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TITLE: JOURNAL OF MOLECULAR BIOLOGY
PUBLISHER/PLACE: Academic Press London
VOLUME/ISSUE/PAGES:1994 Jan 21;235(3):871-80 871-80
DATE: 1994
AUTHOR OF ARTICLE: Leuba SH; Zlatanova J; van Holde K
TITLE OF ARTICLE: On the location of linker DNA in the chromatin fib
ISSN: 0022-2836
OTHER NOS/LETTERS: Library reports holding volume or year
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J. Mol. Biol. (1994) **235**, 871–880

On the Location of Linker DNA in the Chromatin Fiber

Studies with Immobilized and Soluble Micrococcal Nuclease

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The structure of chicken erythrocyte chromatin fibers has been probed using micrococcal nuclease, both membrane-immobilized and free in solution. Under the extremely mild digestion conditions used, the linker DNA is almost completely protected against digestion with either immobilized or free enzyme in the 30 nm fibers, whereas it is readily accessible in the more extended structures. Control experiments with glutaraldehyde-fixed chromatin fibers gave essentially the same results. Experiments with fibers of intermediate degree of condensation revealed a direct relationship between the degree of compaction and the resistance of linker DNA to digestion. Our results favor models in which access to the linkers is limited by local steric hindrance due to the high compaction, rather than by internalization in the center of the fibers.

Keywords: chicken erythrocytes; chromatin; higher order structure; linker DNA; immobilized micrococcal nuclease

1. Introduction

Since the first recognition of the existence of a folded "30 nm fiber" conformation of chromatin under physiological conditions, the nature of this structure has been the subject of intense controversy (for recent reviews, see van Holde, 1988; Thoma, 1988; Widom, 1989; Tsanev *et al.*, 1992). Many models have been suggested but only limited data are available; most of the results are derived from physical and electron microscopic studies. What does seem clear is that the individual core particles are oriented with their cylinder axes roughly perpendicular to the fiber axis; furthermore, most workers seem to agree that there are about six nucleosomes per turn, in a helix with pitch of approximately 10 nm. The greatest controversy, and the major difference among the models, concerns the disposition of the linker DNA between nucleosomes. In many models the linker is internal to the fiber, in some cases crossing back and forth across the fiber axis, in other cases being internally looped. Some models suggest instead that the linker lies coiled between adjacent nucleosomes.

Closely tied to the problem of the location of the linker DNA is the question of the location of the "linker histones" (the H1/H5 family of lysine-rich histones). These seem to be necessary for the formation of the regular 30 nm fiber (e.g. see Thoma *et al.*, 1979) and are, from much evidence, known to be associated with the linker DNA. Thus, if the linker DNA is internalized, so should be the linker histones.

It has seemed to us that one way of approaching these questions would be through the use of immobilized enzymes to cleave either the linker DNA or the linker histones. Both seem accessible to free enzymes, at least under the rather aggressive conditions usually employed in digesting chromatin. Such, however, are not very revealing experiments, for either nucleases like micrococcal nuclease (MNase§) or proteases like trypsin are small proteins and might be expected to easily penetrate into the fiber structure. Furthermore, the rapid disintegration of the fiber which occurs during most enzymatic digestion studies will rapidly obliterate any subtle effects of structure. Therefore, we decided to utilize immobilized enzymes, and to work under conditions of very mild digestions so that we could observe the initial stages of the process, before

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§ Abbreviation used: MNase, micrococcal nuclease

0022–2836/94/030871–10 $08.00/0

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the responding structure had been destroyed. We felt that the use of immobilized enzymes should allow unambiguous discrimination between external and other alternative locations of the chromatin constituents under study.

In a recent paper, we have described studies of the digestion of linker histones in chicken erythrocyte chromatin using immobilized trypsin and chymotrypsin (Leuba *et al.*, 1993). In this paper, we present parallel experiments in which linker DNA in this type of chromatin is cleaved by immobilized MNase, under conditions corresponding to different chromatin conformations. We include studies using free enzyme, under comparable conditions, yielding somewhat surprising results.

2. Materials and Methods

(a) *Preparation of chromatin*

Chromatin was prepared essentially as described by Ausio *et al.* (1989) with a reduction in the amount of MNase used to avoid significant amounts of residual activity in the final preparation. Briefly, chicken erythrocyte nuclei at 65 A_{260}/ml were digested with 0·09 unit MNase/ml (Worthington) for 2 h at 37 °C. The nuclei were then hypotonically lysed in 0·25 mM EDTA (pH 7·5) and the soluble chromatin was extensively dialyzed *versus* 10 mM Tris·HCl (pH 7·5) and stored at −80 °C. The length of the DNA in chromatin was checked by electrophoresis in 1%(w/v) agarose gels in TAE buffer (40 mM Tris·HCl (pH 8·0), 40 mM acetic acid, 1 mM EDTA: Maniatis *et al.*, 1982). Samples of chromatin were mixed with 2× sodium dodecyl sulfate sample buffer (Laemmli, 1970) and incubated at 37 °C for 15 min prior to electrophoresis. In some cases, the chromatin preparations contained low molecular mass fragments (mono-, di-, tri-, etc. nucleosomes); to separate the high molecular mass material, these preparations were subjected to gel filtration on a Sephacryl S-1000 column (Pharmacia) (25 mm × 750 mm; buffer 10 mM Tris·HCl (pH 7·5); flow rate 100 ml/h, fraction volume 10 ml).

(b) *Preparation of glutaraldehyde-fixed chromatin*

Glutaraldehyde fixation of chromatin was done as described by Thoma *et al.* (1979) with some modifications. Chromatin at a concentration of 0·1 mg/ml was fixed either in 10 mM Tris·HCl (pH 7·5), or in the same buffer containing 10 or 80 mM NaCl, by the addition of 0·1% (v/v) glutaraldehyde and constant inversion of the solution overnight at 4 °C. The glutaraldehyde-fixed chromatin preparations in these 3 different salt concentrations were then dialyzed extensively *versus* 10 mM Tris·HCl (pH 7·5) and stored on ice.

(c) *Immobilization of MNase on membranes*

MNase (Worthington) was immobilized on Immobilon membranes (Millipore) using one of the protocols of the membrane manufacturer. 20 µl of MNase (45,000 units/ml) were pipetted onto a 24 mm disk of the membrane at room temperature and allowed to dry. The membranes were incubated in capping solution (0·1% (w/v) gelatin in 1.0 M sodium bicarbonate (pH 9·5)) for 1 h and then washed in 0·1% (v/v) Tween 20 in 0·01 M sodium phosphate (pH 7·4) for 15 min. Capping and washing were carried out at 4 °C. The membranes were blotted on Whatman filter paper and used immediately. All chromatin and DNA samples in a single experiment were digested with filters from the same batch.

(d) *Digestion of chromatin with immobilized MNase*

Chromatin preparations were digested with immobilized MNase at 25 °C either in 10 mM Tris·HCl (pH 7·5), or in the same buffer containing different amounts of NaCl, as defined in the text or the Figure legends. To avoid formation of aggregates, digestion was performed on diluted chromatin solutions (0·015 mg/ml). At the times indicated in the Figure legends, portions (0·2 ml) were removed, quick-frozen at −80 °C, lyophilized, resuspended in 2× SDS sample buffer by a 15 min incubation at 37 °C, and analyzed in 1% agarose gels as described above. Plasmid DNA was digested in parallel experiments under the same conditions to determine the effect of the different ionic conditions on the activity of the enzyme. Partially or totally linearized (*Eco*RI) pBR322 or its derivative pML2αG (Nishioka & Leder, 1979) (a kind gift from Dr F. Rougeon, Institute Pasteur, Paris) were extensively dialyzed *versus* Tris·HCl before being brought to the desired salt concentration.

(e) *Digestion of chromatin with soluble MNase*

Digestion with soluble MNase was with 0·25 to 5 unit/ml. All other conditions were exactly the same as those used in the experiments with the immobilized enzyme.

(f) *Digestion of glutaraldehyde-fixed chromatin with immobilized or soluble MNase*

Chromatin that had been glutaraldehyde-fixed under the various salt conditions and extensively dialyzed *versus* Tris·HCl (pH 7·5) was digested with immobilized or soluble MNase in Tris·HCl (pH 7·5) as described for the unfixed material. Samples (0·2 ml) were taken at the indicated times, lyophilized, resuspended in 1% SDS, 10 mM EDTA, 0·5 mg proteinase K/ml, 10 mM Tris·HCl (pH 7·5), incubated for 2 h at 65 °C, mixed with an equal volume of 2× SDS sample buffer, and analyzed in 1% agarose gels.

(g) *Quantitative analysis of agarose gels*

Photographic negatives of the ethidium bromide-stained agarose gels were scanned using an Applescanner (Apple) and then quantitatively analyzed using the Image (NIH) software. Rates of digestion of native chromatin were determined by measuring the accumulation of mononucleosome-sized DNA (intensity of bound ethidium bromide in arbitrary units) at the indicated times. Rates of digestion of pure DNA were estimated from the disappearance of the linear and relaxed-circular plasmid DNA with time of digestion (the amount of undigested material in these 2 bands at zero time point was considered as 100%). Rates of digestion for the glutaraldehyde-fixed chromatin were determined by measuring the amount of DNA (in arbitrary units) that corresponded to the sum of bands from 1 to 6 nucleosomes. This was done because even after the proteinase treatment, the fixed preparations failed to give the clear-cut digestion patterns that were obtained with the native material; in such samples the amount of monomer-sized material could not be measured with the desired precision.

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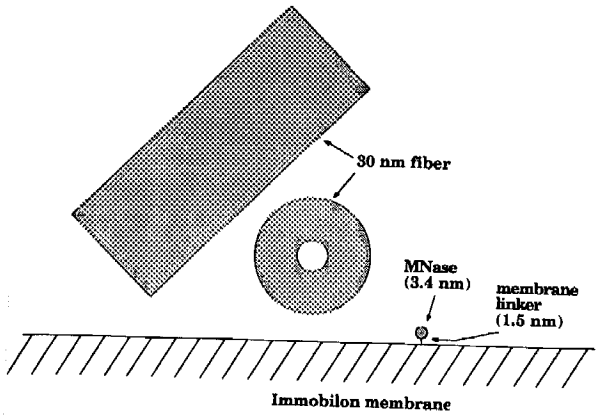

Figure 1. Schematic representation of the relative dimensions of the chromatin fiber, the immobilized enzyme and the membrane (drawn to scale). The 30 nm fiber is presented in cross-sectional (circle) and side (rectangle) views. The existence of a central "hole" (small white circle in center of cross-sectional view, actual dimensions 8 nm) is still a matter of controversy. The immobilized MNase will cleave only linkers situated on the outside of the fiber; alternative linker locations (in the fiber interior or in the walls of the cylinder between adjacent nucleosomes) will not allow digestion.

3. Results

(a) *Experimental system*

The experiments reported in this study were designed to address the question as to whether and to what extent the accessibility of the linker DNA in chromatin to endonucleolytic attack depends upon the conformation of the chromatin fiber. Both immobilized and soluble MNase were used as probes for the accessibility of the linker DNA to digestion. We felt that the immobilization of the enzyme on solid supports would offer several advantages over the use of soluble enzymes. First, it should prevent the enzyme molecules from penetrating into the interior of the fiber, even at times when the fiber is "breathing", i.e. when the regularity of its structural organization is temporarily and locally perturbed. Second, the use of immobilized enzyme can allow extremely mild digestion conditions, which avoid gross changes in the fiber structure during the initial phases of digestion. Third, the reaction can be stopped instantly by removing the chromatin solution from the solid support (Immobilon membrane). Figure 1 shows schematically the relative dimensions of the chromatin fiber, the enzyme and the membrane surface to which it is immobilized. At the same time, we felt it essential to also use soluble enzyme for control experiments because if linker DNA in the compact fiber is accessible from the fiber ends or interior, soluble enzyme might be able to cleave it even if the immobilized enzyme cannot. On the other hand, no difference between the action of the immobilized and soluble enzymes is expected with the extended fiber.

Usually, soluble chromatin is obtained by "mild" digestion of nuclear chromatin with MNase, followed by lysis of the nuclear membrane under low ionic strength conditions, which releases the solubilized chromatin fibers. The MNase added during the initial digestion remains in the preparation and can continue to slowly digest isolated chromatin even in the absence of added Ca^{2+} (results not shown). This background of soluble MNase activity would compete with the mild digestions used in this study. To avoid this complication, we routinely used drastically reduced levels of MNase during chromatin preparation (1/500 of the usual quantity). The resulting chromatin was virtually devoid of any residual MNase activity; it could also then be shown to contain no detectable endogenous endonuclease activity. No degradation of chromatin DNA was observed for as long as 16 hours at 25 °C in the absence of added enzyme.

The experiments were performed on high molecular mass soluble chromatin fragments (referred hereafter to as "long" chromatin), with different preparations containing on the average between 8 to 10 kb of DNA, or 40 to 50 nucleosomes. Care was taken that no material below 5 kb was present: lower molecular mass fragments were eliminated through gel filtration on a S-1000 column (see Materials and Methods). This was necessary in order to: (1) minimize possible end-effects during the digestion, and (2) to ensure formation of regularly folded higher order structure. The number of nucleosomes present in our initial chromatin preparations would secure the formation of a 30 nm fiber of approximately 80 nm in length (assuming 6 nucleosomes per turn).

To study the accessibility of the linker DNA in fibers of different structural characteristics, the digestions were performed under the following three conditions: (1) in 10 mM Tris without salt; (2) with addition of 10 mM NaCl; or (3) with addition of 80 mM NaCl. In the absence of salt the chromatin fiber exists in its most extended conformation, the "open zig-zag", with the linker DNA entering and exiting the nucleosomes on the same side; addition of salt to 5 to 10 mM NaCl leads to "closing" of the zig-zag, bringing the nucleosomes in close proximity (Thoma, 1988). Finally, 80 mM NaCl condenses the fiber to its 30 nm structure, indistinguishable from that observed in the nucleus under physiological salt conditions (Thoma, 1988). As 80 mM NaCl is also known to cause redistribution of linker histones (Caron & Thomas, 1981), and this redistribution might affect the behavior of the linker DNA to which they are bound, we initially attempted to use divalent ions to condense the fiber (0·35 mM $MgCl_2$, Ausio *et al.* (1984), or 0·35 mM $CaCl_2$, Borochov *et al.* (1984)). These ions, however, have major effects on the activity of the enzyme, as well as on the structure of the fiber. Thus, Mg^{2+}, as expected from previous studies (e.g. see Cuatrecasas *et al.*, 1967), inhibited the enzymatic activity almost completely. On the other hand, addition of even moderate levels of Ca^{2+} enhanced activity to a degree that made the comparisons of the parameters of the digestion under the different conditions very difficult. Moreover, the presence of divalent ions in the

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chromatin preparations even in the micromolar range might induce some degree of compaction of the fiber (see, for example, Ausio *et al.*, 1984; Borochov *et al.*, 1984) and thus, preclude the possibility of studying fibers in their wholly extended state. Therefore, we avoided addition of any divalent ions, relying instead on the low enzyme activity present in MNase preparations that have not had Ca^{2+} actively removed by EDTA dialysis (see Cuatrecasas *et al.*, 1967). The potential linker histone redistribution did not seem to cause any problem, as clear differences in the accessibility of the fibers in the different structural states were observed (see below); such differences would have been blurred if linker histone redistribution affected the results. Furthermore, as shown below, the same results were obtained with fibers fixed with glutaraldehyde, which completely prevents redistribution.

(b) *The linker DNA is readily accessible to immobilized MNase in the extended and closed zig-zag conformations, but not in the condensed state*

Long chromatin fibers were subjected to digestion with immobilized enzyme in Tris, in 10 mM NaCl, or in 80 mM NaCl. Pure DNA preparations were hydrolyzed in parallel under the same ionic conditions and with filters from the same batch. The results are shown in Figure 2A and B. Inspection of the gel patterns indicates that: (1) an extremely mild digestion was achieved: only about one-half of the initial high molecular mass material was digested to oligonucleosomes within four hours. Although the continuity of the DNA is not considered essential for the formation/maintenance of the higher-order structure (Finch & Klug, 1976; Thoma *et al.*, 1979), we have tried to secure conditions to preserve the structure as much as possible so that the results will not be compromised by a general disruption of the fiber. (2) The digestion pattern is characterized by the appearance of the nucleosome "ladder" which is typical of results from digestion using soluble enzyme in the presence of added Ca^{2+} ions (van Holde, 1988; Tsanev *et al.*, 1992). Since our long chromatin preparation had been treated with EDTA during the isolation procedure and extensively dialyzed *versus* Tris·HCl before the start of the experiment, the Ca^{2+} necessary for this digestion presumably was associated with the MNase.

The result most important to the aim of this study is the almost complete lack of digestion of the condensed fiber (80 mM NaCl). The quantitative comparisons between the digestions under the different conditions are presented in Figure 3. The fibers in Tris and 10 mM NaCl were digested with similar kinetics as judged by the appearance and accumulation of a monomer-sized DNA band on the gels (Fig. 3A). At the same time, no monomer, nor trace of a nucleosomal ladder could be detected in 80 mM NaCl; some slight signs of digestion are detectable only in the upper portion of the gel at long digestion times. The observations are complicated by the fact that the activity of the enzyme is

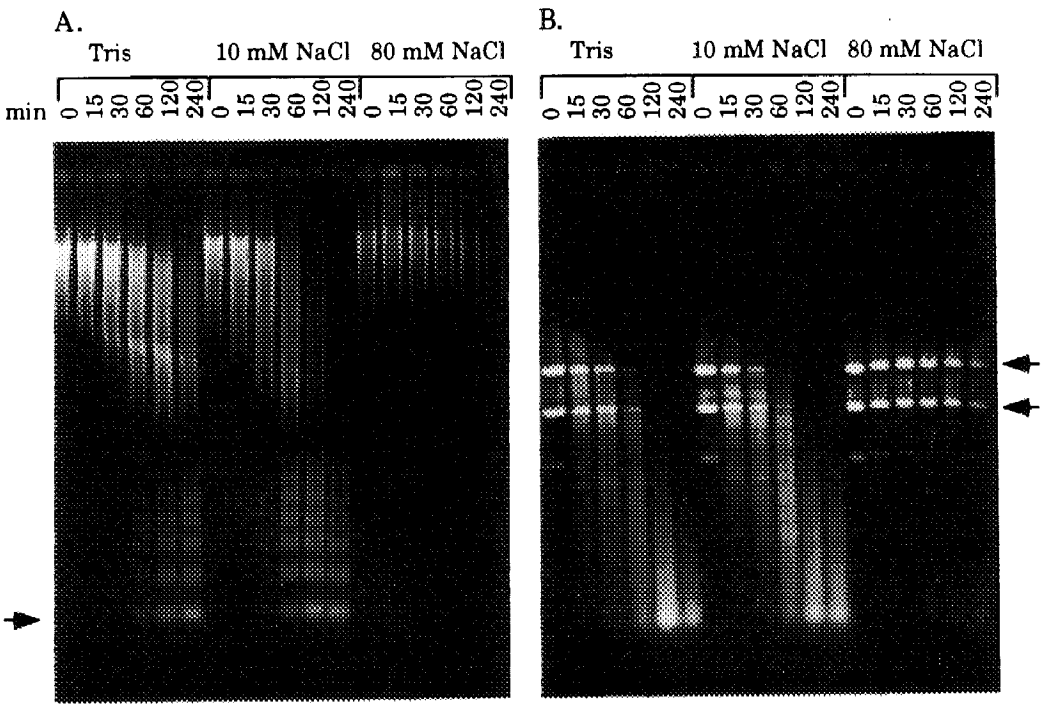

Figure 2. Agarose gel electrophoretic analysis of the digestion of long chromatin (A) and pure DNA (B) with immobilized MNase. The digestion conditions are denoted above the respective lanes. The consecutive lanes within each group are 0, 15, 30, 60, 120 and 240 min of digestion (left to right). Equal amounts of material were digested under the 3 conditions. The position of the monomer-sized DNA band in A is denoted by an arrow. The bands marked by arrows in B are the closed relaxed and linear bands of plasmid pML2αG; the sum of these 2 were used for the quantitations in Fig. 3.

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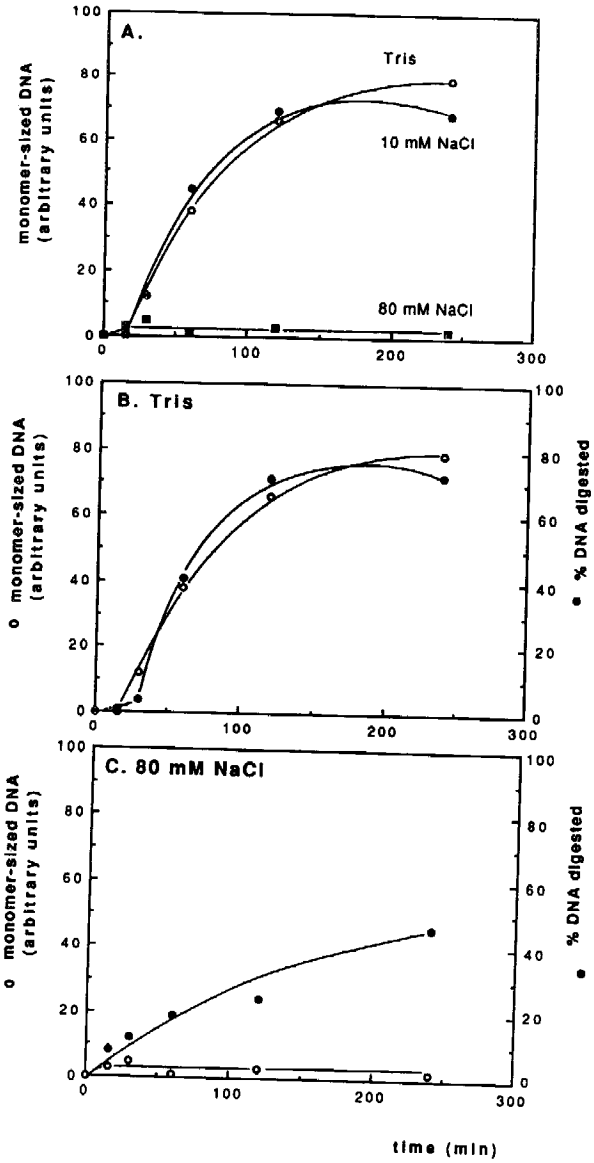

Figure 3. Quantitation of the digestion patterns shown in Fig. 2. The gels were scanned and quantified as described in Materials and Methods. A, Accumulation of monomer-sized DNA band during the digestion of chromatin with immobilized MNase. Digestion conditions: (o) Tris; (●) 10 mM NaCl; (■) 80 mM NaCl. B and C, Accumulation of monomer-sized DNA band in the chromatin preparations (o) and % DNA digested in the pML2αG preparations (●): B, Tris; C, 80 mM NaCl.

somewhat sensitive to the salt concentration. Therefore, meaningful interpretation of any structure-dependent effects required direct comparisons of the digestion kinetics for the chromatin fiber and pure DNA under the various conditions. This was achieved by carrying out, under exactly the same conditions, parallel digestions of plasmid DNA. The digestion of chromatin was monitored by following the accumulation of mononucleosome-sized DNA; the digestion of naked DNA was followed by measuring the loss of the closed relaxed and linear forms of purified plasmid DNA. As can be seen from Figure 3B, the kinetics of digestion of the pure DNA and the kinetics of accumulation of monomer-sized DNA follow similar paths for the low ionic strength conditions, including the presence of a "lag" period, as originally observed by Cuatrecasas *et al.* (1967) at very low Ca^{2+} concentrations. In 80 mM NaCl, free DNA is still digested, albeit more slowly than in low salt. For example, in 120 minutes about 65% of the DNA has been digested in Tris, and only about 25% in 80 mM NaCl, a two- to threefold decrease in rate. In contrast, the amount of monomer generated by digestion of chromatin in 80 mM NaCl is completely negligible in comparison to that generated in Tris in the same time period (Fig. 3C).

(c) *The linker DNA in glutaraldehyde-fixed long chromatin fiber is also accessible to immobilized MNase only in the more extended conformations*

The experiments described above, although showing reproducibly that the linker DNA is much more protected in the 30 nm fiber than in the extended fiber, are complicated by the dependence of the activity of the enzyme on salt concentration. To completely avoid this potential source of complication, we have repeated the experiments with immobilized enzyme using glutaraldehyde-fixed fibers. Glutaraldehyde fixation has been widely used in the chromatin field to fix the conformations of the fiber, induced by different salt conditions, without affecting such characteristics of the fiber as its general shape (Thoma *et al.*, 1979; Russanova *et al.*, 1987), or the orientation of the nucleosomes relative to the fiber axis (Russanova *et al.*, 1987).

The fibers were brought to each of the desired conformations in the manner described above, that is, incubated in Tris buffer with 0, 10 mM or 80 mM NaCl, and fixed in 0·1% glutaraldehyde. They were then dialyzed against Tris and all samples digested under exactly the same conditions, i.e. in Tris buffer. Pure plasmid DNA samples were digested in parallel. The results are presented in Figure 4. The most extended fixed fiber was attacked the fastest, followed by that fixed as a closed zig-zag; finally, no significant digestion could be seen in the fiber fixed as a condensed structure. Keeping in mind that all digestions were performed under identical ionic conditions, the difference in susceptibility among the fibers fixed under the three different conditions is unequivocally structure-related. Some influence of the glutaraldehyde fixation could not be excluded, as the 10 mM NaCl-fixed fibers now displayed a slightly slower digestion kinetics than the Tris-fixed ones, in contrast to identical behavior of the unfixed fibers in Tris and 10 mM NaCl (see above). This might be expected if the zig-zag linker exhibited significant "breathing" in 10 mM salt, which would be prevented by glutaraldehyde fixation.

(d) *The linker DNA in higher-order structure fibers is protected also against soluble MNase*

The experiments described above show that the higher-order fiber is organized in such a way that

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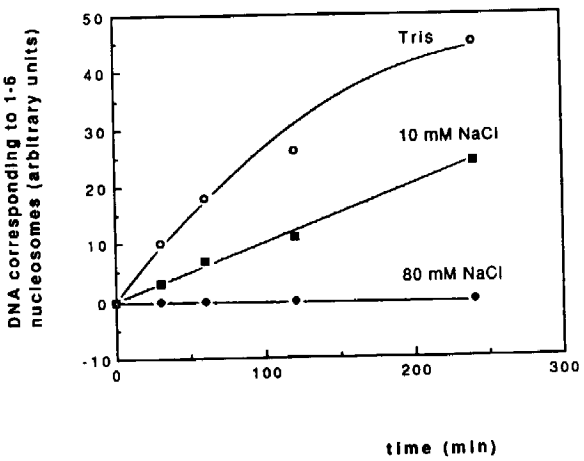

Figure 4. Quantitation of the digestion patterns of glutaraldehyde-fixed long chromatin fibers with immobilized MNase. Three chromatin preparations were prepared: one in 10 mM Tris·HCl (pH 7·5), a second one in the same buffer containing 10 mM NaCl, and a third one in the buffer containing 80 mM NaCl. These were then fixed with glutaraldehyde, dialyzed extensively against Tris·HCl (pH 7·5) and subjected to digestion with immobilized MNase. The products of digestion were analyzed in agarose electrophoretic gels and quantified as described in Materials and Methods. The curves represent the accumulation of digestion products in the region corresponding to 1 to 6 nucleosomes.

the linker DNA is not accessible to endonucleolytic cleavage by immobilized MNase. Keeping in mind that the immobilized enzyme can only attack externally situated linkers, one obvious interpretation of these results would be that the linker DNA is completely internalized into the interior of the 30 nm fiber, as postulated in some models (see Introduction). The same protection of the linker DNA could, however, be observed if the linker DNA becomes folded between nucleosomes during compaction. If this were the case, protection should be generated against soluble nuclease as well. The enzyme is a globular protein of only 17 kDa (Taniuchi *et al.*, 1967) and therefore about 3·4 nm in diameter. It should be capable of penetrating and gradually diffusing into the interior of the 30 nm fiber, particularly from fiber ends. Thus, if the linker DNA is protected simply by being internalized in the higher-order structure, the soluble enzyme should be able to cleave it even though the immobilized enzyme cannot. If the linker is protected by being sequestered between nucleosomes, it should be inaccessible to both forms of the enzyme.

The digestion conditions were chosen by trial so as to make the rate of digestion of DNA and chromatin comparable to that achieved with the immobilized enzyme (only 1/2000 of the quantity immobilized onto the filters was used in the experi-

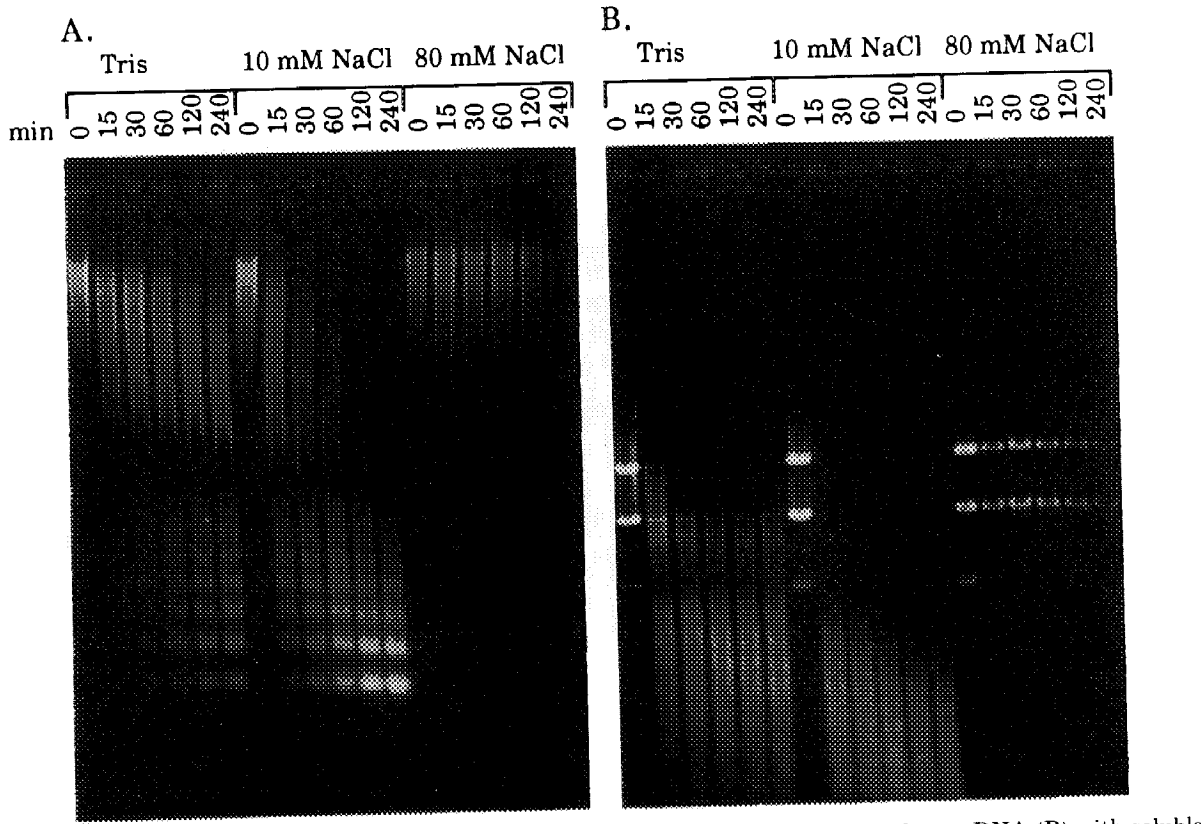

Figure 5. Agarose gel electrophoretic analysis of the digestion of long chromatin (A) and pure DNA (B) with soluble MNase. Only 0·017 to 0·034 unit of MNase per μg of DNA were used so as to secure rates of digestion comparable to those obtained with the immobilized enzyme. For further details see the legend to Fig. 2.

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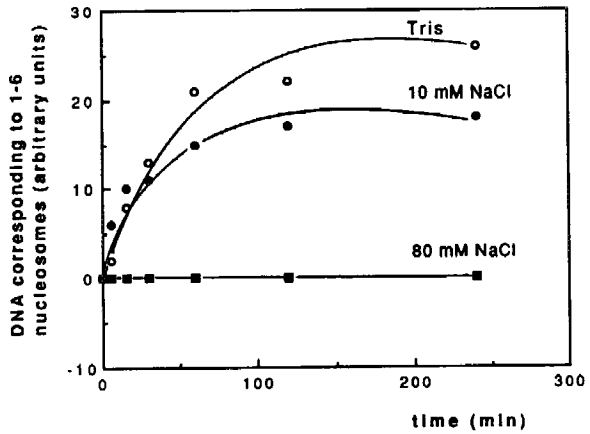

Figure 6. Quantitation of the digestion patterns of glutaraldehyde-fixed long chromatin fibers with soluble MNase. For further details see the legends to Figs 4 and 5.

ments with the soluble enzyme). The electrophoretic patterns obtained upon digestion of chromatin are presented in Figure 5A and the parallel control digestions of DNA in Figure 5B. In general, both the extended and the closed zig-zag fibers were digested with the production of the typical nucleosomal ladder and with similar rates (the rate of digestion of chromatin correlated with the rate of digestion of the pure DNA controls). As in the case with the immobilized MNase, the soluble enzyme also did not digest the higher-order structure to any significant degree under the mild conditions employed. Once again, the enzyme activity was somewhat inhibited in 80 mM NaCl (compare the digestion of pure plasmid DNA under the three ionic conditions, Fig. 5B), but the inhibition was only partial in the case of DNA and almost total in the case of chromatin. This result shows that the lack of digestion of the linker DNA in the condensed fiber is a consequence of steric inaccessibility of the linker to either immobilized or free enzyme.

The experiments with the soluble enzyme were repeated with fibers glutaraldehyde-fixed under the three conformational states. This was again done to avoid complications in the interpretation, due to the sensitivity of the enzyme activity to the ionic environment. The results (Fig. 6) reiterated those obtained on the unfixed fiber: the linker DNA in the more extended fibers was accessible to digestion while that in the condensed fiber was not.

Finally, a trivial explanation that the similar results with the immobilized and soluble enzyme could be due to a gradual release of the MNase from the filters during the course of digestion, was excluded by control experiments, which showed that no significant activity comes off the filters under our experimental conditions (data not shown).

(e) *The accessibility of the linker DNA diminishes gradually upon gradual compaction of the fiber*

The somewhat unexpected results with the soluble enzyme suggested that most probably

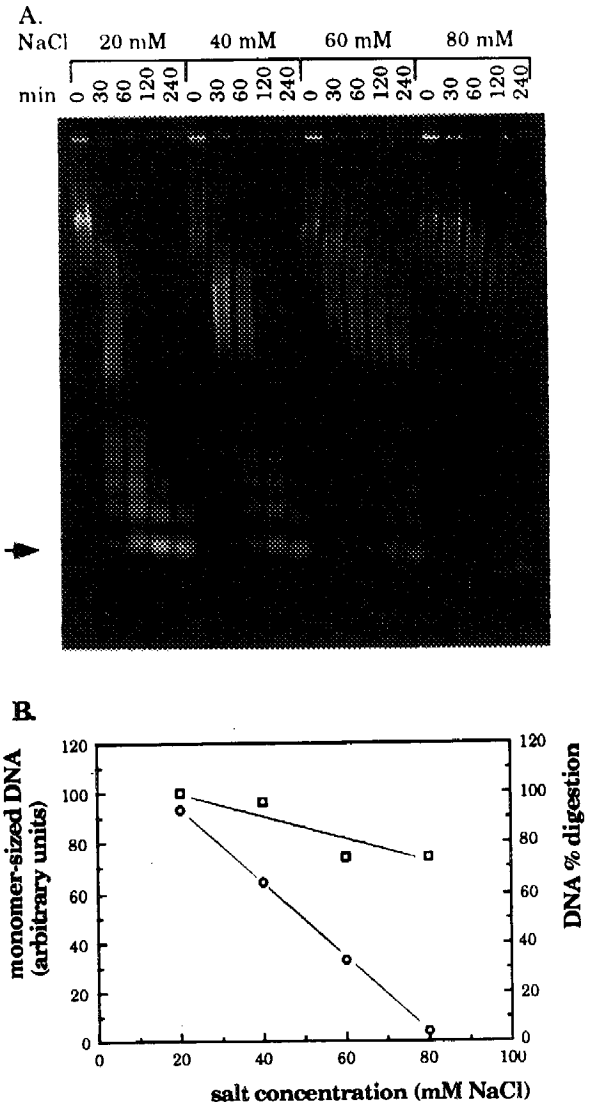

Figure 7. A, Comparative agarose gel electrophoretic patterns for chromatin fibers with an intermediate degree of compaction. Chromatin preparations were made 20, 40, 60 or 80 mM with respect to NaCl and digested with immobilized MNase as usual. The consecutive lanes within each group are 0, 30, 60, 120 and 240 min of digestion (left to right). The position of the monomer-sized DNA band is denoted by an arrow. B, Quantitation of the amount of pure DNA digested for 120 min under the different ionic strength conditions (the electrophoretic gel is not shown) (□), and of the amount of monomer-sized DNA accumulated for the same time period in the respective chromatin samples (lanes 4, 9, 14, and 19 of the gel shown in A (o).

the protection against digestion observed with the higher-order structure fiber did not reflect a simple internalization of the linker but resulted from local steric hindrance by densely packed chromatin components. However, it could be argued that access to the interior from fiber ends is somehow blocked by proteins and thus protection could be offered against both immobilized and free nuclease. But if the linker was protected in this way, then its protection should not become evident until the fiber was

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almost completely folded. To test for such a possibility we used a series of salt concentrations ranging from 0 to 80 mM NaCl, to induce progressive folding of the fiber from an extended state, through several intermediately compacted structures, to the fully condensed state (Thoma *et al.*, 1979). As seen in Figure 7A, the progressive folding of the fiber led to a progressive decline in the accessibility of linker DNA to digestion. Long before the final condensed state was reached, i.e. long before the fiber acquired the regular shape, characteristic of the 30 nm fiber (see Thoma *et al.*, 1979), the linker DNA became partially protected. Moreover, the degree of protection correlated with the degree of compaction. To eliminate the trivial explanation that the gradual protection of the linker might only be a reflection of the gradual inhibition of the enzyme activity with increasing salt concentration, the data obtained on chromatin were compared to those obtained in parallel DNA digestions. The comparison shown in Figure 7B is based on the data for the 120 minute time point; this point was chosen because it was on the linear portion of the chromatin digestion curves for all conditions, and it corresponded to the accumulation of maximal amounts of monomer-sized DNA (further digestion led to some reduction in the intensity of this band). This result shows that the progressive protection of linker DNA against digestion is a truly structure-related phenomenon, which cannot be accounted for by salt inhibition of MNase.

4. Discussion

(a) *On the location of the linker DNA in the higher-order chromatin structure*

In this work we have systematically studied the kinetics and patterns of digestion of chromatin fibers in different conformational states with immobilized and soluble MNase in an attempt to determine the location of the linker DNA in the higher-order structure. We have used extremely mild digestion conditions in the absence of added divalent ions, which have permitted, for the first time, the distinction to be made in the response of the chromatin fiber to nuclease in the different conformations. Experiments performed with either "native" or glutaraldehyde-fixed fibers show that in the higher-order structure induced by 80 mM NaCl, the linker DNA is protected almost completely against cleavage with both the immobilized and soluble enzyme under these mild digestion conditions. Control experiments were performed with fibers of an intermediate degree of compaction in an effort to distinguish between the two most probable explanations of the observed protection: internalization of the linker DNA into the fiber or simple steric hindrance to the enzyme as a consequence of fiber compaction. The controls support the second possibility, which was first suggested by the results with the soluble enzyme. The soluble enzyme was expected to digest the linker DNA even in the compact fiber, if the linker protruded into the fiber interior. Though H1 and H5 bind to the linker DNA, they do not necessarily protect it as evidenced by the digestion of the linker in nuclear chromatin or in soluble "native" fiber under the conditions routinely used in the literature.

How can our results be incorporated into existing models of the higher-order structure of the chromatin fiber? They are difficult to reconcile with any of the crossed-linker models (for reviews, see Felsenfeld & McGhee, 1986; Thoma, 1988; van Holde, 1988; Widom, 1989; Freeman & Garrard, 1992; Tsanev *et al.*, 1992) which postulate that the linker DNA is entirely in the interior of the fiber. They are also difficult to reconcile with one specific feature of the solenoidal model of Finch & Klug (1976) and Thoma *et al.* (1979): the requirement that the linker DNA forms reversed loops inside the fiber (see Butler, 1984). The problem with all of these models is that one would expect greater accessibility to free enzyme, which should be able to enter and digest from solenoidal ends to a greater degree than immobilized enzyme. Our results, taken together with data on the existence of a ten base-pair cutting pattern of linker DNA (e.g. see Lohr & van Holde, 1979; Karpov *et al.*, 1982; Bavykin *et al.*, 1990), would more strongly support solenoidal models in which the linker is immobilized between nucleosomes, such as the supercoiled linker models of McGhee *et al.* (1983) or Bavykin *et al.* (1990). Such models would explain both the progressive increase in protection as the fiber is compacted and the identical results obtained with immobilized and free enzyme. Our view that the linker is not internalized in the higher-order structure is also generally compatible with the twisted-ribbon model of Woodcock *et al.* (1984); it is, however, difficult to reconcile the way in which this model envisages the condensation process with the gradual loss of accessibility of the linker to digestion observed by us. Our view on the way the linker DNA is located in the condensed 30 nm chromatin fiber, forming together with the nucleosomes the walls of the solenoid and not protruding into the interior, can also incorporate features reflecting increase in the fiber diameter as a function of linker length as documented by some studies (e.g. see Williams *et al.*, 1986).

The progressive protection that we observe upon folding into the higher-order structures is entirely in accord with the recent observations from Widom's laboratory, that the linker DNA in dinucleosomes bends or folds as conditions for chromatin condensation are approached (Yao *et al.*, 1990, 1991). Such a folding could lead to a structure in which the curved linker DNA lay in the space between two adjacent nucleosome cores.

Despite the fact that our data favor one class of models over another, they cannot be said to conclusively prove or disprove any models. What they do demonstrate is a surprizing resistance of condensed chromatin to digestion under mild conditions.

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(b) *On the location of the linker histones in the higher-order structure of chromatin fiber*

A question closely related to the location of the linker DNA concerns the location of the linker histones themselves. The literature on this issue, which has been mainly approached *via* immunochemical studies, is extremely controversial (see Zlatanova (1990) for a review; Banchev *et al.* (1990), and Leuba *et al.* (1993) for detailed discussions). The results from a study in which the location of the globular domain of histone H5 in the extended and condensed fiber was studied by specific antibodies attached to bulky ferritin molecules (so as to create a probe too large to penetrate into a condensed fiber) were interpreted as an indication of internalization. In these studies, however, the immunochemical reaction was low even in the fully extended fiber and gradually diminished with the increase of salt concentration, reaching background levels before the fibers could attain regular higher-order structure. The same gradual decrease in the intensity of the reaction was observed with free non-ferritin conjugated antibodies. (In a sense, the ferritin-linked antibody can be considered as a probe analogous to our immobilized MNase, and the free antibody, to the soluble enzyme.) So, in our view, the results of Dimitrov *et al.* (1987) should be reinterpreted, keeping in mind the general steric hindrance to the probes that develops upon compaction of the fiber. Moreover, the experiments were performed only on glutaraldehyde-fixed fibers and this treatment has been shown by others (Thibodeau & Ruiz-Carrillo, 1988), to cause artifacts in immunochemical studies, probably because some antigenic determinants become modified in the crosslinking reaction. The carefully performed study of Thibodeau & Ruiz-Carrillo (1988), based on the use of a series of monoclonal antibodies against the globular domain of H5, reached the opposite conclusion, i.e. that the accessibility of this domain did not change upon compaction of the fiber. A similar conclusion was inferred from the use of antibodies specific to histones H1 and H1° and mouse liver chromatin (Banchev *et al.*, 1990).

Recently, we have approached this issue using immobilized trypsin and chymotrypsin as specific probes for the unstructured tails and the globular domains, respectively, of the linker histones in chicken erythrocyte chromatin (Leuba *et al.*, 1993). We concluded that the N and C-terminal portions of histone H1 remained accessible, and hence did not change in location, upon compaction of the fiber; this behavior was in contrast to that of histone H5 whose tails became significantly inaccessible in the 30 nm fiber. On the other hand, no definitive conclusion could be reached concerning the location of the globular domain of either histone, as the phenylalanine that was the specific target of chymotrypsin turned out to be inaccessible even in the mononucleosomal particle.

The location of the linker DNA between the surfaces of the nucleosomes would be consistent with the behavior of the linker histones, with no significant changes in accessibility to specific immunochemical and enzymatic probes upon compaction of the fiber, if one assumes that these histones are located on the external surface of the fiber. One feature difficult to explain at the moment is the partial protection of the unstructured tails of histone H5 in the higher-order structure, as contrasted to the lack of protection of the tails of histone H1 (Leuba *et al.*, 1993). Further studies are necessary to resolve this interesting question.

An important set of data to be explained with respect to the location of the linker histones is the formation of H1-H1 polymers upon chemical crosslinking of soluble and nuclear chromatin (for references see Thoma, 1988). As similar proximities exist in low (15 mM) and high (75 mM) ionic strength (Thomas & Khabaza, 1980), most probably the main contacts between the H1 molecules in the polymer are along the superhelical turns rather than between them (a similar view was expressed by Thoma *et al.* (1979)). In such a case it is not necessary to invoke positioning of the H1 molecules in the middle of the fibers, as usually suggested, in order to explain the formation of the H1 polymers.

S.L. and J.Z. contributed equally to this work. K.v.H. is the recipient of an American Cancer Society Research Professorship. The skillful assistance of V. Stanik with the chromatin preparations is greatly acknowledged. This research was supported by NIH grant GM22916 and NIEHS grant ES04766 to K.v.H.

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Edited by P. von Hippel

(Received 10 June 1993; accepted 21 September 1993)

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