Transitions between in situ and isolated chromatin

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SUMMARY

We show that the mechanism by which chromatin displaying higher-order structure is usually isolated from nuclei involves a transition to an extended nucleosomal arrangement. After being released from nuclei, chromatin must refold in order to produce the typical chromatin fibers observed in solution. For starfish sperm chromatin with a long nucleosome repeat (222 bp), isolated fibers are significantly wider than those in the nucleus, indicating that the refolding process does not regenerate the native higher-order structure. We also propose that for typical eukaryotic nuclei, the concept that the native state of the (inactive) bulk of the genome is a chromatin fiber with defined architecture be reconsidered.

Key words: chromatin, 30 nm fiber, nuclease digestion, nucleus, ultrastructure

INTRODUCTION

The genomic DNA of most eukaryotes is bound to histones and other proteins forming the complex known as chromatin (van Holde, 1988). As the fundamental subunit of chromatin, the nucleosome has an invariant structure that has been extensively studied (Richmond et al., 1984). Individual nucleosomes are connected by linker DNA that varies in length among species and cell types. There is now compelling evidence that topologically constrained ‘domains’ of chromatin involving from 150 to 1500 nucleosomes constitute functional units within the nucleus (e.g. see Goldman, 1988; Travers, 1992), and that the structural organization of chromatin is important in gene regulation (Felsenfeld, 1992). It also appears that the level of compaction of chromatin is correlated with the transcriptional competence both of individual domains (Bonifer et al., 1991) and of larger heterochromatic segments of the genome (Gartler and Riggs, 1983; Babu and Verna, 1987; Kellum and Schedl, 1991; Pardue and Hennig, 1990). For example, domains are envisaged as in an ‘open’ transcriptionally competent conformation, or in an inactive ‘closed’ state in which the chromatin is condensed, and the nucleosomes closely packed (van Holde, 1988; Bonifer et al., 1991). Consequently, the principles that govern chromatin compaction and the arrangement of nucleosomes in the compact state are of considerable interest.

Extensive work on isolated polynucleosomes has demonstrated that chromatin compaction in vitro may be modulated by the ionic environment, through charge shielding and neutralization of the DNA (Widom, 1986; Clark and Kimura, 1990). Accompanying the compaction process in vitro is a well-characterized morphological transition from a low-salt ‘beads on a string’ state to a compact fiber in which the 11 nm diameter nucleosomes aggregate into a compact 30-40 nm diameter fiber (van Holde, 1988; Widom, 1989). Compact fibers form spontaneously if the monovalent salt concentration is raised to between 50 mM and 150 mM, and provided the linker histone H1 is present (Thoma et al., 1979; Ruiz-Carillo et al., 1980). If the charge on the DNA is shielded further by raising the monovalent ion concentration above about 150 mM, or neutralized sufficiently with di- or polyvalent cations, the chromatin tends to precipitate from solution (Widom, 1986; Clark and Kimura, 1990).

The relevance of these in vitro morphological states and transitions to the in vivo properties of chromatin depends on the degree to which structural congruence can be demonstrated between them. At present, although the cumulative evidence in support of congruence is incomplete and rather circumstantial, the concept that compact chromatin fibers, such as are found in vitro at physiological ionic strengths, are the default state of chromatin in vivo has gained wide acceptance. X-ray scattering studies of intact nuclei often, but not always, show a reflection corresponding to a spacing of 35-45 nm, attributed to the center-to-center spacing of chromatin fibers (Langmore and Schutt, 1980; Langmore and Paulson, 1983; Williams et al., 1986). However, in only a few cell types, most notably the mature avian erythrocyte (Davies et al., 1974; Olins and Olins, 1972; Brasch et al., 1972), can ‘30 nm’ chromatin fibers be seen readily in situ. The majority of nuclei and metaphase chromosomes, when examined in intact cells, show little if any indication that the chromatin has a basic compact fiber organization.
Instead, the chromatin usually appears rather amorphous in texture. Under certain artificial conditions both chromo-
somes and interphase chromatin can be induced to adopt a
fiber morphology. For example, if metaphase chromosomes
or interphase nuclei are spread on a water surface or oth-
erwise exposed briefly to lowered ionic conditions, com-
 pact chromatin fibers are revealed (Gall, 1963; Ris and
Kubai, 1970; Bahr, 1977; Rattner and Hamkalo, 1978; Bel-
mont et al., 1989).

The present study was aimed at developing a better un-
derstanding of the relationship between chromatin in solution
and in intact nuclei. Since the usual method of isolating chro-
matin is to prepare nuclei and then expose them to an exoge-
nous nuclease, we have undertaken a careful examination of
the processes leading to the release of polynucleosomes. By
observing nuclei and isolated chromatin under identical ionic
conditions, we have found that the in situ compact fiber mor-
phology is extremely labile and rapidly lost under conditions
that allow access by exogenous nucleases and the release of
soluble polynucleosomes into the medium. Despite the loss
of fiber morphology in the isolated nuclei, the polynucleo-
somes released from them assume a typical compact fiber
shape. These results suggest that the processes of chromatin
digestion and release do not simply transfer the native chro-
matin higher-order structure to the external medium, but
involve changes in chromatin organization. We discuss the
implications of these findings for studies of isolated chro-
matin, and for chromatin as a substrate for in vivo processes
that depend on the binding of ligands to specific DNA
sequences.

MATERIALS AND METHODS

Buffers used

Artificial sea water (ASW): 420 mM NaCl, 9 mM KCl, 9.3 mM
CaCl₂, 22.9 mM MgCl₂, 25.5 mM MgSO₄, 21.5 mM NaHCO₃,
PH 8.0. HEPES buffer (HB): 150 mM NaCl, 15 mM HEPES,
PH 8.0. Tris isolation buffer (TIB): 150 mM NaCl, 10 mM Tris, PH
8.0, 2 mM EDTA, 0.2 mM PMSF. Magnesium isolation buffer
(MIB): 0.25 M sucrose, 10 mM triethanolamine (TEA), PH 7.4,
2 mM MgCl₂, 10 mM PMSF. Digestion buffers (DB): 15 mM
HEPES, PH 8.0, 1 mM CaCl₂, 0.2 mM PMSF plus monovalent
salts to bring the total ionic concentration to: 36 mM (DB36), 17
mM KCl, 4 mM NaCl; 48 mM (DB48), 26 mM KCl, 7 mM NaCl;
70 mM (DB70), 44 mM KCl, 11 mM NaCl; 100 mM (DB100),
68 mM KCl, 17 mM NaCl; 150 mM (DB150), 108 mM KCl, 27
mM NaCl.

Isolation of sperm and nuclei

Patiria miniata were obtained from Marinus, Inc. (Long Beach,
CA) and maintained at 12-14°C. Dissected male gonads were
placed in ASW and gently mixed to liberate sperm. For detergent
isolation of nuclei, sperm were released into TIB (Green and
Poccia, 1988), centrifuged at 3300 g for 5 min at 0°C and resus-
pended in TIB plus 0.5% Triton X-100. After 10 min on ice with
occasional vortexing, the liberated nuclei were pelleted at 650 g,
and washed 3 times with TIB. Alternatively, nuclei were isolated
by a modification of the method of Thomas et al. (1986). Sperm
in ASW were washed three times in MIB, then twice in 0.25 M sucrose, 60
mM KCl, 15 mM NaCl, 0.15 mM spermine, 0.5 mM spermidine,
10 mM TEA, PH 7.4, 10 µM PMSF.

Nuclei from sea urchin (Strongylocentrotus purpuratus) sperm
were prepared as described by Green and Poccia (1988), and
chicken erythrocyte nuclei as described by Ruiz-Carrillo et al.
(1980).

The DNA content of nuclear preparations was estimated from
the absorbance at 260 nm of aliquots solubilized in 1 M NaOH,
using the relationship 1 A₂₆₀=50 µg/ml DNA.

Protein analysis

Isolated nuclei were resuspended in sample buffer (0.1 M Tris,
PH 6.8, 2.5% SDS, 2.5 M Urea, 5 mM EDTA, 2.5% 2-mercap-
toethanol, 25% glycerol, 0.01% Bromophenol Blue), boiled for 1
min, and aliquots applied to 18% SDS-polyacrylamide gels (Korn-
berg and Thomas, 1974). The gels were run at 30 mA for 7 hours
and stained with Coomassie blue.

Chromatin isolation

Starfish sperm nuclei (5 units of A₂₆₀) were washed 2× by cen-
trifugation in TIB without EDTA at 1,500 g for 4 min, resus-
pended in DB of the appropriate ionic strength, 1.3 units micro-
coccal nuclease (Worthington) was added, and the nuclei
incubated at 0°C for 1 hour. Samples were then raised to 21°C
and the reaction stopped after 0, 2, 5, 10 or 20 min by adding
EDTA to 4 mM and placing on ice. Nuclei were pelleted and the
 supernatants (S1) retained. The pellets were resuspended in release
buffer consisting of the digest buffer without CaCl₂, plus 4 mM
EDTA, incubated overnight at 4°C, centrifuged at 2,000 g for 4
min and the supernatants (S2) collected.

DNA extraction and separation

Post-digestion, 2 mg/ml proteinase K was added to nuclei, and
incubated overnight at room temperature. After phenol extrac-
tion and ethanol precipitation, samples were heated to 60°C for 15 min
in 10% glycerol, 89 mM Tris, 89 mM boric acid, 2 mM EDTA,
0.1% SDS, Bromophenol Blue, loaded onto 1.2% SDS-agarose
gels (Varshavsky et al., 1978) run at 100 V for 3 hours, stained
with ethidium bromide and photographed by UV transillumina-
tion. The average nucleosome repeat length was calculated from
the slope of a linear least-squares fit of dinucleosome through
nonanucleosome DNA sizes (in bp) versus oligomer number
(Morris, 1976; Woodcock et al., 1976).

Preparation and use of micrococcal
nuclease/FITC conjugate

Micrococcal nuclease was obtained using an expression vector
carrying the cloned wild-type nuclease (a generous gift from A.
S. Miltman) as described (Serpersu et al., 1986). In order to label
the nuclease for fluorescence microscopy, a protocol for the con-
jugation of antibodies to fluorescein (Harlow and Lane, 1988) was
adapted as follows: fluorescein isothiocyanate-isomer 1 (FITC,
Sigma), 1 mg/ml in dimethylsulfoxide, was added to 1 mg/ml
micrococcal nuclease in 0.1 M Na₂CO₃ (pH 9.0), to a final con-
centration of 25 µg/ml. After overnight incubation at 4°C, NH₄Cl
was added to 50 mM, and the mixture loaded onto a Sephadex
G-10 gel filtration column (Pharmacia) equilibrated with 5 mM
HEPES (pH 8.0). The enzyme/FITC conjugate fractions were col-
lected, and the extent of conjugation measured optically using the
ratio of absorbance at 280 nm and 495 nm (usually approximately
1.0). The activity of the enzyme/FITC conjugate was reduced
about fourfold compared to unconjugated enzyme.

For digestion experiments, 5 units of A₂₆₀ nuclei were equili-
brated in 100 µl of digest buffer on ice. Micrococcal
nuclease/FITC equivalent to about 20 units of native enzyme
was added, and incubated for 1 hour. After addition of EDTA to 5
nM, nuclei were pelleted at 1,500 g for 3 min, resuspended in fresh buffer and the fluorescence intensity of nuclei with respect to the medium was recorded. Parallel samples were prepared for electrophoresis.

Determination of nuclear permeability using fluorescent dextrans

Fluorescent dextrans (500 µg/ml) from 4,100-497,000 M, (Sigma), were added to 5 units A, nucleic acid on ice. The samples were incubated for 30 min, pelleted at 1,500 g for 3 min, then resuspended in fresh buffer. Ascorbic acid was added to 1 mM to reduce fluorochrome bleaching (Kochanski and Borisy, 1990), and drops of the suspension were applied to glass slides. Penetration into nuclei was evaluated by comparing the intensity of fluorescence within nuclei relative with that of the surrounding medium.

Light and electron microscopy

Phase-contrast, differential interference contrast (DIC), fluorescence observations and photomicrography were performed using a Nikon Optiphot microscope. For electron microscopy, soluble chromatin from the S2 fraction after digestion with micrococcal nuclease in DB48 was adjusted to the desired ionic strength, fixed in 0.1% glutaraldehyde for 12 hours at 4°C (Thoma et al., 1979). Samples were adsorbed to low-discharged carbon-coated grids (Woodcock et al., 1981), washed 3x with water and stained with 2% (aq.) uranyl acetate (UA), or 2.5% (aq.) aurothioglucose (ATG) (Woodcock et al., 1991b).

Samples of sperm as whole cells, at various points in nuclear isolation, or during nuclease digestion, and S2 supernatants were embedded by either low-temperature or conventional methods. Low-temperature dehydration, embedding and polymerization in Lowicryl K11M was carried out as described previously (Horowititz et al., 1990). Conventionally prepared samples were fixed by addition of glutaraldehyde (GA) to 2%, held overnight at 4°C, and post-fixed in 0.1% osmium tetroxide (OsO4) for 1 hour at 0°C. After 4 washes in distilled water, cells were taken up into 1% agar held at 45°C, cooled, and cut into pieces for dehydration in ethanol, and subsequent embedding in Spurr’s resin. Spurr’s resin sections were stained with uranyl acetate followed by Sato’s Triple Lead (Sato, 1967).

Histone content and nucleosomal organization

The total protein content of the isolated P. miniata sperm nuclei is shown in Fig. 2A, with proteins from the sperm nuclei of the sea urchin Strongylocentrotus purpuratus, and chicken erythrocyte nuclei for comparison. In agreement with work on other starfish (Zalenskaya et al., 1980; Massey and Watts, 1992) the core histones from P. miniata sperm nuclei show a migration pattern typical of somatic nuclei, and do not contain the larger histone H2B present in sea urchin sperm (von Holt et al., 1984). P. miniata sperm do, however, have a large sperm-specific histone H1, a feature apparently shared by all echinoderm sperm. Histone H2A in both echinoderm sperm examples stained less efficiently with Coomassie Blue than other histones, a common occurrence among echinoderms (personal communication, G.R. Green, Biology Department, Amherst College). The amount of non-histone nuclear proteins is very low.

Digestion of isolated sperm nuclei with micrococcal nuclease yields a typical nucleosomal ladder of DNA fragments (Fig. 2B) from which a nucleosome repeat of 222 bp, s.d. 5 bp, is obtained (Giannasca and Woodcock, 1988). Vodicka et al. (1990) recently reported a similar value of 225 bp for the same species. This nucleosomal repeat is intermediate between the 240 bp typical of sea urchin sperm and the 212 bp of the avian erythrocyte (van Holde, 1988).

Digestion and release of chromatin

The processes of nuclease digestion and chromatin release from isolated nuclei were examined over a range of ionic strengths, and morphological changes monitored. Isolated nuclei were washed in isolation buffer without EDTA and resuspended in digestion buffer (DB) consisting of KCl,
NaCl and HEPES buffer to give total concentrations of monovalent ions ranging from 36 mM (DB36) to 150 mM (DB150), plus 1 mM CaCl₂ to promote nuclease activity (see Materials and Methods for details). In the DB150, nuclei maintained their acrosomal depression, while exposure to DB100 or DB70 resulted in heterogeneity in size and shape, with nuclei tending to become rounded. Further reduction of ionic strength (DB48 or DB36) yielded more swollen nuclei that had largely lost the acrosomal depression, and showed an increase in diameter to 4 µm.

After equilibration of nuclei in the desired medium, micrococcal nuclease was added and pre-incubated for 1 hour at 0°C. Aliquots were removed at 0, 5 and 20 min after raising the temperature to 21°C, and the reaction stopped by adding EDTA on ice. Two supernatants were collected, representing the initial release during digestion (S1), and after an overnight incubation at 4°C in the same ionic strength buffer without CaCl₂ (S2). The combined S1 and S2 values are presented in Fig. 3. The amount of chromatin released was dependent on the ionic strength of the digestion and release buffers, the lowest value (<2%) occurring at the highest ionic strength. At lower ionic strengths, a progressive increase in release was observed, ultimately exceeding 17% at 36 mM monovalent ions. The released material in control samples in which the enzyme was omitted never exceeded 1%.

The extent of digestion at the different ionic strengths was similar to that shown below in Fig. 6 for nuclease conjugated

<table>
<thead>
<tr>
<th>Diameter</th>
<th>Fibers in nuclei</th>
<th>Isolated fibers</th>
</tr>
</thead>
<tbody>
<tr>
<td>Low-temperature embedded</td>
<td>31.0, s.d. 2.1</td>
<td>40.4, s.d. 2.0</td>
</tr>
<tr>
<td>Cryosectioned</td>
<td>30.5, s.d. 2.3</td>
<td>-</td>
</tr>
<tr>
<td>Epoxy embedded</td>
<td>23.9, s.d. 1.9</td>
<td>42.0, s.d. 2.0</td>
</tr>
<tr>
<td>Negatively stained</td>
<td>-</td>
<td>-</td>
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</tbody>
</table>

Values are in nm s.d. = standard deviation.
to fluorescein. At all ionic strengths tested except the highest (150 mM monovalent ions), a similar rate and level of digestion was obtained as judged by the relative amounts of oligonucleosomal DNA, but at 150 mM the extent of digestion was greatly reduced. Cuatrecasas et al. (1967) found that micrococcal nuclease was unaffected by monovalent salts below a concentration of 300 mM. The reduced digestion we observed at 150 mM (Fig. 6, below) is therefore unlikely to be caused by salt inhibition of enzyme activity.

Conformation of isolated chromatin

The chromatin released from nuclei after micrococcal nuclease digestion at different ionic strengths was examined by electron microscopy, and the DNA sized by electrophoresis. For these studies, we wished to maximize the production of large polynucleosomes, and reduced the amount of enzyme about 15-fold from that used to monitor digestion. With DB24 and DB36, irregular, partially unfolded fibers were obtained, while at 48 mM ionic strength, compact chromatin fibers of regular diameter were obtained. The preponderance of high molecular mass DNA in the released chromatin (Fig. 4) demonstrates that the fibers observed by electron microscopy did not arise from aggregates of oligonucleosomes (e.g. see Perez Grau et al., 1982).

In order to examine the response of isolated chromatin to changes in ionic strength, chromatin released from digested nuclei in 48 mM monovalent ions was adjusted to 24, 36, 48, 70, 100 and 150 mM monovalent ions and, after equilibration, fixed and negatively stained with uranyl acetate or the neutral, non-ionic aurothioglucose (ATG). As anticipated from earlier studies (Thoma et al., 1979; Woodcock et al., 1984), at the lower ionic strengths of 24 mM and 36 mM fibers appeared largely unfolded, with irregular discontinuities in fiber structure similar to that observed for *Echinus esculentus* sperm chromatin (Hill et al., 1991), while at 48 mM and 70 mM, the transition to the compact chromatin fiber appears complete (Fig. 5). Evidence of internal order in the fibers was infrequent. At ionic strengths of 100 mM and 150 mM, chromatin fibers with smoother contours showed less internal structure compared to those fixed at lower ionic strengths (Fig. 5F,G). Side-to-side and end-to-side aggregation of chromatin fibers was often observed at these ionic strengths. Diameters were measured of fibers fixed at ionic strengths of 48 mM and 36 mM, and at 100 mM and 150 mM for monovalent ions, and with both UA and ATG stains. No consistent or significant relationship between fiber diameter and ionic strength or stain method was found, the overall mean diameter being 42.0 nm, s.d. 2.0 nm.

We checked to see whether the 25% difference in diameter between the isolated (approx. 40 nm) and in situ (approx. 30 nm) chromatin fibers (in low-temperature-embedded and cryosectioned nuclei) was due to the rather different preparation conditions. Chromatin released from nuclei in 48 mM digestion buffer was embedded by low temperature methods and thin sections were obtained. The sections contained fibers similar in morphology and diameter (40.4 nm, s.d. 2.0 nm; Table 1) to their negatively stained counterparts (Fig. 5D).

Characterization of the chromatin isolation process

Having established that the intact sperm nucleus contains clearly defined chromatin fibers (Fig. 1B,C), and that com-

**Fig. 3.** Release of chromatin (S1 + S2 fractions) from starfish sperm nuclei following nuclease digestion in buffers of differing ionic strength. The zero time point shows the chromatin released after 60 min at 0°C.

**Fig. 4.** Long chromatin DNA released following micrococcal nuclease digestion of starfish sperm nuclei in DB48. The enzyme level was reduced approximately 15-fold from that used for Fig. 2B. Lanes 1-3, S1 fraction after 5, 10 and 20 min of digestion, representing 6.4%, 9.4% and 14.6% of starting material, respectively. Lanes 5-7, S2 fraction after 5, 10 and 20 min of digestion, representing 3.8%, 5.8% and 7.8% of starting material, respectively. Molecular mass markers (lanes 4 and 8), in base pairs, from the top are 4072, 3054, 2036, 1635, 1018, 516, 394, 344 and 298.
Pact fibers can be released into solution (Fig. 5), we investigated the key steps in the isolation process, beginning with the purification of nuclei, and with particular attention to conditions that might contribute to the change in fiber diameter. Of numerous isolation methods tested, one based on the procedure of Green and Poccia (1988) utilizing TIB (150 mM NaCl, 10 mM Tris (pH 8.0), 2 mM EDTA, 0.2 mM PMSF) and Triton X-100 proved to be the most effective, resulting in nuclei that retained only vestiges of nuclear envelope (Fig. 7A).

Permeability of isolated nuclei
Because of the strong effect of ionic strength on digestion and release, especially the dramatically reduced digestion in the 150 mM buffer (Figs 3, 6), we examined the accessibility of isolated nuclei to the nuclease enzyme, and to a range of dextrans of different size. We used fluorescein covalently linked to micrococcal nuclease and a number of commercially available dextran-fluorescein conjugates, and recorded the ability of the conjugates to enter the nuclei (see Materials and Methods). In experiments with the nuclease conjugate, the extent of digestion was also monitored by removing samples from which DNA was extracted and separated on gels.

The DNA gels (Fig. 6) indicated a pattern of digestion very similar to that of the unconjugated enzyme. At the lower ionic strengths, the rate of digestion was similar, but at 150 mM monovalent ions, digestion was greatly reduced. The fluorescence data showed that the digestion results were correlated with differences in enzyme penetration. Nuclei equilibrated in 36 mM to 100 mM ionic strength...
were readily penetrated by the enzyme conjugate, but at 150 mM penetration was greatly retarded (Table 2). Results with the fluorescent dextrans confirmed the observations with micrococcal nuclease: as the monovalent ionic strength was increased, the permeability of the nuclei decreased (Table 2). At 150 mM, fluorescent dextrans of only 4,100 $M_r$ and smaller were able to enter the nuclei without restriction, while at 36 mM dextrans ten times larger in molecular mass readily entered the nuclei. Under the conditions tested, dextrans of 65,600 $M_r$ and larger failed to enter nuclei. From the calculated Stoke’s radii of dextrans (Peters, 1983, 1984), we estimate that for nuclei in 36 mM monovalent ions, free diffusion is limited to particles $\sim$9.3 nm in diameter or smaller, while particles $>\sim$11.3 nm are completely excluded. For nuclei equilibrated in 150 mM monovalent ions, the corresponding sizes are $\sim$4.6 nm and 6.6 nm.

**Ultrastructural observations on isolated nuclei and chromatin**

The complete process of digestion and release that produced the isolated chromatin fibers described above was also monitored at the ultrastructural level. Isolated nuclei in TIB, in DB48 before and after digestion, and both pelleted nuclei and pooled S1 and S2 supernatants in release buffer, were prepared by low-temperature methods, and thin sections examined. After isolation and equilibration in TIB, the nuclear contents had undergone a dramatic change from the native state: ordered arrays of compact fibers were absent, and the chromatin assumed a uniform, diffuse appearance. Staining with the DNA-specific osmium ammine-B reagent (Olins et al., 1989; Derenzini and Farbegoli, 1990; Horowitz and Woodcock, 1992) showed that nucleosome-associated DNA was present but no longer arranged in compact fibers (Fig. 7A). The complete absence of the compact fiber morphology of the chromatin persisted through all subsequent steps in digestion and release, the nuclear contents becoming progressively more diffuse. However, in marked contrast, chromatin released from these nuclei after digestion was observed, in negative stains or in thin sections, to be in the form of compact fibers (Fig. 5).

Given the requirement for di- and polyvalent cations for the maintenance of nuclear structures in some systems (Belmont et al., 1987, 1989), we tested the possibility that the loss of compact fiber morphology was due to the lack of such ions in the isolation buffers. Nuclei prepared and equilibrated in isolation buffers containing divalent ions and polyamines (Thomas et al., 1986; and see Materials and Methods) were examined. Again, the native compact fiber arrangement was lost, and the nuclear contents appeared to be a rather uniform mass of nucleosomes (Fig. 7B,C). Tilney (1976) observed a similar loss of chromatin fiber morphology following isolation of nuclei using a variety of procedures from sperm of the echinoderm *Thyone briareus*.

![Table 2. Permeability of starfish sperm nuclei to micrococcal nuclease and dextrans](image)

<table>
<thead>
<tr>
<th>Ionic strength of buffer (mM)</th>
<th>36</th>
<th>48</th>
<th>70</th>
<th>100</th>
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<tr>
<td>Dextran-FITC Dye Diameter (nm)*</td>
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<tr>
<td>4,100 4.6</td>
<td>++</td>
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<tr>
<td>9,000 5.4</td>
<td>++</td>
<td>++</td>
<td>++</td>
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<tr>
<td>65,600 11.3</td>
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<tr>
<td>156,000 18.1</td>
<td>-</td>
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<td>497,000 44.4</td>
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<tr>
<td>Nuclease-FITC 4.0†</td>
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</table>

Nuclei were incubated with fluorescein-conjugated probes, washed and examined by fluorescence microscopy.
++ fluorescence within nuclei similar to or above background level;
+-weak fluorescence within nuclei, lower than background; -, no fluorescence within nuclei.

†Estimated from value of free enzyme diameter determined by Cotton et al. (1979).

Fig. 7. Loss of in situ chromatin fiber morphology after isolation of nuclei in TIB (A) and MIB (B,C). Samples were low-temperature embedded and stained with the DNA-specific osmium ammine-B reagent. At higher magnification (C) a mass of fibrils and approx. 10 nm particles characteristic of linker DNA and nucleosomes is seen. Bars: 0.5 µm (A) and (B); 50 nm (C).
DISCUSSION

A primary goal of this study was to trace the process by which large assemblies of nucleosomes are released from nuclei for study in vitro, and to monitor the retention of higher-order structure during these events. The starfish sperm nucleus provides a useful system for this purpose, having a normal complement of histones (Fig. 2A), a typical nucleosomal organization (Fig. 2B), and a well-defined compact chromatin fiber morphology in intact cells (Fig. 1B,C). Also, standard techniques can be used to isolate nuclei, digest them with micrococcal nuclease, and release polynucleosomes that assume a compact fiber morphology in vitro, under appropriate salt conditions (Fig. 5). The observation of fibers with a similar general morphology in situ and after isolation leads to the assumption that the process of digestion and release simply involves nuclease cutting followed by diffusion from the nucleus. Our results show that this does not occur. Isolated nuclei from which polynucleosomes can be released have lost the compact fiber organization (Fig. 7), containing instead a mass of nucleosomes. Moreover, the permeability properties of isolated nuclei would prevent the diffusion of compact fibers from them (Table 2).

Structural transitions in chromatin

The loss of compact fiber morphology occurred under all conditions tested and was not confined to nuclei that also underwent swelling or other loss of the native size and shape. After loss of fiber morphology, nucleosomes were still present but no longer distributed in a pattern that led to the recognition of individual fibers (Fig. 7). This loss of fiber morphology under conditions allowing nuclease digestion appears to be a general one, not restricted to this species or cell type. We have previously shown that long compact fibers can be released from the nucleated erythrocytes of *Necturus maculosus* after digestion in 60 mM KCl, 15 mM NaCl, 15 mM HEPES, pH 8.0 (Woodcock et al., 1991a,b). Observation of these nuclei in the same buffer prior to adding nuclease shows that, here also, the compact fiber morphology is lost (not shown). Similar behavior is seen with avian erythrocyte nuclei. Although in this case fibers are often preserved after the initial nuclear isolation (e.g. see Brasher et al., 1972; Olins and Olins, 1972; Davies et al., 1974; Langmore and Paulson, 1983; Horowitz et al., 1990), we find that once such nuclei are allowed to equilibrate in a buffer that allows digestion and release to take place, fibers are no longer present (not shown). We suggest that in no case has it yet been proven that isolated chromatin fibers have been generated from nuclei retaining the compact fiber morphology.

At present the forces that govern the intranuclear arrangements of nucleosomes are poorly understood, although there is evidence from in vitro studies that the positively charged N termini of the core histones interact with the DNA of neighboring nucleosomes (Hansen et al., 1989). In the concentrated milieu of the nucleus, this type of interaction is likely to occur both between and within chromatin fibers, while the H1-DNA interactions that are required for fiber formation and stability in vitro (Allan et al., 1986) will be largely within individual fibers. In considering the loss of fiber morphology observed here, one possibility consistent with the available evidence is that changes in the balance between these inter-fiber and intra-fiber interactions (as a result of an altered concentration of ions) lead to a greater diffusional freedom for the individual nucleosome, now restricted primarily by linker DNA continuity. As the relative strength of inter-fiber interactions increased, fiber outlines would first become blurred, then finally lost. The path of a DNA strand would continue to trace out a fiber-like trajectory, but there would no longer be any other structural discrimination between fibers. The overall distribution of nucleosomes would then approach the ‘liquid’ state proposed by McDowall et al. (1986) on the basis of their examination of frozen hydrated sections of metaphase chromosomes.

Nuclear permeability

Once chromat has entered an ‘amorphous’ state, nuclease digestion and release of polynucleosomes is strongly influenced by the ionic strength of the medium. At 150 mM monovalent ions, nuclei retain their native size and shape, and the chromatin is much more resistant to nuclease attack than at 100 mM ions or less (Fig. 3). Our experiments with fluorescein-nuclease show that this resistance is not due to protection of linker DNA within a fiber, but to the restricted permeability of the whole nucleus to micrococcal nuclease (Table 2). As the ionic strength is lowered, nuclear permeability increases, allowing increased entry of the micrococcal nuclease enzyme, and resulting in a similar extent of digestion over the range of 36 mM to 100 mM monovalent ions (Fig. 6). Nevertheless, the amount of soluble chromatin released is inversely related to ionic strength. This effect is most simply explained by the increase in nuclear permeability that occurs as the ionic strength is lowered. Since the nuclear envelope is largely removed during isolation (Fig. 7A), it is unlikely to influence these permeability studies.) During the transition from 150 mM to 36 mM monovalent ions, the exclusion limit for dextrans increases from approx. 6.6 nm to 11.3 nm, concomitant with a nuclear diameter increase from approx. 3 μm to 4 μm (over 2-fold volume increase). We suggest that the amount of chromatin solubilized is dependent on the ability of the cut fragments to diffuse within and escape from the isolated nuclei. Since the exclusion size limit for dextrans is considerably smaller than the 30-40 nm diameter of a compact chromatin fiber, it is most unlikely that compact fibers could diffuse from these nuclei.

Compact fibers in solution

In view of the above, why is soluble chromatin found in the compact fiber form? We suggest that upon release from nuclei polynucleosomes undergo a rapid dilution from the very high intranuclear concentrations, effectively eliminating inter-fiber interactions. Under these conditions intra-fiber interactions dominate and, if the ionic conditions are appropriate, the fiber condenses and assumes the compact state. Is the isolated fiber in solution identical to its original in situ state? This has been a difficult question to answer rigorously, but given the similarities of gross morphology and biochemical composition, and in the absence of contrary data, isolated fibers have been assumed to mirror the
in vivo state. Our results, indicating an obligatory conformational transition, as well as a change in diameter between the in situ and in vitro states, suggest that the question should be re-examined.

**Micrococcal nuclease as a probe for chromatin higher-order structure**

Our results suggest that micrococcal nuclease cannot be used to obtain information about the organization of chromatin fibers in nuclei. Although reduced enzymatic activity occurs in DB150 (Fig. 3), consistent with, for example, a decreased linker DNA accessibility in a compact chromatin fiber, the effect is accounted for by the reduced inaccessibility of the whole nucleus (Table 2). Moreover, the absence of fiber morphology at all ionic strengths (Fig. 7) indicates that the chromatin substrate for the enzyme is inappropriate for studies of fiber organization.

**Chromatin fiber diameter**

The diameter of the compact chromatin fiber has become an important issue in testing the different proposed models of fiber architecture (Felsenfeld and McGhee, 1986). Those models in which the linker DNA traverses the fiber (Williams et al., 1986; Bordas et al., 1986) predict a linear relationship between linker length and fiber diameter. It has been well documented that the diameters of isolated compact fibers are proportional to the DNA linker length (Athey et al., 1990; Williams and Langmore, 1991), and a similar relationship for in situ fibers has been claimed by Alegre and Subirana (1989). In the latter study, chromatin fiber diameters, measured after embedding isolated nuclei in epoxy resin, were recorded for erythrocyte nuclei (repeat length 212 bp) in nine different buffers, and a correlation was obtained between divalent cation concentration and fiber diameter, the maximum being 29.0 nm, s.d. 2.9 nm, in 1 mM MgCl$_2$, 10 mM Tris, pH 7.0. For *Holothuria tubulosa* sperm, repeat length 227 bp, two buffers were used, the maximum diameter being 28.4 nm, s.d. 1.8 nm. In view of the similar maximum values for the chicken erythrocyte and *H. tubulosa* sperm, the sparse information on mouse thymus, and the different buffer conditions used for each species, we think that a relationship between linker length and in situ fiber diameter remains to be proven. Our diameter values for the isolated fibers (Table 1) are consistent with the diameter/linker length relationship, but the significantly smaller approx. 30 nm for in situ fibers prepared by low-temperature methods is not. Three pieces of evidence suggest that the values for in situ fibers are not the result of preparation-induced shrinkage. First, comparisons of similar scale components of the sperm axoneme with their negatively stained counterparts reveal no shrinkage after low-temperature embedding, consistent with other studies showing superior preservation of structure after low-temperature preparation (Carlemalm et al., 1982). Second, isolated compact fibers have a similar diameter whether embedded in Lowicryl (40.4 nm, s.d. 2.0 nm) or negatively stained (42.0 nm, s.d. 2.0 nm). Third, cryosection of sperm cells contain fibers with a diameter of 31.9 nm, s.d. 2.6 nm (Table 1). Thus, it seems unlikely that the observed difference in diameter between the in situ and isolated chromatin fibers is a trivial result of the different methods used for ultrastructural research. It is not yet clear what structural differences lead to the change in diameter, but an important future goal will be to determine how the processes leading to fiber formation and maintenance in vitro differ from those operating in the intact cell.

**Isolation of higher-order chromatin**

There are obvious advantages for studying subcellular components, including higher-order chromatin structures, in vitro. For chromatin, it is not clear what isolation strategies are optimal, or whether any techniques yet exist that permit the isolation of poly nucleosomes that retain their in vivo higher-order conformation. Even if it were possible to isolate nuclei that retained the native chromatin morphology in a state accessible to exogenous nuclease, the ionic strengths needed to prevent decondensation would promote the mobility of histone H1 (Caron and Thomas, 1981; Thomas and Rees, 1983; Williams and Langmore, 1991), depleting the isolated material. In addition, the problems of nuclease penetration and release of polynucleosomes discussed here would have to be taken into account. Until more effective isolation methods for chromatin are developed, it is important to recognize the limitations of available techniques, and it may be advantageous to place more emphasis on studies using intact cells.

**Morphology of chromatin in general**

The starfish sperm belongs to a limited class of cells in which compact chromatin fibers are readily seen in the intact nuclei. More typically, compact chromatin fibers are not seen in thin sections of either interphase nuclei or chromosomes fixed in situ, but may be observed (at the appropriate ionic strength) following nuclease digestion of isolated nuclei. Also, under a variety of conditions involving short exposure to lowered ionic strength media, compact fibers may be observed in whole chromosomes (e.g. see Bahr, 1977; Rattner and Hamkalo, 1978) or interphase nuclei (e.g. see Gall, 1963; Kirschner et al., 1977). One explanation for the infrequent observation of chromatin fibers in situ is that techniques for contrasting chromatin are insufficient to resolve close-packed fibers in the presence of other nuclear components. With the refinement of DNA-specific staining in combination with low-temperature embedding (Derenzini and Farabegoli, 1991; Horowitz and Woodcock, 1992), this argument is becoming less attractive. In light of the present study, we propose that the state of chromatin in vivo be considered in terms of transitions in chromatin morphology related to the balance between inter- and intra-fiber interactions. In solution, all chromatin appears to behave in a similar fashion, inter-fiber interactions being minimal unless the salt concentration is sufficient to promote precipitation (Widom, 1986; Clark and Kimura, 1990). Under the conditions prevailing in the typical nucleus or chromosome, we suggest that inter-fiber interactions are sufficiently strong to prevent the structural discrimination between fibers, but that sudden changes in ionic conditions, such as a rapid drop in ionic strength, can
produce a transient appearance of fibers. If the native state of chromatin in most nuclei and chromosomes does not involve compact fibers, then the key to the accessibility of a given gene or chromatin domain would be the permeability of the local chromatin mass, rather than the specific architecture of the canonical compact fiber.

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REFERENCES


Transitions during chromatin isolation


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