domain of histone H1 (see Figure 2A1, lane δ) [19,20] which runs slightly faster than histone H2A. Extensive micrococcal nuclease digestion of trypsinized nuclei produces a typical nucleosome 'ladder' (Figure 2A2, lanes c and δ) showing that the removal of the linker histone

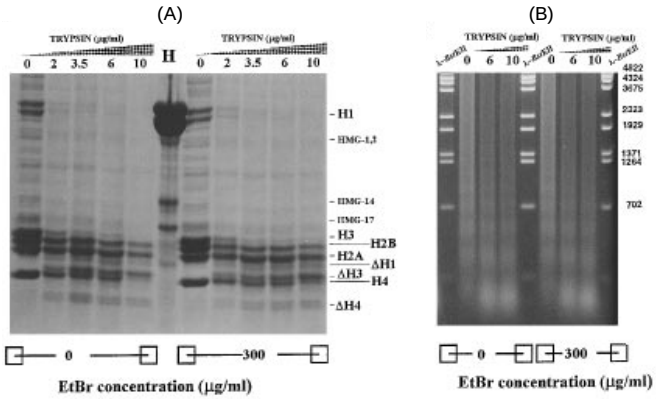

Figure 3 Protein composition and the nucleosome repeat of the trypsin-digested nuclei after treatment with ethidium bromide

(**A**) Electrophoretic analysis in SDS/13%-polyacrylamide gel of proteins extracted from trypsin-treated HeLa S3 nuclei after incubation for 60 min with ethidium bromide at 0 or 300 μg/ml (for more details see the legend to Figure 2). Lane H contains a 0.4 M NaCl protein extract obtained from chicken erythrocyte nuclei as described in [34]. (**B**) Micrococcal nuclease digestion analysis of the trypsin-treated HeLa nuclei after incubation for 60 min with ethidium bromide at 0 or 300 μg/ml.

'tails' does not result in any noticeable disassembly of individual nucleosomes. Higher levels of trypsin digestion (up to 10 μg/ml) result in the progressive degradation of the histone H3 and H4 'tails', while histones H2A and H2B remain mainly intact (Figure 2B1, lanes c, δ1 and δ2; see also Figure 3A). The pattern of proteolysis observed under our *in situ* digestion conditions is almost identical to the patterns of digestion observed for the digestion of isolated chromatin fragments *in vitro* [21,22]. It should be pointed out here that because of the lysine- and arginine-rich nature of the N- and C-terminal domains of histones, digestion of chromatin with trypsin starts with the removal of these regions ('tails') [23]. The identification of the proteolytic fragments (as in Figure 2) arising from sequential trypsin digestion has been well documented [24]. As before, micrococcal nuclease digestion also produced a nucleosomal 'ladder' (Figure 2B2, lanes c, δ1 and δ2). However, the size of the mononucleosome DNA fragments was slightly smaller (about 6 ± 2 bp) when compared with that of the nucleosomes from the control nuclei.

Analysis of the nuclease susceptibility of ethidium bromide-treated nuclei

To examine the pattern of chromatin higher-order folding, the trypsin-digested and intact nuclei were divided into aliquots and titrated with ethidium bromide. The nuclear suspensions thus obtained were incubated for 40–60 min to allow the binding to reach an equilibrium. The nuclei were then fixed with glutaraldehyde to preserve the resulting chromatin conformation(s). Previously we have shown that transient alterations in chromatin higher-order organization can be preserved by mild glutaraldehyde fixation [25,26]. Using sucrose gradients, Thoma et al. [5] have shown that such glutaraldehyde fixation does not alter the state of higher-order folding of chromatin (see Figure 1 in [5]). Therefore, glutaraldehyde fixation of nuclei should be able to preserve the changes induced by the binding of ethidium bromide. Upon subsequent removal of the intercalator, the fixed nuclei were examined for chromatin folding using micrococcal nuclease as a structural probe [12]. After nuclease digestion, the fixed

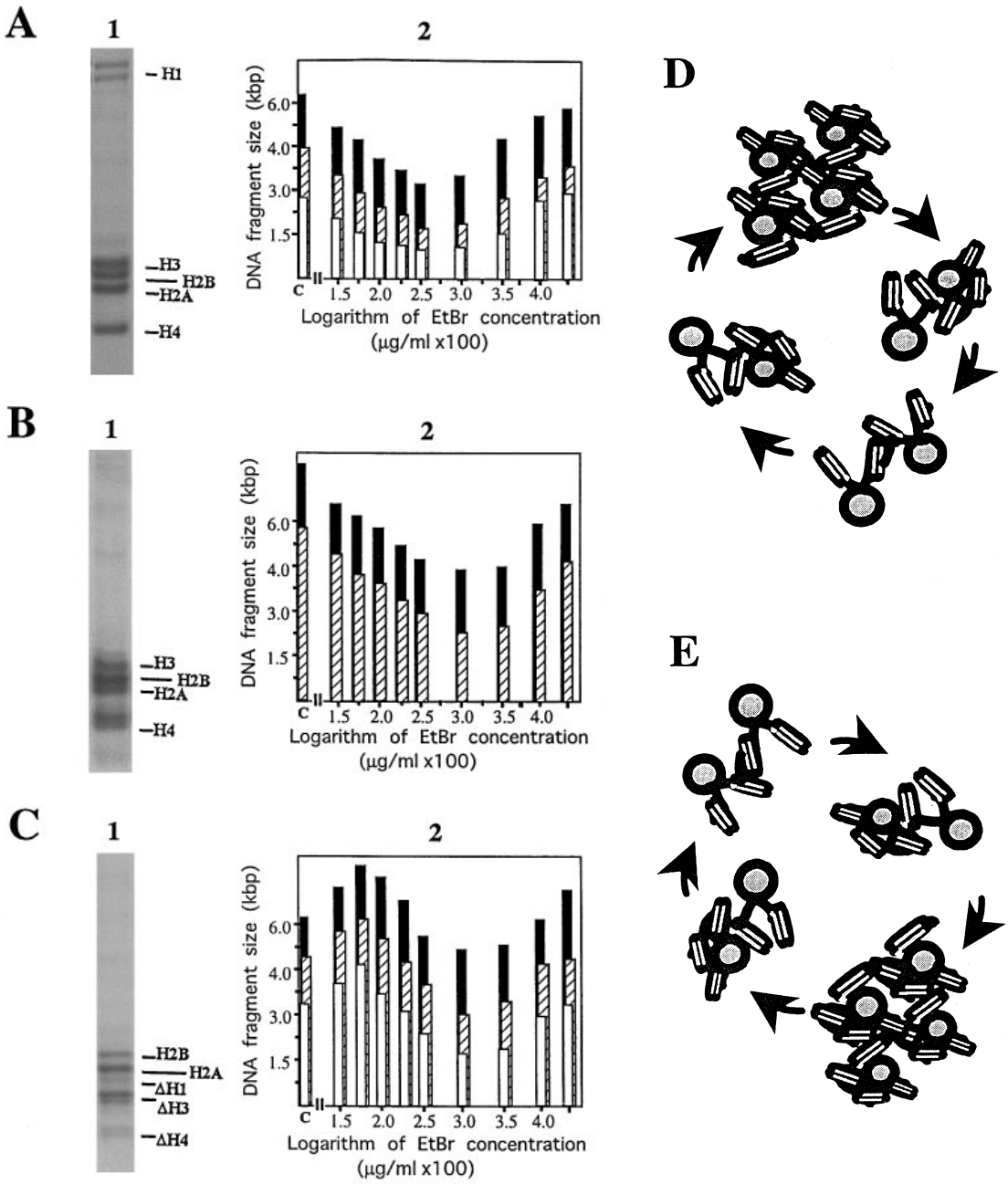

Figure 4 Diagrams summarizing the different micrococcal nuclease digestion patterns of chromatin with intact histones (A2), chromatin in which the C- and N-terminal regions of histone H1 have been digested by trypsin (B2), and chromatin lacking histone H3/H4 'tails' obtained upon further trypsin digestion (C2)

Isolated HeLa S3 nuclei were treated with 10 μg/ml of trypsin. Trypsinized nuclei were incubated with various amounts of ethidium bromide for 1 h and fixed by glutaraldehyde. Fixed nuclei were digested with different amounts of micrococcal nuclease (10–30 units/ml) and then DNA was released by extensive treatment with proteinase K. After isolation, DNA was analysed in 0.25% agarose gels. A schematic representation illustrating the folding of the nucleosome filament corresponding to (**A**2) and (**B**2) is shown in (**D**), and in (**E**) for (**C**2). The 'phase' of the folding pattern of chromatin with intact histones is shifted with respect to that of chromatin with truncated H3/H4 histones. In the first case, the folding cycle due to the unwinding of the linkers (induced by ethidium bromide intercalation) starts with a folded chromatin state as shown in (**D**). In contrast, in the second case, the chromatin folding cycle starts with an unfolded conformation, as shown in (**E**). The protein composition of the chromatin used in these experiments is shown in (**A**1), (**B**1) and (**C**1). The micrococcal nuclease concentrations represented by the bars are as follows: closed bar, 10 units/ml; hatched bar, 15 units/ml; and open bar, 30 units/ml. C: control (DNA from ethidium bromide untreated nuclei).

nuclei were denatured by phenol/chloroform treatment followed by extensive (overnight) incubation with proteinase K. This procedure ensured that nearly 100% of nuclear DNA could be extracted, thus ruling out the possibility of any differential chromatin extraction. It is worth noting that in the absence of phenol pretreatment the DNA yields of the extraction were significantly lower (results not shown). Figure 2 shows the analysis, by agarose gel electrophoresis, of the DNA from the

partial micrococcal nuclease digestion of the ethidium bromide-treated nuclei. In native nuclei (Figure 2A, gel 'c'), the increase in ethidium bromide concentration (up to 8–10 μg/ml) causes a significant concentration-dependent decrease in the average size of digested DNA fragments. This increased accessibility to micrococcal nuclease is probably the result of a progressive 'unfolding' of the chromatin higher-order structures. Further increase in the ethidium intercalation (above approx. 10 μg/ml up to 300 μg/ml) results in a progressive increase of the average size of the DNA fragments, up to values which are comparable with those of the ethidium bromide untreated nuclei. This second transition presumably reflects the refolding of the extended nucleosome fibre. The effect of ethidium intercalation most likely reflects the structural transition of chromatin through the chromatin folding 'cycle' (Figure 1) as a result of the changes in the nucleosome linker twist, i.e. in the relative rotational angle between consecutive nucleosomes [27]. The nuclease digestion pattern of the H1-depleted nuclei (Figure 2A, gel 'δ') does not differ significantly from that of the control nuclei (Figure 2A, 'c'), except that chromatin with trimmed H1 histones shows decreased sensitivity to the micrococcal nuclease, as evidenced by the overall increase in the DNA fragment size (see Figures 4A2 and 4B2). However, as can be seen in Figure 2(B; gels 'δ1' and 'δ2') and also in Figure 4(C2), the nuclease digestion pattern dramatically changes in chromatin lacking the histone H3 and H4 'tails'. A similar 'wave'-like distribution of the DNA fragment sizes can be observed in both cases. However, in the latter case, the 'wave' has a different phase to that of chromatin with intact histones. This effect is most likely the result of some kind of reorganization of the higher-order chromatin structure occurring upon removal of histone H3 and H4 'tails'. Figure 4 summarizes the results of the nuclease digestion patterns obtained with native chromatin (consisting of intact histones) (Figure 4A2) and after progressive removal of the C- and N-terminal domains of histone H1 (Figure 4B2) and further removal of the histone H3 and H4 'tails' (Figure 4C2).

Protein composition and nucleosome repeat of the ethidium bromide-treated nuclei

It has been shown previously that intercalation of ethidium bromide in DNA may result in a selective release of weakly bound chromosomal proteins, such as the high-mobility-group proteins and linker histones [28,29]. Even a partial release of H2A·H2B dimers was reported [15]. However, it is important to point out that all these effects were observed at ethidium bromide concentrations which were at least 10-fold higher than those used in the present study. Under our experimental conditions the analysis of the protein composition of chromatin lacking histone 'tails' (Figure 3A) revealed no evidence for any intercalator-mediated release of either the four core histones or histone H1. In addition, a more detailed study on the protein composition of ethidium bromide-treated nuclei did not reveal any evidence for the specific release of high-mobility-group proteins (W. A. Krajewski and J. Ausió, unpublished work). Micrococcal nuclease digestion produced a nucleosome ladder for all the chromatin samples analysed (see Figure 3B), suggesting that the basic nucleosome structure and chromatin organization were also preserved upon ethidium treatment.

Earlier studies have also shown that ionic binding of the ethidium bromide to nucleosome core particles [30,31] and even to naked DNA [32] is negligible within the range of concentrations used in the present study. At these concentrations ethidium intercalation neither affects the bending stiffness of DNA [33], nor the intrinsic torsional stiffness of linear DNA [34,35], thus ruling out any of these effects on the interpretations of our results.

DISCUSSION

A mechanistic model for chromatin folding

It has been shown recently that alterations in the nucleosomal linker twist may significantly influence the way in which the nucleosome fibre folds into its higher-order structure *in vivo* [12] as well as *in vitro* [14]. Woodcock et al. [27], have presented a set of computer-generated models for the folding of the chromatin fibre based on the variation in the linker DNA length which in turn gives rise to changes in the relative rotational angle and the distance between adjacent nucleosomes. The folded structures, arising from the progressive changes in the rotational angle between consecutive nucleosomes at a given DNA linker length (Figure 1B), 'cycle' from a symmetrical organization with completely extended beads-on-a-string (relative rotational angle 180 °) to that of fibres with the most densely packed nucleosomes (relative rotational angle 250 °–280 °) (Figure 1C). Thus, the folding of the nucleosome filament into its higher-order structure and the periodicity of its folding pattern, are derived from the intrinsic properties of the nucleosome-linker structure [27]. The precise location in the folding 'cycle' may be regulated by the rotational angle between the adjacent nucleosomes (Figure 1). Alterations in their linker settings must have the effect of shifting the location within this 'cycle'.

Role of the histone H3-H4 C- and N-terminal regions in the unfolding of trypsin-digested chromatin

Despite the fact that many hypotheses have been proposed [36,37] to account for the role(s) played by the lysine-rich terminal regions of the core histones, there is still very little known about their role(s) in the regulation of chromatin fibre folding. Post-translational modifications of these histone regions have often been correlated with the processes of chromatin activation and structural reorganization [2,3,37].

The ethidium bromide intercalation results on native chromatin (Figure 2, gel 'c') presented in the preceding section are fully consistent with the model described above as has been discussed previously [12]. Within this context, the simplest interpretation for the *in situ* trypsinization results (Figure 2, gels δ1 and δ2 and Figure 4C2) is that the extent of chromatin folding in the 30 nm chromatin fibre is strongly affected by the 'tail' domains of histones H3 and H4 (Figure 4). As seen in Figure 4, the removal of histone H1 C- and N-terminal domains did not change the 'shape' of the nuclease digestion pattern (Figure 4B2) (except for an overall decrease in nuclease sensitivity of this chromatin; see the Results section). This is in contrast to the change which is observed upon removal of the histone H3/H4 'tails' (see Figure 4C2). Within the frame of the experimental background used by us [12] and the chromatin folding model presented in the preceding sections, this change could be explained by a shift in the 'phase' of the chromatin folding cycle induced by the ethidium bromide intercalation (see the legend to Figure 4 for more details). Accordingly, chromatin lacking the C- and N-terminal regions of histones H3/H4 would exhibit a less folded conformation (Figure 4E) at the onset of the ethidium bromide titration as compared with intact chromatin or chromatin with 'trimmed' H1 (Figure 4D).

These results are in very good agreement with the early data from Allan et al. [38] and further emphasize the critical role of the N-terminal regions of histone H3 in the folding of the chromatin fibre [21,39]. Furthermore, these results also agree with our

previous *in vitro* data on trypsin-digested chromatin [40] and acetylated oligonucleosomal fragments [41,42].

The constraints imposed by the histone 'tails' on the folding of the nucleosome fibre might have important implications for the mechanisms of gene activation. Modifications of the terminal regions of these histones could lead to the unfolding of the compact chromatin structure. Such unfolding is most likely a prerequisite for most of the processes occurring on DNA.

We would like to acknowledge the Institute of Developmental Biology, Russian Academy of Science, for generosity in providing information on intercalation-mediated chromatin folding (and corresponding methodological approaches that were unpublished by the time this work was performed [12]). We would also like to thank Maree Roome for her skilful typing of the manuscript and Philip Rice for technical assistance and careful reading of the manuscript. This research was supported by NSERC grant OGP0046 399 and British Columbia Health Care Research Foundation grant 94-1. W.A.K. was a recipient of a postdoctoral NATO Science–NSERC Fellowship (1994).

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Received 12 September 1995/5 February 1996; accepted 9 February 1996