AN ABSTRACT OF THE THESIS OF

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Title: On the Location of the Linker Histones and the Linker DNA in the 30 nm Fiber of Chromatin

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Kensal van Holde

Understanding the structure of the 30 nm fiber in chromatin is relevant to understanding eukaryotic replication and transcription. The major controversy among the models of the fiber concerns the disposition of the linker DNA, the DNA between adjacent nucleosomes, and the location of the linker histones. To determine if the location of the linker histones and the linker DNA was internal or external, chromatin was digested with immobilized proteases and nucleases. The chromatin was probed either in a low salt extended 10 nm fiber of nucleosomes or in progressive compactions (addition of increasing amounts of salt) to form a condensed (30 nm) fiber.

Digestion experiments performed on linker histones either in chicken erythrocyte chromatin, or free in solution or bound in mononucleosomes revealed: (1) Histone H5 is more protected than histone H1 in the fiber; (2) The N- and C-terminal portions of H1 do not change their

accessibility upon compaction of the fiber; the tails of H5, however, become significantly internalized in the 30 nm fiber; (3) phenylalanine in the globular domain of both H1 and H5 is inaccessible both in the fiber and in mononucleosomes. Sedimentation velocity measurements demonstrate that the conformation of the fiber at all its different condensation states is highly sensitive to cuts in even a few of the linker histone molecules.

The structure of these chromatin fibers has also been probed using micrococcal nuclease, both membrane-immobilized and free in solution, under extremely mild digestion conditions. The linker DNA is almost completely protected against digestion in the 30 nm fibers, whereas it is readily accessible in the more extended structures, independent of whether immobilized or free enzyme is employed. To circumvent complications due to the sensitivity of the enzyme to the salt concentration, control experiments were performed in which chromatin fibers were glutaraldehyde-fixed under different ionic conditions and then digested in low salt. The results were very similar to the above. 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. These results support models for chromatin structure in which access to the linkers is limited by local steric hindrance, rather than by internalization in the core of the fibers.

On the Location of the Linker Histones and the Linker DNA in the 30 nm Fiber of Chromatin

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Sanford H. Leuba

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Professor of Biochemistry and Biophysics in charge of major

Redacted for Privacy

Chairman of Department of Biochemistry and Biophysics

Redacted for Privacy

Dean of Graduate School

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Chapter 1

Introduction

Each eukaryotic chromosome contains a single continuous DNA molecule (Kavenoff and Zimm, 1973). This DNA molecule is compacted with proteins in sequential hierarchic structures. The lowest level of packing is the nucleosome, the basic unit of chromatin. The nucleosome core particle has 146 bp of DNA wrapped in 1.75 left-handed superhelical turns around an octamer of core histones (two each of H4, H3, H2B, and H2A) (van Holde, 1988). The nucleosome core particle has dimensions of a disk with 11 nm diameter and 5.5 nm thickness (Pardon et al., 1977a, 1977b; Suau et al., 1977; Braddock et al., 1981; Richmond et al., 1984).

The delineation of eukaryotes from prokaryotes is the packaging of eukaryotic DNA with histones. The histones are extremely basic eukaryotic nuclear proteins rich in lysine and arginine, poor in aromatic amino acids, and lacking tryptophan (van Holde, 1988). The proportion of lysines and arginines can be used to classify the histones: the arginine-rich histones H3 and H4, the slightly lysine-rich histones H2A and H2B, and the lysine-rich histones, the H1/H5 family (van Holde, 1988). The histones have a globular domain flanked by random coiled N- and C- terminals. The histones have varying levels of evolutionary stability. H4 is extremely conserved; it has only two conservative substitutions between pea and cow (DeLange et al., 1969a, 1969b). While H4 and H3 are conserved in length and in amino acid sequence, H2A and H2B are more variable (van Holde, 1988). The most variable parts of H2A and H2B are their N- and C- terminal tails. The

evolutionary variability of the histones is subject to the general rule that in globular proteins the most conservative amino acid residues are those situated in the internal part of molecule and not in contact with the solvent; the most variable are the external sites (Smith, 1975). The order of evolutionary stability of histones (arginine rich > slightly lysine-rich > lysine rich) is correlated with localization of these histones in the nucleosome where a H3/H4 tetramer forms its inner core; two H2A/H2B dimers flank the tetramer; and H1 is external (see below) (Tsanev et al., 1992). While there is some variability in sizes of H2A and H2B, the number of basepairs (bp) of DNA around the octamer in a nucleosome core particle is an invariant 146 bp.

The next step up from the core particle is the chromatosome. Chromatosomes were first observed as a ~160 bp pause in digestion of chromatin with micrococcal nuclease (Simpson, 1978). Analysis of these particles found them to have all the components of a core particle plus somewhat more DNA and one molecule of the linker histones (the H1/H5 family of lysine-rich histones). Sealing off two turns (166 bp) of DNA around the octamer, H1 binds to the ends of the DNA as it enters and exits the nucleosome core particle (Allan et al., 1980).

The linker histones are represented in each cell by a family of closely related molecular species (Cole, 1987). The H1 complement can vary during development and differentiation, with some specific H1 subtypes present only in certain cell types. The best studied example of a cell-specific H1 subtype is histone H5, present in nucleated erythrocytes of birds and some fish (Neelin et al., 1964; Miki and Neelin, 1975). This histone has been implicated in the process of terminal differentiation as a factor in the shutting down of transcription and replication in the mature erythrocyte.

The linker histones are characterized by a highly asymmetrical distribution of the various types of amino acid residues along the polypeptide chain. They adopt, when dissolved in the presence of salt, a well defined three dimensional structure that consists of a short, randomly coiled basic N-terminal tail, an apolar globular domain of about 80 amino acid residues and a long, basic, randomly coiled C-terminal tail (Hartman et al., 1977; Aviles et al., 1978). The different structural domains are thought to perform different roles in structuring the nucleosome and the fiber. Thus, while the globular domain seals off the two turns of DNA in the nucleosome (Allan et al., 1980), it is thought that the C-terminal tail interacts with DNA to form higher order structures (Allan et al., 1986; for review see Zlatanova and Yaneva, 1991). The function of the short N-tail is less well known; it is believed that it serves to precisely place, or anchor, the globular domain with respect to the nucleosome (Allan et al., 1986).

The chromatosome together with the DNA between adjacent chromatosomes, the linker DNA, make up a nucleosome. Under low ionic conditions less than 10 mM M^+ (monovalent cations), a continuous chain of nucleosomes has been characterized as a 10 nm fiber (Thoma et al., 1979). Addition of salt to 80 mM M^+ (Thoma et al., 1979) or 0.35 mM M^{++} (divalent cations) (Ausio et al., 1984) fold the 10 nm fiber of nucleosomes into a 30 nm fiber (see Figure 1.1).

It is now widely accepted that most chromatin in the eukaryotic nucleus is organized into 30 nm fibers (van Holde, 1988). Since eukaryotic replication (and possibly transcription) encounters chromatin-contained DNA initially compacted in such a form, there is considerable interest about the structure of this fiber. A variety of models have been proposed for

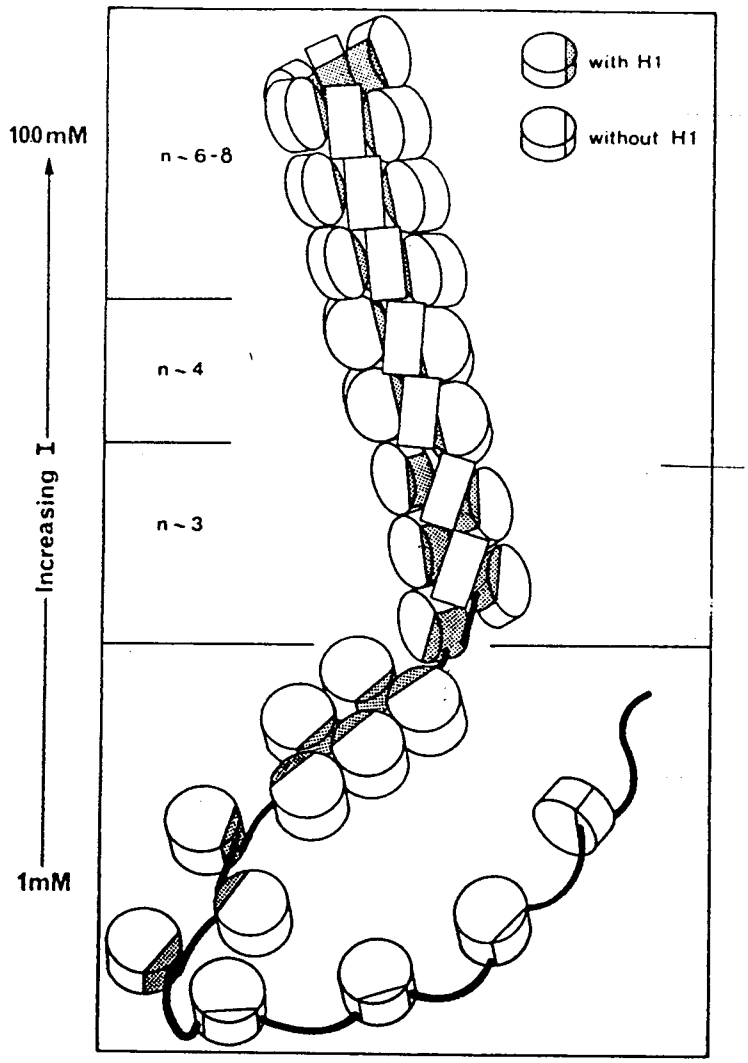


Figure 1.1 Idealized drawing of helical superstructures formed by chromatin containing H1 with increasing ionic strength (Thoma et al., 1979). The open zigzag of nucleosomes (bottom left) closes up to form helices with increasing numbers of nucleosomes per turn (n). When H1 is absent (pictured at bottom right), no zigzags or definite higher-order structures are found.

Table 1.1
Some general models for the 30 nm fiber (van Holde, 1988).

Fig.	Distinguishing features	Location of linker DNA	References
1.1	Continuous coiled chain of nucleosomes, variable number per turn	Inside of fiber	Thoma et al.(1979)
—	Twisted ribbon of zigzag chain, with "crossover" of linkers	Close to fiber surface	Worcel et al. (1981)
1.2	Continuous coil (as Thoma et al.) with linker coiled as continuation of nucleosome DNA coil	Variable; distribution depends on linker length	McGhee et al. (1980,1983)
1.3	Helix of zigzag chain; no linker "crossover"	Close to fiber surface	Woodcock et al. (1984)
1.4	Continuous coil, with linker as reverse loop inside	Inside of fiber	Butler (1984)
—	Layered zigzag structure	Some inside, some external	Subirana et al. (1985)
1.5	Helix (left-handed) of zigzag chain (2-start)	Inside of fiber	Williams et al. (1986)

the 30 nm fiber (for reviews see Felsenfeld and McGhee, 1986; Sayers, 1988; Thoma, 1988; Widom, 1989; Freeman and Garrard, 1992; Tsanev et al., 1993; also see Table 1.1), based mainly on results from physical studies and electron microscopic observations; however, none of them is universally accepted. 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

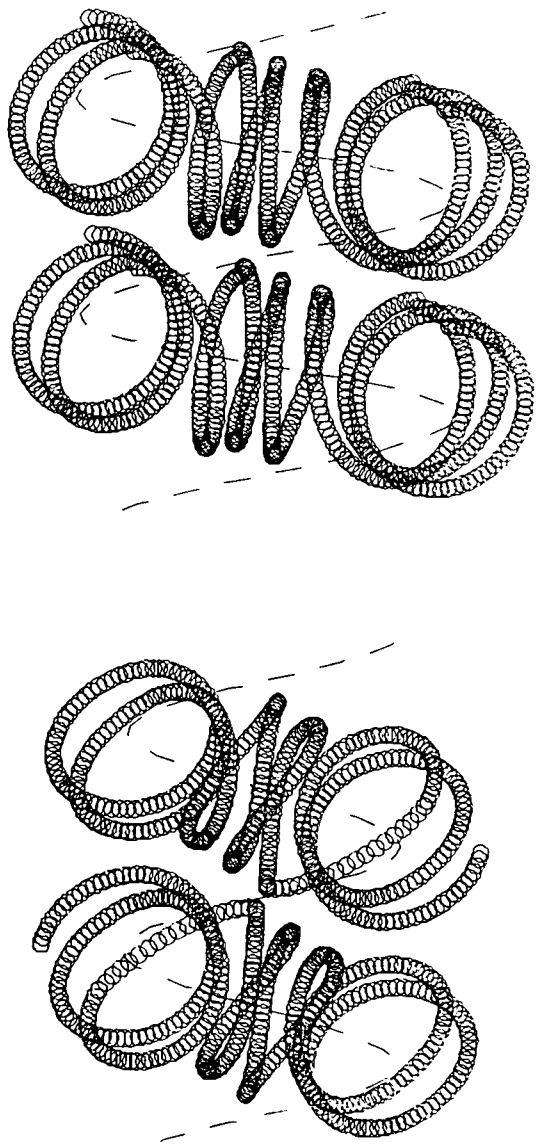


Figure 1.2 The 30 nm fiber model of McGhee et al. (1983). The chromatosomes are proposed to lie radially from the solenoid axis. The linker DNA between nucleosomes is supercoiled about the helix (dashed line) that passes through the chromatosome centers. For clarity, only the three nucleosomes on the front surface of the fiber are seen. The histone cores are also omitted. The top model is of sea urchin sperm with a linker length of 77 bp. The bottom model is of HeLa with a linker length of 20 bp.

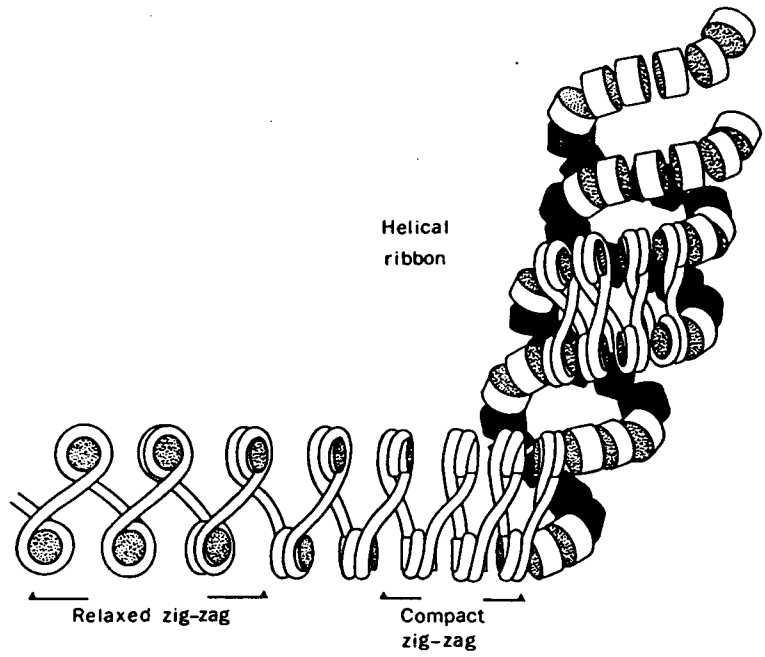


Figure 1.3 The 30 nm fiber model of Woodcock et al. (1984). The zigzag fiber is compacted and then wound into a two-start helix. For clarity, the DNA is shown only in places and nucleosomes are shaded in various ways.

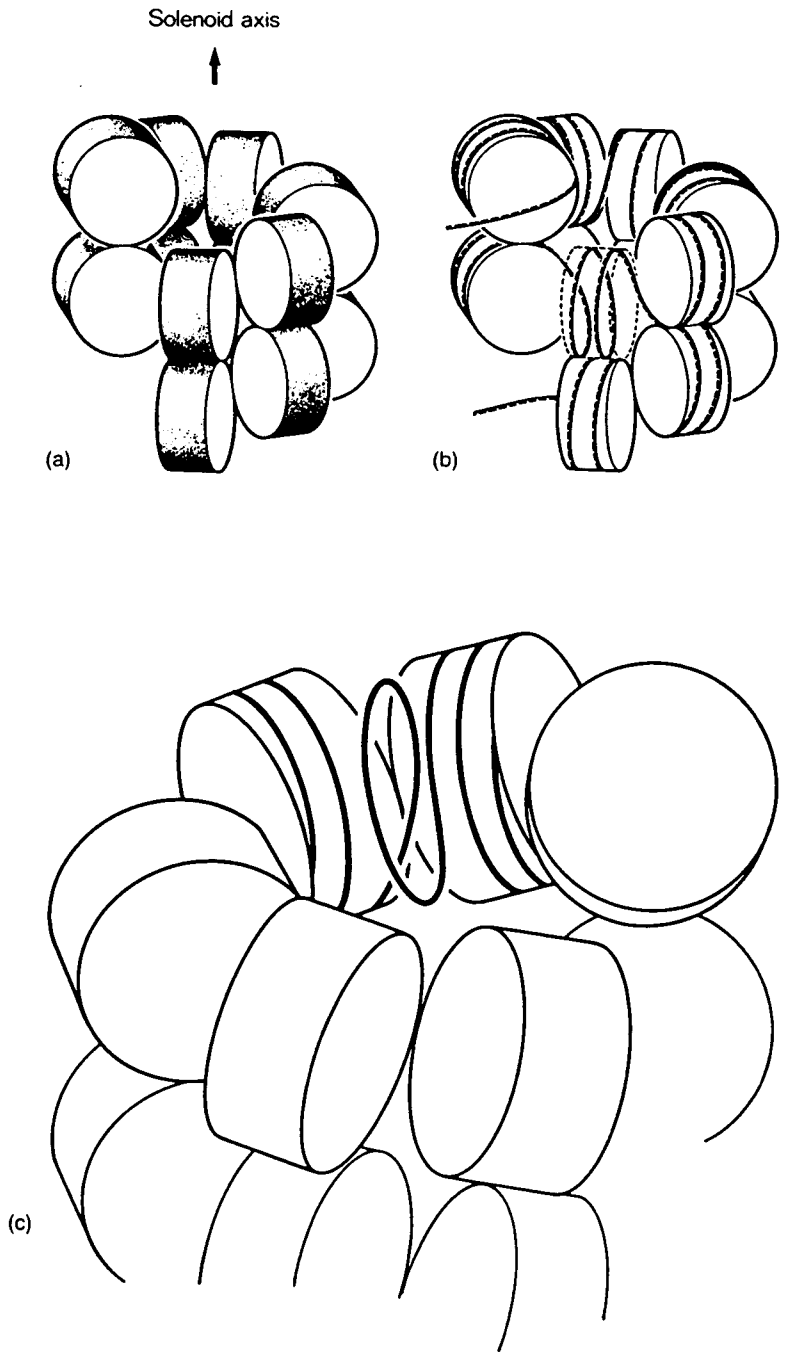


Figure 1.4 30 nm fiber models proposed by Butler (1984). Structures (a) and (b) are specifically for short-repeat chromatin (~165 bp). Note that DNA passes directly from one chromatosome to the next. This is a right-hand helix. Model (c) is applicable to chromatin with repeats of greater than 176 bp. It is a left-hand helix, in which the linker DNA makes internal reverse loops. In both structures, the lysine-rich histones would be confined to the inside of the helix.

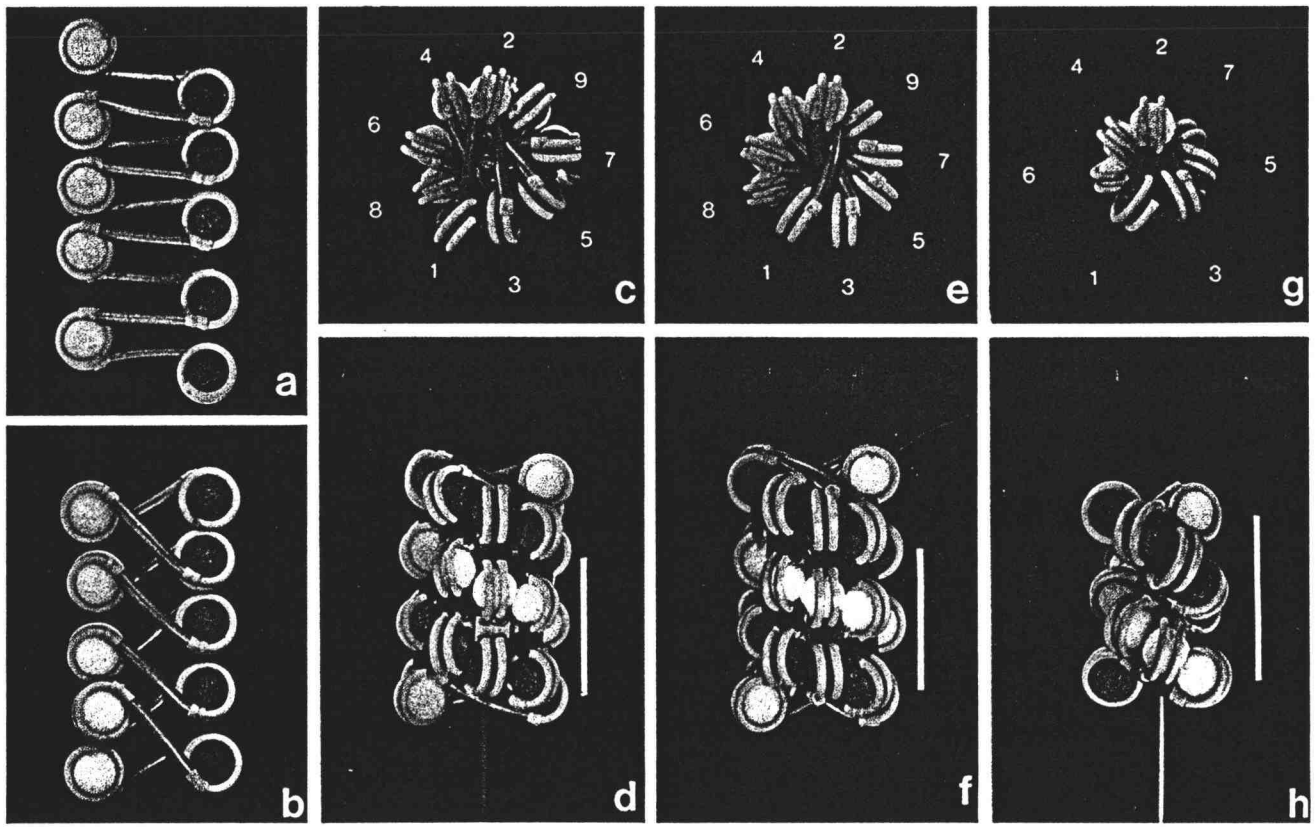


Figure 1.5 30 nm fiber models of Williams et al. (1986). This is a crosslinker model with a two-start helix. Odd and even nucleosomes are shaded and white, respectively. Numbers indicate the sequence of nucleosomes. (a-f) show models with a linker length of 48 bp. (a) and (b) are extended ribbons without and with crossover of the linker DNA. (c) and (d) show a solenoid of ribbon without crossovers of linker DNA. (e) and (f) show a solenoid of ribbon with crossovers of linker DNA. (g) and (h) show a solenoid without linker DNA crossovers with a 22 bp linker length.

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 DNA is internal to the fiber (see Figs. 1.1, 1.4 and 1.5), in some cases crossing back and forth across the fiber axis (see Fig. 1.5), in other cases being internally looped (see Fig. 1.4). Some have the linker DNA close to the surface of the fiber (see Fig. 1.3). Some models suggest instead that the linker lies coiled between adjacent nucleosomes (see Fig. 1.2). Closely tied to the problem of the location of the linker DNA is the question of the location of the linker histones. These seem to be necessary for the formation of the regular 30 nm fiber (see, for example Thoma et al., 1979; Thoma and Koller, 1981; Allan et al., 1981, 1986; Thoma, 1988) 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. A serious obstacle in resolving the higher order structure of the chromatin fiber lies in the lack of knowledge as to the disposition of the linker DNA and where and how the linker histones are located.

High resolution polyacrylamide gels of the products from nuclease and chemical cleavage of the DNA in a core particle show the cutting to occur approximately every 10 bp (Lutter, 1979, 1981; Hayes et al., 1990, 1991). As the double stranded DNA winds around the octamer, the side of the helix facing the core histones is protected while the side 5 bp and 180° away is more exposed to digestion. This approximate 10 bp ladder extends not only beyond core particles but also beyond the nucleosomal repeat size. Several labs (Lohr et al., 1977; Lohr and van Holde, 1979; Karpov et al., 1982; Strauss and Prunell, 1983; Lohr, 1986) have found a significant number of linker lengths between adjacent nucleosomes to be 10 n ± 5 bp (where n is

an integer). Being half a turn, 5 bp can be interpreted as requiring adjacent nucleosomes to be rotated 180° in orientation to each other, perhaps as in

Table 1.2
Some selected core particle and nucleosomal repeat lengths (van Holde, 1988).

Tissue, cell	Length of core particle DNA	Nucleosomal repeat length	References
Yeast	148 ± 3 bp	165 bp	Lohr and van Holde (1979) Lohr and Ide (1979)
Pea	145 bp	195 bp	Grellet et al. (1980)
HeLa	146 ± 3 b	188 ± 1 bp	Levinger and Varshavsky (1980) Compton et al. (1976)
Strongylocentrotus purpuratus (sea urchin) sperm		260 bp	Simpson and Bergman (1980)
Chicken erythrocytes	145 ± 3 bp	210 ± 3 bp	Hörz and Zachau (1980)
Rat liver	146 b	196 ± 1 bp	Prunell et al. (1979) Compton et al. (1976)

the zig-zag the form seen in EM micrographs (Thoma et al., 1979). In concordance with the 10 n ± 5 rule are observations of a 10 bp cutting pattern in the linker DNA (Karpov et al., 1982; Bavykin et al., 1990) that have been interpreted as indicating that the linker DNA forms a continuous supercoil between nucleosomes. Work in Jon Widom's laboratory shows EM micrographs of isolated dinucleosomes (Yao et al., 1990, 1991). In low salt the two nucleosomes stand apart separated by the extended strand of

linker DNA; but as the salt is increased, the two nucleosomes touch while the linker DNA disappears, likely to between the two nucleosomes. This is the strongest evidence that the linker DNA can be coiled between adjacent nucleosomes.

While the length of DNA in a core particle is an invariant 146 bp, the nucleosomal repeat length varies among species and cell types. As can be seen from Table 1.2, nucleosomal repeat lengths can vary from as low as 165 bp in yeast to as high as 260 bp in certain sea urchin species. These differences of nucleosomal repeat length become significant in the next level of packing, the 30 nm fiber.

The issue of the location of the linker histones in the fiber is of immediate importance to the understanding of the higher order structure of chromatin in the nucleus. Do they lie within the fiber, on its surface, or both? The data, obtained thus far, mainly from immunochemical studies (for review see Zlatanova, 1990), are highly controversial (see pp. 73 and 74 in Chapter 5 for discussion).

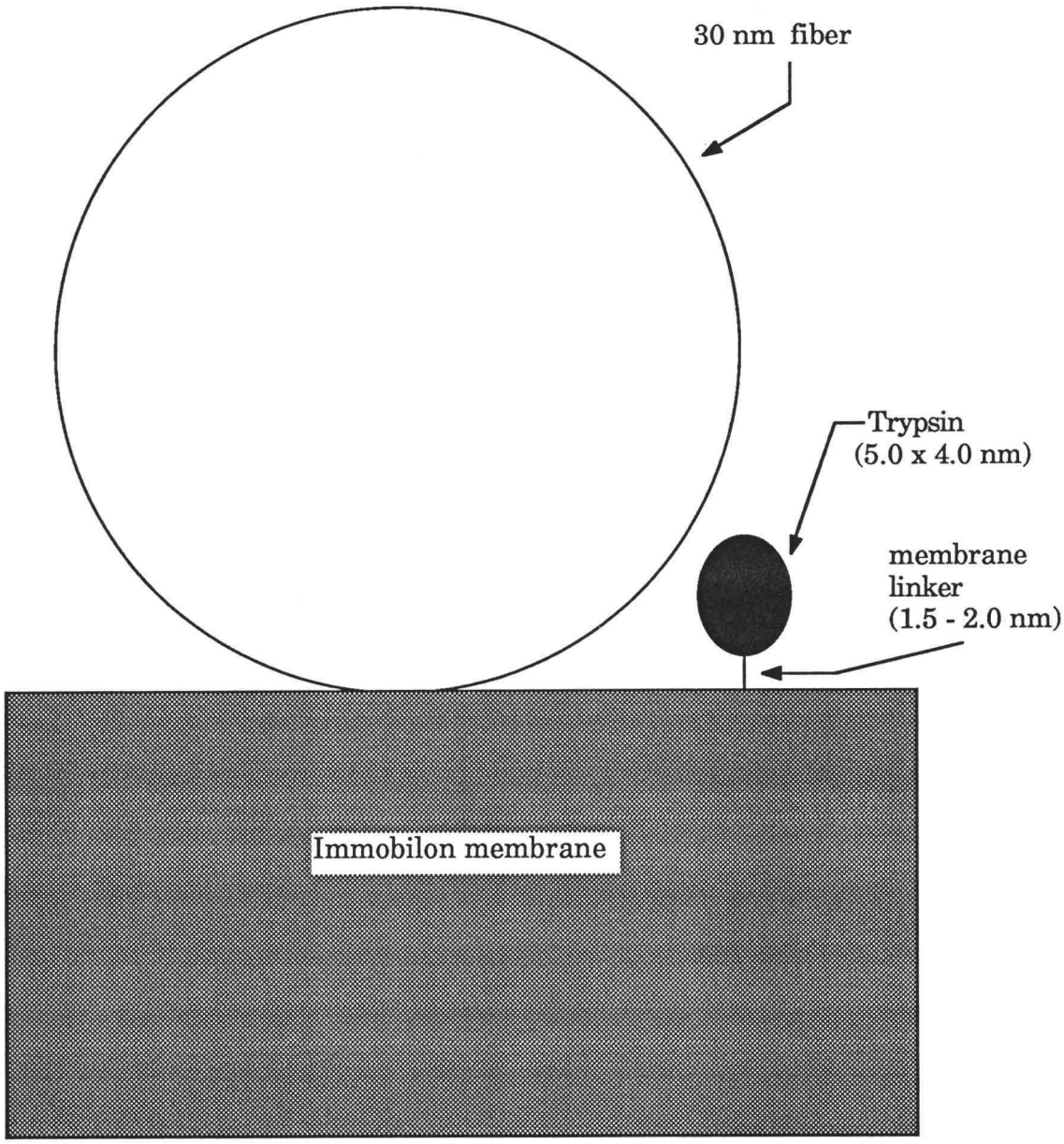
It seems that one way of approaching these questions would be through the use of immobilized enzymes to cleave either the linker DNA or the linker histones. Both seem accessible to free enzymes, at least under the rather aggressive conditions usually employed in digesting chromatin. Such, however, are not very revealing experiments, for both nucleases like micrococcal nuclease (MNase) and 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 the initial structure. Therefore, immobilized enzymes were utilized, and work was done under conditions of very mild digestions so that the initial

stages of the process could be observed before the responding structure had been destroyed. The use of immobilized enzymes should allow unambiguous discrimination between external and internal location of the chromatin constituents under study (see Figure 1.6). The proteolytic enzymes were chosen so as to distinguish between the location of the terminal portions of the molecules (trypsin) and the globular domains (chymotrypsin). Figure 1.7 shows the possible sites of cleavage for trypsin and chymotrypsin for H1 and H5. The preferential sites of trypsin cleavage are in the extended and exposed N- and C- terminal tails of the linker histones in chromatin (Böhm and Crane-Robinson, 1984). Chymotrypsin preferentially cleaves the phenylalanine in the globular domain of the linker histones (Bradbury et al., 1975; Singer and Singer, 1976; Hartman et al., 1977).

In this thesis I present studies of the digestion of linker histones in chicken erythrocyte chromatin using immobilized trypsin and chymotrypsin and parallel experiments in which linker DNA in this type of chromatin is cleaved by immobilized MNase, under conditions corresponding to different chromatin conformations. Included are studies using free MNase, under comparable conditions, yielding somewhat surprising results.

Figure 1.6

Comparison of the sizes of the 30 nm chromatin fiber and immobilized trypsin.



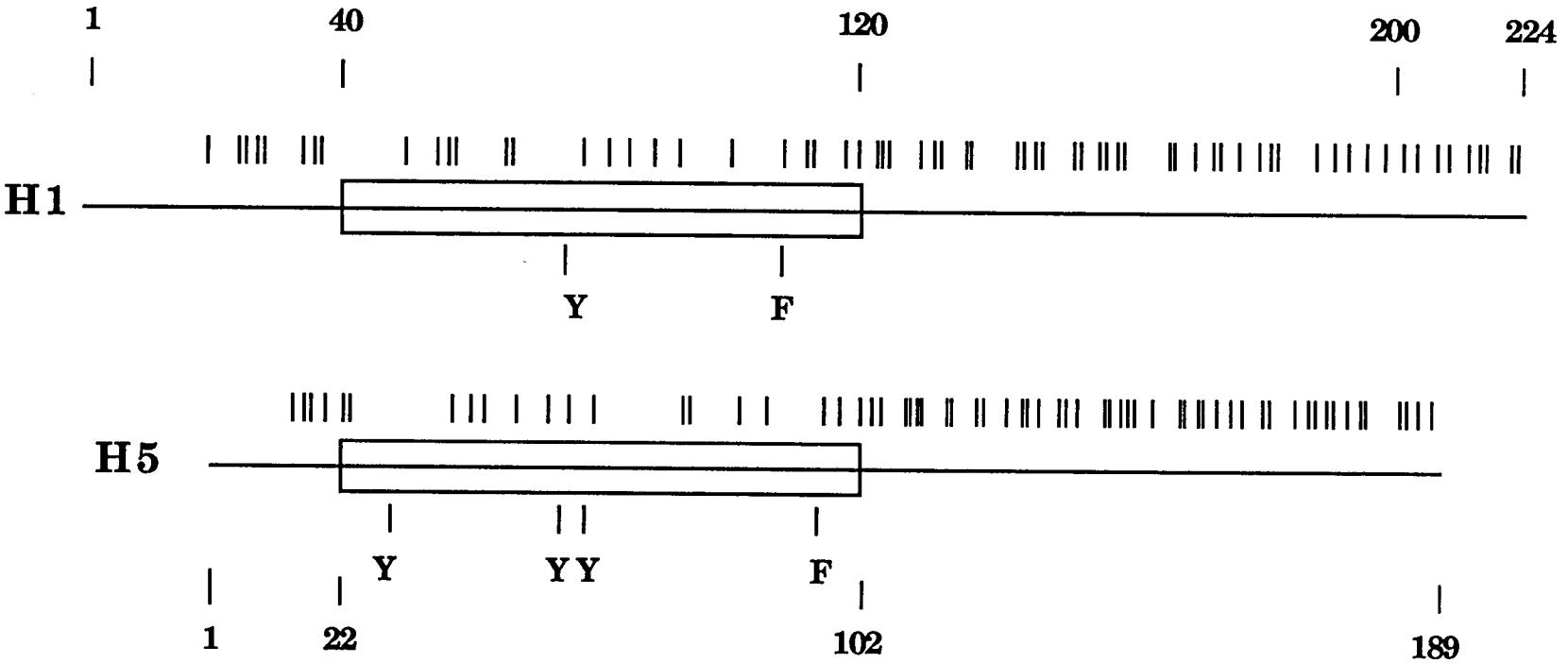


Figure 1.7

Sites of lysines and arginines (above) and aromatic amino acids phenylalanine (F) and tyrosine (Y) below in lysine-rich histones. Sequence data for H1 from Shannon and Wells (1987) and for H5 from Bohm and Crane-Robinson (1984). The preferential trypsin cleavage sites are the lysines and arginines in the tails (1-40 and 120-224 for H1 and 1-22 and 102-189 for H5) (Bohm and Crane-Robinson). The chymotrypsin preferential site is the phenylalanine (F) in the globular domain (Bradbury et al., 1975; Singer and Singer, 1976; Hartman et al., 1977).

Chapter 2

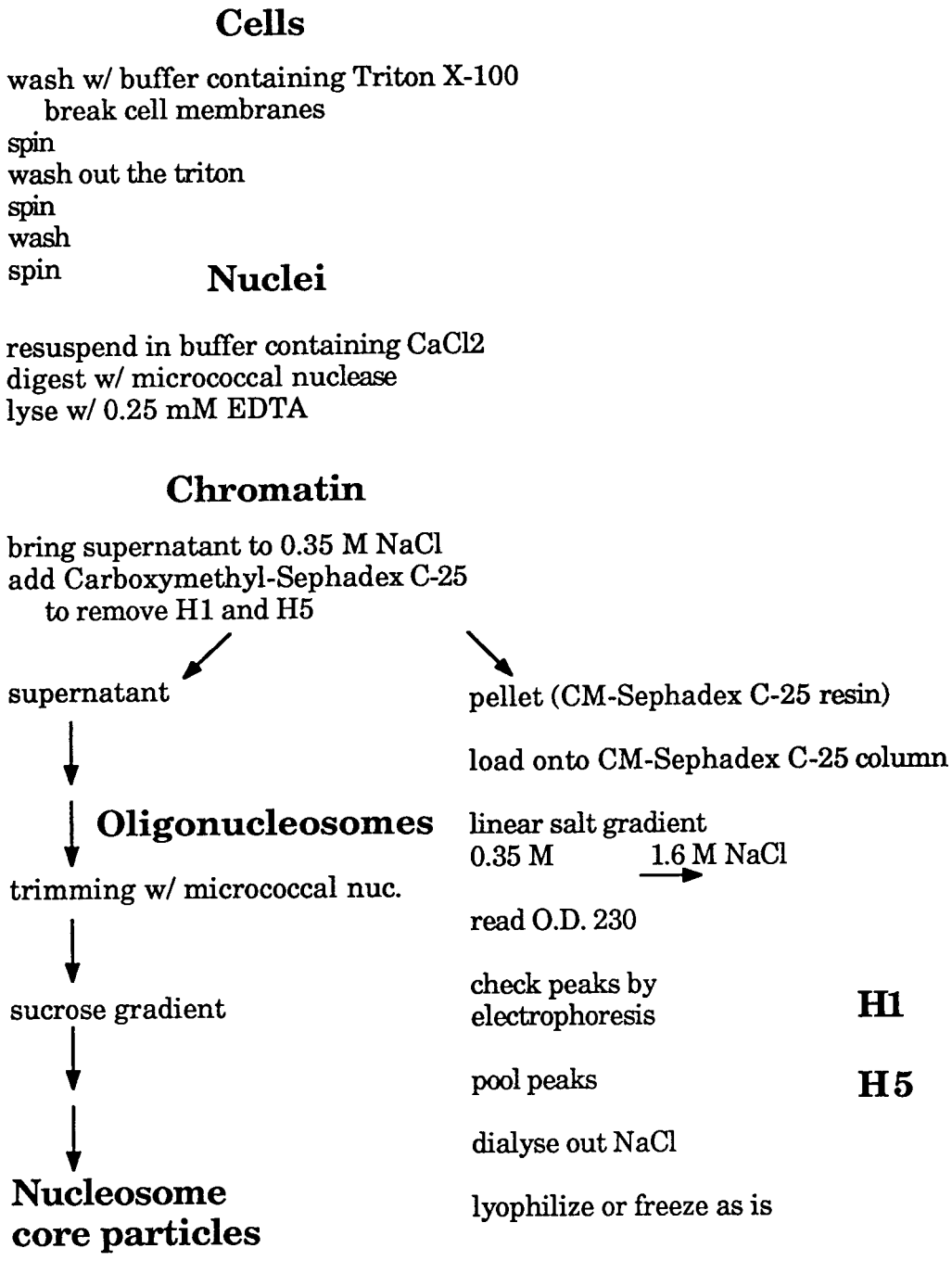
Purification of Histones H1 and H5

Experimental approach

Most of the methods used in the past to prepare histone H1 from different chromatin sources have taken advantage of the selective solubility of these histones in 5% perchloric acid (Johns, 1974). Whether such a low pH method of extraction might have altered the conformational properties of the protein has been a point of major concern. Brand et al. (1981) have reported irreversible changes in the circular dichroic and hydrodynamic parameters of histone H1 subjected to acid treatment. Salt-extracted H1 was titrated either with acid from pH 7 to pH 2.5 and then back titrated to pH 7 or with base to pH 11 and then back to pH 7. In both cases there were significant irreversible losses of alpha helical content as measured by the circular dichroism at 222 nm. Titrations with salt, however, were reversible as measured by circular dichroism. Acid titrations from pH 7 to pH 2 and then back to pH 7 of salt extracted H1 also caused an irreversible decrease in the sedimentation coefficient from 2.2 to 1.5 S. Therefore, acid can cause irreversible changes in both the secondary and tertiary structure of the protein.

Modified amino acid residues that are particularly sensitive to acid are phosphorylated lysines, histidines and arginines containing P-N linkages. P-N linkages were discovered in histones by R. A. Smith and collaborators (Smith et al., 1973, 1974; Chen et al., 1974, 1977; Bruegger et al., 1979; Fujitaki et al., 1981) and found to have half-lives of only minutes in

Figure 2.1 A general method to prepare nuclei, chromatin, nucleosome core particles and the lysine-rich histones.



10 mM mineral acid at room temperature (Smith et al., 1978). Bruegger et al. (1979) demonstrated activity of an enzyme in vitro which forms ε-N phospholysines in H1, and also reported the presence of phosphoarginine in H1. Such acid-labile linkages would not likely have been found in acid-extracted histones. It has also been reported that there is a difference in the antigenic determinants between salt and acid-extracted calf thymus H1 (Mihalakis et al., 1976).

For studies of structure it is extremely important that experiments be done with molecules in their native confirmation. Hence for all the work with H1 in our laboratory and for all the above mentioned reasons, it was necessary to have a method to produce large amounts of these histones in a native nondenatured state free of other histones.

Advantage has been taken of the fact that H1 histones can be stripped from chromatin with a CM-Sephadex C-25 ion exchanger (Libertini and Small, 1980) and this same resin has been used to further fractionate and isolate these proteins in a nondenatured native conformation.

Fractionation of H1 histones from chromatin

The method described here (for experimental details see App. 3 and 4) for isolating H1 fractions from chromatin may be employed as an integral part of a more general methodology designed for simultaneous isolation of chromatin and/or nucleosomes, histone octamers, and linker histones (see Figure 2.1). Chicken erythrocytes are washed several times in a buffer containing the detergent Triton X-100 which lyses the cell membrane. Rinsed to remove the Triton X-100 and cellular debris, the resulting nuclei are resuspended in a digestion buffer containing $CaCl_2$ and digested for 5 min with micrococcal nuclease. The aim of this initial

digestion is to cut out large pieces of the chromatin within the nuclei. Chromatin is then obtained by hypotonic lysis of the nuclei and is thereafter brought to 0.35 M NaCl. The chromatin thus obtained is then mixed with CM-Sephadex C-25, which under the ionic strength indicated will bind most of the linker histones (Libertini and Small, 1980). Under these conditions greater than 95% of the core histones (H3, H4, H2A, H2B) are not bound to the resin but remain attached to the DNA as nucleosome cores. After the extraction step, the resulting slurry is centrifuged to remove the unbound "stripped" chromatin fraction (in the supernatent). The pellet of resin is then washed with 10 mM Tris-HCl, pH 8.8, containing 0.35 M NaCl in order to remove most of the chromatin which might be trapped among the resin beads. At this stage it is very important that clumps be removed from the suspension during each washing step. The clumps arise from some large chromatin aggregates, and they may severely clog the column if they are not removed. The number of clumps actually depends on the extent of the micrococcal nuclease digestion of the nuclei before hypotonic lysis. In the experiments reported here, the following digestion conditions were routinely used: 15 units of micrococcal nuclease (Worthington)/mg DNA for 5 min at 37°C. These conditions represent a good compromise in terms of the number of clumps formed and the ability for further manipulation of the chromatin sample in order to obtain nucleosome core particles and/or histone octamers (Tatchell and van Holde, 1977). Indeed, with the enzyme-to-substrate ratio used here, only a few clumps are produced. After this washing procedure, the resin cake is resuspended in an equal volume of the 0.35 M NaCl buffer and carefully layered on top of a column previously prepared with the same resin and equilibrated with the same buffer. After

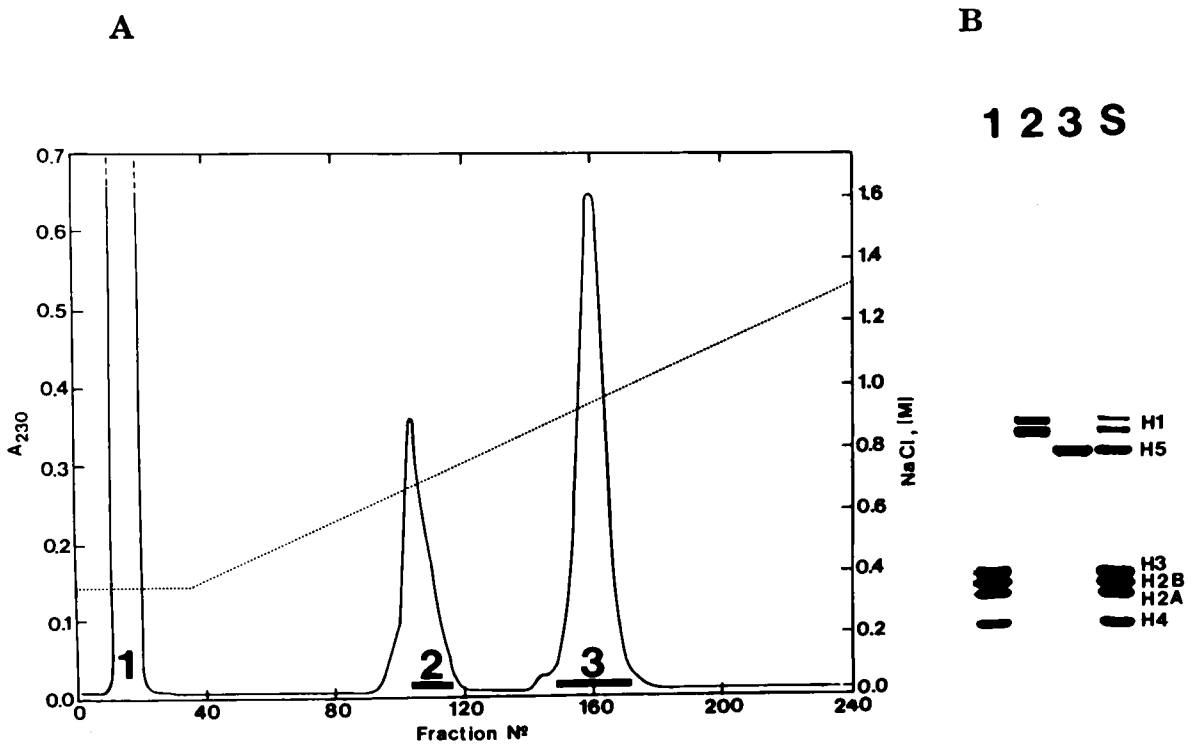


Figure 2.2 Chicken erythrocyte linker histone chromatography and electrophoresis. (A)Elution profile of the material stripped from chicken erythrocyte chromatin using CM-Sephadex C-25 in the presence of 0.35 M NaCl. The resin cake (~2.4 g of resin) after stripping was layered on top of a 2.5 X 26 cm CM-Sephadex C-25 column which had been previously equilibrated in 0.35 M NaCl, 10 mM Tris-HCl, pH 8.8. The column was eluted with a 1 L, 0.35 to 1.6 M NaCl linear gradient in the same buffer at a flow rate of 30 ml/hr. Fractions were collected at 8 min intervals. (B) SDS-polyacrylamide gel electrophoresis of the fractions pooled from the corresponding peaks in A. (S, starting chicken erythrocyte histones before stripping.)

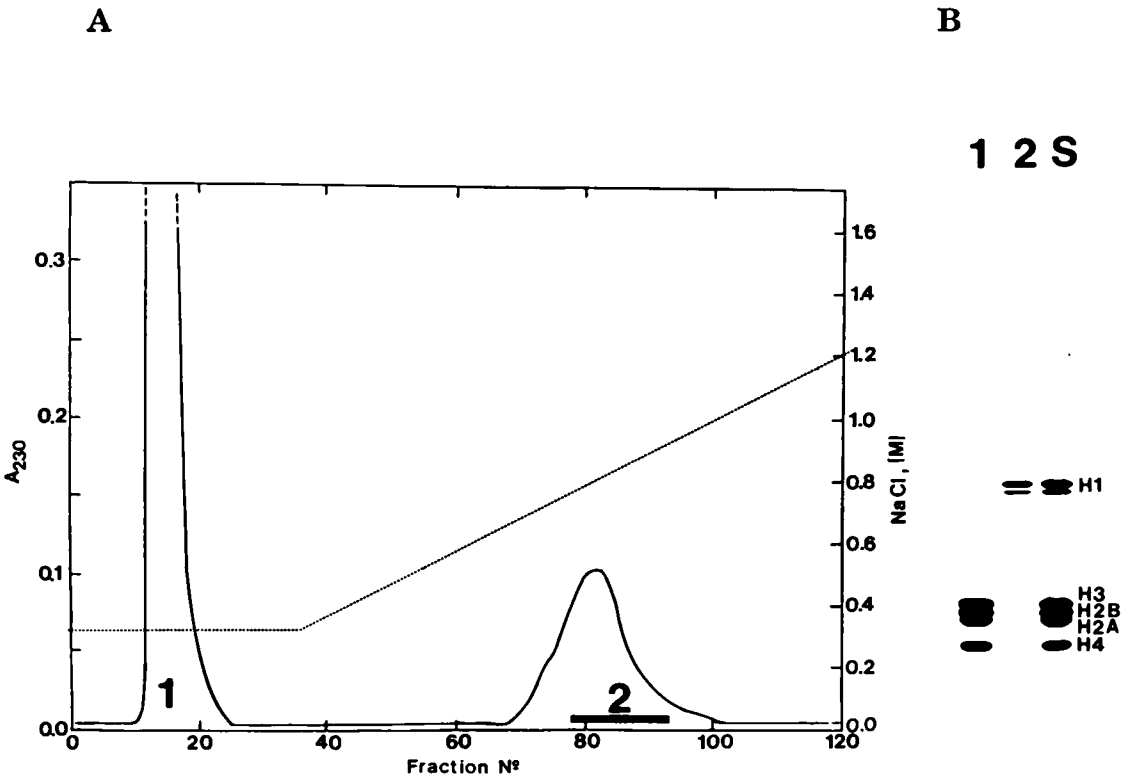


Figure 2.3 BB88 (Mouse leukemia cells) linker histone chromatography and electrophoresis. (A) Elution profile of the material stripped from BB88 chromatin, under the same experimental conditions as those in Fig. 2.2. The column dimensions were 2 X 20 cm and the elution was performed at 20 ml/hr using a 300 ml, 0.35 to 1.6 M NaCl linear gradient in 10 mM Tris-HCl, pH 8.8. (B) SDS-polyacrylamide gel electrophoresis of the corresponding peaks. (S, starting BB88 histones before stripping.)

the resin has settled the column is eluted with a 0.35 to 1.6 M NaCl gradient in 10 mM Tris-HCl, pH 8.8.

Figures 2.2A and 2.3A show the elution profiles obtained for chicken erythrocyte linker histones and for histone H1 from BB88, a mouse leukemia cell line. The flow through peak of these columns has a strong absorbance at 260. The material in this peak consists of some residual chromatin which remains interspersed in the beads of the CM-Sephadex C-25 resin after the washing steps. The electrophoretic analysis of the proteins of this region shows stoichiometric amounts of the four core histones and the absence of linker histones (see Fig. 2.2B, lane 1). Linker histones are eluted from the column at different positions along the salt gradient. With chicken erythrocyte histones, histone H1 elutes at ~0.68 M NaCl, whereas H5 elutes at higher ionic strength (~0.93 M NaCl, Figs. 2.2A and 2.2B, lanes 2 and 3). By decreasing the slope of the gradient, the peak corresponding to histone H1 in Fig. 2.2A (peak 2) can be further resolved into two overlapping peaks (data not shown). This allows one to separate the two histone H1 fractions indicated by arrows in Fig. 2.2B, lane S. This separation is most likely due to small differences in the number of basic amino acids present in these two fractions. Note, however, that none of the histone fractions isolated in this way correspond to a pure histone H1 variant. A total of six histone variants have been described for the histone H1 complement of chicken erythrocytes (Shannon and Wells, 1987). Figures 2.3A and 2.3B show the results obtained with BB88. Approximately 50 to 60 mg of total linker histones (~15 mg H1 and ~40 mg H5) was obtained from 100 ml of chicken blood and ~1.3 mg of histone H1 was obtained from 1 liter of (1.0×10^6 cells/ml) BB88. The whole procedure takes about 24 hrs, with the chromatin preparation and stripping taking around 10-12 hrs and the

column fractionation carried out overnight (~10-12 additional hrs). In order to obtain fractions free of other histones, it is very important that aliquots along the peak be analyzed electrophoretically before the fractions are pooled. This is necessary because of the presence of trace amounts (5%) of histone H2A-H2B dimers which tend to comigrate with the leading edge of the histone H1 peak at ~0.65 M NaCl, 10 mM Tris-HCl, pH 8.8. This small contamination which is responsible for the slight asymmetry exhibited by the peaks (see Figs. 2.2A, lane 2, and 2.3A, lane 2), can alternatively be removed by decreasing the slope of the salt gradient.

The method described here allows very rapid and relatively simple fractionation and isolation of large quantities of linker histones from chromatin from different sources. The isolated protein fractions exhibit a high degree of purity by SDS gel electrophoresis (data not shown) and are obtained under nondenaturing conditions. The method thus offers the advantage of recovery of the histone proteins in their native state, an important consideration for the further use of these proteins in studies in which the native conformational parameters need to be preserved. In this sense, the method represents an advantageous alternative to the widely used procedures in which selective perchloric acid extraction is used to prepare histone H1 (Johns, 1974).

Chapter 3

Results from Immobilized Trypsin and Immobilized Chymotrypsin Digestion of Chromatin

Experimental approach

The experiments reported here were designed to address the question of whether and to what extent the location of the linker histones in chicken erythrocyte chromatin changes as a function of the transition of the chromatin fiber from extended to progressively more condensed conformations. Immobilized proteolytic enzymes, trypsin and chymotrypsin, were used as probes to compare the accessibility of the linker histones in the fiber, free in solution or in isolated mononucleosomal particles. The use of immobilized endopeptidases possesses several advantages over the use of soluble enzymes: (1) The reaction can be stopped instantly by simply removing the protein-containing solution from the membrane. (2) It allows extremely mild digestion conditions so that the digestion of H1 and H5 can be monitored before any significant digestion of the core histones takes place; parallel physical studies can yield information of the structural changes in the fiber accompanying the initial digestion of a fraction of linker histone molecules. (3) The immobilization on a solid surface prevents the enzyme molecule from penetrating into the interior of the fiber, even at times when the fiber is "breathing", i.e. temporarily and locally opening up and closing down. This, in turn, avoids the necessity to fix fiber structure against breathing using chemical cross-linking agents such as glutaraldehyde. Control experiments were performed to make sure that the enzymes remained attached to the

membrane even after prolonged incubation under conditions used for digestion.

All experiments were performed on high molecular mass soluble chromatin fragments, containing about 75±30 nucleosomes (the average S value in Tris determined by analytical centrifugation was about 58±7; the average molecular mass of the DNA estimated by agarose gel electrophoresis was 15±6 kbp). No material of DNA size below 2 kbp was present in the preparations. The use of high molecular mass fragments was required in order to: (1) avoid possible end-effects that might significantly distort data obtained on oligonucleosomes and (2) ensure proper formation of condensed structures (at least 6 nucleosomes are necessary to form higher order structure (Bates et al., 1981; McGhee et al., 1983). Control experiments showed that the chromatin preparations did not contain significant endogenous proteolytic activities.

To study the accessibility of chromatin-contained linker histones in fibers of different structural characteristics, comparisons were done of the proteolytic digestion patterns of chromatin dissolved in 10 mM Tris without salt, with addition of 10 mM NaCl, or with additions of 0.35 mM $MgCl_2$. As well documented in the literature (reviewed in Thoma, 1988; see also Fig. 1.1), in the absence of salts the chromatin fiber exists as an extended "open zig-zag", with the linker DNA entering and exiting the nucleosomes on the same side; addition of salt to 5-10 mM NaCl leads to "closing of the zig-zag", bringing the nucleosomes in close proximity. Finally, in the presence of 0.35 mM $MgCl_2$ the fiber condenses into the 30 nm fiber structure (Ausio et al., 1984), indistinguishable from the one observed in the nucleus under physiological salt conditions. The choice of Mg^{++} ions instead of the more commonly used Na^{+} ions for chromatin condensation was made in order to

avoid the redistribution of linker histones known to occur at the NaCl concentrations necessary to achieve chromatin condensation (Caron and Thomas, 1981) and was based on a careful physical study of the condensation process driven by either Na^+ or Mg^{++} (Ausio et al., 1984). Sedimentation velocity measurements (App. 11) showed that the median sedimentation coefficient of the chromatin fiber was about 57, 85 and 140 S in Tris, 10 mM NaCl and 0.35 mM $MgCl_2$, respectively (Fig. 3.10), as expected from gradual condensation of the fiber.

Addition of divalent ions to concentrated chromatin solutions often brings about aggregation, and it was important to work under conditions where no significant precipitation of chromatin took place during the course of the experiment. This was achieved by using relatively diluted chromatin solutions (about 100 μg of DNA/ml) and keeping the Mg^{++} concentration at the lowest value that would still assure condensation (see Fig. 3.10). Measurements of the A_{260} of the chromatin preparations at different times during proteolytic digestion following removal of any precipitated material by centrifugation showed that even 8 h after the addition of Mg^{++} only about 5% of the material had precipitated.

Trypsin digestion

Trypsin digestion of long chromatin

Trypsin, either free in solution or immobilized on collagen membranes, has been widely used in studies which attempted to correlate chromatin fiber unfolding with digestion of H1 and core histones (see Böhm and Crane-Robinson, 1984 for a review of earlier experiments, Hacques et al., 1990 for a more recent study). Because the N- and C-tails of the molecules of the linker histones are extended in conformation and rich in

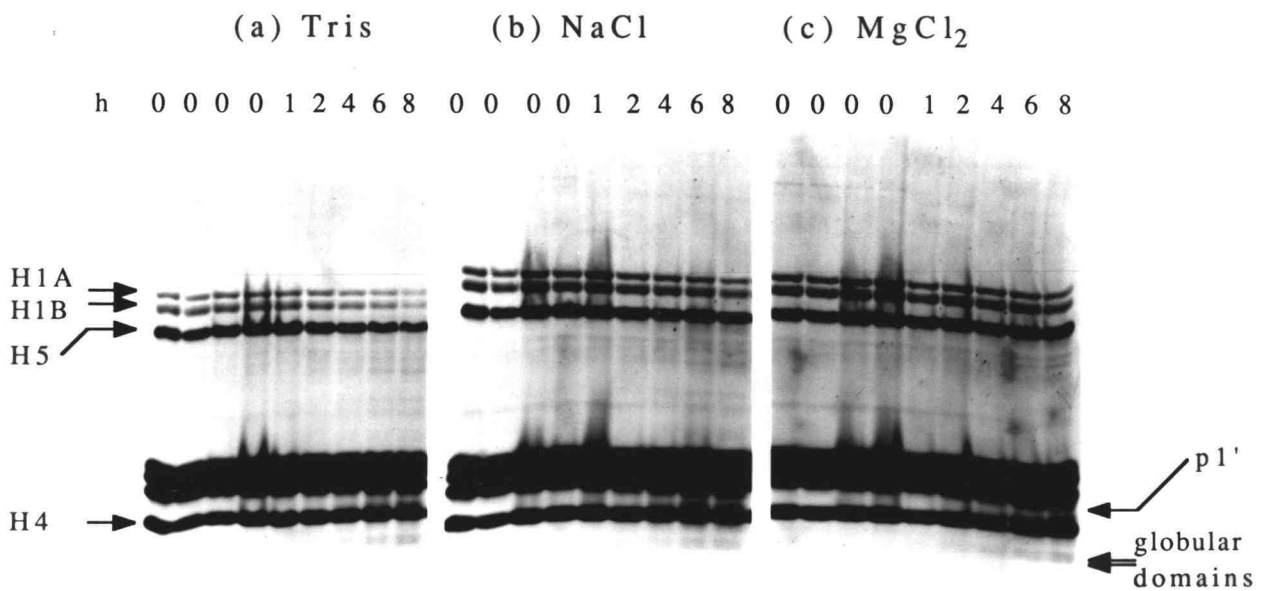


Figure 3.1 SDS gel electrophoretic patterns of long chromatin digested with immobilized trypsin at 25°C. Chromatin was dissolved in 10 mM Tris-HCl (pH 7.5) (a), or in the same buffer containing 10 mM NaCl (b) or 0.35 mM $MgCl_2$ (c). Time of trypsin treatment (h) is denoted above the lanes. The positions of the two major H1 fractions resolvable by SDS-gel electrophoresis are marked by arrows to the left, as are the positions of histones H5 and H4. The positions of the globular domains of H1 and H5, as well as that of the major digestion product of histone H3 (P1') are marked to the right.

lysines and arginines, trypsin degrades them quickly, giving rise in each case to a relatively stable fragment encompassing the central structured globular domain of the histone molecule (Allan et al., 1980).

A typical pattern of immobilized trypsin digestion of long chromatin fibers in the three structural states is presented in Figure 3.1. It is clear that the digestion was extremely mild: even at 8 h only about 50% of the linker histone molecules had been digested. At the same time the trypsin-resistant globular domains of both H1 and H5 became evident, as bands migrating below H4. Because these studies were confined to early digestion time points, the relative kinetics of the accompanying digestion of the core histones was difficult to follow; in accordance with most published data (Böhm and Crane-Robinson, 1984), H3 was found to be the histone attacked immediately following degradation of some of the linker histone molecules, as judged by the appearance of the proteolytic fragment P', situated just above H4. As attempts to localize linker histones with respect to the fiber higher order structure will be compromised in preparations where the fiber is destroyed due to proteolysis of the core histones, specific care was taken to include in the analysis of the linker histone accessibility only time points of digestion before visible degradation of the core histones had occurred.

The digestion patterns were quantified by scanning of the gels and determining the area under the histone peaks. To compensate for possibly different protein loadings in the individual lanes, all values for H1 and H5 content were normalized to the amount of H4 present in the respective lanes (H4 was used for normalization as there were no signs of degradation of this very stable histone even late during digestion and as it was well separated from the other histones in electrophoretic gels). The results of such quantitation are presented in Figure 3.2. As can be seen, histone H5

Figure 3.2 Quantitation of the trypsin digestion patterns of long chromatin, presented in Fig. 3.1. The electrophoretic gels were scanned and quantitation was performed as described in App. 12. For easier interpretation the data are presented as pairwise comparisons between histones H1 and H5 under the three conditions: (a) 10 mM Tris-HCl (pH 7.5), (b) 10 mM NaCl, and (c) 0.35 mM $MgCl_2$. Additionally, the curves for each linker histone under the three conditions are plotted on the same graph: (d) histone H1 and (e) histone H5. The values on the ordinate represent the amount of undigested linker histone relative to the amount of histone H4, the corresponding value for the 0 time point being taken as 100%.

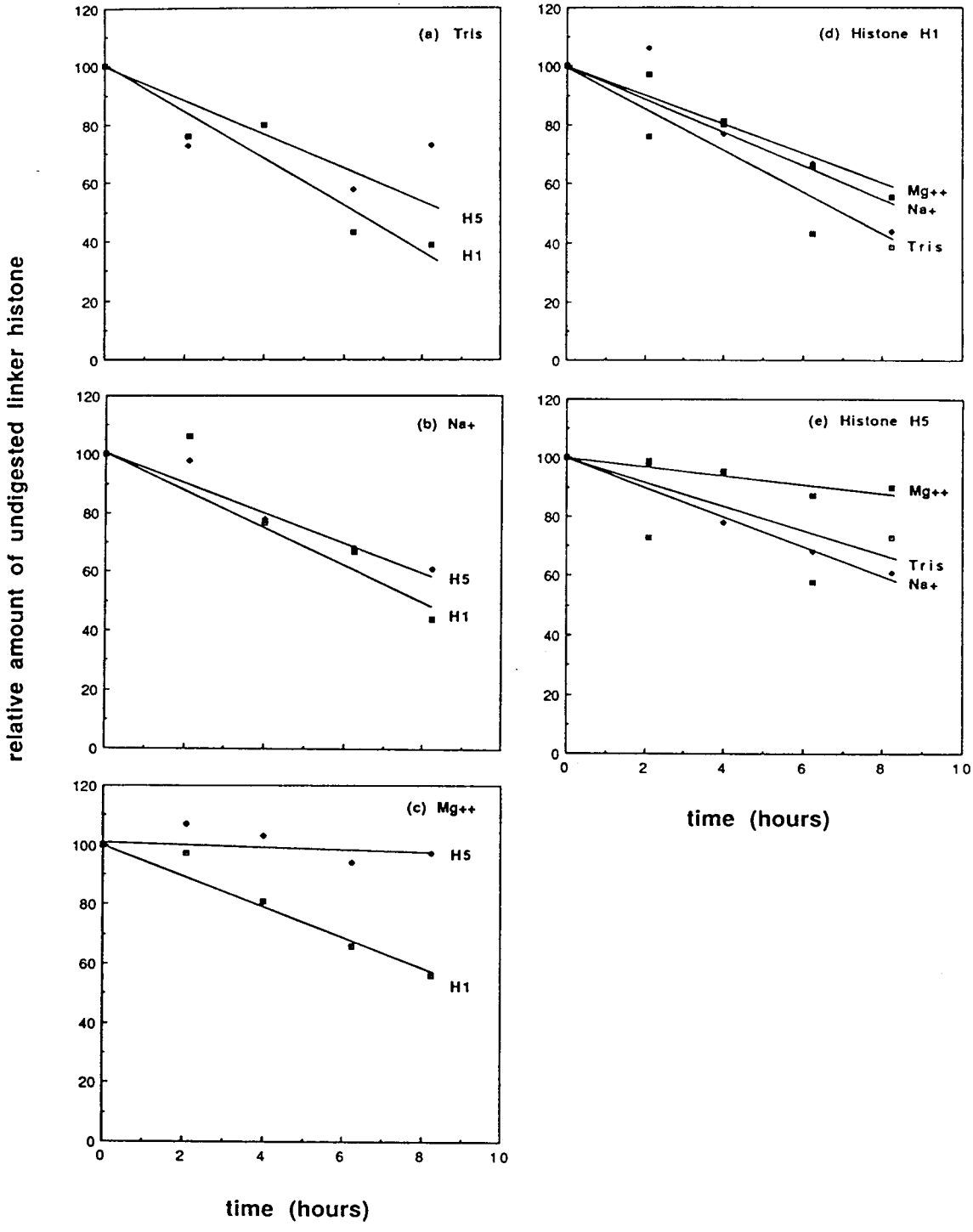

relative amount of undigested linker histone
(a) Tris
H5
H1
(b) Na+
H5
H1
(c) Mg++
H5
H1
(d) Histone H1
Mg++
Na+
Tris
(e) Histone H5
Mg++
Tris
Na+
time (hours)
time (hours)

Figure 3.2

was somewhat more protected than histone H1 under all three conditions tested: Tris, 10 mM NaCl and 0.35 mM $MgCl_2$. In the more extended conformations of the fiber (Tris, 10 mM NaCl), there was only a slight, albeit highly reproducible, protection; on the other hand, H5 was much more protected in 0.35 mM $MgCl_2$. That the higher protection of H5 in the condensed fiber was due to the formation of the higher order structure is especially evident from comparisons among the three conditions for each histone. While H1 seemed to be equally accessible to digestion under all conditions, H5 apparently became more protected only in the Mg^{++}-containing solution. The data shown in Figures 3.1 and 3.2 are representative of those observed in six independent experiments using several different preparations of immobilized trypsin.

Trypsin digestion of free linker histones in solution

There are possible trivial explanations of the results reported above. First, it could be that H5 is intrinsically more resistant than H1 to trypsin digestion even when free in solution. Second, it is possible that the presence of Mg^{++} ions specifically decreases the rate of digestion of H5 in comparison to that in Tris or in NaCl. To check for these possibilities, an equimolar mixture of purified H1 and H5 was subjected to digestion with immobilized trypsin under the salt conditions used to differently structure the long chromatin fiber. The results for Tris and $MgCl_2$ are presented in Figure 3.3. Histones H1 and H5 were digested at indistinguishable rates either in the absence of salt or in the presence of 0.35 mM $MgCl_2$. Similar digestion curves were obtained using 10 mM NaCl (not shown). Thus, the differences observed with long chromatin-contained linker histones reflect features of accessibility to the enzymatic probes related to chromatin structure rather

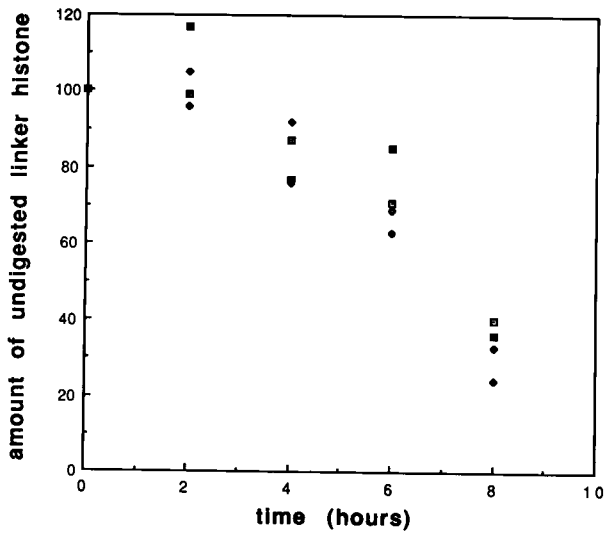


Figure 3.3 Quantitation of the digestion of the linker histones free in solution in Tris-HCl (pH 7.5) and in 0.35 mM $MgCl_2$ at 25°C. An equimolar mixture of purified H1 and H5 was subjected to trypsinization, run on SDS-polyacrylamide gels and the amount of linker histones remaining at the position of the intact molecules was quantified by setting the amount present at the start of digestion as 100%. The symbols are : ⊡, H1 in Tris; ◆, H1 in Mg^{++}; ◘, H5 in Tris; ◇, H5 in Mg^{++}.

than differences inherent in the interaction of the free histone molecules themselves with the enzyme.

The linker histones are structured in all conformations of the fiber

Any study aimed at determining the role or location of the linker histones in the chromatin fiber should be performed under conditions in which the histone is structured in its native tertiary conformation. It is known that these molecules exist as random coils in solution in the absence of salt and that the transition of freely dissolved linker histones to their organized structures requires the addition of salt (100 mM NaCl; Smerdon and Isenberg, 1976). Consistent with this, control digestion experiments with the *free* linker histones did not show any evidence for a trypsin-resistant protein core under any of the three ionic conditions studied. Therefore, it was important to make sure that the linker histones were structured while chromatin-bound. That binding of H1/H5 to DNA in chromatin might mimic higher salt concentrations has been long been suspected since some of the positive charges on the molecules (particularly those in the C- and N-terminal tails) are neutralized by interaction with the DNA (see the discussion by Thoma, 1988). In fact, experiments with H1/DNA model systems have shown that a trypsin-resistant folded globular domain could be detected at salt concentrations as low as 15 mM NaCl (Clark and Thomas, 1986).

A careful examination of the trypsin digestion patterns (Fig. 3.1) revealed the gradual appearance of two closely migrating bands below H4, corresponding to the globular domains of H1 and H5. The rate of accumulation of these domains was proportional to the rate of disappearance of the intact H1 and H5 bands (Fig. 3.4). Significantly,

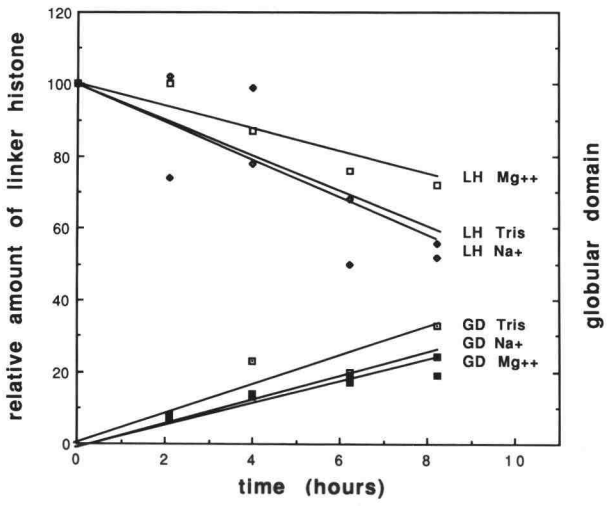


Figure 3.4 Time course of digestion of the chromatin-contained linker histones (LH) and appearance of the globular domain (GD) under the three different salt conditions: 10 mM Tris-HCl (pH 7.5), 10 mM NaCl, and 0.35 mM $MgCl_2$ (marked as Tris, Na^+ and Mg^{++} on the graph). The data for the digestion of histones H1 and H5 have been presented as common curves; the same holds true for the globular domains of the two proteins. The figures for the globular domains represent the proportion of total intact linker histones that appears as globular domains (it was considered that the globular domain encompasses around 40% of the size of the molecule of an "averaged" linker histone molecule).

globular domains became detectable and had similar kinetics of accumulation under all three conditions. This implies that the linker histones are structured even in the absence of salt when chromatin-bound, and that the differences observed in the digestion patterns and sedimentation behavior (see below) of the three distinct fiber structures are not due to transitions in the structure of the linker histones themselves.

Chymotrypsin digestion

As mentioned above, trypsin cleaves most readily in the positively charged tails of the linker histone. Therefore the results obtained upon trypsinization of chromatin provide, primarily, insight into the accessibility of these tails in the fiber under different conformations. On the other hand, not much can be said from these studies about the globular domains whose relative stability will make them disappear only very late during trypsin digestion at a point when the chromatin fiber is no longer intact. To study the accessibility of the globular domains, advantage was taken of the fact that α-chymotrypsin preferentially cleaves to the carboxyl side of the single phenylalanine situated in the globular domains of both H1 and H5 (see Fig. 1.7). This residue lies at positions 105, 108, and 109 for the different subfractions of chicken H1 (Shannon and Wells, 1987), and at position 94 in chicken erythrocyte H5 (Böhm and Crane-Robinson, 1984). Chymotrypsin digestion has the advantage that the C-terminal half of the molecule resulting from the preferential cleavage adjacent to this phenylalanine residue migrates upon SDS polyacrylamide gel electrophoresis between the core histones and H1 and can be easily monitored (Losa et al., 1984, see also Fig. 3.7).

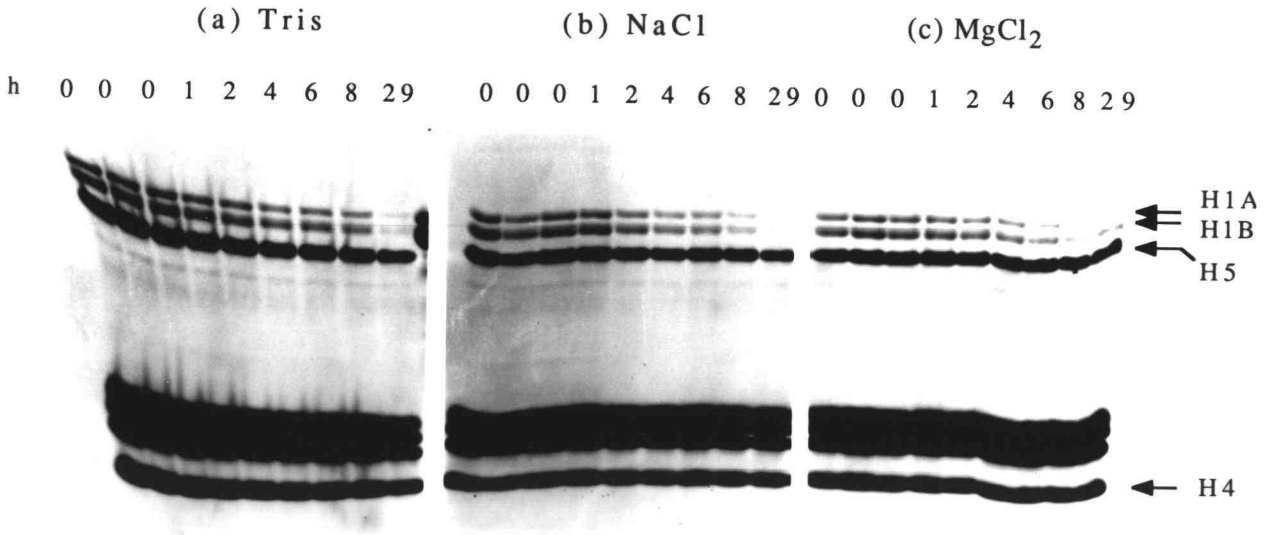


Figure 3.5 SDS gel electrophoretic patterns of long chromatin digested with immobilized chymotrypsin. Chromatin was dissolved in 10 mM Tris-HCl (pH 7.5) (a), or in the same buffer containing 10 mM NaCl (b) or 0.35 mM $MgCl_2$(c) and the digestion was carried out at 37°C. Time of chymotrypsin treatment (h) is denoted above the lanes. The positions of H1 (see legend to Fig. 3.1), H5, and H4 are marked to the right.

Figure 3.6 Quantitation of the chymotrypsin digestion patterns of long chromatin, presented in Fig. 3.5. The electrophoretic gels were scanned and quantitation was performed as described in App. 12. For easier interpretation the data are presented as pairwise comparisons between histones H1 and H5 under the three conditions: (a) 10 mM Tris-HCl (pH 7.5), (b) 10 mM NaCl, and (c) 0.35 mM $MgCl_2$. The values on the ordinate represent the amount of undigested linker histone relative to the amount of histone H4, the corresponding value for the 0 time point being taken as 100%.

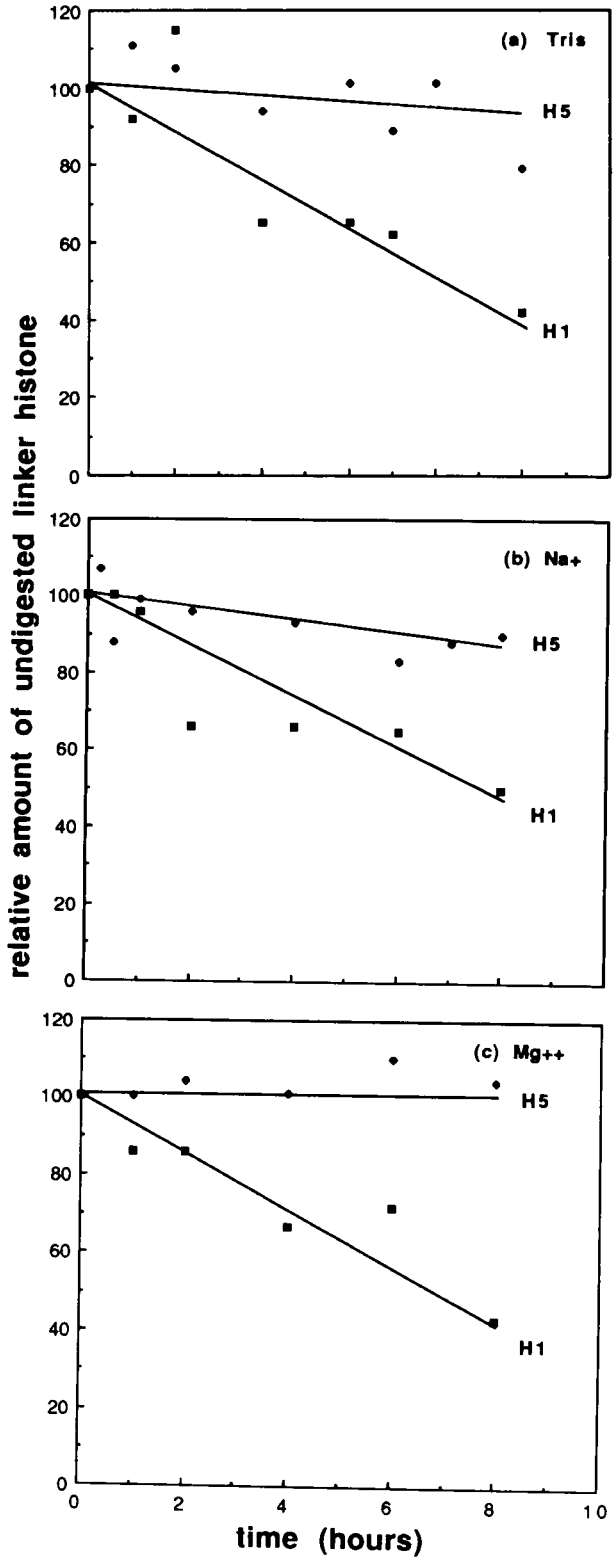

(a) Tris
H5
H1
(b) Na+
H5
H1
(c) Mg++
H5
H1
relative amount of undigested linker histone
time (hours)
0
20
40
60
80
100
120
0
2
4
6
8
10

Figure 3.6

Histones H1 and H5 in chromatin behaved very differently with respect to chymotrypsin digestion: whereas H1 was digested at a rate comparable to the rate of its digestion with trypsin, H5 was much more resistant, showing only very slow digestion in Tris, or in NaCl, and no detectable digestion in $MgCl_2$ (Figs. 3.5 and 3.6). The experiments were repeated several times, performing the reaction at 10, 25 or 37°C with the same result. Only when the digestion was continued for about 30 h at 37°C was some slight degradation of H5 visible, the relative amount of undigested protein still being between 60 and 90% for the three conditions (the corresponding value for H1 remaining intact at this time point was between 0 and 25%). The negative result with H5 could not be due to inactivity of the enzyme, as H1 in the same samples was readily digested. To make sure that the difference was not due to H5 being refractory to chymotrypsin digestion even when free in solution, free H1 and H5 histones were subjected to hydrolysis with immobilized chymotrypsin. The free linker histones were digested extremely efficiently, with the intact molecules disappearing within 2 min of digestion at 25°C. To properly monitor the kinetics of digestion and the proteolytic products, the reaction with the free histones had to be performed at 10°C and time points had to be taken much more frequently than in the case of trypsin digestion (Fig. 3.7). The rate of digestion of both H1 (Fig. 3.7(a)) and H5 (Fig. 3.7(b)) was indistinguishable for the proteins dissolved in Tris, NaCl, and $MgCl_2$. Furthermore, when the two proteins were digested simultaneously in an artificial equimolar mixture, H5 was digested only slightly more slowly than H1 (data not shown).

The observation that the protection of H5 and the digestion of H1 with chymotrypsin was independent of the conformation of the fiber, taken

Figure 3.7 SDS gel electrophoretic patterns of purified histones H1 and H5 digested with chymotrypsin at 10°C. (a) Histone H1; (b) Histone H5. The purified histones were dissolved in 10 mM Tris-HCl (pH 7.5) (left panels), in 10 mM NaCl (middle panels), and in 0.35 mM $MgCl_2$ (right panels), respectively. The time of digestion is denoted above the lanes. Total chicken erythrocyte histones were used as markers (denoted M above the respective gels) in order to facilitate the identification of the C-terminal halves of the molecules, resulting from the preferential digestion at the sole phenylalanine in the globular domains (the positions of the C-terminal halves are denoted to the right).

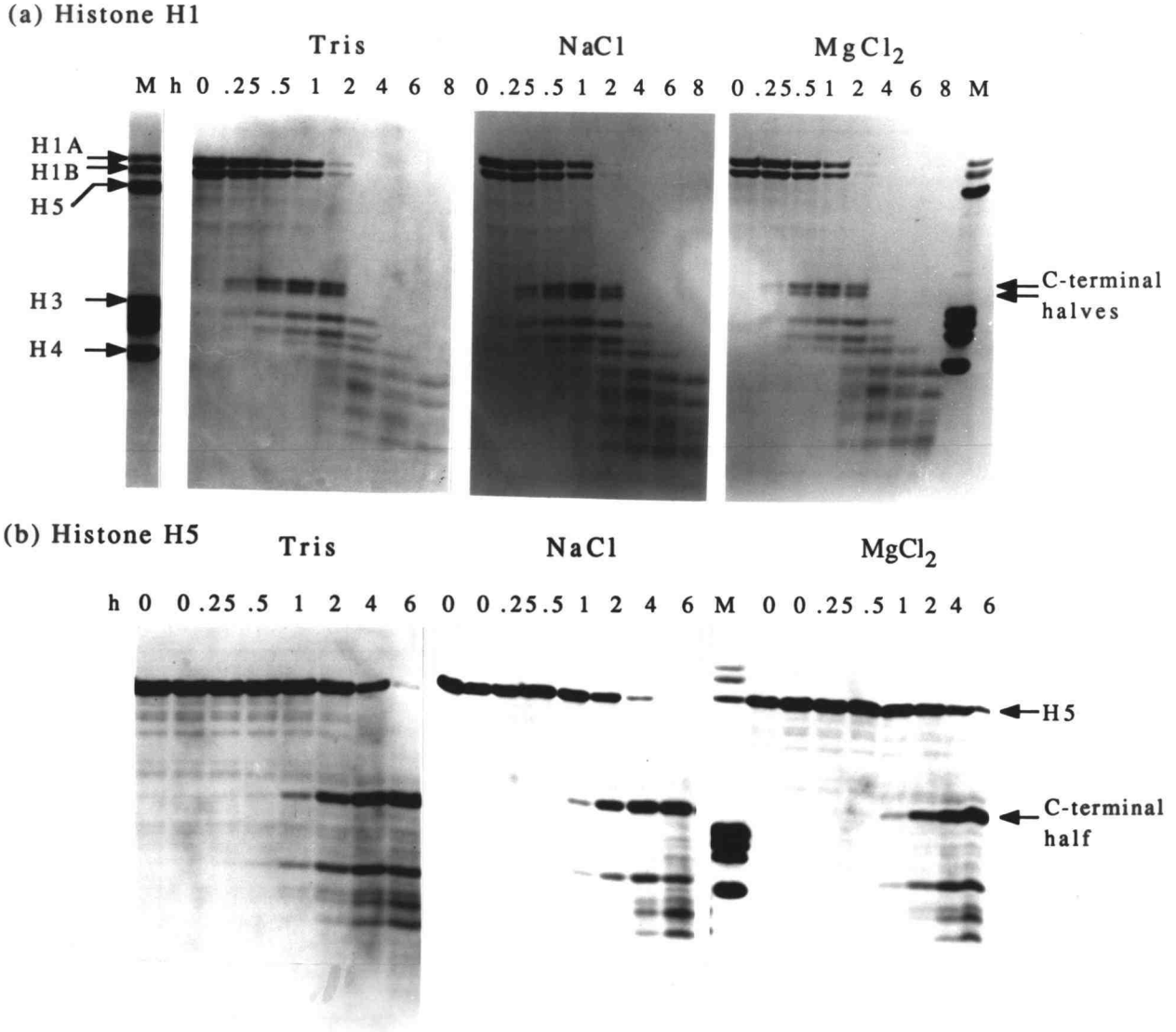

(a) Histone H1
Tris
NaCl
MgCl2
M h 0 .25 .5 1 2 4 6 8 0 .25 .5 1 2 4 6 8 0 .25 .5 1 2 4 6 8 M
H1A
H1B
H5
H3
H4
C-terminal halves
(b) Histone H5
Tris
NaCl
MgCl2
h 0 0 .25 .5 1 2 4 6 0 0 .25 .5 1 2 4 6 M 0 0 .25 .5 1 2 4 6
H5
C-terminal half

Figure 3.7

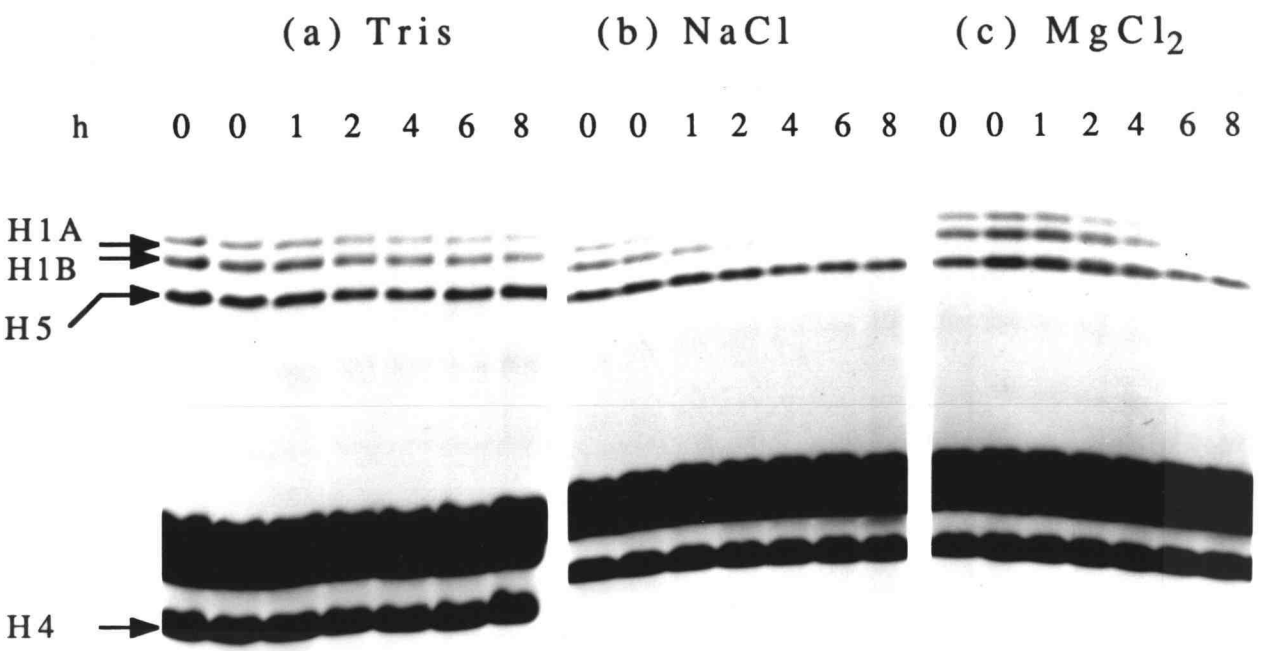


Figure 3.8 SDS gel electrophoretic patterns of isolated mononucleosomal particles digested with immobilized chymotrypsin. Monosomes, obtained and characterized as described in App. 9, were dissolved in 10 mM Tris-HCl (pH 7.5) (a), or in the same buffer containing 10 mM NaCl (b) or 0.35 mM $MgCl_2$ (c) and the digestion was carried out at 37°C. Time of chymotrypsin treatment (h) is denoted above the lanes. The positions of H1 (see legend to Fig. 3.1), H5, and H4 are marked to the left.

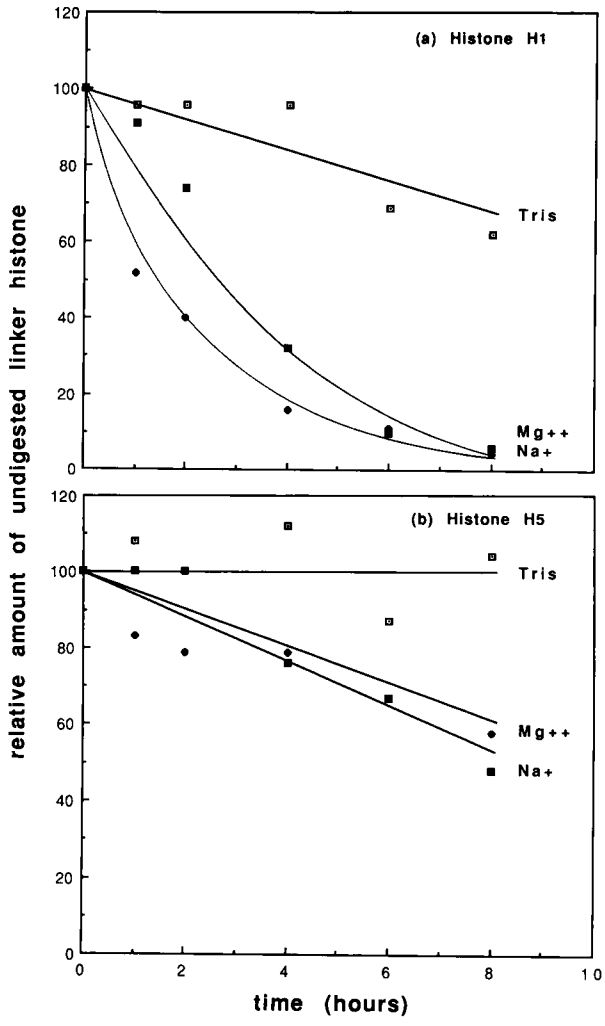


Figure 3.9 Quantitation of the chymotrypsin digestion patterns of isolated mononucleosomal particles, presented in Fig. 3.8. The electrophoretic gels were scanned and quantitation was performed as described in App. 12. For easier interpretation the data are presented separately for each linker histone: (a) histone H1 and (b) histone H5, under the three conditions: 10 mM Tris-HCl (pH 7.5), 10 mM NaCl, and 0.35 mM $MgCl_2$ (marked on the graphs as Tris, Na^+ and Mg^{++}, respectively). The values on the ordinate represent the amount of undigested linker histone relative to the amount of histone H4, the corresponding value for the 0 time point being taken as 100%.

together with the ease of attack of the free histone, suggested two possibilities: either that phenylalanine, which is the preferential site of attack, is not accessible even in the mononucleosome or that it becomes inaccessible upon formation of the fiber itself, independent of what particular three dimensional conformation the fiber has adopted. To distinguish between these two possibilities, isolated H1/H5 containing mononucleosomes were digested (App. 9 and 10). The results are shown in Figures 3.8 and 3.9. Three points deserve attention. (1) H5 was more stable than H1 under all conditions (2) Both linker histones were much more stable in Tris than in 10 mM NaCl or 0.35 mM $MgCl_2$. (3) In no case were digestion products seen that corresponded to the C-terminal halves of H1 and H5. The lack of C-terminal halves upon digestion of monosomes was similar to the pattern obtained upon digestion of long chromatin (see Fig. 3.5). This implied that the enzyme degraded the histone by attacking at sites other than the phenylalanine in the globular domain. The lack of attack at this phenylalanine even in the nucleosome unequivocally shows that the globular domain of H5 is so situated in the nucleosome that the phenylalanine is buried inside the structure and is thus inaccessible to proteolytic attack by the immobilized enzyme. The sites attacked were possibly multiple, as no discrete bands for digestion products were seen anywhere in the gel. Since the phenylalanines in H1 and in H5 are in different primary sequence location within the globular regions of H1 and H5 (see Fig. 1.7), the phenylalanine in H5 may be closer to nucleosomal DNA which prevents chymotrypsin cleavage of this amino acid.

Sedimentation analysis of the trypsin digestion of chromatin

The issue of whether the initial cleavages introduced into the molecules of the linker histones by mild trypsin treatment already cause structural perturbations of the 30 nm fiber has been the matter of considerable controversy. Thus, Böhm and Crane-Robinson (1984) argue that a single cut in even a small fraction of the molecules might result in breakdown of the supercoil, so that subsequent cuts do not reflect the supercoil geometry. Thus, only initial rates of digestion could possibly provide insight into fiber structure. A contrary opinion is expressed by Marion et al. (1983a) who assert that the cleavage of H1 does not affect higher order chromatin structure and that only the digestion of the terminal regions of H3 leads to unfolding of the fiber. However, in this and other studies by the latter group (Marion et al., 1983b; Hacques et al., 1990), digestion was performed at low ionic strength conditions (1 mM Na phosphate, 0.2 mM EDTA, pH 7.4) in which the chromatin fiber is known to exist in an extended conformation (Thoma and Koller, 1981; Losa et al., 1984; reviewed in Thoma, 1988); the positive birefringence of the undigested preparation served as the only criterion for a vaguely defined "very compact conformation", presumably the 30 nm fiber. In view of this controversy it was important to look for possible changes in the fiber structure taking place during the mild digestion procedure. To that end the sedimentation behavior of the initial chromatin preparation was studied under the three conditions both before digestion, and also later, during the course of digestion, with the results presented in Figure 3.10.

The data are presented as integral distributions of S, calculated according to the van Holde and Weischet analysis (1978), which corrects for the effects of diffusion (see App. 11). All sedimentation analysis is

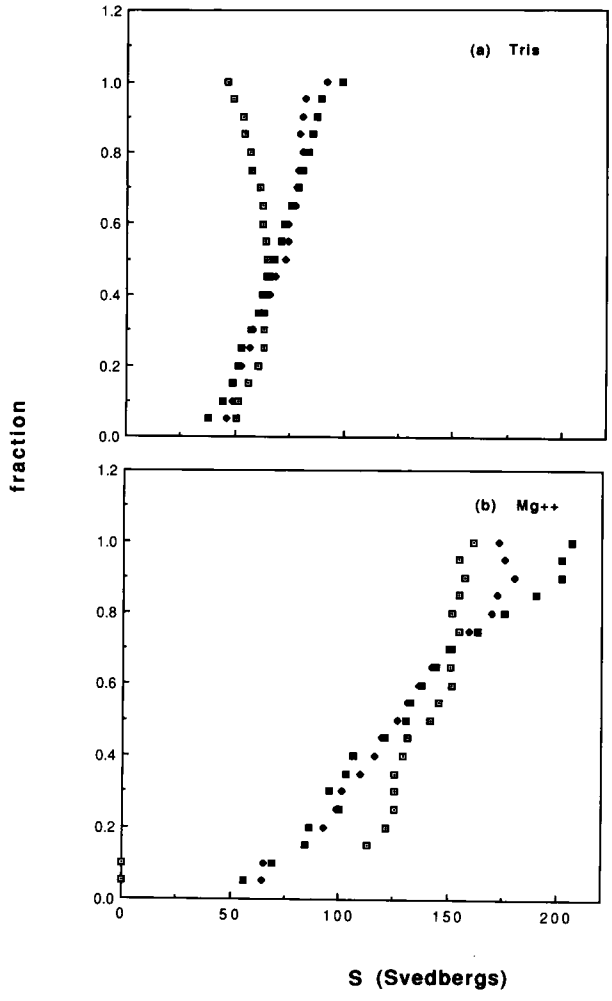


Figure 3.10 Integral distribution of the sedimentation coefficients of the chromatin fiber in (a) 10 mM Tris-HCl (pH 7.5), and (b) 0.35 mM $MgCl_2$ in the course of a trypsin digestion experiment. Digestion was at 25°C and samples for analytical centrifugation were withdrawn at 0 (□), 1.5 (◆), and 4 (■) h. The kinetics of digestion was followed by SDS gel electrophoresis as illustrated in Figs. 3.1 and 3.2.

complicated by diffusion of the boundary. van Holde and Weischet analysis allows extrapolations of the data that removes this boundary spreading. The apparent sedimentation coefficients are determined at integral points across the sedimentation boundary and extrapolated to infinite time according to a theoretical analysis. Since heterogeneity spreading goes as the first power of time, and diffusion as the 1/2 power, the extrapolation yields true S values corrected for the diffusion. The integral distribution of these sedimentation coefficients reflects the hydrodynamic behavior of all the molecules in solution (i. e. all points along the boundary). Hence, a narrow integral distribution of S shows a relatively homogeneous population of macromolecules while a wide integral distribution of S indicates heterogeneity.

As Figure 3.10 shows, the distribution at zero digestion time (undigested material) was relatively sharp, although the apparent sharpness is, in part at least, a consequence of concentration dependence of S. This concentration-dependence artifact was most pronounced with the highly extended fibers run at low salt. The mean S value for undigested chromatin increased from about 58 S in Tris to about 85 S in 10 mM NaCl (not shown) and to about 140 S in 0.35 mM $MgCl_2$, reflecting the increasing condensation of the fiber. Under all three conditions, even limited digestion produced a marked broadening of the sedimentation coefficient distribution. This was evident after only 1.5 h of digestion, when only about 10-20% of the linker histones had been cleaved and the core histones were still intact.

The changes in S-distribution indicated a more complex process than simple unfolding of the fiber, for a portion of the material increased in S. This might reflect either a small amount of aggregation, or a collapse of

previously extended fiber regions into a more globular, condensed conformation. The latter interpretation is more likely, since the increase in S was most pronounced when the molecules had been initially folded into very asymmetric 30 nm fibers.

Chapter 4

Results from Immobilized and Soluble Micrococcal Nuclease Digestion of Chromatin and Glutaraldehyde-fixed Chromatin

Experimental approach

The experiments reported in this study were designed to address the question of whether and to what extent the accessibility of the linker DNA itself in chromatin to endonucleolytic attack depends upon the conformation of the chromatin fiber. They complement, therefore, the previously described studies of accessibility of the linker histones. Both immobilized and soluble micrococcal nuclease (MNase) were used as probes for the accessibility of the linker DNA to digestion. 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 in any way, 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). On the other hand, it was essential to also use soluble enzyme for control experiments because if linker DNA is accessible from the fiber ends or interior, soluble enzyme might be able to enter and cleave it even if immobilized enzyme cannot.

Usually, soluble chromatin (see App 3 and Chapter 2) 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^{++} (results not shown). This background of soluble MNase activity would compete with the mild digestions used in this study, especially in the cases when immobilized enzyme is used. To avoid this complication, drastically reduced levels of MNase were routinely used during chromatin preparation (1/500 of the usual quantity; see App. 13). 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 hr at 25°C in the absence of added enzyme. This result confirmed year-long observations in this laboratory concerning the stability of chicken erythrocyte chromatin.

The experiments were performed on high molecular mass soluble chromatin fragments (referred hereafter to as "long" chromatin), with different preparations containing on average between 8 to 10 kbp of DNA, or 40 to 50 nucleosomes (App. 13). Care was taken that no material below 5 kbp was present: lower molecular mass fragments were eliminated through gel filtration on an S-1000 column (App. 13). 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 the 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 5-10 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 and Thomas, 1981), and this redistribution might affect the behavior of the linker DNA to which they are bound, I 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^{++}, as expected from previous studies (e.g., Cuatrecasas et al., 1967), inhibited the enzymatic activity almost completely. On the other hand, addition of even moderate levels of Ca^{++} 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 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, I avoided addition of any divalent ions, relying instead on the low enzymatic activity present in MNase preparations that have not had Ca^{++} actively removed by EDTA dialysis (see

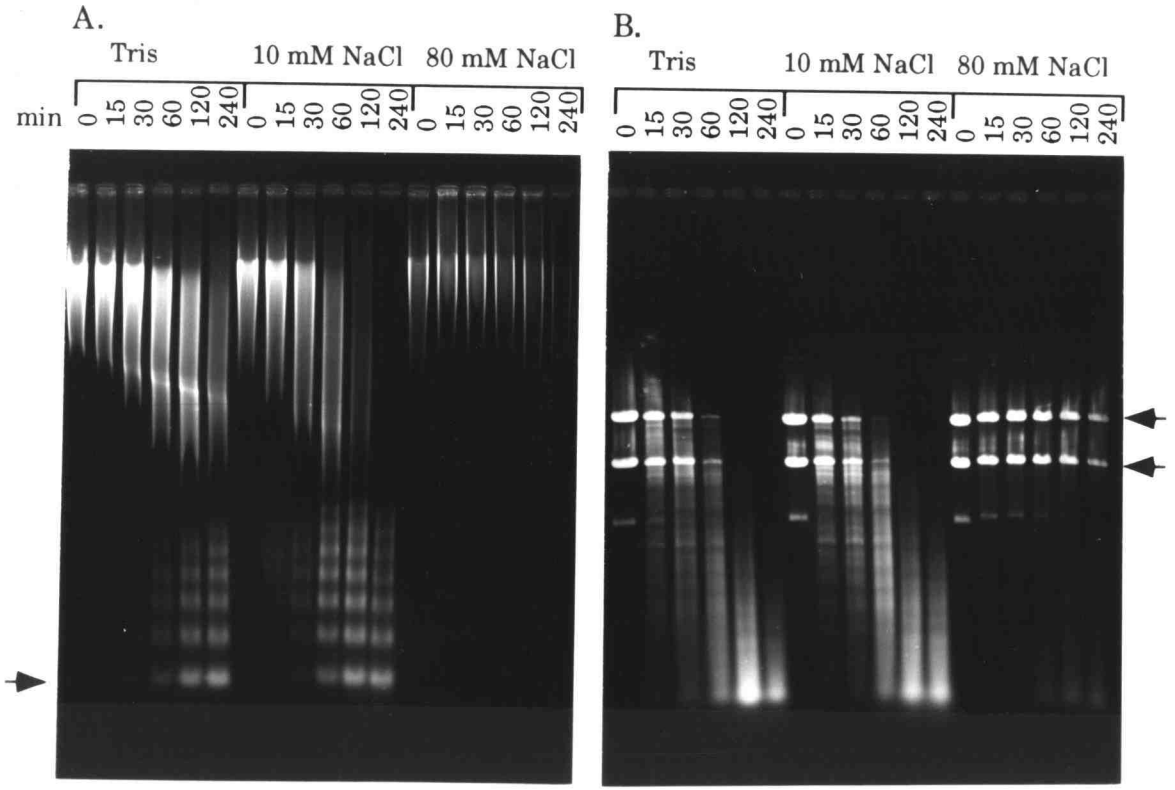


Figure 4.1 Agarose gel electrophoretic analysis of the digestion of long chromatin (A) and pure DNA (B) with immobilized MNase. The digestion conditions are denoted above the respective lanes. The consecutive lanes within each group are 0, 15, 30, 60, 120 and 240 min of digestion (left to right). Equal amounts of material were digested under the three 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 two were used for the quantitations in Figure 4.2.

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.

The linker DNA is 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 the immobilized enzyme in Tris, in 10 mM NaCl, or in 80 mM NaCl (App. 15 and 16). 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 4.1A and B. Inspection of the gel patterns indicates that: (1) An extremely mild digestion has been achieved: only about one half of the initial high molecular mass material was digested to oligonucleosomes within 4 hr. Although the continuity of the DNA is not considered essential for the formation/maintenance of the higher order structure (Finch and Klug, 1976; Thoma et al., 1979), I 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^{++} ions (van Holde, 1988; Tsanev et al., 1992). Since the long chromatin preparation had been treated with EDTA during the isolation procedure and extensively dialyzed versus Tris-HCl before the start of the

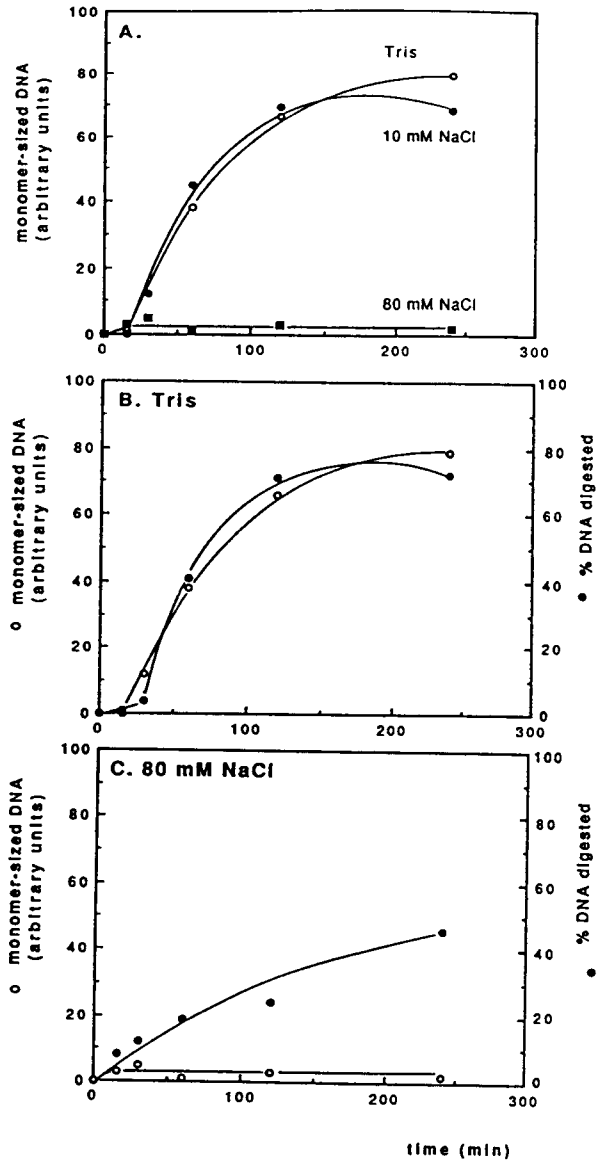


Figure 4.2 Quantitation of the digestion patterns shown in Figure 4.1. The gels were scanned and quantified as described in App. 19. (A) Accumulation of monomer-sized DNA band during the digestion of chromatin with immobilized MNase. Digestion conditions: (o) Tris; (●) 10 mM NaCl; (■) 80 mM NaCl. (B) 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.

experiment, the Ca^{++} 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 4.2. 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 (Figure 4.2A). 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 times. The observations are complicated by the fact that the activity of the enzyme is 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. As can be seen from Figure 4.2B, 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^{++} concentrations. In 80 mM NaCl, free DNA is still digested, albeit more slowly than in low salt. For example, in 120 min about 65% of the DNA is digested in Tris, and only about 25% in 80 mM NaCl. 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 (Figure 4.2C).

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 undesirable dependence of the activity of the enzyme on salt concentration. To completely avoid this potential source of complication, the experiments were repeated with the 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 (App. 14). They were then dialyzed against Tris and all samples digested under exactly the same conditions, i.e., in Tris buffer (App. 18). Pure DNA samples were digested in parallel. The results are presented in Figure 4.3. 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 shown to be 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

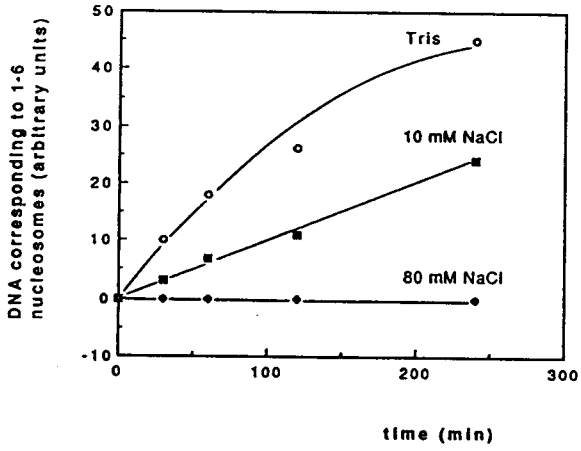


Figure 4.3 Quantitation of the digestion patterns of glutaraldehyde-fixed long chromatin fibers with immobilized MNase. Three chromatin preparations were prepared: one in 10 mM Tris-HCl, pH 7.5, a second one in the same buffer containing 10 mM NaCl, and a third one in the buffer containing 80 mM NaCl. 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 App. 19. The curves represent the accumulation of digestion products in the region corresponding to 1-6 nucleosomes.

might be expected if the zig-zag linker exhibited significant "breathing" in 10 mM salt, which would be prevented by glutaraldehyde fixation.

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 the linker DNA is not accessible to endonucleolytic cleavage by immobilized MNase. Keeping in mind that the immobilized enzyme can only attack externally located linkers, one obvious interpretation of these results would be that the linker DNA is completely internalized into the core 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 inaccessible to the enzyme because the enormous compaction of the fiber causes steric hindrance to the approach of the enzyme to the linker, wherever the linker may be located. It seemed that an obvious way to distinguish between these two possibilities was to use soluble MNase. 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 diffusing into the core of the fiber, particularly from fiber ends. Thus, if the linker DNA is protected simply by being internalized in the core of the higher order structure, the soluble enzyme should be able to cleave it even though the immobilized enzyme cannot. Somewhat unexpectedly, however, this turned out not to be the case.

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 experiments with the soluble enzyme; App. 17). The

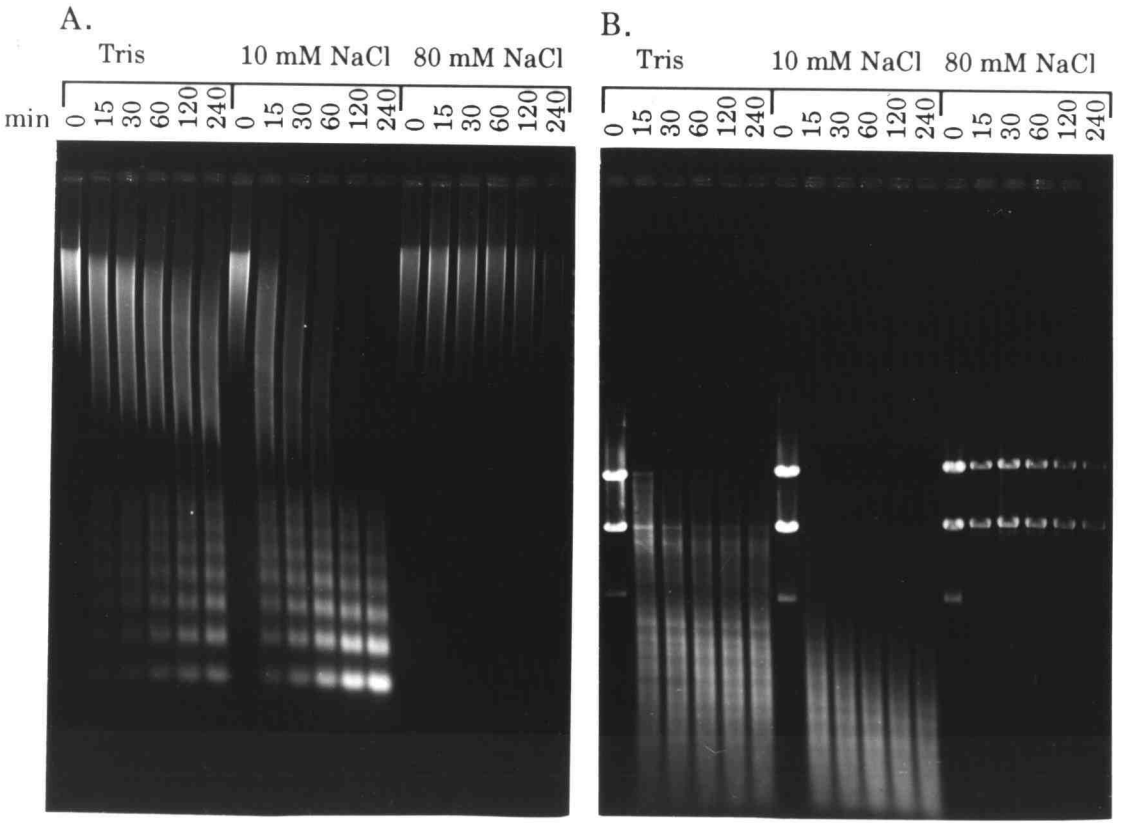


Figure 4.4 Agarose gel electrophoretic analysis of the digestion of long chromatin (A) and pure DNA (B) with soluble MNase. Only 0.017-0.034 units of MNase per mg of DNA were used so as to secure rates of digestion comparable to those obtained with the immobilized enzyme. For further details see legend to Figure 4.1.

electrophoretic patterns obtained upon digestion of chromatin are presented in Figure 4.4A and the parallel control digestions of DNA in Figure 4.4B. 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. Once again, the enzyme activity was somewhat inhibited in 80 mM NaCl (compare the digestion of pure DNA under the three ionic conditions, Figure 4.4B), 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 (App. 14 and 18). This was again done to avoid complications in the interpretation, due to the sensitivity of the enzyme activity to the ionic environment. The results (Figure 4.5) 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 the experimental conditions (data not shown).

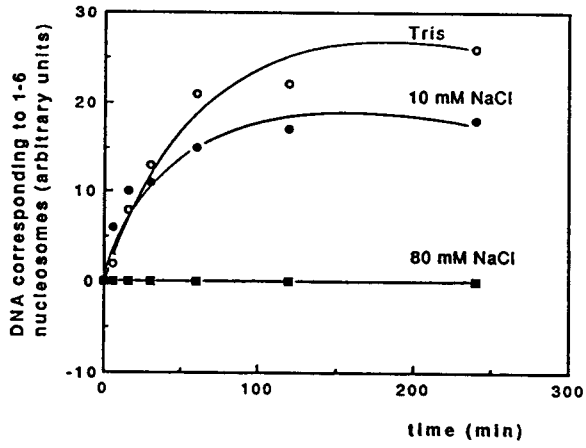


Figure 4.5 Quantitation of the digestion patterns of glutaraldehyde-fixed long chromatin fibers with soluble MNase. For further details see legends to Figures 4.3 and 4.4.

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 the protection against digestion observed with the higher order structure fiber did not reflect internalization of the linker, but resulted from local steric hindrance by chromatin components. If the linker was protected only because it was internal and completely surrounded by nucleosomes, then little protection should become evident until the fiber was almost completely folded. To test for such a possibility, a series of salt concentrations ranging from 0 to 80 mM NaCl was used, 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 4.6A, 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 4.6B is based on the data for the 120 min 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

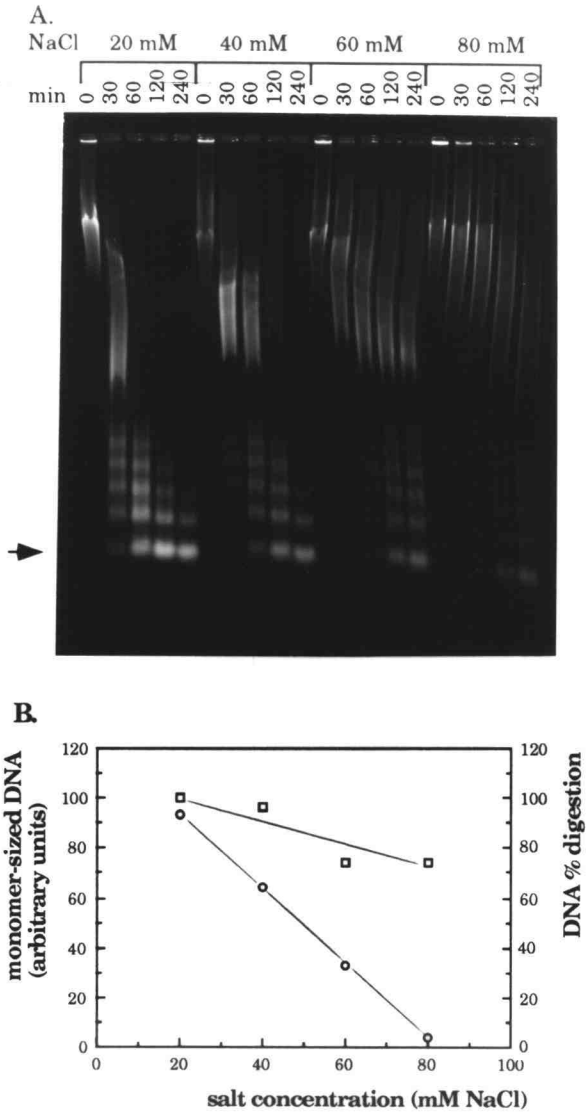


Figure 4.6 Micrococcal nuclease digestion of progressively compacted fibers. (A) Comparative agarose gel electrophoretic patterns for chromatin fibers with intermediate degree of compaction. Chromatin preparations were made 20, 40, 60 or 80 mM with respect to NaCl and digested with immobilized MNase as usually. 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).

progressive protection of linker DNA against digestion is a truly structure-related phenomenon, which cannot be accounted for by salt inhibition of MNase.

Chapter 5

Discussion

The results from these studies lead to several important insights concerning the location of the linker histones and the linker DNA in different structural forms of the chromatin fiber. In analyzing these results it is important to keep in mind that, according to the manufacturer, enzymes attached covalently to Immobilon membranes are positioned about 1.5-2.0 nm from the membrane surface. Therefore, only limited penetration into the 30 nm fiber should be possible (see Fig. 1.6).

Histone H5 is more protected than histone H1 in the chromatin fiber

Under all conditions tested, H5 contained in chromatin was digested with immobilized trypsin more slowly than was histone H1. No such difference was detected upon digestion of the free linker histones. As under the mild digestion conditions trypsin attacked only the N- and C-tails of the molecules, this result implies that histone H5 and histone H1 differ in the way their tails are located in the fiber (see Fig. 1.7). The case for H5 being differently located in the fiber is made especially strong by the results of chymotrypsin digestion. The almost complete resistance of fiber-contained H5 to digestion under all ionic conditions, together with its "normal" digestion in the monosome particle, implies that it is the formation of the fiber itself (the presence of adjacent nucleosomes) that makes H5 completely protected from chymotrypsin attack.

To the best of my knowledge, these results constitute the first observation that the two major linker histone types in chicken erythrocyte

nuclei are located in a distinguishable manner. This difference in location might be the molecular basis for the different role of these histones in affecting transcription and replication (see Introduction). It should be noted that the stronger protection of H5 in the fiber in comparison with H1 is reminiscent of a similar difference observed immunochemically between H1 and $H1^0$, a histone H1 subfraction typically present only in nondividing differentiated cell types (Panyim and Chalkley, 1969). Banchev et al. (1990) reported that $H1^0$ was much less exposed to antibody binding than was H1 in mouse liver chromatin. The difference in location observed between H1, on one hand, and H5 and $H1^0$, on the other, might be relevant to the different functions of these different H1 subtypes in chromatin of cells differing in their state of proliferation and/or differentiation.

A potentially interesting incidental observation is that in the nucleosome itself, H5 in Tris behaves differently than in Mg^{++} or Na^+ (Chapter 3, Fig. 3.9(b)). There is no explanation at the present; clarification of this point requires additional experimentation.

The N- and C-terminal tails of histone H1 do not change their location significantly upon compaction of the chromatin fiber

The data presented in Chapter 3, Figures 3.1 and 3.2(d), show that under mild digestion conditions trypsin attacks the tails of histone H1 in a manner independent of the chromatin fiber structure. This observation implies that the location of these portions of the molecule does not change upon compaction of the fiber which, in turn, suggests that at least the majority of H1 molecules do not become internalized in the 30 nm fiber.

The issue of H1 location in chromatin fibers of different conformation has so far been studied by immunochemical approaches, with conflicting

results (reviewed in Zlatanova, 1990). Some authors (Takahashi and Tashiro, 1979; Russanova et al., 1987) reported that H1 in the folded fiber was not accessible to antibody binding. Using antibody populations against the intact molecule and against its globular domain, Russanova et al. (1987) found that the globular domain was always "hidden" in the fiber, while the tails were accessible at the extended state but protected in the condensed fiber. However, other immunological studies (Banchev et al., 1991) did not detect any change in the accessibility of H1 in different fiber conformations. The antibody population used responded to antigenic determinants located both in the globular domain and in the C-tail. My results are in accordance with those of Banchev et al. (1990), despite the entirely different approaches used in the two studies.

The tails of histone H5 become partially inaccessible (internalized) in the 30 nm fiber

One of the major findings of this work is that upon formation of the 30 nm fiber, the accessibility of the terminal portions of the H5 molecules to immobilized trypsin decreases. Significantly, this behavior of histone H5 differs from that of H1, the accessibility of whose N- and C-tails does not change upon fiber condensation. The protection from digestion is not complete which implies one of two things: (1) either all H5 molecules become somewhat less accessible due to steric hindrance by chromatin components becoming tightly packed upon condensation or (2) that some of the molecules become internalized in the fiber, while others remain on the outside. To discriminate between these two possibilities would require other approaches. To the best of my knowledge, these data are the first in the literature to provide information on the behavior of the H5 tails in

condensation. Most immunochemical studies concerning histone H5 have made use of antibodies directed against epitopes in the immunodominant globular domain (see Zlatanova, 1990).

Phenylalanine in the globular domains of H1 and H5 is buried inside the structure both at the level of the nucleosome and in the fiber

As trypsin digestion could give insights only into the location of the terminal domains of the linker histone, the study was complemented by the use of immobilized chymotrypsin which preferentially attacks adjacent to the single phenylalanine residue of the globular domain. At all conformations of the fiber and in the isolated H1/H5-containing nucleosomes, this amino acid was completely inaccessible to the enzyme and no attack was observed even at 37°C, and at prolonged digestion times although other sites were cleaved. Although H1 in chromatin and in nucleosomes and H5 in nucleosomes were attacked by chymotrypsin, cleavage was not adjacent to the phenylalanine in the globular domain, since no C-terminal halves of the molecules were observed in the gels: the cleavage evidently involved multiple other amino acid residues situated away from the phenylalanine. These results imply that the globular domain both in the nucleosome and in the fiber is situated in such a way that its phenylalanine is inaccessible to the immobilized enzyme. An alternative explanation for the lack of the C-terminal half could be that other sites become equally accessible to the enzyme when the linker histone is bound to the nucleosome or to the fiber. This possibility seems less likely, as it would require drastic changes in the specificity of the enzyme or in the conformation of the protein.

The observation that the accessibility of the phenylalanine in the globular domain was not a function of the fiber conformation differed from the results reported by Losa et al. (1984). The explanation for this discrepancy might be connected to the fact that soluble chymotrypsin was used in that study. The length of the cross-link between the enzyme and the membrane in this study (15-20 Å) would severely restrict its action to molecules in its immediate vicinity. A possible alternative explanation that the difference is due to the fact that rat liver chromatin contains only H1 as opposed to the presence of both H1 and H5 in chicken erythrocytes seems less likely.

On the location of the linker histones in the higher order structure of chromatin fiber

The literature on the location of the linker histones in the higher order structure of chromatin fiber, which has been mainly approached via immunochemical studies, is extremely controversial (see Zlatanova, 1990, for a review; Banchev et al., 1990, for a detailed discussion). 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. The immunochemical reaction with the fiber gradually lessened with increasing ionic strength and was already negligible at 20-30 mM NaCl, a salt concentration where the fiber is still a closed zig-zag. 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 immobilized MNase,

and the free antibody - to the soluble enzyme.) So, in the view of this thesis, 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 and 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 and 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^{o} and mouse liver chromatin (Banchev et al., 1990).

The results demonstrate that chymotrypsin must be used with caution as a globular domain probe for studies of higher order structure, since the preferentially cleaved, globular domain-contained phenylalanine is inaccessible even in the nucleosome. It is clear that alternative probes, including antibodies of high reactivity, possibly immobilized to create large but sensitive probes should be sought to resolve this issue.

Linker histone digestion and ordered chromatin structure

The sedimentation velocity experiments carried out in this work show that the integrity of almost all of the linker histone molecules seems to be required for the proper maintenance of ordered chromatin structure. It is of importance that most of the change observed in the distribution of S occurs early during the digestion (1.5 hr), when only a small fraction ($\leq$5%) of H1 or H5 has been cleaved; the distribution at 4 hr shows little further

change. These results are in much better agreement with the position of Böhm and Crane-Robinson (1984) than with that of Hacques et al. (1990).

There are two possible explanations for the effect of linker histone cleavage on chromatin structure. First, the changes in the fiber structure that was observed upon digestion may be caused by dissociation of the globular domain from the fiber and thus the initial cleavages in the histone molecules might be accompanied by complete loss of individual H1 or H5 molecules from the fiber. Alternatively, if the globular domain remains bound to chromatin after initial cleavages in the linker histone molecules, the changes in the fiber structure might reflect the inability of the globular domain by itself to maintain long range interactions required to form higher order structures, as proposed earlier by Allan et al. (1986).

My experimental approach does not allow determination of whether the globular domain still remains bound to chromatin after digestion of the tails. Such a discrimination would require separation of the unattached digestion products from the long chromatin fiber. Alternatively, the differences in the behavior of the globular domain when the protein is free in solution or attached in chromatin with respect to chymotrypsin, could be used to approach this question. This would require consecutive trypsin and chymotrypsin treatments under carefully controlled conditions. After the initial trypsin digestion which releases the tails, the globular domain may or may not be bound to the fiber. The following digestion with chymotrypsin would then ascertain whether there is any free globular domain in solution with an accessible phenylalanine.

The sedimentation velocity measurements performed during the course of trypsin digestion suggest that linker histones are important in maintenance not only of the higher order structure of the chromatin fiber,

as accepted hitherto, but of ordered fiber structure in general, independent of its particular condensation state. Indeed, the broadening of the distribution of the sedimentation coefficient with time of digestion, which reflects changes in the fiber structure, is observed not only in 0.35 mM $MgCl_2$, but also in Tris and in 10 mM NaCl. This observation, together with the known fact that the linker histones are involved in the structuring of the nucleosomal particle itself (Allan et al., 1980) points to the importance of this histone class at all levels of chromatin organization.

On the location of the linker DNA in the higher order chromatin structure

In this work I 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. These experiments may be considered to complement those of the linker histones described above. Extremely mild digestion conditions were used in the absence of added divalent ions. They have permitted, for the first time, 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 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 structure or simple steric hindrance to the enzyme by components of the condensed structure. 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 these 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 and McGhee, 1986; Thoma, 1988; van Holde, 1988; Widom, 1989; Freeman and Garrard, 1992; Tsanev et al., 1992) which postulate that the linker DNA is entirely in the interior of the fiber. They are also incompatible with one specific feature of the solenoidal model of Finch and Klug (1976) and Thoma et al. (1979) - the requirement that the linker DNA forms reversed loops inside the fiber (Butler, 1984). These results, taken together with data on the existence of a ten base pair cutting pattern of linker DNA (e.g., Lohr and van Holde, 1979; Karpov et al., 1982; Bavykin et al., 1990), would support solenoidal models in which the linker is immobilized between nucleosomes and on their surfaces. Such supercoiled linker models (McGhee et al., 1983; Bavykin et al., 1990) would explain both the progressive increase in protection as the fiber is compacted and the fact that identical results were obtained with immobilized and free enzyme. The 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 in this thesis. The view of 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., Williams et al., 1986).

The progressive protection that was observed 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 folding could lead to a structure in which the curved linker DNA lay in the space between two adjacent nucleosome cores.

The location of the linker DNA between nucleosomes and on their surfaces would be consistent with the behavior of the linker histones, which show 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 (Chapter 3, Figs. 3.1 and 3.2). 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 cross-linking 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 and 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.

On the mechanism of chromatin folding during the formation of the higher order structure

From the data of these studies on the changes of accessibility of the linker DNA and the linker histones upon the salt-dependent condensation of the nucleosome filament, a model for the way in which the fiber folds can be proposed. It can be envisaged that the conformation of the extended fiber resembles that of an extended telephone cord, which, upon increasing the salt concentration progressively compacts, without any other significant changes of the parameters of the fiber except for a reduction in the pitch. Such a compaction mechanism is equally possible with a one-start solenoid (of the kind proposed by Finch and Klug, 1976, or by McGhee et al., 1983) and with a two-start helix (of the kind proposed by Woodcock et al., 1984). It is difficult to say how and with the participation of what factors the initial transition from a zig-zag arrangements of the nucleosomes at low ionic strength to a helical arrangement of the nucleosomal filament would be triggered and carried out. More sophisticated experiments have to be designed in order to solve this fascinating problem.

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APPENDIX

Appendix

1. Cell culture

BB88 mouse leukemia cells (BB88) were obtained from the American Type Cell Collection (Rockville, MD). They were grown in Ham's F12:Dulbecco's modified Eagle's (1:1) medium with 10% calf serum, 120 μg/ml penicillin G, 200 μg/ml streptomycin, 25 μg/ml ampicillin, and buffered with 15 mM Hepes (pH 7.2) in spinner culture at 37°C.

2. Trypsin and chymotrypsin immobilization on membranes

Diphenyl carbamyl chloride treated trypsin (Sigma type XI) and α-chymotrypsin (Sigma type II) were immobilized on Immobilon (Millipore) membranes using one of the protocols of the membrane manufacturer: immobilization was carried out at 4°C; a solution containing 1 mg of enzyme/ml of coupling buffer (0.5 M potassium phosphate, pH 7.4) was incubated with 24 mm diameter disks of the membrane for 1 hr. Immobilization is done by covalent bonding of the primary amines of the enzyme to the membrane. Membranes were incubated in capping solution (0.1% gelatin in 1.0 M sodium bicarbonate, pH 9.5) for 2 hr and finally incubated for 30 min in wash solution (0.01 M sodium phosphate, pH 7.4, containing 0.1% Tween-20). In all incubations solutions were agitated to keep the membranes away from walls of the vessels. Membranes were then dried on Whatman filter paper and stored in sealed Petri dishes at 4°C.

3. Preparation of chromatin for digestion with trypsin or chymotrypsin

Chromatin was prepared as described by Ausio et al., 1989. All buffers were made 0.1 mM in PMSF (phenylmethylsulfonylfluoride)

immediately before use. Fresh chicken blood was centrifuged at low speed at 4°C. The cell pellet was extensively washed in membrane lysis buffer (0.1 M KCl, 50 mM Tris-HCl, pH 7.5, 1 mM $MgCl_2$, 0.5 % Triton X-100) until the pellet was white. The pellet was resuspended in micrococcal nuclease digestion buffer (0.1 M KCl, 50 mM Tris-HCl, pH 7.5, 1 mM $CaCl_2$) and mildly digested with micrococcal nuclease (Worthington). The nuclear suspension was 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. Soluble chromatin was analyzed for histone content in 15% polyacrylamide/SDS slab gels in the discontinuous buffer system described by Laemmli, 1970. The length of the DNA in the chromatin was checked by electrophoresis on 1% agarose in TAE (40 mM Tris-HCl, pH 8.0, 40 mM acetic acid, 1 mM EDTA; Maniatis et al., 1982). Finally, the sedimentation behavior of the chromatin was determined by analytical ultracentrifugation (see below).

4. Preparation of histones H1 and H5

The chromatin fraction recovered from hypotonic lysis of the nuclei against 25 mM EDTA (pH 7.5) (see above) was diluted to an A_{260} ~20, using the same buffer. The solution was then brought to 0.35 M NaCl by dropwise addition, under stirring, of a 4 M NaCl stock. Stripping of the linker H1 histones, as well as most of the nonhistone proteins, was achieved by addition of 12 mg of CM-Sephadex C-25/ml of suspension (Libertini and Small, 1980). After gently shaking for 2 hr at 4°C (avoid using magnetic stirrers), the CM-Sephadex C-25 chromatin suspension was spun down at 10,000 x g in a Sorvall SS-34 rotor for 20 min at 4°C. The clear supernatent was used to prepare either nucleosomes (Ausio et al., 1989) or histone

octamers (Tatchell and van Holde, 1977). The resin cake from the pellet was washed twice with and was finally resuspended in and equal volume of the initial column buffer (0.35 M NaCl, 10 mM Tris-HCl, pH 8.8, containing 0.1 mM PMSF) and then carefully loaded on top of a CM-Sephadex C-25 column which had been previously equilibrated with the same buffer. This fractionation procedure is based on that originally described by Johns (1974) except that the borate-NaOH (pH 9.0) buffer has been replaced by Tris-HCl, pH 8.8. It is very important that this fractionation procedure be performed immediately after the stripping step; a delay may result in proteolytic degradation of the H1 fractions. The column was subsequently eluted with a linear 0.35 to 1.6 M NaCl gradient in 10 mM Tris-HCl (pH 8.8) at a flow rate of 6 ml/cm^2/hr. For more details see the figure legends in Chapter 2, Figs. 1 and 2.

5. Preparation of H5 globular domain (GH5)

The method for preparing GH5 was derived from the methods of Banchev et al., 1991, and Thomas et al., 1992. 10 mg of H5 (about 0.5 mg/ml) in 0.5 M NaCl, 10 mM Tris-HCl (pH 7.5) were digested with trypsin (Sigma) at a w/w ratio of 1:250 enzyme/substrate for 20 min at 25^{o}C. The mixture was diluted to 0.3 M NaCl, PMSF was added to 0.5 mM, and the solution was loaded onto a 10x0.7 cm CM-Sephadex C-25 (Pharmacia) column previously equilibrated with 0.3 M NaCl, 10 mM Tris-HCl, pH 7.5, 0.5 mM PMSF. The column was washed until the absorbance at 230 nm was below 0.04. GH5 was eluted with a 100 ml linear gradient of 0.3 - 1.0 M NaCl in 10 mM Tris-HCl, pH 7.5, 0.5 mM PMSF in 50 drop fractions. Aliquots were checked on SDS/polyacrylamide gels (19) before pooling fractions which were extensively dialyzed versus 10 mM Tris-HCl (pH 7.5) at 4^{o}C before use.

6. Plasmid DNA

Plasmid DNA was obtained by the alkaline lysis procedure (Maniatis et al.,1982) and further purified by either cesium chloride gradient (Maniatis et al.,1982) or according to a modification of the protocol described in (Maniatis et al.,1982). In the second procedure the DNA was phenol extracted, precipitated with ethanol and treated with DNase-free RNase. The plasmid was then separated from the RNA degradation products on an A15 M (BioRad) gel filtration column (3x45 cm) in 0.5 M NaCl, 10 mM Tris-HCl, pH 7.5, 0.25 mM EDTA. Fractions containing the plasmid were ethanol precipitated, and the pelleted DNA was dissolved in 10 mM Tris-HCl, pH 7.5, 1 mM EDTA. The DNA was treated with calf thymus topoisomerase I (BRL-GIBCO) under the conditions recommended by the manufacturer, phenol-chloroform extracted and precipitated by ethanol.

7. Formation and analysis of histone-DNA complexes

Relaxed DNA was dissolved in binding buffer (10 mM Tris-HCl, pH 7.5, 1 mM EDTA, 1 mM DTT, 5% glycerol (22), modified by addition of 0.5 mg/ml BSA (Sigma) and containing 0.6 M NaCl. The appropriate amount of histone in the same binding buffer without NaCl was mixed slowly with the high salt DNA solution. Vortexing assured that the reaction mixture was homogeneous with respect to NaCl concentration at all times and the molarity of salt lowered smoothly to a desired value (25-30 or 100-125 mM). After mixing the samples were left at room temperature for 30 min and then loaded onto 1% agarose gels; electrophoresis was performed in 0.04 M Tris-acetate, pH 7.7, 1 mM EDTA, for 1300 Vhr. The DNA in the gel was visualized by ethidium bromide staining; photographs were taken on

Polaroid 55 Professional Instant Sheet Film, and the negatives were scanned on a Zeineh SL-504-XL densitometer. Densitograms were enlarged by 100% on a photocopier and the peaks were cut out and weighed. The amount of DNA in a band was taken to be proportional to the weight of the corresponding peak.

8. Binding of GH5 to pBR322

The binding of the isolated GH5 to pBR322 was assayed by gel filtration of the mixture through Chroma Spin-100 columns (Clontech Labs, Inc., Palo Alto, California) that were previously equilibrated with binding buffer (see above), containing 25 mM NaCl.

9. Preparation of nucleosomes

Nucleosomes containing the full complement of histones were prepared with minor modifications of the method of Ausio et al., 1989. Soluble chromatin at 7.5 mg/ml was made 1.0 mM with respect to $CaCl_2$ and digested with 2 units/ml of micrococcal nuclease (Worthington) for 5 hr at 25°C. Digestion was checked by electrophoresis on 1.5% agarose in TAE (Maniatis et al., 1982). Enzyme digestion was stopped by adding EDTA to 10 mM, cooling to 4°C, and dialyzing versus 10 mM Tris-HCl, pH 7.5, 10 mM EDTA, 0.1 mM PMSF. The digested sample was loaded onto a Sephacryl (Pharmacia) S-300 column (200 cm x 5 cm) which had been equilibrated with the same buffer. Fractions were checked for free DNA and nucleosome monomer content on 4% native polyacrylamide gels as described in Ausio et al., 1989. DNA size was monitored by incubating the sample in an equal volume of dissociation sample buffer (0.4% SDS, 0.04% bromophenol blue, 20% glycerol, 40 mM Tris-HCl, pH 8.0, 1 mM EDTA; Juan Ausio, personal

communication) for 30 min at 37°C before loading onto 4% native polyacrylamide gels. Histone content was checked on SDS/polyacrylamide gels. Column fractions containing nucleosome monomers with a DNA distribution of 200 ± 10 bp and both core and linker histones were extensively dialyzed versus 10 mM Tris-HCl, pH 7.5.

10. Trypsin and chymotrypsin digestion of chromatin

Chromatin aliquots were removed from the -80°C freezer and melted. Precipitated material was removed by centrifugation at 10,000 x g in a microcentrifuge (Eppendorf) for 5 min. Digestion was performed at a relatively low concentration (0.1 mg of DNA/ml) to prevent aggregation artifacts. Digestions of nucleosomes were also at 0.1 mg/ml. Digestions of linker histones free in solution were at 0.1 mg of protein/ml. Enzyme-immobilized membranes were immersed in the reaction solution 15 min prior to the addition of substrate. Samples were digested in 10 mM Tris-HCl (pH 7.5) or in the same buffer containing either 10 mM NaCl or 0.35 mM $MgCl_2$. 0.5 ml aliquots were taken at the indicated times, frozen, lyophilized with a SpeedVac (Savant), resuspended in 2x SDS sample buffer and analyzed on SDS/polyacrylamide gels.

11. Sedimentation velocity

Sedimentation velocity measurements were done as in Ausio et al., 1989, on a model E analytical ultracentrifuge (Beckman) equipped with a photoelectric scanner and a multiplexer. Soluble chromatin had an A_{265} of 0.7-0.9 and was centrifuged at 12,000 rpm at 20 (±1) °C. The temperature was kept constant within each run to within 0.1°C using the RTIC temperature control unit. Data were collected on a computer interfaced

with digitized scanner output (Demeler, 1992) and analyzed by the method of van Holde and Weischet, 1978, to determine the integral distribution of the sedimentation coefficients.

12. Quantitative analysis of histone gels

SDS gels or their photographic negatives were scanned on a soft laser scanning densitometer (Zeineh). Densitograms were enlarged on a photocopier and the peaks were cut out and weighed. To compensate for different loadings, the amount of linker histones was normalized to the amount of histone H4 in each lane. Chromatin-contained histone H4 was chosen as a reference protein as it is relatively stable to enzymatic cleavage and under the mild digestion conditions used in this study (see Chapter 3, Fig. 1) its amount remained constant with time of digestion. Results were presented as percentage of the amount (relative to H4) of the respective linker histone at the zero time point of digestion. In the case of linker histones free in solution, the amount of H1 and H5 at the zero time point was taken as 100%. To increase the accuracy of determination of the (relative) amounts of linker histones at the zero time point, several zero time points were loaded on each gel and the determinations averaged. The peaks were approximated as Gaussian curves as suggested by Staynov and Crane-Robinson, 1988. An estimated accuracy of estimation of the area under the peaks was better than ± 20%. Because measurements of this kind are subject to significant experimental error, each experiment was repeated a number of times.

13. Preparation of chromatin for digestion with MNase

Chromatin was prepared essentially as described in 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 units/ml MNase (Worthington) for 2 hrs 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% agarose gels in TAE buffer (40 mM Tris-HCl, pH 8.0, 40 mM acetic acid, 1 mM EDTA; Maniatis et al., 1982). Aliquots of chromatin were mixed with 2xSDS (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) (25x750 mm; buffer 10 mM Tris-HCl (pH 7.5) flow rate 100 ml/hr, fraction volume 10 ml).

14. 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% glutaraldehyde and constant inversion of the solution overnight at 4°C. The glutaraldehyde-fixed chromatin preparations in these three different salt concentrations were then dialyzed extensively versus 10 mM Tris-HCl (pH 7.5) and stored on ice.

15. Immobilization of MNase on membranes

MNase (Worthington) was immobilized on Immobilon membranes (Millipore) using one of the protocols of the membrane manufacturer: 20 ml 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% gelatin in 1.0 M sodium bicarbonate, pH 9.5) for 1 hr and then washed in 0.1% 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 immediately used.

16. 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, aliquots (0.2 ml) were removed, lyophilized, resuspended in 2xSDS sample buffer, incubated at 37°C for 15 min, 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 (EcoRI) pBR322 or its derivative pML2αG (Nishioka and Leder, 1979) (a kind gift of Dr. F. Rougeon, Institute Pasteur, Paris) were extensively dialyzed versus Tris-HCl before being brought to the desired salt concentration.

17. Digestion of chromatin with soluble MNase

Digestion with soluble MNase was with 0.25-0.5 units/ml. All other conditions were exactly the same as those used in the experiments with the immobilized enzyme.

18. 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. Aliquots (0.2 ml) were taken at the indicated times, lyophilized, resuspended in 1% SDS, 10 mM EDTA, 0.5 mg/ml proteinase K, 10 mM Tris-HCl (pH 7.5) incubated for 2 hr at 65°C, mixed with an equal volume of 2xSDS sample buffer, and analyzed in 1% agarose gels.

19. 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 two bands at 0 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.

20. Interaction of the globular domain of H5 with supercoiled pBR322

In experiments with model systems performed in our lab, it has already been shown that linker histones preferentially bound cross-overs in superhelical DNA (Krylov et al., 1993). The interpretation that the linker histones recognize and bind preferentially to DNA cross-overs is in accordance with recent X-ray diffraction studies suggesting two specific DNA-binding sites in the globular regions of H5 (Ramakrishnan et al., 1993). As the globular domains of H5 and H1 are evolutionarily conserved, it is highly probable that similar DNA binding sites exist in H1. These observations raised the question of whether the globular part of a linker histone by itself could show the preference to cross-overs observed with the intact protein molecules. My contribution to Krylov et al. (1993) was to study this question.

The globular domain of H5 was prepared (see App. 5) by a combination of the methods of Banchev et al. (1990) and Thomas et al. (1992). H5 in 0.5 M NaCl, 10 mM Tris-HCl, pH 7.5, was mildly digested with trypsin to produce the GH5 fragment. O.5 M NaCl was used to fold H5 into its tripartite structure with a folded globular domain and random-coiled N- and C- terminal tails. The mixture was diluted to 0.3 M NaCl and loaded onto a CM-Sephadex C-25 ion exchange column and then eluted with a continuous 0.3-1.0 M NaCl gradient in the same buffer.

Control experiments proved that under the conditions used in the experiment, the globular domain by itself bound to DNA (App. 8). A mixture of purified GH5 and pBR322 (molar ratio of 200) was spun through Chroma Spin-100 gel filtration columns, preequilibrated with the low salt binding buffer. Any free GH5 would be retained in the column, while only bound GH5 would appear in the flow-through fraction. Indeed, as shown in Figure App. 20, the flow-through contained both DNA and GH5 (about 40% of the DNA and 60% of the protein from the initial input were not recovered due to losses to the walls of the vessels and to some retention by the resin).

To study the interaction of the GH5 with DNA molecules of different superhelical densities at the resolution of single topoisomers, the initial superhelical pBR322 population was relaxed with topoisomerase I at 37°C (App. 6 and 7). DNA topoisomer populations were obtained that contained about 65% of completely relaxed (and also some nicked) DNA circles and DNA circles with 1 superhelical turn and decreasing amounts of circular molecules with 2, 3, 4, and in some cases, 5 superhelical turns (about 20, 11, 3-4 and less than 1% of the total DNA in the sample, respectively).

As can be clearly seen in Figure App. 20B, gradual increase of the input GH5/DNA ratio led to consecutive disappearance of DNA bands, starting from the most supercoiled, accompanied by the formation of aggregated material which did not enter the gel. This demonstrates that the globular domain by itself can bring about preferential loss of the higher superhelical topoisomers, but this occurs only at very high molar ratios of the protein fragment to DNA (compare lanes 7 and 8 , Fig. App. 20B). In the case of intact H5 a molar ratio of 150 caused aggregation of about 70% of the total DNA in the gel (Fig. App. 20B, lane 8); similar levels of aggregation required a molar ratio of 400-500 in the case of GH5.

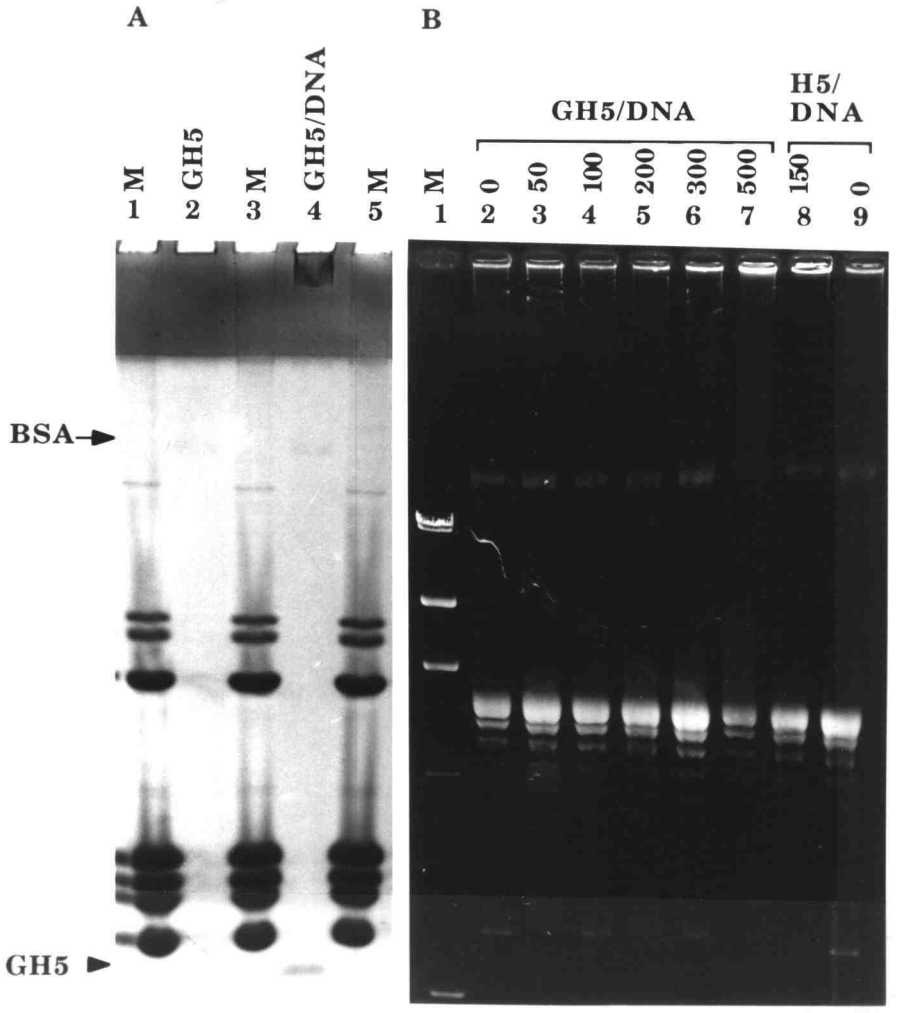

Figure App. 20 Interaction of GH5 with relaxed pBR322. (A) SDS/polyacrylamide gel electrophoretic analysis of the flow-through fractions of Chroma Spin-100 columns. GH5 was allowed to interact with pBR322 in binding buffer, containing 25 mM NaCl at a molar ratio (GH5/DNA) of 200 and spun through the column, equilibrated with the same buffer. Lanes 1, 3, and 5: total chicken erythrocyte histones as markers; lane 2, flow-through fraction of a control sample, containing GH5 and no DNA; lane 4, flow-through fraction of a sample, containing both GH5 and DNA. The arrowheads point to the band of BSA, present in the binding buffer and to the GH5, as marked. (B) Agarose gel electrophoresis of GH5/DNA complexes formed at 25 mM. Lane 1, molecular mass marker; lanes 2 and 9, free DNA; lanes 3-7, GH5/DNA complexes at the molar ratios indicated above the lanes; lane 8 - intact H5/DNA complex, molar ratio of 150.

The fact that preferential aggregation of higher topoisomers can be observed even with the globular portion of H5 must mean that the ability to bind to two DNA duplexes is retained even in this truncated protein. However, the fact that much higher levels of GH5 are required to produce comparable effects suggests that the C-terminal and/or N-terminal tails of linker histones strongly stabilize such interactions.

This view of how the molecules of the linker histones interact with superhelical DNA is reminiscent of that of Singer and Singer (1976). Their experiments with isolated fragments 72-217 and 106-212 (see Fig. 1.7), comprising respectively most of the globular domain and the whole C-terminus and just the C-terminus, indicated that the globular domain was involved in the recognition of superhelicity. However, these experiments did not address the issue of which feature(s) of superhelical DNA was actually recognized. Singer and Singer (1976) postulated two components in the interaction: initial recognition of the superhelicity by the globular domain and subsequent stabilization of the interaction in consequence of the binding of the highly charged C-terminus.