Partial denaturation of small chromatin fragments: direct evidence for the radial distribution of nucleosomes in folded chromatin fibers

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Accepted 2 April; published on WWW 27 May 1998

SUMMARY

To examine the internal structure of chromatin fibers, we have developed procedures for partial denaturation of small chromatin fragments (8-30 nucleosomes) from chicken erythrocytes. Electron micrographs of samples prepared under conditions that cause nucleosome dissociation show rods and loops projecting from short compact fibers fixed by glutaraldehyde in 1.7 mM Mg2+. According to previous studies in our laboratory, these images correspond to the top view of partially denatured fibers. Our results indicate that rods and loops consist of extended duplex DNA of different lengths. DNA in loops is nicked, as demonstrated by experiments performed in the presence of high concentrations of ethidium bromide. Length measurements indicate that the radial projections of DNA are produced by unfolding of nucleosomal units. Loops are formed by DNA from denatured nucleosomes in internal positions of the fiber; DNA from denatured nucleosomes in terminal positions form rods. Our micrographs show clearly a radial distribution of DNA loops and rods projecting from fibers. Rods are orthogonal to the surface of the chromatin fragments. Considering that the high ionic strength used in this study (0.8-2.0 M NaCl) neutralizes the electrostatic repulsions between rods and fiber, this observation suggests that rods are extensions of nucleosomes radially organized inside the fiber. The position of the entry points of DNA loops into the fiber could be influenced by constraint on loops, but our results showing that the arc that separates these points in dinucleosome loops is relatively short suggest that consecutive nucleosomes are relatively close to each other in the folded fiber.

Key words: Chromatin fiber, Chromatin folding, Nucleosome

INTRODUCTION

Eukaryotic chromatin is organized according to various hierarchical levels of DNA folding (Wolffe, 1995; Koshland and Strunnikov, 1996). The fundamental structural unit of chromatin, the nucleosome, contains two turns of DNA (about 165 bp) wrapped around a histone octamer (Richmond et al., 1984; Arents et al., 1991; Arents and Moudrianakis, 1993; Luger et al., 1997), and has associated with it a single molecule of histone H1 (Ramakrishnan et al., 1993; Pruss et al., 1996; Travers and Muylldermans, 1996; Crane-Robinson, 1997). Nucleosomes are connected by linker DNA and form fibers of 30-40 nm in diameter (Thoma et al., 1979; Bradbury and Baldwin, 1986; Gerchman and Ramakrishnan, 1987; Koch et al., 1988; Athey et al., 1990; Woodcock, 1994; Zlatanova et al., 1994) which are probably involved in the packaging of DNA in interphase and metaphase chromosomes (Pienta and Coffey, 1984; Rattner and Lin, 1985; Manuelidis, 1990; Saitoh and Laemmli, 1993; Belmont and Bruce, 1994).

The structure of the folded chromatin fiber has been difficult to study (Widom, 1989; Woodcock and Horowitz, 1995; van Holde and Zlatanova, 1995, 1996). Using transmission electron microscopy, the location of nucleosomes in the fiber is only clearly seen when chromatin has an extended conformation at low ionic strength. Several laboratories have observed that under these conditions chromatin appears as a zigzag chain of nucleosomes in which linker DNA is completely extended (Thoma et al., 1979; Losa et al., 1991; Arents and Moudrianakis, 1993; Luger et al., 1997), and has associated with it a single molecule of histone H1 (Ramakrishnan et al., 1993; Pruss et al., 1996; Travers and Muylldermans, 1996; Crane-Robinson, 1997). Nucleosomes are connected by linker DNA and form fibers of 30-40 nm in diameter (Thoma et al., 1979; Bradbury and Baldwin, 1986; Gerchman and Ramakrishnan, 1987; Koch et al., 1988; Athey et al., 1990; Woodcock, 1994; Zlatanova et al., 1994) which are probably involved in the packaging of DNA in interphase and metaphase chromosomes (Pienta and Coffey, 1984; Rattner and Lin, 1985; Manuelidis, 1990; Saitoh and Laemmli, 1993; Belmont and Bruce, 1994).

To overcome the difficulties encountered in the study of the organization of nucleosomes in these compact structures of high molecular mass, several laboratories have examined the structure of small chromatin fragments (Bartolomé et al., 1994) and complexes containing few nucleosomes (Yao et al., 1990, 1996).
1991; Bednar et al., 1995). The results obtained in all these microscopy studies, together with indirect data obtained using other techniques (McGhee et al., 1983; Widom and Klug, 1985; Bradbury and Baldwin, 1986; Koch et al., 1987; Williams and Langmore, 1991; Graziano et al., 1994) have led to the suggestion of various models for folded chromatin fibers. At present, there are two kinds of models for the organization of nucleosomes in the folded chromatin fiber. (i) In the solenoidal models linker DNA is folded and consecutive nucleosomes form a helix (Finch and Klug, 1976; McGhee et al., 1983; Butler, 1984; Widom and Klug, 1985; Bartolomé et al., 1994; Graziano et al., 1994; Daban and Bermúdez, 1998). (ii) In the crossed-linker models (Staynov, 1983; Subirana et al., 1985; Williams et al., 1986; Bordas et al., 1986; Koch, 1989; Atthey et al., 1990), and in the variable zigzag nucleosomal ribbon models (Woodcock et al., 1993; Leuba et al., 1994), consecutive nucleosomes are roughly in opposite positions with respect to the fiber axis and linker DNA is extended into the fiber interior.

We have previously shown, using transmission electron microscopy (Bartolomé et al., 1994), that in the presence of 1.7 mM Mg$^{2+}$ rotary-shadowed chromatin fragments from chicken erythrocytes containing from about 6 to 35 nucleosomes are circular structures with approximately the same diameter (33 nm). Our results from unidirectional shadowing experiments showed that the height of these structures increases with the number of nucleosomes. These height measurements, and the results showing that the electrophoretic mobility of small chromatin fragments in the presence of 1.7 mM Mg$^{2+}$ decreases only slightly with molecular mass (Bartolomé et al., 1995), led us to the conclusion that under these conditions chromatin fragments are highly packed and form short folded fibers. This high compactness favours the vertical placement of the folded fibers on the carbon film of the electron microscopy grid (Bartolomé et al., 1994). In this work, we have taken advantage of the possibility to obtain images corresponding to the top view of short folded fibers to study directly the distribution of nucleosomes within the fiber. Following an experimental approach similar to that of Paulson and Laemmli (1977) for the study of metaphase chromosomes, we have developed different procedures to remove histones from small chromatin fragments and have examined the products by electron microscopy. The resulting images show radially distributed DNA loops and threads with a free end attached to the remaining part of the folded fiber. Length measurements indicate that these radial projections of DNA are produced by unfolding of nucleosomal units.

### MATERIALS AND METHODS

#### Preparation and fixation of small chromatin fragments

Chicken erythrocyte chromatin was prepared as described (Bartolomé et al., 1994). In brief, nuclei were digested at 37°C for 30-90 minutes with micrococcal nuclease (Sigma; 1 unit/mg of DNA) in 90 mM KC1, 30 mM NaCl, 0.5 mM spermidine, 0.15 mM spermine, 0.4 mM phenylmethylsulfonyl fluoride (PMSF), 10 mM triethanolamine-HCl, pH 7.4, 1 mM CaCl$_2$. Soluble chromatin of relatively low molecular mass was extracted overnight at 4°C with 40 mM NaCl, 1 mM EDTA, 0.4 mM PMSF, 10 mM triethanolamine-HCl, pH 7.4, concentrated to 5-6 mg of DNA/ml with a Diaflo PM10 concentrator (Amicon), and fractionated by a 5-20% sucrose gradient containing 1 mM EDTA, 0.4 mM PMSF, 10 mM triethanolamine-HCl, pH 7.4. The selected chromatin fractions were further concentrated (~5 mg of DNA/ml with Centricon 10, Amicon) and purified by electrophoresis on 0.5% agarose gels in the presence of 90 mM Tris-borate, pH 8.3, and 1.7 mM MgCl$_2$ as previously described (Bartolomé et al., 1995). After electrophoresis, the gel was stained with ethidium bromide (1 µg/ml) and chromatin bands were visualized by ultraviolet transillumination. In some experiments duplicate bands were neither stained with ethidium bromide nor irradiated by ultraviolet light. Selected bands were excised from the gel, washed twice (for 1 hour each time) with 1.5 ml of a buffer containing 90 mM triethanolamine-borate, pH 8.6, 0.5 mM PMSF, 1.7 mM MgCl$_2$, and, unless otherwise indicated (see below), crosslinked with 1.5 ml of 0.1% glutaraldehyde in the same buffer for 15 hours at 4°C. The resulting gel slice, plus 50 µl of the crosslinking buffer, were transferred to an Ultrafree-CL tube with a 0.45 µm membrane (Millipore), and centrifuged at 2,500 g for 30 minutes. The eluted chromatin fragments were used directly or treated with the denaturing agents indicated below before electron microscopy. Part of the chromatin of the different experiments was used to analyse the length of DNA on 0.7% agarose gels. In this study we have used samples containing fragments with about 8, 19 and 30 nucleosomes. Long chromatin fragments presented in Fig. 6 were prepared as described by Bartolomé et al. (1994) and purified by electrophoresis as indicated above for small chromatin fragments.

#### Denaturation of chromatin fragments

Generally, the small chromatin fragments used in denaturation experiments were previously crosslinked with 0.1% glutaraldehyde as described in the preceding section, but in some experiments, to facilitate further chromatin denaturation, we used lower concentrations of glutaraldehyde (from 0.04 to 0.08%). All denaturation experiments were performed in the presence of 90 mM triethanolamine-borate, pH 8.6, 0.5 mM PMSF, 1.7 mM MgCl$_2$. Part of the chromatin samples (20 µg of DNA/ml) were digested with trypsin (Sigma, type IX) before the treatment with high concentrations of NaCl (see below). These samples were digested for 2 hours at room temperature with trypsin (11 µg/mg of DNA). Digestion was stopped by the addition of bovine pancreas trypsin inhibitor (Sigma; 2 µg/µg of trypsin). To study the denaturing effect of NaCl, chromatin samples were incubated with the indicated concentrations of NaCl (see figure legends) for 3-6 hours at 4°C. Finally, part of the samples were treated with different concentrations of ethidium bromide (see Figs 2, 7, 8) for about 2-3 hours at room temperature. The concentration of chromatin in NaCl and ethidium bromide denaturation experiments was 1-6 µg of DNA/ml; the resulting samples were used directly in electron microscopy experiments.

#### Electron microscopy and data analysis

Spread preparations of chromatin samples were obtained using carbon-coated copper grids pretreated with Alcian blue as described elsewhere (Aragay et al., 1991). The grids were rotary shadowed with platinum-carbon at an angle of about 7°. Micrographs were obtained at a magnification of 30,000 with a H7000 (Hitachi) transmission electron microscope operated at 75 kV. Magnifications were calibrated using a grating with 54,000 lines/inch (Bal-Tec). The thickness of bars, loops and reference DNA molecules was measured with a ruler on magnified projections of micrographs. For these measurements we used preparations of partially denatured chromatin fragments containing linear and circular naked DNA molecules of about 3,000 bp as internal references. The contour length of bars and loops were obtained from the projected micrograph images using a digitizer. Images from the original micrographs were acquired with a Horizon (Agfa-Gevaert) scanner and printed in negative contrast using a Stylus Pro (Seiko Epson) color printer.
RESULTS

Denaturation produces bars and loops projecting from chromatin fibers

We have investigated different procedures for the denaturation of small chromatin fragments. As can be seen in Fig. 1, a decrease in glutaraldehyde concentration, below the normal concentration used for this crosslinking agent (0.1%), results in the formation of soft structures that can be unfolded in the presence of 2 M NaCl. This high concentration of NaCl produces the complete dissociation of native uncrosslinked nucleosomes (Aragay et al., 1991). The electron micrographs in Fig. 1a-d show that under these conditions most of the chromatin is unfolded. Fig. 1k is an example corresponding to a particle completely denatured. In some samples (Fig. 1e-j) the material projecting from the denatured chromatin fragments forms loops of different lengths.

Partial unfolding can be obtained using chromatin fragments crosslinked with 0.1% glutaraldehyde in the presence of 1.7 mM Mg^{2+}. Treatment of these samples with different concentrations of NaCl (Fig. 2b,d,e) and ethidium bromide (Fig. 2c) gives rise to relatively small loops and threads with a free end which are often relatively straight (see below, Fig. 3) and generally are called rods or bars in this work. Trypsin alone is unable to induce significant denaturation of chromatin fragments (see Fig. 2a), but this enzyme has been included in many samples to facilitate the removal of histones from crosslinked structures. We have analysed 1,307 short chromatin particles showing different degrees of partial denaturation: 22.3, 2.8 and 1.1% of these particles show, respectively, 1, 2 and 3 or more loops; and 39.9, 28.8 and 14.9% show 1, 2 and 3 or more bars, respectively. Note that, as can be seen in the different figures presented in this work, there are structures that have both loops and rods.

Although all the chromatin fragments treated with the denaturing agents used in this work are structurally altered,
under the conditions indicated in Fig. 2, the integrity of the folded fragments is preserved to some extent. It can be seen that the micrographs obtained with partially denatured chromatin contain circular structures similar to those observed previously in our laboratory (Bartolomé et al., 1994, 1995) with preparations of native chromatin fragments. According to our previous observations these circular structures correspond to the top view of short chromatin fibers and we will show below that the study of the images obtained with partially unfolded structures gives information about the internal structure of folded fibers.

Rods and loops consist of extended duplex DNA
The NaCl concentrations used in this study range from 0.8 to 2 M. These conditions produce nucleosome dissociation (Aragay et al., 1991). Thus, presumably, the material in bars and loops considered in the preceding section is DNA that was packed in nucleosomes inside the fibers before unfolding. In fact, the structures that are completely denatured (e.g. Fig. 1k) have a length that is roughly in agreement with the values expected for the DNA of the corresponding chromatin fragments. Furthermore, we have measured the thickness of bars and loops (see Table 1). Both structures have approximately the same thickness as that obtained with linear naked DNA included in the electron microscopy preparations as an internal reference (see Fig. 2a,d,e). These measurements have been done from metal-shadowed preparations, in which the diameter of the DNA is significantly increased by metal deposition. However, from our data it is possible to distinguish between linear double stranded DNA and tightly interwound circular DNA (see Fig. 2c and Table 1). Thus, our results indicate that bars and loops are formed by extended duplex DNA.

Rods and loops are produced by unfolding of nucleosome units
Selected electron micrographs presented in Figs 3, 4 show that partially denatured structures contain bars and loops of different lengths. The histograms corresponding to all the samples measured in this study (Fig. 5) show that both bars and loops have a discontinuous distribution of lengths. Only the rods and loops that are clearly attached to the chromatin fiber and can be easily followed along their entire length without discontinuities have been considered for these measurements. Assuming that duplex DNA has a rise of 0.34 nm per bp, the results obtained for the DNA bars in Fig. 5A...
correspond to about 168 bp for the mean of the first peak. This result is similar to the value obtained for the first peak of loop length measurements (182 bp; Fig. 5B). Since it seems reasonable to consider that part of the linker DNA could be attached inside the fiber after denaturation, these values are compatible with the length expected for DNA of a single nucleosome (206–210 bp for chicken erythrocytes; Williams et al., 1986; Williams and Langmore, 1991; Leuba et al., 1994). The mean of the second peak found for loops corresponds to 324 bp, a value that is very similar to the mean of the small peak (326 bp; partially hidden by the main peak) of the histogram of bar lengths and, presumably, is due to the unfolding of DNA of two consecutive nucleosomes. The small number of loops corresponding to the third peak makes difficult the determination of its mean length. We have calculated that the mean corresponding to this peak is 509 bp. This value could correspond to the DNA of three consecutive nucleosomes denatured by our treatments.

Taken together these results suggest that loops are formed by DNA from nucleosomes in internal positions of the folded chromatin fibers, and bars correspond to denatured nucleosomes in the ends of the folded fibers. Fig. 6 shows longer fibers containing bars and loops produced by partial denaturation by treatment with 2.0 M NaCl. It can be seen that in this case the images correspond to a side view of the fibers and that bars are located exclusively in the terminal regions. We have found 67 long fibers having bars: 58 show 1 bar and 9 have 2 bars; in all cases bars are in the terminal regions, and in fibers with two bars each bar is at one end. This demonstrates that as expected the release of free-ended DNA is from the terminal nucleosomes.

In principle, the above considerations exclude the possibility of formation of more than two bars per fiber. Nevertheless, we have observed short fibers with three or four bars (see for instance Fig. 2c). The break of DNA in loops, favoured by the more intense digestion required to prepare short fragments, could produce the observed extra bars. On the other hand, the spontaneous association of small chromatin fragments observed in our previous studies (Bartolomé et al., 1995) can give rise to structures with the same shape as normal folded fibers but containing four terminal nucleosomes. Fibers containing associated fragments could originate partially denatured structures with more than two bars.

Denaturation in the presence of ethidium bromide indicates that DNA in loops is nicked

The relatively high degree of denaturation of chromatin fragments shown in Fig. 7 is due to the presence of ethidium bromide. The high concentrations of ethidium bromide used in this study can induce nucleosome dissociation (McMurray and van Holde, 1986). In addition, this intercalating agent could produce supercoiled DNA in loops. In fact, control circular DNA included in our preparations containing ethidium bromide forms tightly interwound structures (see Fig. 2c) with thickness significantly higher than that observed for normal duplex DNA (Table 1). To our surprise, DNA in loops of denatured fragments has the same thickness as extended duplex DNA (see above and Table 1) and is not supercoiled in the presence of high concentrations of ethidium bromide.
ethidium bromide. On the other hand, bars could be produced from loops having its DNA tightly interwound due to the presence of high concentrations of ethidium bromide. However, our thickness measurements (Table 1) also indicate that DNA in bars is not supercoiled.

One possibility to explain these results is that histones, or fragments of these proteins, remain bound to DNA in denatured structures and preclude ethidium bromide binding. Nevertheless, the results presented in Fig. 8 show that the presence of increasing amounts of ethidium bromide in our samples produces a significant increase in the length of DNA in loops. Since the observed increase in length (47%) is approximately the value expected for DNA fully saturated with ethidium bromide (Cantor and Schimmel, 1980), these results indicate that this dye is able to bind DNA in loops by intercalation. From these observations it can be suggested that we do not detect the expected supercoiling in the presence of intercalated ethidium bromide, because loops contain nicked DNA. Nicks could be produced by micrococcal nuclease used in the preparation of small chromatin fragments. It has been demonstrated by Cockell et al. (1983) that this enzyme can introduce single-stranded nicks during nucleosome digestion. Furthermore, according to early studies on DNA photonicking (Brunk and Simpson, 1977), the ultraviolet illumination used for the visualization and excision of chromatin bands from preparative electrophoretic gels could also produce nicking. However, since we have obtained the same results using samples that have not been irradiated with ultraviolet light, it can be concluded that nicks are produced by the nuclease treatment.

Radial distribution of bars and loops

It can be seen in many micrographs (Figs 2, 3, 4, 7) that loops

![Fig. 7. Gallery of electron micrographs of small chromatin fragments treated with different concentrations of NaCl and ethidium bromide. Chromatin fragments containing ~8 nucleosomes in 1.7 mM Mg²⁺ were crosslinked with 0.1% glutaraldehyde. Samples in d-f were digested with trypsin before the treatment with NaCl and ethidium bromide. Samples were treated with 0 M (b,c), 1.0 M (a,e-h), and 2.0 M (d,i-l) NaCl, and 0.01 µg/ml (a), 5.0 µg/ml (j,k), 8.1 µg/ml (d), 25.2 µg/ml (f), 50.4 µg/ml (b,c), 101 µg/ml (e) and 504 µg/ml (g-i,l) of ethidium bromide. Bar, 100 nm.](image)

![Fig. 8. Length of DNA in loops of partially denatured chromatin fragments as a function of ethidium bromide concentration. The values represented correspond to the mean length of the first peak (i.e. denatured mononucleosomes) of histograms similar to that presented in Fig. 5B but for samples containing the indicated concentrations of ethidium bromide.](image)

![Fig. 9. (A) Values of angle α of bars exiting partially denatured chromatin fragments. Angle α is defined in the inset (positive values for clockwise angles). The mean angle is 0.56° (s.d. 1.9°). (B) Values of angle β (see inset). This angle was measured considering the two lines going from the point at the center of the fiber to the entry and exit points of the loop. The data were obtained from loops with a length corresponding to about two nucleosomes (i.e. loops having a length equal to the mean ± s.d. of the second peak in Fig. 5B, or equivalent samples containing ethidium bromide). The mean angle is 47° (s.d. 16°). The curves correspond to the Gaussian fittings of measurements obtained from samples denatured as indicated in Figs 3, 4, 7.](image)
and bars are radially projecting from the central fiber. Furthermore, the micrographs presented in Fig. 3 show that bars are roughly orthogonal to the surface of the chromatin fragment. The histogram in Fig. 9A demonstrates that the exiting angle of bars (angle α, defined as indicated in the inset of this figure) is approximately 0°. These observations suggest that bars are extensions of structural elements radially organized inside the fiber. Nevertheless, the possibility that the radial projection of rods could be due to electrostatic repulsion between DNA in rods and the chromatin particle or to interaction with the grid surface has to be considered. Almost all our denatured samples containing rods were prepared and spread in the presence of 1.0 to 2.0 M NaCl and 1.7 mM MgCl2 (see Fig. 3b-r). Under these conditions the electrostatic interactions are significantly reduced. Bednar et al. (1994) have shown that the DNA-DNA electrostatic repulsion decreases significantly in the presence of 0.1 M NaCl or 10 mM MgCl2, and causes the supercoiled DNA molecules to change from a loose structure to a tightly interwound superhelix, with the two DNA chains in close contact. Our own results indicate that in the presence of 1 M NaCl supercoiled DNA is tightly interwound (see Fig. 2c and Table 1). In addition, it is well known that the strong electrostatic interaction between histones and DNA is dependent on ionic strength; dissociation occurs from 0.8 to 2.0 M NaCl (see above). Thus, it seems unlikely that, under the high ionic strength conditions used in our samples, the suggested electrostatic repulsion between DNA in rods and the chromatin particle can be responsible for the values obtained for the angle α. It seems also unlikely that the interaction of DNA with the electron microscopy grids can produce a systematic orientation of the rods. Taken together, our results and all these considerations strongly suggest that nucleosomes are radially arranged in the folded fiber and organize the DNA of the projecting bars and loops of partially denatured fibers following the radial patterns seen in our micrographs.

Finally, to obtain information about the relative location of consecutive nucleosomes in the fiber, we have measured the angle corresponding to the arc that separates the two entry points of the DNA loops into the fiber (angle β, defined in Fig. 9B). The observed values of angle β may be influenced by the constraint of the loops. Mononucleosome loops are found frequently in our denatured samples but they are short (62 nm) and presumably have more intense constraint than di- and trinucleosome loops; in this case the mean value of angle β is 45° (s.d. 19). Dinucleosome loops are also found frequently, allowing good statistical determination of angle β (see Fig. 9B; mean angle 47°; s.d. 16). Loops of three nucleosomes are much longer (173 nm), but their frequency is low. We have analysed more than a thousand denatured fibers (see above), but we have found only 14 trinucleosome loops; the mean value of angle β is 77° (s.d. 37). Assuming that constraint on mononucleosome loops is responsible for the similar values of angle β found for mono- and dinucleosome loops, these results indicate that angle β increases with the number of denatured nucleosomes forming the loop. Furthermore, the angle β corresponding to dinucleosome loops can give information about the relative location of two consecutive nucleosomes in the fiber. As compared to the perimeter of the folded fiber (~129 nm), dinucleosome loops are long enough (110 nm) to have entering and exiting positions far from each other. Nevertheless, the low value found for angle β in dinucleosome loops suggests that the internal structural elements that hold the DNA ends of two consecutive nucleosomes are relatively close to each other in the folded fiber.

**DISCUSSION**

Images of partially denatured small chromatin fragments are informative about the internal structure of folded chromatin fibers because, under the conditions used in this study, part of the folded fiber is preserved in almost its original form. According to our previous findings (Bartolomé et al., 1994) these images correspond to the top view of small chromatin fragments and consequently are equivalent to electron micrographs of cross sections of highly packed fibers. Although we cannot exclude the possibility that bars and loops in partially denatured fibers could contain crosslinked histone fragments, our thickness measurements (Table 1), and results indicating the binding of large amounts of ethidium bromide (Fig. 8), allow us to conclude that these structures are formed by extended duplex DNA. The results obtained in the presence of high concentrations of ethidium bromide (Fig. 7) indicate that DNA in loops is nicked and cannot form supercoiled DNA. This unexpected finding has precluded the study of the degree of supercoiling of nucleosomal DNA in chromatin fibers. On the other hand, from these results, it can be speculated that only nicked DNA possesses the structural properties necessary for its projection from the folded fiber under the denaturing conditions used in our experiments.

Our length measurements strongly suggest that denaturation of nucleosome units originates rods and loops in which part of the linker DNA remains attached to internal regions of the folded fiber (Fig. 5). In keeping with this observation, our micrographs also show that bars are roughly orthogonal to the surface of the fiber (Fig. 9A), indicating that they are extensions of DNA from nucleosomes radially organized inside the fiber. This is also inferred from results showing that loops from denatured mono-, di-, and trinucleosomes are radially projecting from the fiber (Figs 4, 7). The possibility that the radial projection of rods and loops could be influenced by the electrostatic repulsion between released DNA and the chromatin particle has been considered above. In the presence of the high concentration of NaCl (and MgCl2) used in this work, it seems reasonable to consider that the electrostatic interactions between the fiber and DNA in rods and loops are neutralized.

The angle β between the DNA entering and exiting the folded fiber in loops corresponding to dinucleosomes is relatively low (Fig. 9B). In the scheme presented in Fig. 10 we indicate the implications of this result in the context of the different chromatin models considered in the Introduction. In crossed-linker models and in models based on a three-dimensional zigzag organization of nucleosomes (Williams et al., 1986; Koch, 1989; Athey et al., 1990; Woodcock et al., 1993; Leuba et al., 1994), consecutive nucleosomes are in opposite positions and consequently loops from denatured dinucleosomes (see Fig. 10B) are expected to have DNA entering and exiting the fiber roughly in opposite positions (i.e., angle β approaching 180°). Although our measurements could be affected by loop constraint (see above), the low value found
in this study for angle $\beta$ of dinucleosomes suggests that consecutive nucleosomes are not located in opposite positions with respect to the fiber axis. Our results are more consistent with structures having the connectivity of solenoidal models (Finch and Klug, 1976; McGhee et al., 1983; Butler, 1984; Widom, 1989; Bartolomé et al., 1994; Daban and Bermúdez, 1998), in which consecutive nucleosomes within the fiber are relatively close to each other (Fig. 10A).

The DNA loops seen in the micrographs presented in this work (Figs 4, 7) have been obtained using conditions that produce the dissociation of histones from DNA. Histone-depleted metaphase chromosomes form long radial loops of DNA (50-100 kb) attached to a central scaffold which retains the characteristic shape of metaphase chromosomes (Paulson and Laemmli, 1977). The radially organized DNA loops observed in our denaturation experiments are attached to partially denatured chromatin fibers which remain structured when they are fixed with 0.1% glutaraldehyde. The simple geometrical organization of the DNA loops seen in our micrographs and in histone-depleted metaphase chromosomes suggests that DNA is packaged in chromosomes following relatively simple patterns. According to previous studies (Marsden and Laemmli, 1979; Pienta and Coffey, 1984; Saitoh and Laemmli, 1993), the folding of DNA of small loops into the chromatin fiber is required for the packaging of large DNA loops, but additional folding levels may be necessary for the formation of fully condensed chromosomes (Rattner and Lin, 1985; Belmont et al., 1987; Filipski et al., 1990; Belmont and Bruce, 1994).

We are grateful to F. J. Alba and F. Gallego for generously providing circular and linear DNA used as internal references. Electron microscopy, scanning of micrographs and image analysis were performed at Servei de Microscopía Electrónica, Servei de Tractament d’Imatges, and Laboratori d’Anàlisi i Fotodocumentació, UAB. This work was supported in part by grants PB92-0602 and PB95-0611 from the Direcció General de Investigació Científica y Tècnica. A. B. was supported by a predoctoral fellowship from the Generalitat de Catalunya. We are indebted to one of the referees of our original manuscript for valuable comments.

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