AN ABSTRACT OF THE THESIS OF

Ahmed H. Hassan for the degree of Master of Science in Genetics presented on April 17, 1997.

Title : Linker Histone/DNA Interactions : *In Vitro* Studies

Abstract approved: Redacted for Privacy

Jordanka Zlatanova

By studying the linker histone/DNA complex *in vitro*, our goal was to gain further insight into the interaction of these histones in chromatin structure, which, in turn, helps us in better understanding critical biological processes such as replication, transcription, and recombination. In direct competition experiments, we have confirmed previous results of the preference of the linker histones in binding to superhelical DNA over linear or nicked circular DNA forms. This binding of linker histones to DNA supercoils in the presence of DNA competitors was examined at varying histone/DNA ratios and different ionic strengths. It was demonstrated that: 1) With increasing H1/DNA ratios, the electrophoretic mobility of the H1/supercoiled DNA complex decreases. 2) With increasing ionic strengths, the mobility of this complex increases. The presence of more protein bound to superhelical DNA explains the first observation. The second observation, however, is explained by a reduction in the amount of protein bound to DNA as well as a change in the conformation of the complex at higher salt concentrations.

The conformational change of the superhelical DNA upon linker histone binding was then investigated. The effect of linker histone binding on the accessibility of sites on superhelical plasmids to single-strand-specific nucleases

(P1 or S1) was studied first. The results show protection of preexisting P1- or S1-sensitive sites at low to moderate linker histone/DNA ratios as well as the appearance of new susceptible sites at higher ratios. The protection of single-strand-specific nuclease-sensitive sites can be explained by a change in the superhelical torsion of the plasmid as a result of linker histone binding. Furthermore, the interaction of the C-terminal domain of the linker histones with AT-rich sites on DNA could (by destabilizing B-DNA structure) make the sites susceptible to nuclease cleavage. This explains the appearance of new susceptible sites at higher linker histone levels.

These changes in the pattern of cleavage by nucleases with increasing linker histone/DNA ratios were further studied by investigating the effect of linker histones on superhelical plasmids upon binding, looking for changes in the linking number of the plasmid DNA. Two classical assays (the topoisomerase I-mediated relaxation assay and the ligase-mediated supercoiling assay) were performed. The results clearly indicate that the linker histones unwind superhelical DNA, with the unwinding angle being about 8° per histone H1 molecule bound to DNA. Even though this unwinding angle is small relative to the unwinding effect of other proteins, it is crucial to consider this effect in the studies of chromatin fiber structure.

Linker Histone/DNA Interactions : *In Vitro* Studies

by

Ahmed H. Hassan

A THESIS

submitted to

Oregon State University

in partial fulfillment of

the requirement for the

degree of

Master of Science

Completed April 17, 1997

Commencement June 1997

Master of Science thesis of Ahmed Humaid Hassan presented on April 17, 1997

APPROVED:

Redacted for Privacy

Major Professor, representing Genetics

Redacted for Privacy

Chairman of Program of Genetics

Redacted for Privacy

Dean of Graduate School

I understand that my thesis will become part of the permanent collection of Oregon State University libraries. My signature below authorizes release of my thesis to any reader upon request.

Redacted for Privacy

Ahmed Humaid Hassan

Acknowledgments

This thesis is dedicated to my parents Amir and Ehteram for their unfailing support, encouragement, and love during my education and throughout my life. They have sacrificed a lot for me so I am greatly indebted to them and I love them deeply with all my heart. I would also like to thank the rest of my family, my Brothers (Changiz and Siroos) and their wives (Saghar and Khadijeh) for their understanding, guidance, and patience, my sisters (Khorsheed, Khavar, and Mahtab) and their husbands (Khosrow, Fathollah, and Amrollah), and my nieces and nephews (Ebraheem, Naseem, Raheel, Akram, Rahela, Asadollah Khan, Raha, Ravieh, Beeta, and the ones that will be added in the future), and my friends for all their love.

I would like to thank my Professors K. van Holde and J. Zlatanova for their intensive help and patience during the research. It was a privilege and an honor to work with them. I would like to acknowledge Dr. M. Ivanchenko for her assistant in the design and performance of complex molecular biology techniques. This work would not have been possible without the support of all of the above people.

TABLE OF CONTENT

TABLE OF CONTENTS (Cont.)

LIST OF FIGURES

LIST OF FIGURES (Cont.)

LINKER HISTONE / DNA INTERACTIONS : *IN VITRO* STUDIES

Chapter 1

Introduction

a) Chromatin and the Nucleosome

Several long DNA molecules inside each eukaryotic cell have to somehow be compacted and compressed to fit into a small nucleus. This compaction is achieved in sequential hierarchic structures with the help of proteins. When naked DNA is partially digested with micrococcal nuclease (MNase) (a relatively nonspecific endonuclease), a smear of DNA fragments is produced on the gel. However, when chromatin is digested with the same endonuclease, the DNA is cleaved in specific nonrandom sites into integral multiples of a unit length, giving a series of bands that are multiples of about 200 bp on electrophoresis gels (van Holde, 1988). These nuclease digestion data, as well as electron microscopy data have shown that chromatin is organized as a linear succession of beaded structures, called nucleosome core particles, which contain the proteins that protect DNA from uniform MNase digestion.

The fundamental structural unit of chromatin, the nucleosome core particle, has the same type of design in all eukaryotes. It consists of 146 bp of DNA wrapped in 1.75 left-handed superhelical turns around an octameric assembly of the four core histones (two of each of H3, H4, H2A, and H2B), as shown in Figure 1.1A (van Holde, 1988). The nucleosome core particle has been well characterized; its diameter and thickness are 11 nm and 5.5 nm respectively (Braddock et al., 1981; Richmond et al., 1984). This winding of the DNA into the bead-like particles is the lowest level of compaction (a compaction ratio of 5). These particles are connected by DNA

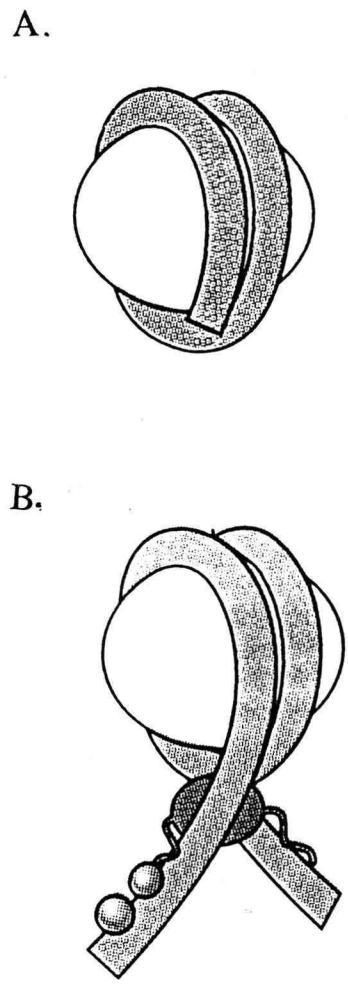


Figure 1.1. Schematic presentation of nuclesome core particle and chromatosome. (A) The nucleosome core particle, consisting of 146 bp of DNA (no linker histone). (B) The chromatosome, consisting of 168 bp of DNA whose ends are sealed by a linker histone (Mathews, C. K. and K. van Holde (1995). Biochemistry, second edition).

(linker DNA) and are the components of euchromatin, heterochromatin, and chromosomes. Thus, the histones play an important part in the folding of the chromatin fibers in the eukaryotic nucleus and this packaging is of general importance for gene function and regulation. In the presence of a fifth histone, H1 and the other members of the lysine-rich histone family (the so-called linker histones), the nucleosome assembly, now called the chromatosome, can condense to form a higher order structure, in which each nucleosome is now associated with 168 bp or about two full superhelical turns of DNA whose ends are sealed by contacts with H1 (Simpson et al., 1978; Boulikas et al., 1980; Allan et al., 1980) (Figure 1.1B). Histone H1 is thought to primarily bind to linker DNA where it enters and leaves the nucleosome core particle. At low salt concentrations (about 10 mM NaCl), a continuous extended chain of nucleosomes is observed. Upon addition of more salt (at about 60 mM), this extended fiber of nucleosome folds into a compacted or compressed fiber (Thoma et al., 1979).

This further condensed structure is the coiling of the series of beads into a helical array to constitute the compacted fiber, which may take the form of a "solenoid", according to some models (Finch and Klug, 1976; Mc Ghee et al., 1983) or an irregular helical zig-zag, according to others (e.g. Woodcock et al., 1984) (Figure 1.2A & B). Other models proposed for the condensed fiber are reviewed by Freeman and Garrard (1992) and Tsanev et al. (1992). The nature of the higher-order structure of the condensed fiber is poorly understood and the lack of a generally accepted view can be attributed to difficulties in identifying the path of the linker DNA as it passes from one nucleosome to the next, as well as to the uncertain location of the linker histones within the fiber. Some recent studies have demonstrated that the linkers entering and exiting the nucleosomes do not cross each other in linker histone-free mononucleosomes, despite the wrapping of 1.75 turns of the DNA in the core particle (Hamiche et al., 1996). The authors explain that this

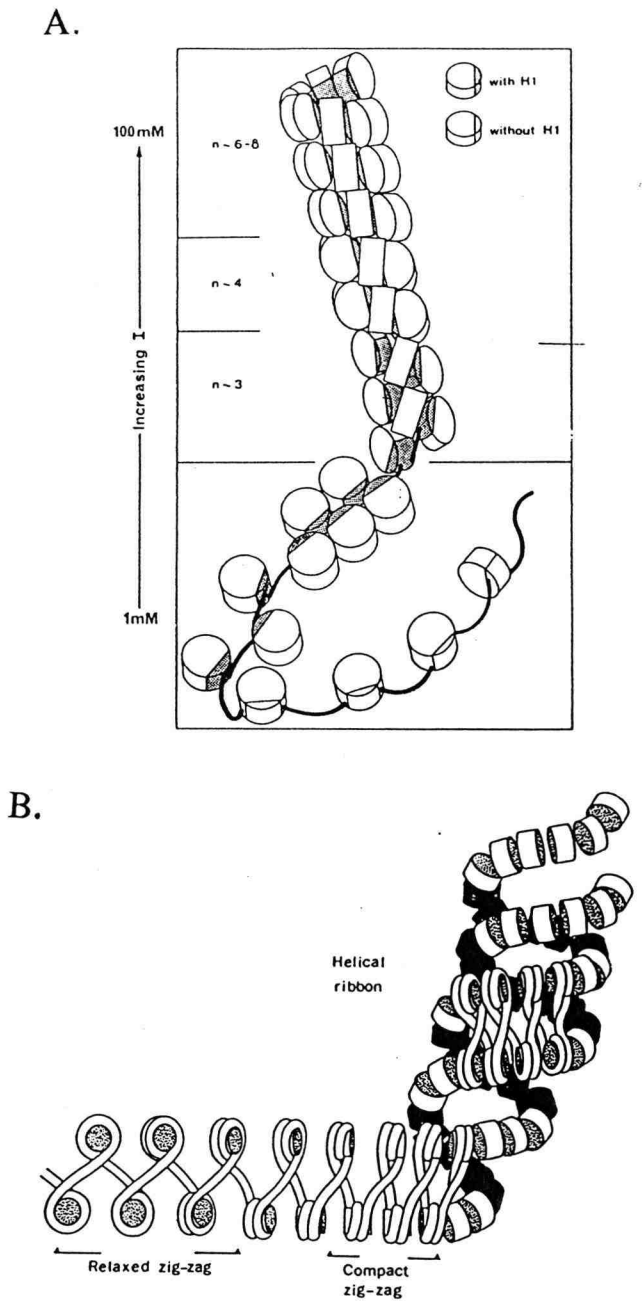


Figure 1.2. Models for the higher-order structure of chromatin. (A) The "solenoid" model proposed for the compacted fiber (Finch and Klug, 1976; Mc Ghee et al., 1984). (B) The "irregular helical zig-zag" model (Woodcock et al., 1984).

absence of crossing is a consequence of the bending of the linker arms in the entry/exit region due to electrostatic repulsion. The addition of GH5 does not lead to crossing of the arms either, according to their studies. However, the presence of the C-terminal domain of H5 has a very important impact on the structure; it bridges the arms together into a stem over a distance of about 30 bp (Hamiche et al., 1996).

At low salt concentrations, when H1 is present on the fiber, a "helical zig-zag" appearance of the extended fiber is observed since the DNA enters and leaves the nucleosome on the same side. However, in H1-depleted chromatin, the entry and exit points of the DNA are more random leading to a "beads-on-a-string" conformation (Thoma et al., 1979). Removal of linker histones, as expected, causes the loss of the "helical zig-zag" structure and produces an extended "beads-on-a-string" structure as demonstrated by scanning-force microscopy (SFM) (Yang et al., 1994). It is, therefore, clear that the linker histone H1 plays a major role in determining chromatin fiber structure even at low ionic strength. Linker histones are also shown to be important in the formation and maintenance of the compacted fiber. With increasing salt concentrations, both H1-containing and H1-depleted chromatin will condense even though the latter will condense randomly, with no definite structure.

Studies have also suggested important functional interactions between the core histone amino termini and the linker histones. These interactions are also important in the folding of the fiber. Garcia-Ramirez et al. have shown that, in the absence of linker histones, the removal of the N-terminal tails of the core histones by trypsin digestion, prevents linker DNA bending and oligonucleosome folding (Garcia-Ramirez et al., 1992).

The extended and the condensed fibers are the first two levels of compaction; however, since these two ensure only intermediate levels of compaction, there must be further compaction. The third level of organization is the packaging of the

condensed fiber itself. This function is also likely to be modulated by accessory proteins.

There are two different types of DNA-binding proteins in chromatin of eukaryotic cells:

1) Histones, which include the five types of proteins mentioned earlier. They are small and extremely basic nuclear proteins, rich in lysine and arginine. The relative proportions of lysine and arginine could be used to classify and characterize the five histone types. The same classes can be virtually recognized in all eukaryotes. H2A and H2B are slightly lysine-rich; H3 and H4 are arginine-rich, and the linker histone family are highly lysine-rich. Some of these histones are well conserved throughout evolution. H3 and H4 are among the most conserved proteins known (van Holde, 1988; Tsanev et al., 1992). For example, H4 in cow and pea have almost identical sequences (they differ only at two sites in 102 residues) (Delange et al., 1969a, b), suggesting perhaps identical functions in all eukaryotes. The H2A and H2B families can also be recognized in all eukaryotes, but show appreciable species-specific variation in sequence. Other histones, like the linker histone H1, show appreciable variation between tissues and between species. They may be also absent in some lower eukaryotes. Thus, the linker histones (especially their N- and C-termini) are the least conserved among the five types of histone proteins.

2) Non-histone chromosomal proteins, which include a variety of proteins such as hormone receptor proteins, nuclear enzymes, polymerases, as well as structural and regulatory proteins. In other words, they include all the proteins of chromatin that are not histones. The High Mobility Group (HMG) proteins comprise a distinct and well-defined family of non-histone proteins (for review see Bustin and Reeves, 1996).

b) The Linker Histones

The linker histones (H1, H1°, H5, etc.) are essential both for the formation of the chromatosome and for the formation of chromatin higher-order structures. They interact with the nucleosome, protecting the entry and exit strands of DNA against nuclease attack, sealing off two turns of nucleosomal DNA around the octamer, thus stabilizing the complex. Despite years of studies, their exact location in chromatin is still unknown.

The H1 histones represent a group of lysine-rich chromosomal proteins with an approximate protein length of 200 amino acid residues. The linker histones have a high lysine to arginine ratio (about 15) and are highly basic (the net charge of the protein is about +50 to +60). These lysine-rich histones in physiological conditions consist of three clearly distinct and different domains: a basic, short (20-40 residues) N-terminal (nose) which varies considerably in length between H1 subtypes, a longer (about 100 residues), highly basic C-terminal (tail), and a central nonpolar globular domain (head) of about 80 amino acid residues (Figure 1.3A). The N- and C-terminal regions contain many lysine, proline, and arginine residues, while the globular domain contains mostly hydrophobic amino acid residues. This globular domain is highly conserved in evolution and is essential for binding to the nucleosome. The C-terminal tail of H1 is believed to interact with linker DNA, assuming a segmented α-helical conformation upon binding; this tail is essential for the formation of higher order structures (Allan et al., 1986; Clark et al., 1988). This conclusion was reached by secondary structure predictions and Circular Dichroism (CD) measurements. The α-helical segments may track one of the grooves of DNA. Since the C-terminal is highly basic (positively charged), it is expected to partially neutralize the negative charge of linker DNA, facilitating bending in the structure on close approach of linkers. Allan and coworkers showed that the globular domain alone is not sufficient to induce salt-dependent chromatin folding, while the

combination of the globular and the C-terminal domains is. This is direct evidence for the importance of the C-terminal in chromatin folding (Allan et al., 1986; Zlatanova and Yaneva, 1991; Strauss and Maher, 1994). The function of the N-terminal nose is less well understood; it is thought that the N-terminal directs and anchors the globular domain to properly position it with respect to nucleosomes in the fiber (Allan et al., 1986).

Lysine-rich histones are represented by different forms (subfractions or subtypes) with different molecular mass, amino acid composition, and properties in each cell (Cole, 1987). H1 histones are the most heterogeneous of the histone proteins, and within any one organism, they represent a set of nonallelic subtypes. Vertebrate species generally have between four and six different H1 subtypes and the expression of these is differentially regulated throughout development, through the cell cycle, and during the differentiation of particular cell lines. Different H1 subtypes are probably functionally distinct; they differ in their abilities to condense DNA, dinucleosomes, and H1-depleted chromatin. The best studied tissue-specific H1 subtype is histone H5, which accumulates during the process of terminal differentiation of nucleated erythrocytes of birds and some fish (Neelin et al., 1964; Miki and Neelin, 1975). It is thought that H5 is involved in chromatin compaction and the shutting down of transcription and replication in this cell type. It also binds more tightly to chromatin than H1. Some tissue-specific H1 subtypes are observed only in the testis, like H1t (Seyedin and Kistler, 1980), while others are seen during embryonic development, like H1α and H1cs in sea urchin (Newrock et al., 1978). There are also species-specific H1 subtypes.

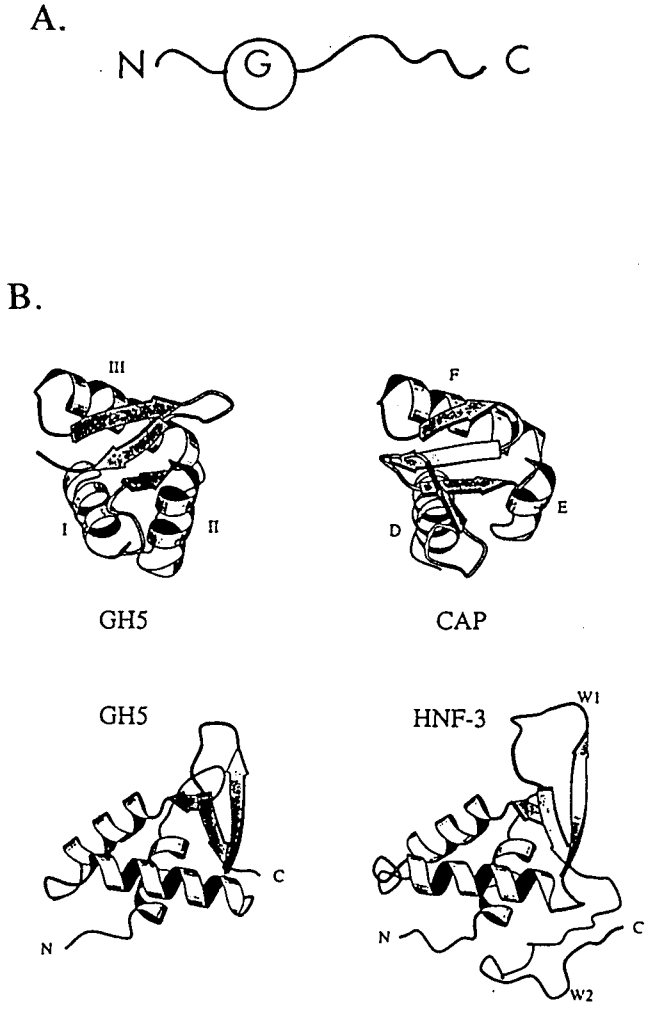


Figure 1.3. Schematic presentation of the three domains of the linker histones and comparing their globular domain to other DNA binding proteins. (A) Schematic presentation of the three domains (the globular, N-, and C-terminals) of the linker histones. (B) schematic diagram of the globular domain of histone H5 based on crystallography data, and comparison of this structure with the DNA binding domain of bacterial DNA binding protein CAP and with the DNA recognition motif of the *Drosophila* transcription factor HNF-3γ (Ramakrishnan et al., 1993).

c) The Three-Dimensional Structure of the Globular Domain of the Linker Histones and its Interaction with DNA

Since the globular domain of linker histones H1 and H5 is essential for binding to the nucleosome and since it protects the nucleosome from micrococcal nuclease in the same way as does the full-length protein, understanding the structure of this domain seems to be the first step in resolving some of the controversies about the function of linker histones in chromatin. The tertiary structure of the globular domains of both H1 and H5 has been determined using NMR techniques (Clore et al., 1987; Cerf et al., 1994). The crystal structure of the globular domain of H5 (GH5) has also been solved to 2.5 Å resolution (Ramakrishnan et al., 1993). The two structures (from NMR and crystallography) are slightly different; no β-sheets are observed in the NMR structure of GH5, while there are three β-sheets in the crystal structure (Figure 1.3B). Besides this difference, which is most likely due to different sampling conditions in the two experiments, the structures match well.

The globular domain of H5 has a "winged-helix motif" and consists of a three-helix bundle (I-III), with a β-hairpin at the C-terminus. Residues 44-46 between helices I and II are also in β-strand form. Thus, three antiparallel β-sheets are present in the globular domain of GH5. The GH5 structure is very similar to that of the DNA binding domain of the bacterial DNA binding protein CAP (catabolite gene activator protein), and also to the DNA recognition motif of the *Drosophila* transcription factor HNF-3γ (Schultz et al., 1991; Ramakrishnan et al., 1993; Clark et al., 1993) (Figure 1.3B). In fact, helices I, II, and III of GH5 can superimpose extremely well with the corresponding helices D, E, and F of CAP, suggesting that GH5 recognizes DNA similar to CAP. Helix III ("the recognition helix") of GH5, like helix F of CAP, may bind to DNA by fitting into the major groove (Figure 1.4). Even though there is little sequence homology between GH5 and CAP, CAP being a prokaryotic sequence-specific DNA-binding protein that binds to DNA as a dimer

inducing a bend in the DNA, and GH5, being a eukaryotic protein that binds as a monomer with no known sequence specificity, their three-dimensional structures are very similar (Ramakrishnan et al., 1993); this suggests that their DNA binding properties may be similar too.

The amino acid residues of GH5 that are supposedly involved in DNA binding are highly conserved in the structure of the protein. Lys 69 and Arg 73 can interact with the phosphates of one strand of DNA and Lys 85 can interact with the other strand (Figure 1.4). These residues are positioned in helix III, and all have counterparts in CAP on which this binding model is based. In addition, these residues are protected against chemical modification in chromatin. If Lys 85 is mutated to glutamine or glutamic acid, GH5 no longer protects 168 bp of nucleosomal DNA from micrococcal nuclease digestion suggesting involvement of Lys 85 in DNA binding (Buckle et al., 1992). His 25 and His 62, which were shown to be crosslinked to DNA in chromatin, are also probably involved in the binding (Mirzabekov et al., 1989).

Simultaneous H1 binding to two DNA duplexes appears to be achieved through two different DNA-binding domains (Dravers et al., 1992; Thomas et al., 1992). The globular domain of H5 (GH5) contains one binding domain which is related to that of CAP and HNF-3γ forkhead, with helix III believed to be the major binding site of GH5 to DNA. A secondary less defined binding site in GH5 has also been proposed. Four positively charged residues, Lys 40, 52, and Arg 42, and 94 (residues of helix II) could interact with a second duplex of nucleosomal DNA (Figure 1.4). These residues are on the opposite face of GH5, about 30 Å away from Lys 69 and Arg 73 in the recognition helix (Ramakrishnan et al., 1993), and are highly conserved in the H1 family (Wells and Brown, 1991). This model predicts that if these basic residues are replaced by neutral ones, GH5 should no

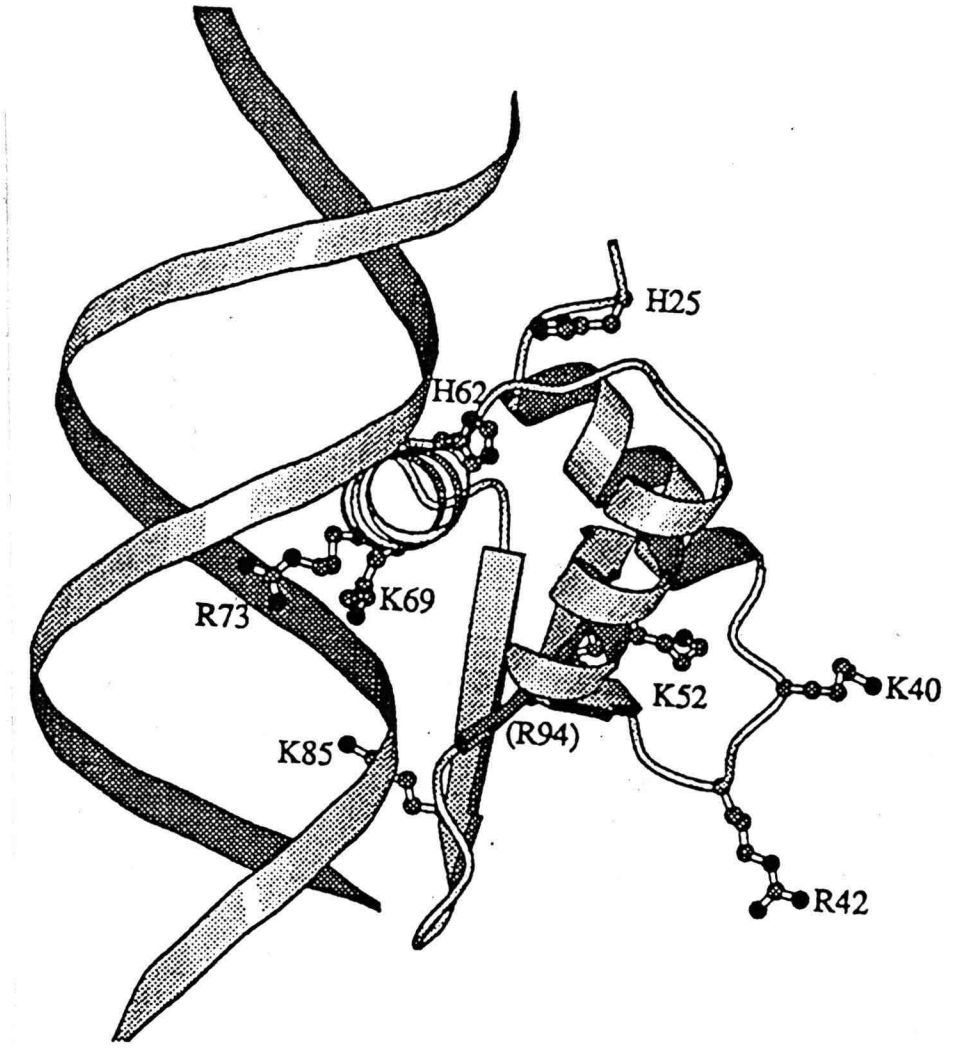


Figure 1.4. Schematic presentation of how GH5 may bind to a duplex of DNA on the basis of the structural similarity between GH5 and other DNA binding proteins. Amino acid residues that are important for binding (Lys 85, Lys 69, Arg 73, Lys 40, Lys 52, Arg 42, Arg 94, His 62, and His 25) are shown (Goytisolo et al., 1996).

longer bind to two duplexes of DNA. Indeed, when the positive charges at one or both of the sites in GH5 were removed by mutagenesis, the cooperative GH5/DNA binding, as well as the chromatosome protection in the nucleosome was reduced or abolished completely (Goytisolo et al., 1996). Another GH5/DNA binding model proposed earlier by Segers et al. (1991) suggesting a simultaneous minor and major groove binding, with helix III binding to major and helix II binding to minor grooves, is thus less plausible. Since the structure of GH1 is very similar to that of GH5 (three helices and turn-like structures), and the helices align perfectly (Cerf et al., 1994), it is reasonable to expect that their interaction with DNA is quite similar.

d) Binding of the Linker Histones to the Nucleosome and to Free DNA

Conflicting data and some controversy exist about the binding of GH5 and the full-length protein to DNA and its location in the nucleosome. It is known that binding of linker histones or their globular domain to linker DNA protects an additional 20 bp of the linker, that is in immediate proximity to the 146 bp of DNA in the nucleosomal core, against MNase digestion. Using protein/protein and protein/DNA crosslinking, it was demonstrated that H1 lies close to the nucleosomal core, probably at or near the dyad axis (Belyavsky et al., 1980; Espel et al., 1985). In the case of a nucleosome reconstituted onto DNA containing the *Xenopus* somatic 5S rRNA gene, protection of the additional 20 bp by linker histones is asymmetric (15 bp protection at one end and 5 bp at the other end); crosslinking studies of GH5 to DNA in the reconstituted nucleosome have led the authors to suggest that perhaps linker histones do not bind at the DNA entry/exit but that they bind at a different position with respect to the nucleosome, away from the dyad either on the outside or on the inside of DNA close to the octamer core (Hayes and Wolffe, 1993; Hayes et al., 1994; Pruss et al., 1995). However, numerous earlier studies suggest the

protection of 10 bp of DNA at each end of the chromatosome after linker histone (or their globular domain) binding and most researchers favor this symmetrical protection of linker DNA. The asymmetric protection of linker DNA may thus be a special feature of the *Xenopus* 5S rRNA gene sequence. Using other DNA sequences, to test this asymmetric protection, will be useful in gaining insight into the problem of the location of linker proteins and their protection of DNA. Since there is much evidence that H1 binds to linker DNA at the point where the DNA double helix enters and exits from the core particle, it is widely believed that a linker histone sits on or close to the crossover of DNA strands interacting with both the entering and exiting strands. Protection experiments in some studies (Staynov and Crane-Robinson, 1988) have suggested that GH5 binds directly to the dyad. Since it is also known that DNA at the dyad has a minor groove at the exposed surface, Ramakrishnan et al. (1993) suggest a model in which the recognition helix fits into a wide minor groove rather than a major groove.

Since it is believed that linker histones interact mainly with linker DNA, many researchers have used free DNA instead of the chromatin fiber in their studies for simplicity (Zlatanova and Yaneva, 1991). Both with the chromatin fiber and with free linear DNA, chemical crosslinking has demonstrated the formation of H1/H1 polymers (Ring and Cole, 1983; Clark and Thomas, 1986). In addition, the saturation of DNA sites with H1 at 0.5 M NaCl is about the same for chromatin and free DNA (Diez-Caballero et al., 1981), and the compaction of the fiber occurs at about the same salt level where H1/free DNA aggregates are formed. The above observations suggest that perhaps H1/free DNA system could be an appropriate model for studying the interaction of linker histones with linker DNA and their role in chromatin.

At different ionic strengths, the appearance of the histone/"naked" DNA complex in the electron microscope changes, ranging from thin DNA filaments

(histone molecules are sandwiched between two DNA molecules or duplexes) at low salt, to cable-like and donut-shape structures at higher salt (Rodriguez et al., 1991). In addition, at low ionic strength, H1 molecules will bind nonspecifically to DNA and depending on the ionic strength, DNA concentration, and H1/DNA ratio, the cooperativity of H1/DNA interaction will vary (Zlatanova and Yaneva, 1991). With concentrated solutions, multistranded complexes begin to form even at salt concentrations between 20-50 mM. Under these conditions, DNA is either free or present in complexes saturated with H1, indicating cooperativity. However, with dilute solutions, the cooperativity is less extreme at the same ionic strengths (Watanabe, 1986; Clark and Thomas, 1988). The isolated C-terminal domain of H1 also shows a salt-dependent transition toward cooperative binding. At 60 mM NaCl concentrations, the C-terminal domain binds to some of the DNA molecules, leaving the rest free; in other words, it binds in a cooperative manner (Rodriguez et al., 1991). At all ionic strengths with concentrated DNA solutions, histone H5 and the globular domains of H1 (GH1) and H5 (GH5) interact with DNA cooperatively.

Even though a lot of disagreement among researchers exists about the location of the linker histone on the nucleosome, as well as about how the compacted fiber is organized, it is clear that linker histones prefer binding to supercoiled DNA over linear, relaxed circle, or nicked DNA. The binding of linker histones to DNA supercoils in the presence of DNA competitors at different salt concentrations is the focus of chapter 2. In Chapter 3 and 4, the effect of linker histone binding on DNA topology is examined. The overall aim of this thesis is to study how linker histones interact with naked DNA.

Chapter 2

The Effect of Salt on the Linker Histone/DNA Complexes

a) Introduction

The linker histones are known to be involved in the salt-dependent folding of the nucleosomes into higher-order chromatin structure (Thoma et al., 1979; Mc Ghee et al., 1980). To better understand the mechanism of action of the linker histones in chromatin, many studies on H1/DNA complexes have been performed; we have also used this complex as a model for linker histone/DNA interactions in chromatin. It is also known from our direct competition experiments that the linker histones prefer binding to supercoiled DNA over other DNA forms (Ivanchenko et al., 1997). The interaction of histones with supercoiled DNA is important since the DNA in the nucleosome is wrapped superhelically around the histone core. Even though many studies have focused on these interactions, the nature of the interaction and the structure of the complexes are not well understood.

It is believed that histone H1 can form two different kinds of complexes with supercoiled DNA (De Bernardin et al., 1986): 1) Soluble complexes, which are formed at low salt concentrations and low H1/DNA ratios and contain varying amounts of histone H1 bound distributively to all DNA molecules. Electron microscopy shows that these soluble complexes contain thickened regions about 6 nm across interspersed with free DNA. Gel electrophoresis shows that with increasing H1 ratios, the mobility of the supercoiled band is decreased, most likely due to increased bound H1. The H1 molecule preferentially binds to regions of DNA with two helically twisted duplex strands. Once bound, the H1 molecules may interact with neighboring H1 molecules on the same DNA leading to the formation

of an H1 polymer along the superhelical DNA axis; alternatively, each H1 may bind to two DNA duplexes. 2) Cable-like aggregates, which are formed at high salt concentrations through cooperative interactions; H1/DNA complexes as well as free DNA are present in the same reaction mixture. These complexes possess rod-like morphology of relatively uniform width (11 to 15 nm) whose length is about 1.6x shorter than that of the input DNA, or they are circular if the DNA is long enough. Those thick complexes can also be formed at low salt when the H1/DNA input ratio is sufficiently high (De Bernardin et al., 1986; Clark and Thomas, 1986). Under these conditions (high salt or high H1/DNA ratio), interactions of H1 bound to different DNA molecules may occur. From the electron microscopy data, the soluble complexes seem to be more distinct, homogeneous, and better-defined than the aggregates, thus, probably reflecting the binding behavior of H1 in chromatin in a more appropriate and realistic way than do aggregates.

In order to gain further insight into the nature of these histone/DNA complexes, gel retardation experiments using various histone/DNA ratios and different salt (NaCl or $MgCl_2$) concentrations were performed. It was observed that salt concentrations and the nature of the ions significantly affect histone/DNA interactions. Experiments were performed to understand the nature of these salt effects.

b) Materials and Methods

Preparation of Linker Histones. Chicken erythrocyte histone H1 was purified under nondenaturing conditions (Banchev et al., 1991) and checked for purity by SDS-containing polyacrylamide gel electrophoresis (Laemmli, 1970). The concentration of the protein stock solutions was determined by two methods: spectrophotometric (using an extinction coefficient of 1.85 ml/(cm.mg) at 230 nm (Camerini-Otero et

al., 1976)), and scanning of Coomassie-stained polyacrylamide gels, using bovine serum albumin as a standard.

Preparation of Plasmid DNA. Two types of plasmid DNA (pBR322 and pUC19) were used, both prepared by CsCl purification, phenol/chloroform extraction, and ethanol precipitation (Maniatis et al., 1982). Nicked plasmid molecules were prepared by DNase I digestion (0.25 ng of enzyme/μg of DNA) for 25 minutes at 15°C in a buffer containing 50 mM Tris-HCl, pH 7.8, 5 mM $MgCl_2$, 10 mM DTT, and 25 μg/ml BSA. Linear DNA molecules were prepared by digestion of the supercoiled plasmids with *Pst*I in the conditions recommended by the manufacturer (Bio-Rad). Different plasmid forms (supercoiled, nicked, and linear) were combined to produce a mixture containing about the same amounts of the three forms.

Histone/DNA Binding Assay. The histone/DNA complexes were formed by direct mixing of DNA and histones in binding buffer (20 mM MES, pH 6.6 + 1 mM EDTA, 0.15 μg/μl BSA) for 10 minutes at room temperature. Different salt (NaCl or $MgCl_2$) concentrations were present in the reaction mixtures. The complexes were crosslinked with glutaraldehyde (0.1%) overnight at 4°C and analyzed by 1.2% agarose gel electrophoresis. The mobility of the protein/DNA complexes relative to that of a restriction fragment (3,675 bp and 5,686 bp for pUC19 and pBR322 respectively) in the marker DNA (Lambda DNA-*Bst*E II Digest), in the presence of different salt levels, was determined and graphed as a function of histone/DNA ratio.

Immunoblotting. The protein/DNA complexes on the agarose gels were transferred to nitrocellulose membrane as described by Ivanchenko and Zlatanova (1997) and visualized by immunostaining using anti-H1 antiserum. To this end, we used ProtoBlot Western Blot AP Systems (Promega), which is based on the enzyme-linked immunodetection of antigen-specific antibodies using rabbit anti-IgG secondary antibodies conjugated with alkaline phosphatase (AP). Following incubation with the primary anti-H1 antibody (kindly donated by Drs. J. Yaneva and

S. Zacharieva, Institute of Molecular Biology and Institute of Experimental Pathology and Parasitology, Sofia, Bulgaria) and appropriate anti-IgG AP conjugate, color development substrates (NBT and BCIP) were applied and the sites of antigen localization were detected (turning dark purple color as a result of alkaline phosphatase activity). The spots on each membrane were scanned and the band intensity was determined using Adobe Photoshop 3.0 and NIH Image 1.57 programs.

c) Results and Discussion

Figure 2.1A and 2.1B show the titration of a mixture of nicked, linear, and supercoiled pBR322 or pUC19 with increasing histone H1/DNA ratios at different $MgCl_2$ or NaCl concentrations, respectively. Glutaraldehyde is used to fix the H1/DNA complexes, in order to avoid their dissociation during electrophoresis. It is obvious from the gels that the mobility of the supercoiled band was decreased (increased retardation) with increasing H1/DNA ratios. The presence of more protein bound to the superhelical DNA is the cause of this lower mobility of the complex (see Figure 4.5, page 64). Upon increasing H1/DNA ratios further, aggregates were formed, which stayed in the wells and did not enter the gel; the presence of these aggregates plus the soluble complexes in the same reaction suggest cooperativity of H1 binding to DNA. This lower mobility of the complex with increasing H1/DNA ratios is true for all salt concentrations tested.

The salt concentrations used for these experiments ranged from 0 to 60 mM NaCl, or 0 to 5 mM $MgCl_2$. As the ionic strength increased, the electrophoretic mobility of the complex increased (decreased retardation). For example, at 5 mM $MgCl_2$ the mobility of the complex was higher than at 4 mM, which, in turn, was higher than at 3 mM, if the same input histone/DNA ratio lanes are compared.

Figure 2.1. Agarose gel electrophoresis of histone H1/DNA complexes formed at increasing H1 levels at different $MgCl_2$ (A) or NaCl (B) concentrations. Lane M is *Bst*EII-digested lambda DNA used as marker. The amount of H1 in the second lane after lane M (in A) was one molecule of H1 per 83 bp of DNA and was increased in each successive lane to 1/38, 1/25, 1/17, and 1/11. The amount of H1 in the second lane after lane M (in B) was one molecule of H1 per 116 bp of DNA and was increased in each successive lane to 1/78, 1/50, 1/34, and 1/23. The concentration of salt in each set of reaction mixtures is shown above the gels. N and S indicate the positions of the nicked and the supercoiled molecules, respectively.

$MgCl_2$ (mM)

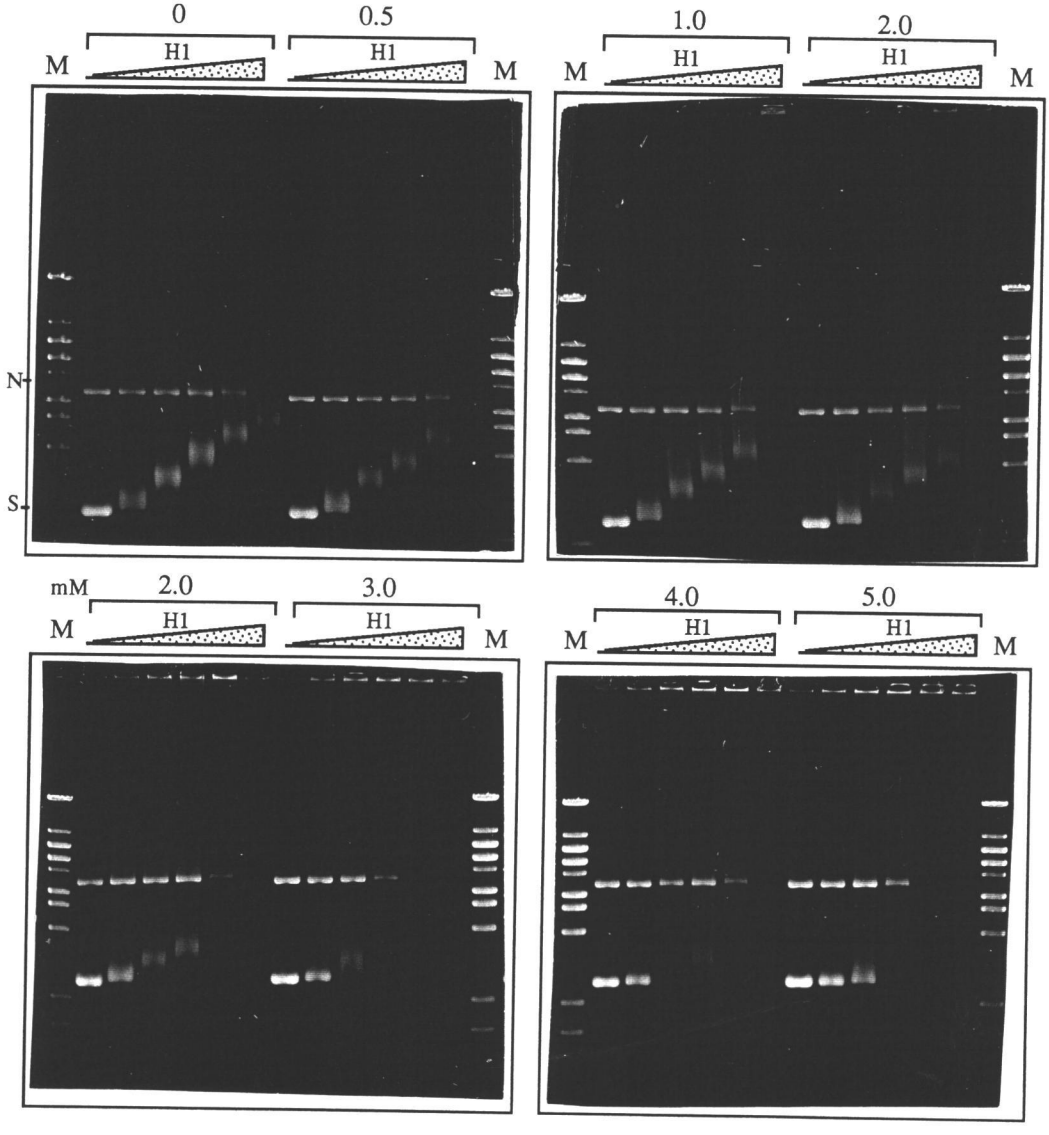


Figure 2.1A

NaCl (mM)

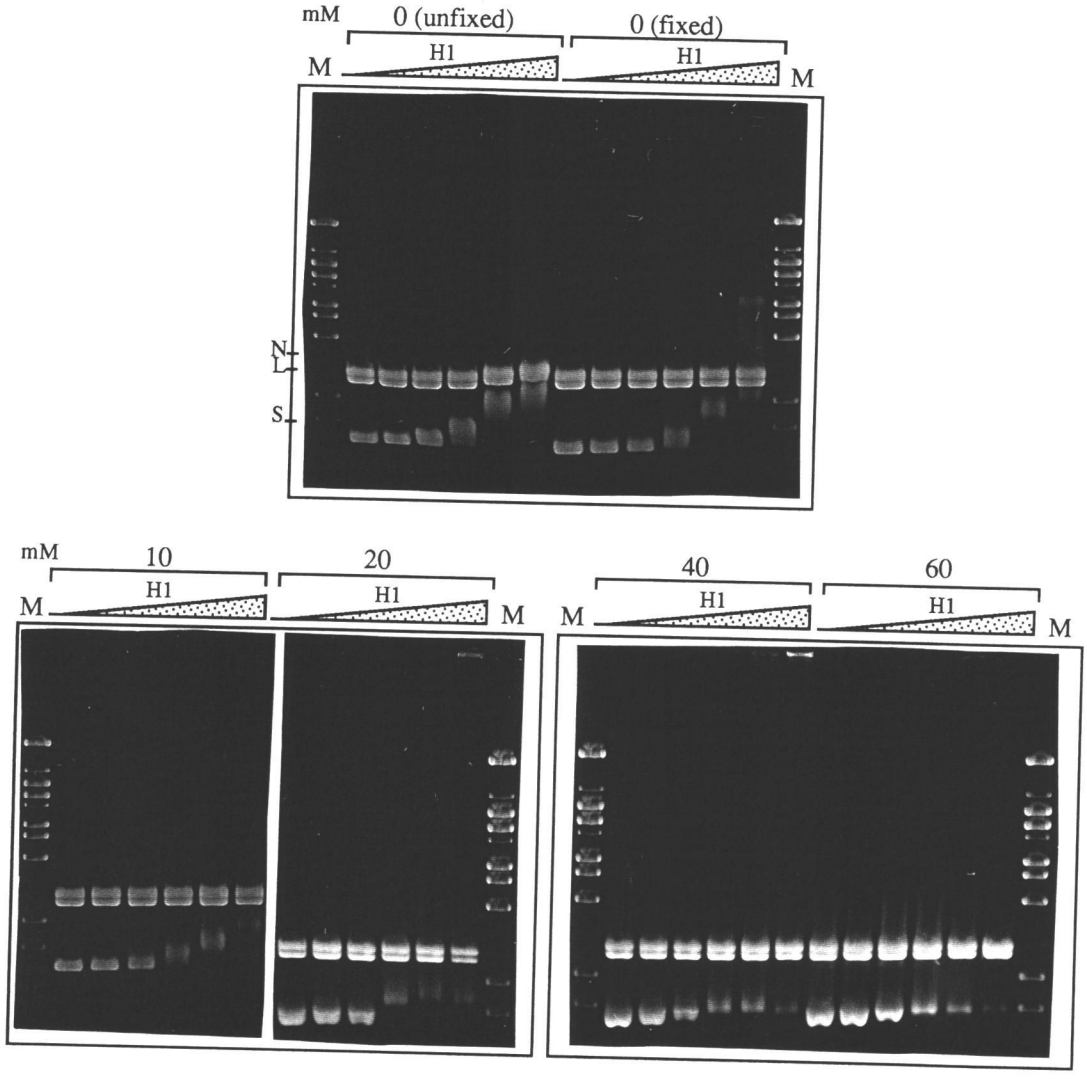


Figure 2.1B

Similarly, at 40 mM NaCl the mobility of the complex was higher than at 20 mM, and so on.

Figure 2.2 shows the quantitative presentation of the results on the gels in Figure 2.1. The relative retardations of the supercoiled band as a function of both H1/DNA (at all salt levels) and salt ($MgCl_2$ or NaCl) (at all protein/DNA ratios) are graphed. The nicked and the linear DNA forms were not retarded with increasing H1/DNA ratios, even though these forms were present together with the supercoiled form. In other words, the supercoiled form competed effectively with these other DNA forms for H1 binding, as expected on the basis of earlier reports. The results clearly show two things: 1) The relative retardation of the H1/DNA complexes increases with increasing H1/DNA ratios at all salt levels. 2) With increasing ionic strengths, the relative retardation decreases at all H1/DNA ratios. The effects of $MgCl_2$ on the H1/DNA complexes was about 6 times greater than the effect of NaCl; with increasing ionic strengths, this difference between the effects of $MgCl_2$ and NaCl increased. This is concluded from the graph of percent relative retardation as a function of ionic strength (Figure 2.3). However, the overall results of the effects of the two different salts on the H1/DNA complex were similar.

The decrease in the mobility of the H1/DNA complex (supercoiled band) with increasing H1/DNA ratios can be explained by the presence of more bound protein to DNA causing the complex to migrate more slowly. Indeed, one would expect such a change from the decrease in the net negative charge of the histone/DNA complex.

On the other hand, the increase in the electrophoretic mobility of the H1/DNA complex observed as a function of increasing salt could be due to two reasons: 1) The amount of protein bound to DNA for a given protein/DNA input ratio is reduced with increasing salt concentration; 2) The complexes formed at the

increasing ionic strengths might have the same relative protein content but may differ in conformation (are more compact and thus more mobile).

In order to find out if the increase in the mobility of the supercoiled band at increasing salt concentrations is a result of decrease in the actual amount of bound H1 at these higher salt levels, we quantified the amount of protein bound to DNA at such salt concentrations. To this end, we separated the complexes formed at one specific H1/DNA ratio at different salt concentrations by electrophoresis, blotted the complexes onto nitrocellulose paper and quantified the amount of H1 present by immunostaining of the blots using anti-H1 antibody. The amount of the DNA in the same complexes was determined on the basis of EtBr-stained gels, scanned to determine the band intensities of the H1/DNA complexes. Figure 2.4 shows these data: with increasing salt, the H1/DNA ratio decreases. This suggests that the lower retardation of the supercoiled band at increasing salt is at least partly due to a decrease in the amount of H1 bound to DNA. The decrease in the binding constant of H1 to DNA with increasing salt is expected, since this binding is essentially electrostatic (Watanabe, 1986).

However, we can not rule out possible conformational changes of the complex with increasing salt levels. Electron microscopy studies have shown that with increasing salt concentration, the structure of the H1/DNA complexes changed and became more compact (De Bernardin et al., 1986). Moreover, recent cryo-electron microscopy studies on superhelical DNA (Adrian et al., 1990; Bednar et al., 1994) showed that the increase of the ionic strength apparently collapses or tightens the interwound DNA superhelix, decreasing its effective diameter (the closest possible approach between two DNA segments), from 12 nm at low ionic strength (10 mM Tris-HCl, 1 mM EDTA) to 4 nm at higher salt (10 mM $MgCl_2$ or 100 mM NaCl). At high salt, the negative charges along the DNA backbone are effectively

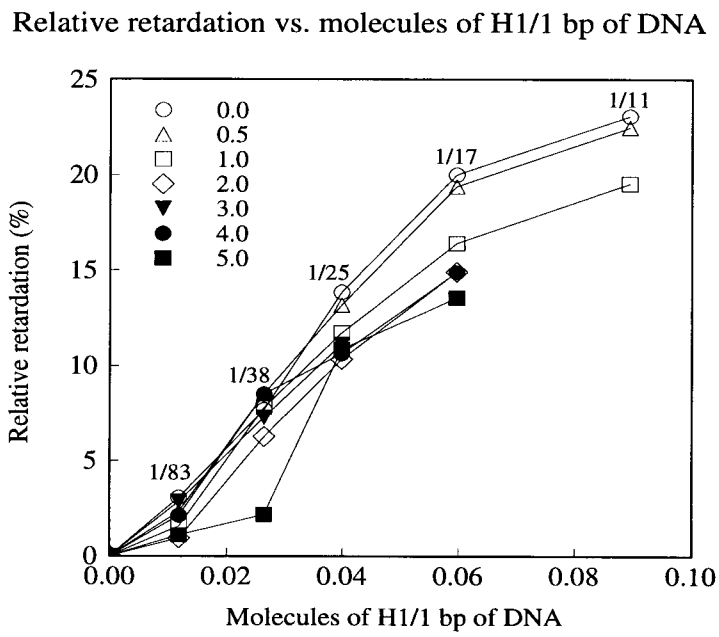
screened or neutralized by the cations, allowing closer contacts between the DNA segments, whereas, at low salt, the repulsion forces keep DNA segments apart.

In addition, two other studies (Shaw and Wang, 1993; Rybenkov et al., 1993) have raised our understanding on the relationship between salt concentration and the effective diameter of DNA. They have shown that the probability of formation of knots during random ring closure increases with salt concentration. This is because of the higher compaction of DNA at higher salt levels leads to higher probability of knot formation. Knotting complexity has also been found to increase with salt concentration. Schlick et al. (1994), using computational studies, have also shown that the conformation and energies of supercoiled DNA depend on ionic strength. At high salt, the supercoiled DNA is very compact, highly bent, and rigid, while at low salt, the DNA supercoils are much more open, loose, and more dynamic. These results are consistent with the previous cryo-electron microscopy data of Adrian et al. (1990) and Bednar et al. (1994). Using Monte Carlo simulations of supercoiled DNA conformation, Vologodskii et al. (1992) and Vologodskii and Cozzarelli (1994) have confirmed the studies on the effect of salt on supercoiled DNA. Moreover, they showed the importance of superhelical stress in DNA in protein/DNA interactions, bringing DNA segments that are far from each other in sequence into close juxtaposition (Vologodskii and Cozzarelli, 1996). Other studies (Fenley et al., 1994; Gebe et al., 1996) have also confirmed that with increasing salt concentrations, the supercoiled DNA adopts a more compacted structure.

Therefore, the results of Monte Carlo simulations, theoretical analysis, and cryo-electron microscopy all show that the conformation of the supercoiled DNA depends strongly on ionic conditions; cations shield DNA negative charges and decrease the repulsion of DNA segments in the tight interwound structure. The effective diameter of the double helix decreases about two folds with increasing Na^+

Figure 2.2. Quantitation of the results on the gels in Figure 2.1. Relative retardation (%) of the H1/DNA complexes as a function of H1/DNA ratios for $MgCl_2$ (A) and NaCl (C) at increasing ionic strengths. (B) and (D) show the graph of relative retardation (%) of H1/DNA complexes as a function of salt concentrations at increasing H1/DNA ratios for $MgCl_2$ and NaCl , respectively. The curves in (D) are exponential fit to the data points.

A .



B .

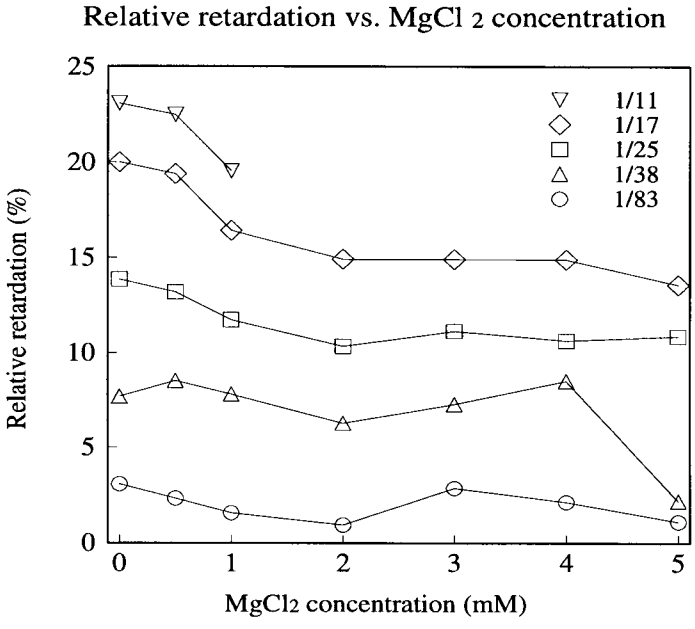
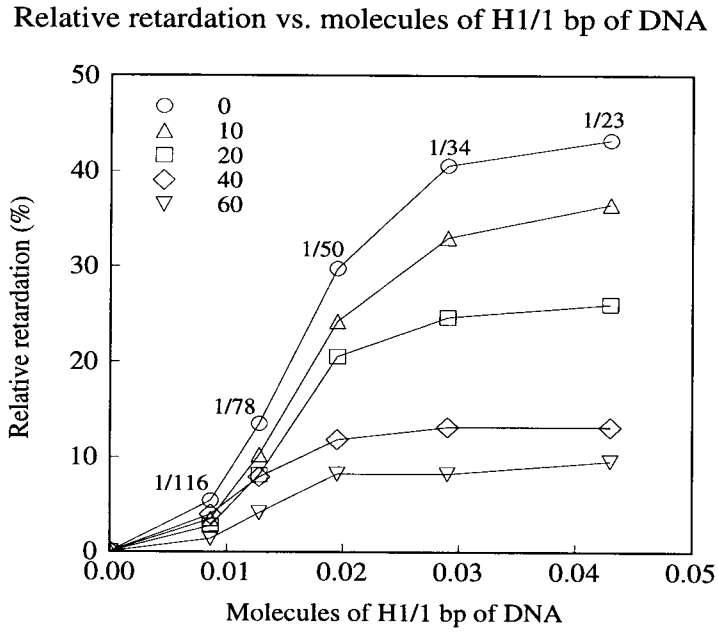


Figure 2.2A, B

C .



D .

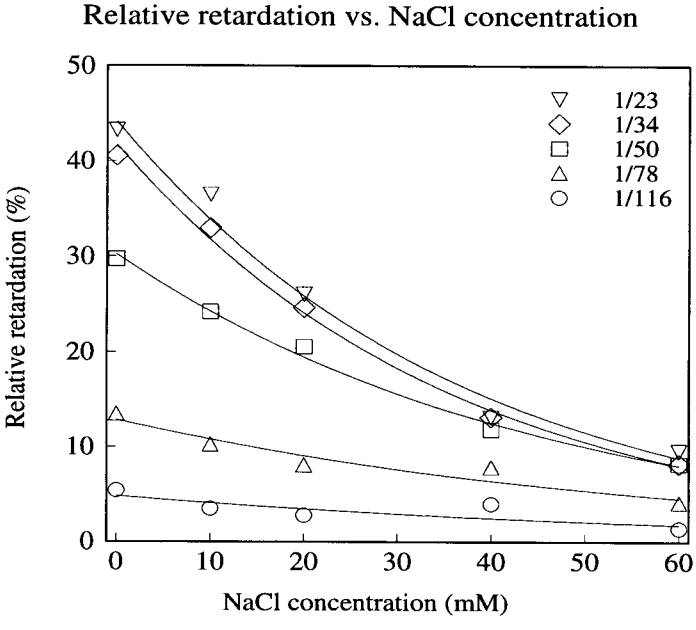


Figure 2.2C, D

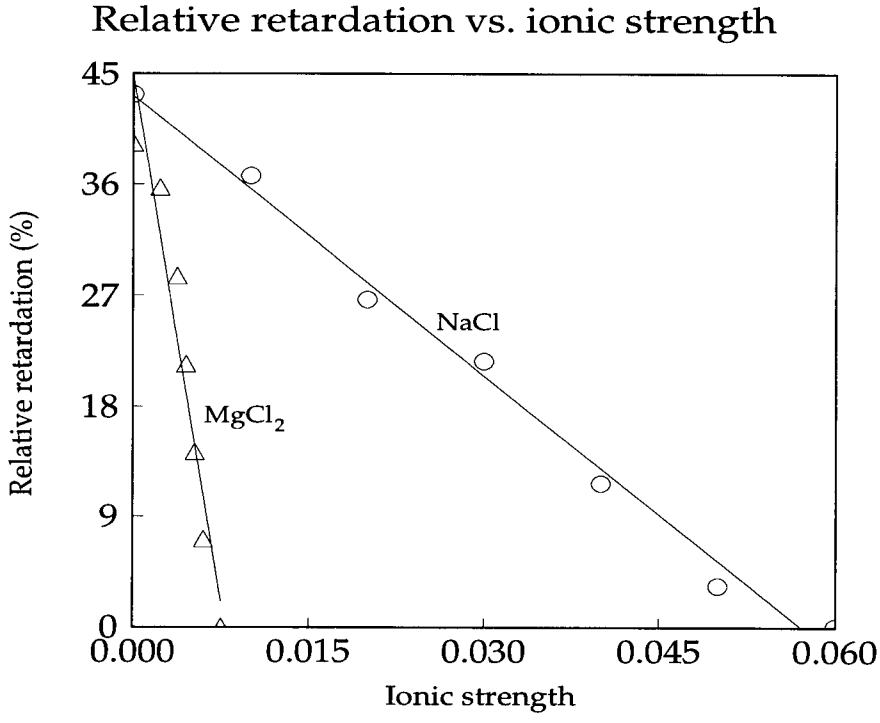


Figure 2.3. Quantitation of the results of Figure 2.4A and B. Relative retardation (%) of the H1/DNA complexes as a function of ionic strength at one H1 molecule per 34 bp of DNA. The lines for both $MgCl_2$ and NaCl are linear fit to the data points. The effect of $MgCl_2$ on the H1/DNA complexes is shown to be about 6x greater than the effect of NaCl; with increasing ionic strength, this difference increases.

Figure 2.4. Selectivity of H1 binding to highly supercoiled DNA at different ionic strengths. (A) Agarose gel electrophoresis of H1/DNA complexes formed at one H1 per 20 bp in the presence of increasing amounts of $MgCl_2$ (from 0 mM to 2.5 mM) and nitrocellulose replica stained with anti-H1 antibody. (B) Agarose gel electrophoresis of H1/DNA complexes formed at one H1 per 20 bp in the presence of increasing amounts of NaCl (from 0 mM to 60 mM) and nitrocellulose replica stained with anti-H1 antibody. The first lane shows the position of the nicked, the linear, and the supercoiled plasmids from top to bottom of the gels. The graphs of the H1/DNA complexes as a function of $MgCl_2$ (C) or NaCl (D) show that with increasing ionic strengths, the H1/DNA ratio decreases. The curve in (C) is logarithmic fit to the data points and the line in (D) is a linear fit to the data points. The units for y-axis is arbitrary and H1/DNA ratios (the y-axis points) are obtained by multiplying the area (in $pixel^2$) of the H1 and DNA bands by their (average density - background) for each pixel. N, L, and S denote the positions of nicked, linear, and supercoiled molecules, respectively.

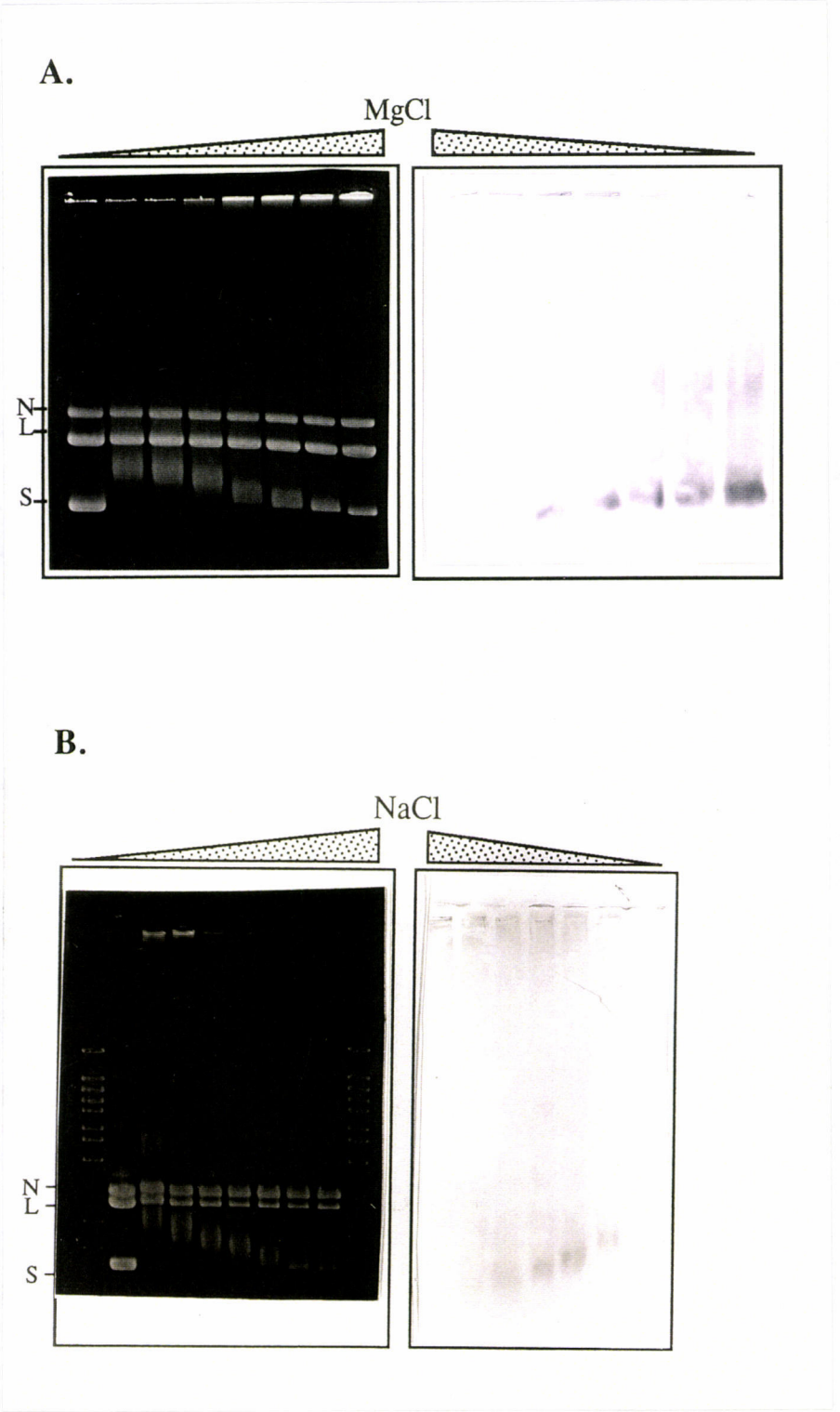
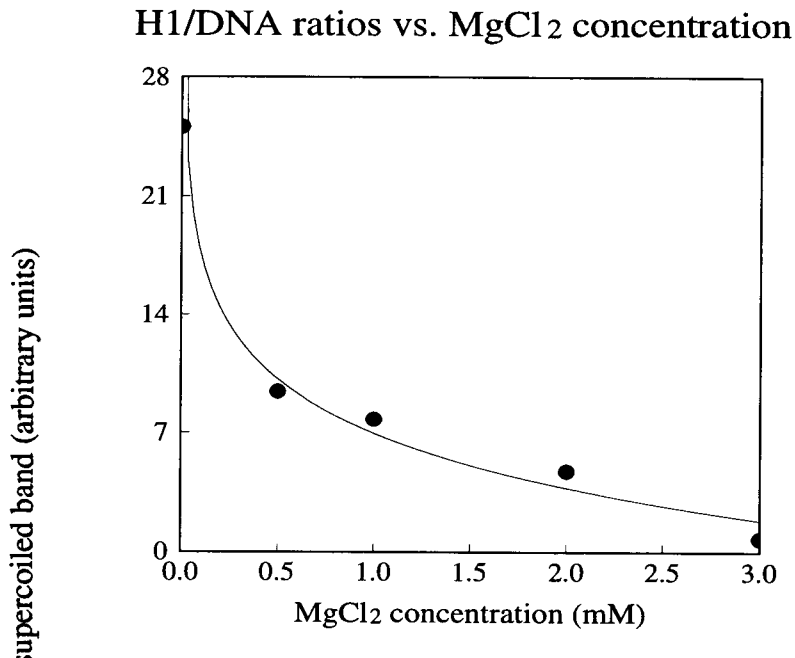


Figure 2.4A, B

C.



D.

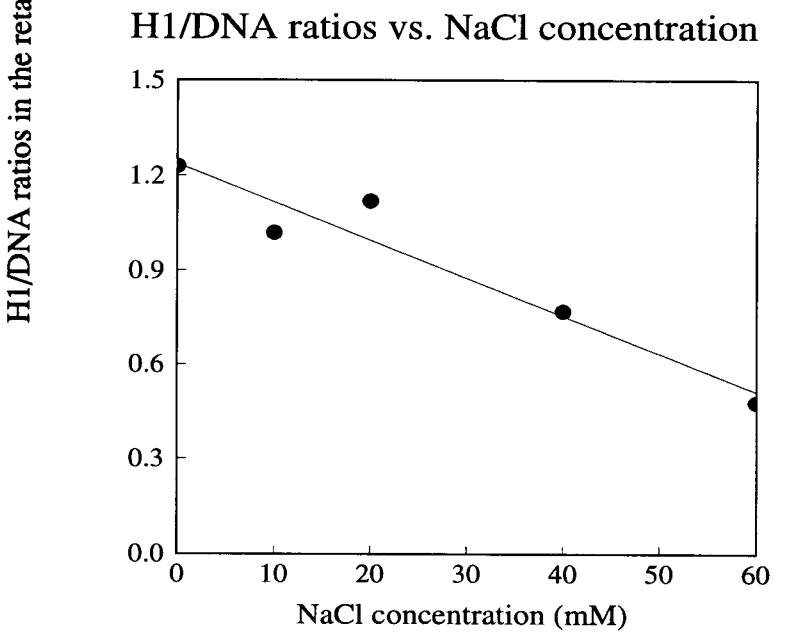


Figure 2.4 C, D

concentration from 10 mM to 60 mM (or 0.5 to 5 mM Mg^{+2}). H1 binding to supercoiled DNA may act in the same way as cations; the positive charges of H1 neutralize some of the phosphates of the DNA resulting in a closer contact between the DNA strands and increasing the potential for braiding the DNA segments together and the formation of the so called "tramline" complexes (Thomas et al., 1992).

It, thus, appears that large conformational changes (compaction) are a general response of supercoiled DNA to increasing the salt concentration. The effects of salt and H1 could be additive in causing compaction of supercoiled DNA.

As mentioned previously, both a decrease in the amount of H1 bound to DNA as well as a compaction of the H1/DNA complex may cause the lower retardation of the supercoiled band at increasing salt levels. Further experiments with linear DNA (at different H1/DNA ratios and varying salt concentrations) would be necessary to better understand and distinguish the effects of these two contributing factors.

Another event happening in the H1/DNA mixtures is the formation of insoluble aggregates. It has been previously shown that titration of pBR322 with H1 fragments containing the C-terminal tail (with most of the positive charges of the molecule) leads to aggregation at much lower ratios than when the globular domain (with or without the N-terminal) is used (De Bernardin et al., 1986). These results support the idea that aggregation occurs as soon as the charges on the DNA are neutralized to some critical level. Upon binding to DNA, H1 reacts with neighboring DNA-bound H1 molecules (via their globular domains) forming a polymer along the axis of the superhelical DNA. This H1/H1 interaction may be either direct (hydrophobic interactions between globular domains) or indirect as a result of conformational changes (bending) of DNA. Thus, H1 polymerization leading to aggregation occurs as a result of the neutralization of DNA phosphate groups (by the

basic C-terminal tail of histones and/or cations). With increasing ionic strength, a shift of the aggregation point towards lower protein/DNA ratios is seen. Thus, cations may partially neutralize the DNA charges leading to protein/protein interactions and aggregation at high salt strengths and the C-terminal might be most important for the binding of H1 to supercoiled DNA, allowing subsequent interaction of the globular domains of neighboring H1 molecules. H1 by itself, which has about 55 positive charges at neutral pH, can not completely compensate the DNA charge at low to moderate levels. This is why salt is required for aggregation. Thus, the charge neutralization of the complex by the help of cations could be an important factor for the aggregation and the compaction of the fiber into higher-order chromatin structure.

The process of packing of chromatin into compacted higher order fibers depends on ionic conditions and the presence of linker histones. The cations as well as the highly basic C-terminal tail of linker histones reduce the electrostatic free energy of the linker DNA, enabling it to become closely packed in the higher order fiber. These activities could clearly contribute to the thermodynamic driving force of chromatin folding. H1 molecules on adjacent nucleosomes in chromatin are in sufficiently close proximity to be chemically crosslinked to each other (Thomas and Khabaza, 1980). H1/H1 interactions may be instrumental in bringing about the salt-induced folding of the nucleosome filament into the compact fiber (Thoma et al., 1979). Condensation and the packing of the linker DNA brings neighboring nucleosomes close together resulting perhaps in their interaction with each other. Chromatin condensation may also bring globular domains of the linker histones into close proximity, at which point the globular domains could polymerize. The above results support the idea that interaction between the globular domains themselves in chromatin may provide the basis for salt-dependent chromatin folding *in vitro*, and stabilization of the compacted fiber *in vivo*.

In summary, we have studied the effect of ionic strength as well as H1/DNA input ratio on the interaction of H1 and supercoiled DNA. It is shown here that the nature of the complexes formed between histones and DNA was dependent on both these factors. Two types of complexes were formed: 1) At lower ionic strengths and low H1/DNA ratios, the H1 molecules seem to bind distributively to all supercoiled DNA molecules, giving soluble complexes that enter the gel. 2) At higher ionic strengths or high H1/DNA ratios, nonsoluble complexes were formed that did not enter the gel and most probably contain more than one DNA molecule. The complex of H1 with DNA may therefore be a useful model for the linker histone/DNA interactions in chromatin, in general. In the next chapter, we will focus on the conformational change of DNA after linker histone binding.

Chapter 3

The Effect of Linker Histones on the Pattern of Single-Strand-Specific Digestion of Supercoiled Plasmids

a) Introduction

Linker histones have been shown to interact preferentially with negatively supercoiled DNA compared to linear or relaxed circular DNA (Vogel and Singer, 1975, 1976; De Bernardin et al., 1986). The interaction of linker histones with DNA is considered to be primarily electrostatic, but structure-specific (Varga-Weisz et al., 1993, 1994; Krylov et al., 1993), as well as sequence-specific (Yaneva et al., 1995) interactions could also play important roles. Thus, for instance, a strong preferential binding of these histones (H1 and H5) to crossovers of double-stranded DNA (Krylov et al., 1993) and to four-way junction DNA (Varga-Weisz et al., 1993, 1994) has been demonstrated. These DNA structures resemble the structure of the DNA at the entry/exit point of the nucleosomal core particle, where linker histones are believed to bind via the two DNA binding sites in their globular domain. The binding of these proteins to DNA cross-overs that exist in superhelical plasmids might explain their preferential binding to superhelical DNA.

Sheflin et al. (1993) have found that HMG1/2 proteins also show a preference to interact with negatively supercoiled DNA. The binding of these proteins (histones and HMGs) to the linker DNA may be a general means of regulating chromatin condensation/decondensation and hence transcription deactivation/activation. The acidic (highly negatively charged) C-terminal of the HMG proteins modulate HMG1/2/core histone interaction to promote nucleosome assembly (Dimov et al., 1990) and HMG1/2/H1 interaction to regulate chromatin condensation (Carballo et al., 1983). Studies by Sheflin et al. (1993) have also

indicated that HMG1/2 proteins affect superhelical plasmids by unwinding negatively supercoiled DNA. In this chapter, I will show and explain the results of the investigation of the relationship between linker histone binding and superhelicity. Another explanation for the preference for supercoiled DNA by linker proteins may be the existence of intrinsic differences in twist between supercoiled and relaxed plasmids. Linker histones may prefer to bind to slightly unwound DNA, perhaps because they unwind that DNA upon binding (see chapter 4).

The change in the pattern of sites accessible to single-strand-specific nucleases (P1 or S1) in supercoiled plasmids upon histone binding was investigated first. S1 or P1 cleave double-stranded DNA where non-B-DNA structures (such as stably unwound AT-rich regions, cruciforms, and B-to-Z junctions) exist. These structures, whose different phosphodiester backbone conformations from standard B-DNA are recognized by the nucleases, have been observed and characterized in supercoiled pBR322 plasmid. Depending on different ionic conditions, temperature, and superhelical density of the plasmid, different sensitive sites in pBR322 will be observed. Since the energy stored in DNA supercoiling directs the presence and stability of these nuclease-sensitive sites, we have looked for changes in the pattern of these sites as indicators of the state of superhelicity. Binding of linker histones to DNA may change DNA superhelicity and, thus, result in changes of the pattern of the sensitive sites. Figure 3.1 shows the scheme of the procedure used here to map the position of P1- or S1-sensitive sites in pBR322 plasmid or its derivative pGCP-36AT, which contains a stretch of $d(AT)_{18}$ insert. These sites are mapped using *Eco*RI and *Ava*I for pBR322 and pGCP-36AT, respectively. For the latter plasmid, since *Eco*RI cleavage site was close to the AT-rich region site, *Ava*I was used instead. Using this procedure, we have observed that the pattern of the sensitive sites in supercoiled plasmids change upon histone binding. Linker histone binding

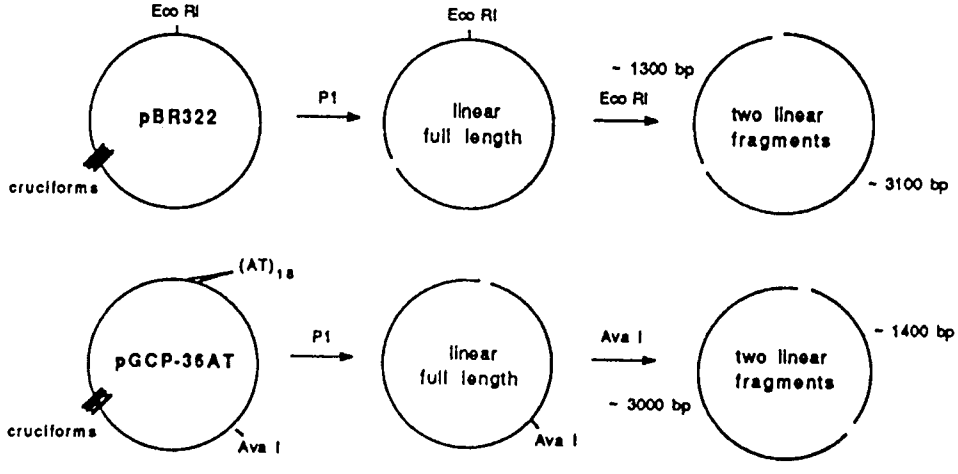


Figure 3.1. Scheme of the procedure used to map the position of P1- or S1-sensitive sites in pBR322 plasmid or its derivative pGCP-36AT, which contains a stretch of $d(AT)_{18}$ insert. These sites were mapped using *Eco*RI and *Ava*I for pBR322 and pGCP-36AT, respectively.

protects some sites from nuclease cutting first, and then new sites are observed at higher linker histone concentrations.

Much of the material in this chapter has appeared in a recent publication, Ivanchenko et al. (1996b). I, Ahmed H. Hassan, am a co-author of this paper.

b) Materials and Methods

Preparation of Linker Histones. Chicken erythrocyte histones H1 and H5 were purified under nondenaturing conditions (Banchev et al., 1991) and checked for purity by SDS-containing polyacrylamide gel electrophoresis (Laemmli, 1970). The globular domains of histone H1 (GH1) and H5 (GH5) were obtained using the method described by Krylov et al. (1993). The concentrations of the protein stock solutions were determined by two methods: spectrophotometric (using an extinction coefficient of 1.85 ml/(cm.mg) at 230 nm (Camerini-Otero et al., 1976)), and scanning of Coomassie-stained polyacrylamide gels, using bovine serum albumin as a standard.

Preparation of Plasmid DNA. Two types of DNA plasmids (pBR322 and pGCP-36AT, in which a synthetic stretch of $(AT)_{18}$ was inserted at the *Hin*dIII site of pBR322) were used. A spontaneously dimerized form of pGCP-36AT was also used. All the plasmids were prepared by CsCl purification, phenol/chloroform extraction, and ethanol precipitation (Maniatis et al., 1982). DNA was preincubated overnight in the appropriate incubation buffer at 16°C to allow the extrusion of the cruciforms (Lilley, 1980).

Single-Strand-Specific Nuclease Cutting: Mapping of the Cleavage Sites. Increasing amounts of histone H1(0-0.8 μg) were added to 0.4 μg of DNA in 60 μl of 20 mM MES-NaOH, pH 6.6, 0.1 mM EDTA, 50 mM NaCl. The ratio of the number of the molecules of histone added to the number of base pairs defines the input ratio. P1 (GIBCO/BRL) digestion was carried out with about 2 units of the enzyme for 1 hour

at 16°C. DNA was then phenol/chloroform extracted and ethanol precipitated, and subjected to restriction nuclease digestion with either *Eco*RI or *Ava*I (Maniatis et al., 1982). For digestion with S1 (GIBCO/BRL), 2 μg of DNA was incubated with increasing amounts of histone H1 in 10 μl of 10 mM Tris-HCl, pH 7.45, 25 mM NaCl, 1 mM $ZnCl_2$ for 15 minutes at room temperature. After addition of 90 μl of 33 mM sodium acetate, pH 4.6, 55 mM NaCl, incubation was continued for another 15 minutes at 37°C. Finally, 8 units of S1 was added and digestion was allowed to proceed for 10 minutes at 37°C. The reaction was stopped by addition of 100 mM Tris-HCl, pH 8.0, 20 mM EDTA, and the DNA was purified and treated with restriction nucleases as above.

Preparation of Topoisomers at Different Average Linking Number. To prepare partially relaxed topoisomers, plasmids were incubated for 4 hours at 37°C with wheat germ topoisomerase I (GIBCO/BRL) in the presence of different concentrations of ethidium bromide (EtBr) (Singleton and Wells, 1982). The reaction conditions were as recommended by the manufacturer. After incubation, EtBr was extracted with butanol, and the DNA was purified by phenol/chloroform extraction and ethanol precipitation.

Gel Electrophoresis. The products of digestion were analyzed by electrophoresis on 1% agarose gels in TBE buffer (Maniatis et al., 1982) at 3 V/cm. Two-dimensional electrophoresis was performed in Tris-acetate/EDTA (Maniatis et al., 1982), or in the same buffer containing 20 mM sodium acetate (Nissen and Reeves, 1995); the concentration of chloroquine in the second dimension was 2.5 μg/ml. Electrophoresis was carried out at 1 V/cm at room temperature.

c) Protection of P1- or S1-Sensitive Sites and Appearance of New Sites in Supercoiled Plasmids by Linker Histone Binding

The pBR322 plasmid molecules, prepared by CsCl purification, are mostly supercoiled; some small amount of nicked molecules are present in the preparations.

Treatment of this pBR322 sample with P1 or S1, resulted in cleavage of sensitive sites and the conversion of all superhelical molecules into nicked or linear DNA molecules. After P1-cutting, digestion of these molecules with *Eco*RI (for pBR322) or *Ava*I (for pGCP-36AT) will show the existence of multiple P1-sensitive sites, as Figure 3.3B shows. At positions 3065, 3221, and 3124, three cruciforms (major, minor, and subminor, respectively) exist (Lilley, 1980), which are cleaved by P1. Another structure sensitive to P1-cutting exists at position 2325 (Sheflin and Kowalski, 1985). In addition to the three cruciforms and the structure at position 2325, other less frequent cutting sites are also present, as weaker bands on the gel indicate (Figure 3.3A, lane 1).

Titration of pBR322 with increasing amounts of histones H5 and GH5 (Figure 3.2A and B), and H1 (Figure 3.3A), followed by P1-cutting and *Eco*RI digestion, demonstrated interesting changes in the pattern of sites susceptible to single-strand-specific nuclease cleavage with changes in the linker histone levels. The globular domain of H5 (GH5) (Figure 3.2B), like the full-length native H5 (Figure 3.2A), protects some sites in pBR322 from P1-cutting initially at low to intermediate histone/DNA ratios. The three cruciforms, cleaved at lower protein levels, were protected at moderate levels. At high H5 (Figure 3.2A) and H1 (Figure 3.3A) levels, new sites susceptible to P1-cutting appeared. As the scheme of figure 3.3B and the gels (Figures 3.2A and 3.3A) show, new P1-sensitive sites appeared at positions 2600-3100, 2150, and 4000 as the level of the linker histones were raised. Linker histones H1 and H5 had the same effect on both the protection of P1-sensitive sites at low levels and the appearance of new sites at higher levels of protein. The position of the new sites in the pBR322 plasmid is the same for both H1 and H5. Titration of pBR322 molecules with GH5, in contrast to titration with the native H5, did not result in the appearance of new P1-sensitive sites at higher protein levels. In other words, with GH5, the bands present at zero protein level

gradually disappeared and no new bands appeared as GH5 levels were increased (Figure 3.2B).

Using S1, instead of P1, a similar effect on the pattern of digestion of pBR322 was observed with slight differences in band intensities. This suggests the effects seen were not due to some peculiarity of P1 digestion and that some intrinsic changes in the pBR322 plasmid occurred upon linker histone binding. Repeating the titration experiment using a pBR322 derivative (pGCP-36AT) further confirmed our result on the effect of linker histones on the cutting of P1-sensitive sites. The $(AT)_{18}$ insert sequence in pGCP-36AT is susceptible to single-strand-specific nucleases (Suggs and Wagner, 1986), since it could adopt a cruciform structure or conformations that are different from A-, B-, or Z-DNA. The scheme of the procedure is shown in Figure 3.1, where *Ava*I is used to map the position of the initial P1-cuttings. Titration with histone H1 (Figure 3.4A) resulted in a gradual protection of the three cruciforms at positions 3065, 3221, 3124, as well as the $(AT)_{18}$ insert. At high H1 levels, new P1-sensitive sites were created corresponding to the sites seen with pBR322. Bands at 3800 bp, as well as bands between 2900 and 3400 bp, corresponding to the band at 2150 and to the sites between 2600 and 3100 bp respectively on the pBR322 map, were observed. Figure 3.4B shows the same titration experiment using a dimerized version of pGCP-36AT plasmid, in which two $(AT)_{18}$ sites are present. Titration of this plasmid with H1 did not result in a complete protection of sites from P1-cutting even at the highest H1 levels. Moreover, no new sites appeared with H1 titration. This could be explained by the presence of two $(AT)_{18}$ inserts, which allow P1 to cut even at high H1 levels. The presence of these readily cleavable sites at all protein levels could prevent the observation of new sensitive sites.

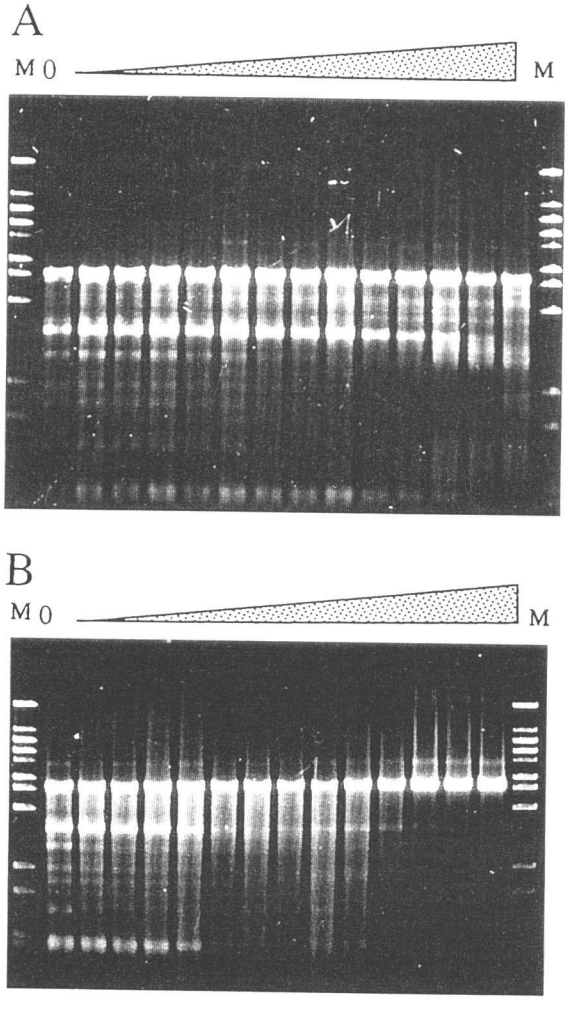


Figure 3.2. Mapping of the position of P1-sensitive sites in pBR322 plasmid by *Eco*RI digestion. Titration of pBR322 plasmid with increasing amounts of histone H5 (A) or GH5 (B) are shown. pBR322 samples were cleaved with P1 in the presence of increasing amount of linker histone, digested with *Eco*RI, and analyzed by 1% agarose gel electrophoresis. Lane M is *Bst*EII-digested lambda DNA used as marker. There is no linker histone in lane 0. The amount of histone H5 and GH5 in the second lane was one molecule of H5 per 2250 bp of DNA (A) and one molecule of GH5 per 950 bp of DNA (B); this amount was increased by 1.5x in each successive lane. The last titration lane had one molecule of H5 per 15 bp (A) and one molecule of GH5 per 7 bp (B).

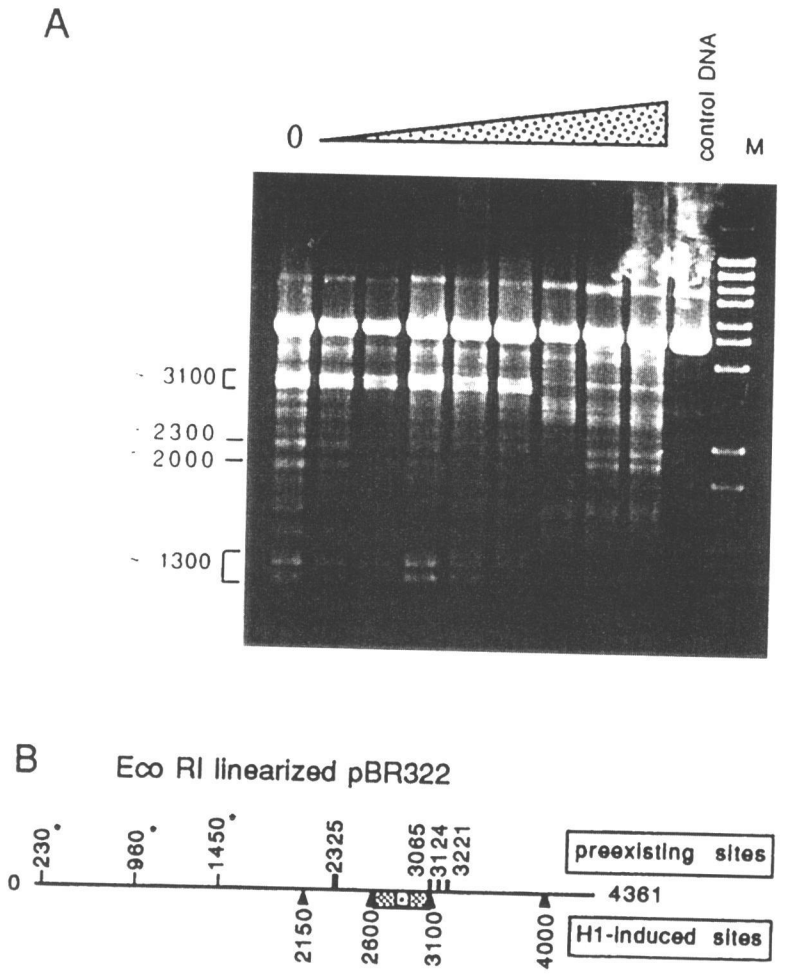


Figure 3.3. (A) Mapping of the position of P1-sensitive sites in pBR322 plasmid by *Eco*RI digestion. Control DNA lane contains *Eco*RI-digested pBR322 (no P1 digestion). There is no histone H1 in lane 0. The amount of H1 in the second lane was one molecule of H1 per 800 bp of DNA and was increased by 1.5x in each successive lane. The last titration lane had one molecule of H1 per 45 bp. The lengths (in base pairs) of the major fragments are shown to the left of the gel. (B) Scheme showing the major P1-sensitive sites (preexisting sites) in pBR322 at native superhelical density and the major H1-induced sites. At positions 3065, 3124, and 3221, three cruciforms identified by Lilley (1980) exist. Another structure sensitive to P1-cutting exists at position 2325, which was identified by Sheflin and Kowalski (1985). In addition to the three cruciforms and the structure at position 2325, other less frequent cutting sites are also present, as bands on the gel (lane 0) indicate. The position 230, 960, and 1450 (marked by asterisks), are potential Z-DNA formation sites; they are not P1-sensitive under the conditions of our experiments.

d) Analysis of the Topoisomers Susceptible to P1 Cleavage at Different H1 Levels

Using two-dimensional gel electrophoresis, one can observe the topoisomers susceptible to single-strand-specific nuclease cleavage in the absence and presence of linker histones. Depending on the degree of superhelical stress (the number of superhelical turns), P1 cleaves the stress-induced sites in a particular plasmid. Figure 3.5A and B show that when P1 was introduced into a mixture of a uniform spread of negatively supercoiled pGCP-AT36 topoisomers (Figure 3.5A), the topoisomers with more than 15 or 16 superhelical turns were cleaved by the nuclease and disappeared from the gel (Figure 3.5B).

Titration of the topoisomers with H1 shows that when the H1 level was increased, the highly negatively supercoiled topoisomers (more than 15 or 16 negative turns) gradually reappeared, indicating that the P1-sensitive sites in these topoisomers were no longer accessible to P1 at increasing H1 to DNA ratios (Figure 3.5C-F). In addition, at very high H1 levels, the highly negatively supercoiled topoisomers disappeared again, suggesting that those topoisomers were once again cleaved by P1 (Figure 3.5G-I). This confirmed our earlier results from the mapping experiments (Figures 3.2 and 3.3): the gradual protection of P1-sensitive sites and the appearance of new sites with increasing protein amounts. How could one explain this alteration in the pattern of sites susceptible to single-strand-specific nucleases upon linker histone binding ?

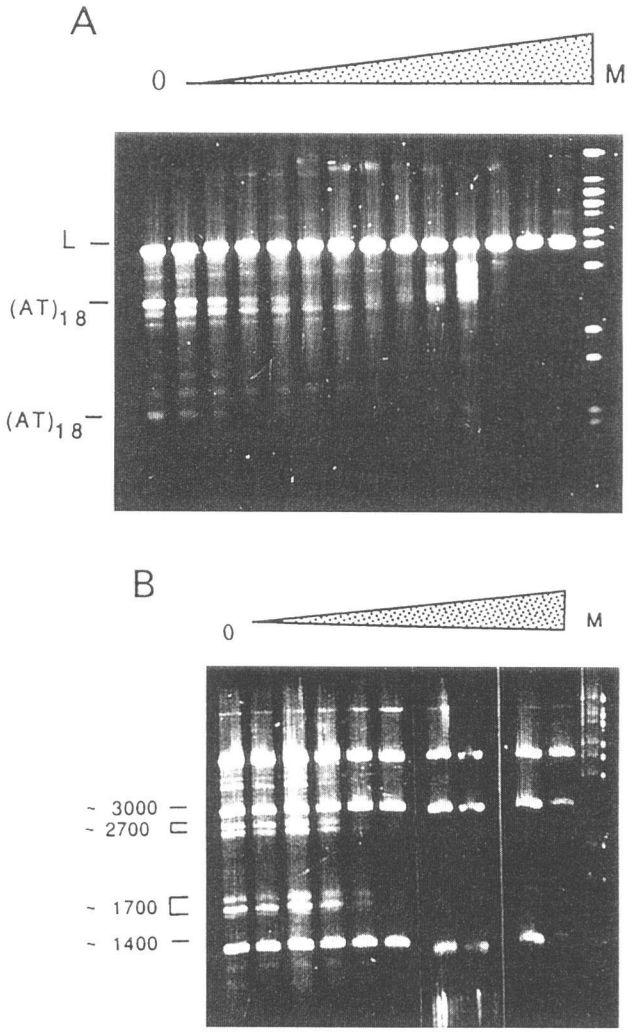


Figure 3.4. Mapping of the position of P1-sensitive sites in pGCP-36AT plasmid (A) or a dimerized version of this plasmid (B) by *Ava*I digestion. As shown in Figure 3.1, *Ava*I is used to digest the plasmids after P1-cutting. Lane M is *Bst*EII-digested lambda DNA used as marker. There is no linker histone in lane 0. The amount of H1 (in A) in the second lane was one molecule of H1 per 2100 bp of DNA and was increased by 1.5x in each successive lane. The last titration lane had one molecule of H1 per 17 bp. Histone H1 amount changed from one H1 molecule per 1500 bp to one H1 per 60 bp in (B). Fragments of about 3000 and 1400 bp were observed with P1 cleavage of the $(AT)_{18}$ insert. Cleavage of the cruciforms present in the pBR322 plasmid, were also seen with these derivative plasmids.

e) Discussion

The disappearance of cleavage sites at intermediate linker histone levels could be explained by either direct histone binding to these sites, resulting in their protection or by a change in the superhelical torsion of the plasmid molecules. Since our results support the latter explanation, we favor that view. Figure 3.5A and B show that in the absence of histone H1, only the higher topoisomers are P1-sensitive and disappear from the electrophoresis gels. It is known that H1 binds to highly supercoiled plasmids with higher affinity (Bina-Stein et al., 1976; Zlatanova et al., 1996). Thus, we would expect that in the presence of H1, the most supercoiled topoisomers would be protected first against P1 cleavage, if the direct H1 binding explanation was correct. However, in the presence of increasing amounts of H1, the least superhelical topoisomers become resistant to P1 cleavage first. This indicates that a change in the superhelical torsion of the plasmid rather than a direct coverage of sites by bound H1 resulted in the loss of sensitivity to P1 (Figure 3.5C-F).

How can we explain the P1-resistance appearance first in the topoisomers at the lower limit of the highly supercoiled plasmid ? If less stress is available to create single-strand-specific sites as a result of H1 binding to the plasmid and absorbing some of the negative superhelical stress in the plasmid, we would expect that the topoisomers at the lower limit of superhelicity would lose their P1-sensitivity first. That is, the less superhelical topoisomers will be P1 resistant first, consistent with Figure 3.5. Therefore, we conclude that the protection of sites at moderate histone levels is more likely due to stress relaxation and disappearance of these sites, rather than direct H1 protection. Results of experiments discussed in chapter 4 indicate that, in fact, H1 unwinds DNA.

How can we explain the creation of new sites at higher linker histone levels? Neither plasmids below 15 superhelical turns (Figure 3.5) nor the globular domain of histones (Figure 3.2B) show creation of new sites. Therefore, these new sites

Figure 3.5. Two-dimensional gel electrophoresis of pGCP-36AT plasmid in the presence of increasing histone H1, showing the change in distribution of topoisomers in the plasmid. A uniform spread of supercoiled topoisomers (from about +3 to about -30 superhelical turns) was prepared by incubating plasmid samples with wheat germ topoisomerase I in the presence of different concentrations of EtBr (Singleton and Wells, 1982). After 4 hours of incubation at 37°C, EtBr was extracted, the DNA was purified, individual samples were mixed, and the population of topoisomers shown in (A) was obtained. Two-dimensional agarose gel electrophoresis was performed in Tris-acetate/EDTA (Maniatis et al., 1982), or in the same buffer containing 20 mM sodium acetate (Nissen and Reeves, 1995); the concentration of chloroquine in the second dimension was 2.5 μg/ml. Electrophoresis was carried out at 1 V/cm at room temperature. (B) Topoisomers with more than 15 or 16 superhelical turns were cleaved by P1 and disappeared from the gel; there is no H1 present in (B). (C-I) P1 cutting in the presence of increasing amounts of H1. The H1/DNA input ratios were 1/1400, 1/700, 1/180, 1/105, 1/70/, 1/45, and 1/33 (one H1 molecule/number of bp), respectively.

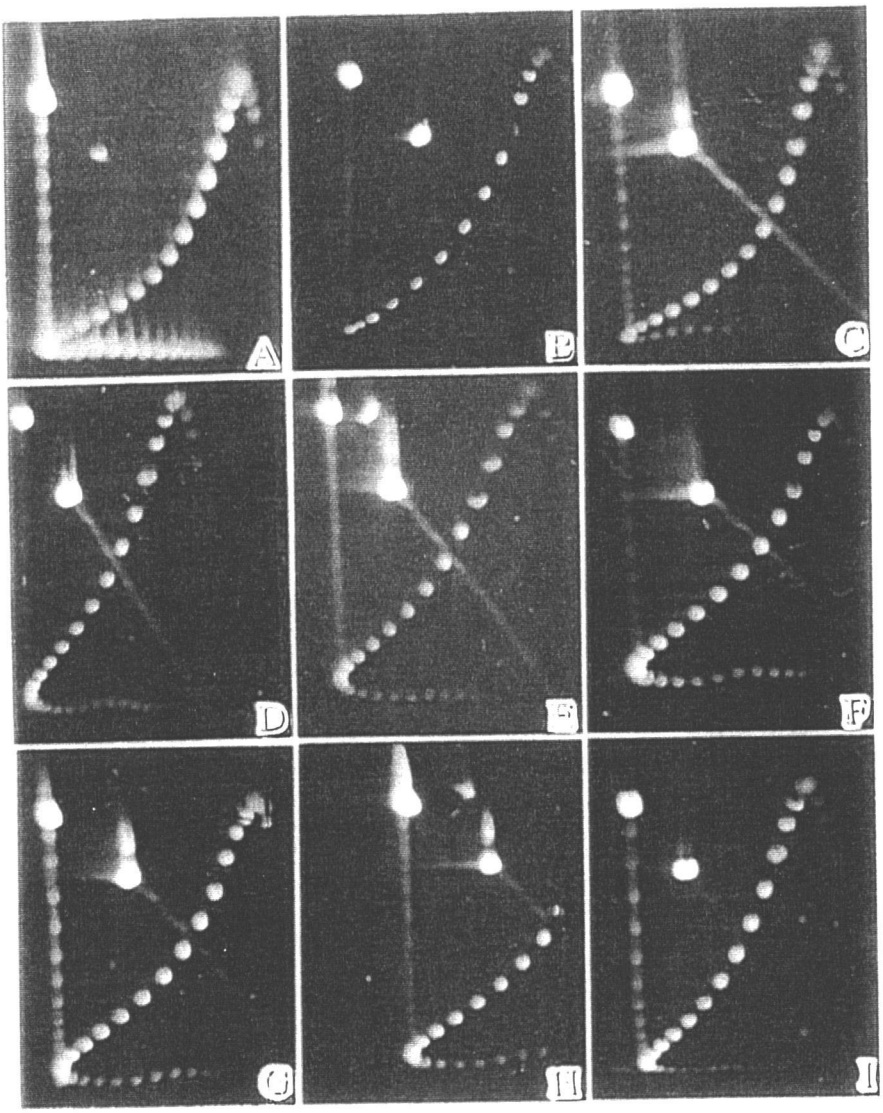


Figure 3.5

require both some level of superhelical torsion and the presence of the intact linker histone, in order to show P1-sensitivity. We believe that the newly appeared sites on the gels at high histone levels contain a high proportion of A and T residues (AT-rich regions). After histone binding and P1 digestion relax the most stable P1-sensitive structures (the cruciforms, etc.), the A (or T) tracts are cleaved and observed on the gel. The interaction of the C-terminal tail of linker histones (which contain DNA-binding motif SPKK) with AT-rich sites on DNA could destabilize B-DNA structure (Takeuchi and Sasamori, 1994), and thus make the structure susceptible to single-strand-specific nuclease cleavage. Support for this conclusion is obtained by the result that the globular domain of linker histone H5 by itself, unlike the full-length native H5, does not produce P1-sensitive sites at higher histone/DNA ratios. The fact that titration with GH5 did not result in the appearance of new P1-sensitive sites, while with H5 it did, suggests the importance of the histone tails in destabilizing the B-conformation of certain nucleotide sequences.

The binding of linker histones to superhelical plasmids change the pattern of sites susceptible to nuclease cleavage. This alteration may be due to both changes in the superhelical torsion of the DNA molecules as well as to stabilization of some P1-sensitive sites by direct linker histone binding. Therefore, this change in DNA superhelicity could play an important role in chromatin fiber condensation upon linker histone binding.

Chapter 4

Linker Histone Binding Unwinds DNA

a) Introduction

Linker histones preferentially bind to superhelical DNA, as mentioned in chapter 3. There, we concluded that binding of histones to superhelical DNA changes the torsion of the DNA molecule by changing its twist or writhe. Titration of superhelical plasmids with increasing linker histone levels results in changes in the pattern of cleavage by single-strand-specific nucleases. The disappearance of P1-sensitive sites at the first half of the titration curve is probably a consequence of absorbing some of the negative superhelical stress in the molecule by the linker histone binding. In other words, histone binding unwinds DNA leading to disappearance of P1-sensitive sites.

Conflicting results from several studies on the binding of linker histones to supercoiled DNA (Stein, 1980; Morse and Cantor, 1986; Sheflin et al., 1993) has led to our investigation of the effect of linker histones on superhelical plasmids upon binding. Two assays (the topoisomerase I-mediated relaxation assay and the ligase-mediated supercoiling assay) were performed in our study in order to find out how the binding of histones effects the superhelicity of DNA. The results, presented below, indicate that linker histones unwind superhelical DNA.

Much of the material in this chapter has appeared in a recent publication, Ivanchenko et al. (1996a). I, Ahmed Hassan, am a co-author of this paper.

b) Materials and Methods

Preparation of Linker Histones. Chicken erythrocyte histones H1 was purified under nondenaturing conditions (Banchev et al., 1991) and checked for purity by SDS-

containing polyacrylamide gel electrophoresis (Laemmli, 1970). The globular domain of histone H1 (GH1) was obtained using the method described by Krylov et al. (1993). The concentrations of the protein stock solutions were determined by two methods: spectrophotometric (using an extinction coefficient of 1.85 ml/(cm.mg) at 230 nm (Camerini-Otero et al., 1976)), and scanning of Coomassie-stained polyacrylamide gels, using bovine serum albumin as a standard.

Preparation of Plasmid DNA. pBR322 plasmid was prepared by CsCl purification, phenol/chloroform extraction, and ethanol precipitation (Maniatis et al., 1982).

Topoisomerase I-mediated Relaxation Assay. Two μg of pBR322 were relaxed by incubation, at 37°C, with 2-6 units of calf thymus topoisomerase I (Life Technologies, Inc.) in 240 μl of reaction mixture containing 50 mM Tris-HCl, pH 7.5, 50 mM NaCl, 0.1 mM EDTA, 1 mM dithiothreitol, 10% glycerol, and increasing amounts of histone H1 for the time specified in the figures. After incubation, NaCl and SDS were added to final concentrations of 1 M and 1%, respectively, and the DNA was purified by phenol/chloroform extraction and ethanol precipitation. The resulting topoisomer distributions were analyzed by two-dimensional gel electrophoresis.

Ligase-mediated Supercoiling Assay. Nicked DNA containing a limited number of nicks per molecule was prepared by digestion of pBR322 with DNase I (0.068 unit/μg of DNA) in the presence of 0.3 mg of EtBr/ml for 1 hour at 30°C (Clark and Felsenfeld, 1991). The reaction was stopped by adding EDTA to 3 mM, and the DNA was purified by phenol/chloroform extraction and ethanol precipitation. The nicked preparation contained no supercoiled topoisomers and only trace amounts of linear molecules. For the ligation reaction, 2 μg of nicked DNA were incubated with 6 units of T4 or *E. coli* ligase (New England Biolabs), in the buffers recommended by the manufacturer; the concentration of $MgCl_2$ was reduced to 2 mM to improve the binding of H1 to DNA. Reactions were allowed to proceed for 1 hour at 32°C

for the T4 ligase and at 25°C for the *E. coli* ligase. After purification, DNA was analyzed by two-dimensional agarose-gel electrophoresis.

Gel Electrophoresis. DNA was analyzed by electrophoresis on 1% agarose gels in TBE buffer (Maniatis et al., 1982) at 3 V/cm. Two-dimensional electrophoresis was performed in Tris-acetate/EDTA (Maniatis et al., 1982), or in the same buffer containing 20 mM sodium acetate (Nissen and Reeves, 1995); the concentration of chloroquine in the second dimension was 2.5 μg/ml. Electrophoresis was carried out at 1 V/cm at room temperature. Gels were washed for several hours to remove the chloroquine, stained with 1 μg of EtBr/ml, and photographed using type 55 positive/negative Polaroid film. Scanning was performed on an EPSON ES-1200C scanner using the Adobe Photoshop 3.0 program (Adobe Systems, Inc., Mountain View, CA).

Immunoblotting. Mixtures of 1.75 μg of pBR322 and correspondingly, 1.6, 3.2, 6.4, and 9.6 μg of H1 were prepared in the ligase buffer and incubated for 15 minutes at room temperature. The protein/DNA complexes on the agarose gels were transferred to nitrocellulose membrane as described by Ivanchenko and Zlatanova (1996) and visualized by immunostaining using anti-H1 antiserum. The spots on each membrane were scanned and the band intensity was determined using Adobe Photoshop 3.0 and NIH Image 1.57 programs.

c) Topoisomerase I-mediated Relaxation Assay

As shown in Figure 4.1, topoisomerase I will relax supercoiled plasmids so that a narrow distribution of topoisomers centered around the relaxed topoisomer ($\Delta L_k=0$) is produced. However, the distribution of topoisomers in the presence of a ligand may not be centered around the relaxed topoisomer. In the presence of EtBr, negative superhelicity remains after the relaxation reaction is complete and the ligand

is removed. Thus, EtBr is considered an unwinding ligand. The opposite is true for netropsin, as indicated in Figure 4.1.

By changing the twist (in the case of the ligands in Figure 4.1) or the writhe (in the case of nucleosome core particles binding to relaxed DNA) of DNA positively or negatively, the ligands will affect DNA superhelicity. Using two-dimensional gel electrophoresis, one can resolve a wide range of topoisomers (from -15 to +15) (Bowater et al., 1992). Using this technique, we obtained interesting information on the binding of linker histones to DNA. The topoisomerase I-mediated relaxation assay (Figure 4.2) shows that with increasing H1 levels, the distribution of topoisomers are shifted to more highly negatively superhelical forms. As Figure 4.2 shows, in the absence of H1, the relative proportions of the supercoiled and relaxed populations depend on the amount of topoisomerase present in the incubation mixtures (compare Figure 4.2A with 4.2B, lanes marked 0). The same overall result is observed as a result of H1 titration in both cases: H1 binding leads to a shift in the distribution to topoisomers to less relaxed forms and thus, apparently unwind the DNA molecules.

To find out if the one hour incubation time was enough to reach the end point of the reaction, the kinetics of the topoisomerase I-mediated relaxation reaction at different levels of histone H1 was followed, with the results shown in Figure 4.3. A fast initial isomerization was observed either in the absence of H1 or in its presence at levels up to 1 molecule of H1 per 40 bp (Figure 4.3, three top panels). Only in the presence of even higher amounts of H1 (1 molecule of H1 per 30 bp), topoisomerase I started relaxing the supercoiled DNA after some time (Figure 4.3, bottom panel). These kinetics experiments show that treating superhelical plasmid molecules with topoisomerase I between 5 to 15 minutes was sufficient to reach the reaction end point. Most of the changes in the topology of the starting superhelical

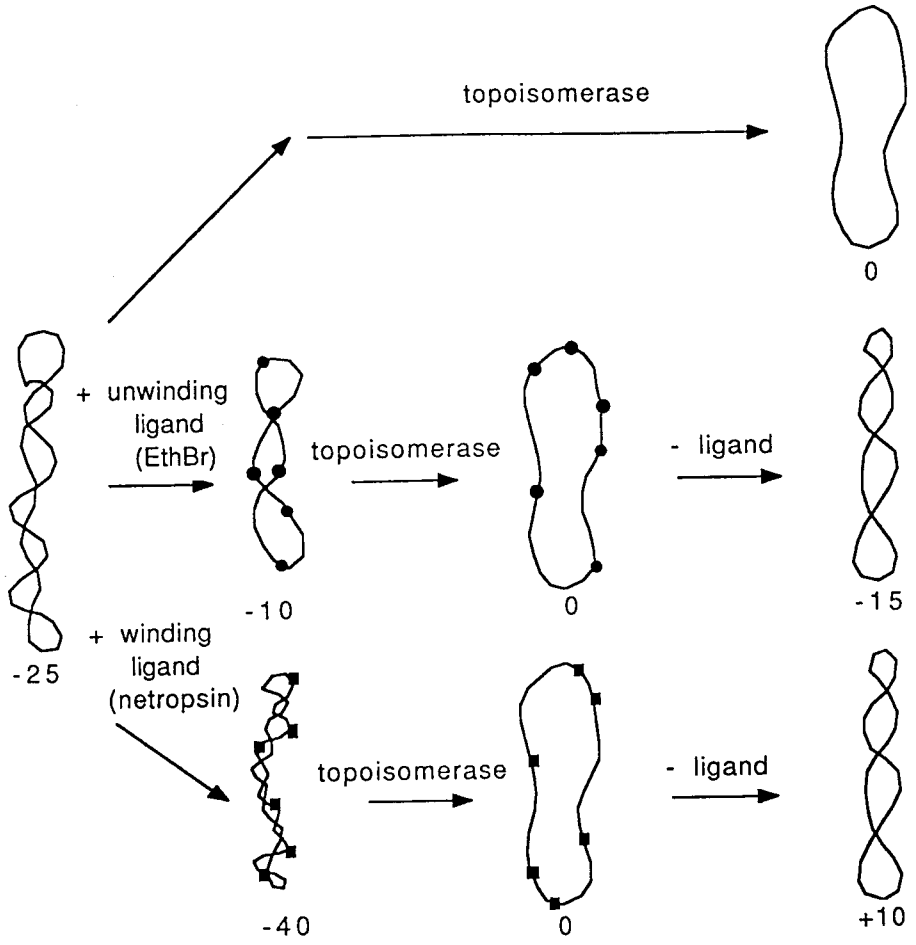


Figure 4.1. Scheme of the principle of the topoisomerase I-mediated relaxation assay. Topoisomerase I will relax supercoiled plasmids so that a narrow distribution of topoisomers centered around the relaxed topoisomer (ΔL_k=0) is produced. However, the distribution of topoisomers in the presence of a ligand that affects superhelicity will not be centered around the relaxed topoisomer. In the presence of EtBr, negative superhelicity remains after the relaxation reaction is complete and the ligand is removed. Thus, EtBr is considered an unwinding ligand. The opposite is true for netropsin.

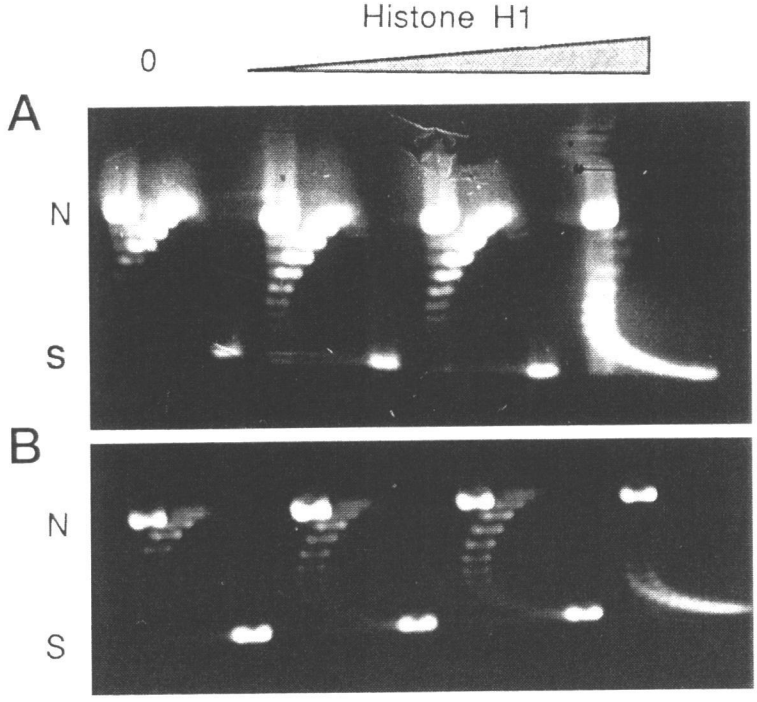


Figure 4.2. Topoisomerase I-mediated relaxation assay. In the presence of increasing amounts of histone H1 (0, 1/220, 1/90, and 1/25 molecule of H1 per bp, respectively, for each consecutive lane), supercoiled pBR322 was relaxed with topoisomerase I for 1 hour at 37°C. Using two-dimensional agarose gel electrophoresis (in Tris acetate/EDTA buffer plus 20 mM sodium acetate) (Nissen and Reeves, 1995) the topoisomers were resolved and analyzed. The concentration of chloroquine in the second dimension was 2.5 μg/ml. Electrophoresis was carried out at 1 V/cm at room temperature. The amount of topoisomerase I used to relax the supercoiled plasmids was 6 (A), or 2 (B) units. N and S indicate the position of nicked and linear supercoiled plasmids, respectively.

population did take place during the initial period of the relaxation reaction. Thus, the changes that occurred at longer times as well as with higher H1 levels can be ignored since they are insignificant. At different H1 levels, when comparing the topoisomer patterns obtained during the initial 5 minutes, the superhelicity end point was different as indicated by Figure 4.3B. A shift is observed in the center of the distributions to increasing negative values with increasing H1 levels.

d) Ligase-mediated Supercoiling Assay

In order to confirm and strengthen our topoisomerase I results, an alternative assay, the ligation assay, was performed. Nicked DNA containing a break in one of the strands and produced by DNase I treatment is the substrate in this assay. Titration of the DNA molecules with histone H1 (or no H1, control) was followed by T4 ligase treatment and H1 removal. In the absence of histone H1, a Boltzmann distribution of topoisomers should be produced (Pulleyblank et al., 1975; Depew and Wang, 1975). However, in the presence of increasing amounts of H1, a change in the distribution of the topoisomers relative to the control (no H1) was detected.

This change was better seen with the globular domain of H1 (GH1) (Figure 4.4B) than with full-length native H1 (Figure 4.4A), partly because the whole protein at higher levels inhibits ligase more than the globular domain alone (Ray et al., 1996). However, both produced the same result: the peak of the distribution of the topoisomers moved to more negative values with increasing H1 (at no H1, under our experimental conditions, all the topoisomers were shown to be positively supercoiled, since the conditions of the reaction and the gel were different). This suggests that H1 unwinds the DNA molecules, consistent with the results from the topoisomerase I-mediated relaxation assay. The same shift is observed when *E. coli* ligase (instead of T4 ligase) or H5 (instead of H1) was used. Figure 4.4C clearly shows that the center of the topoisomer distribution shifts from about +4 in the

Figure 4.3. (A) Kinetics of topoisomerase I-mediated relaxation assay at different histone H1/DNA ratios (0, 1/50, 1/40, and 1/30). Lane M contains the marker ladder of consecutive topoisomers from 0 to about 30 negative superhelical turns (see legends to Figure 3.5). Lane C is the starting supercoiled population. (B) Quantitation of the topoisomer distributions obtained for the 5 min incubation with topoisomerase I. The histone H1/DNA ratios in a, b, and c are 0, 1/50, and 1/40, respectively. The sum of topoisomers -1, 0, and +1 is indicated by a bar at zero, since these topoisomers are not well resolved on the gels. The centers of the distributions are shown by arrows.

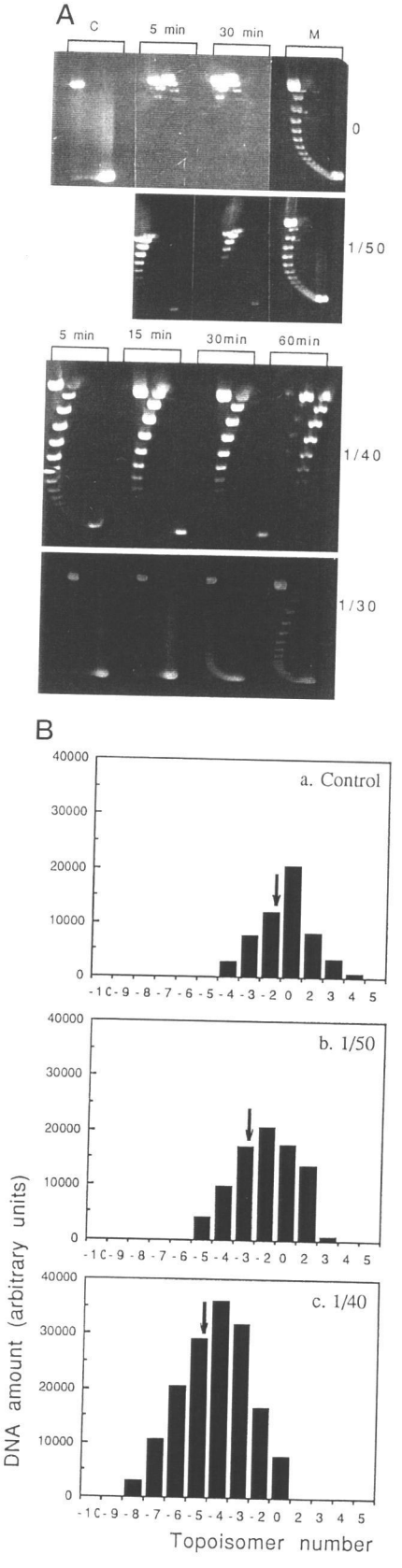

A
C
5 min
30 min
M
0
1/50
5 min
15 min
30min
60min
1/40
1/30
B
a. Control
b. 1/50
c. 1/40
40000
30000
20000
10000
0
-10 -9 -8 -7 -6 -5 -4 -3 -2 0 2 3 4 5
DNA amount (arbitrary units)
Topoisomer number

Figure 4.3

absence of GH1 to about +1 in the presence of 1 molecule of GH1 per 20 bp. Therefore, with certainty, we can now say that linker histones unwind DNA, based on the two different assays performed.

A possible effect of histones on the activity of topoisomerase I may complicate the interpretation of the observed results, using the topoisomerase I assay (see above). In some studies, it has been reported that histone H1 inhibits topoisomerase I (Richter and Kapitza, 1991; Kordiyak et al., 1994), while others show either no significant effect (Bina-Stein and Singer, 1977), or enhancement (Javaherian and Liu, 1983; Turna et al., 1994) of the activity of the enzyme. On the other hand, with the ligase assay, our results can not be affected by possible inhibition of ligase by histones, since that causes more DNA to remain at the position of the nicked circles, as shown in Figure 4.4A. With this ligase assay, any shift in the distribution of the ligated products relative to the control (no H1) will be due only to H1 changing the topology of the DNA molecules.

e) Discussion

Can we estimate the unwinding angle produced by binding of one H1 molecule to either suprcoiled or nicked DNA molecules ? To calculate the unwinding angle, two assumptions should be made here: that all histones are bound and that all DNA molecules bind the same amount of histones. These assumptions are supported by previous studies. Studying the binding of H1 to linear DNA, Watanabe (1986) showed that over 90% of the histones present in the solution would be bound under our conditions. With supercoiled DNA, we can comfortably assume that all the histones are bound. Sucrose gradient experiments performed under similar conditions also show that there is no free H1 at the top of the gradient (not shown).

To confirm the results from the published studies, we performed a semi-quantitative evaluation of the amount of histone present in the H1/DNA complexes.

Figure 4.4. Ligase-mediated supercoiling assay. In the presence of increasing amounts of histone H1 (0, 1/70, 1/50, and 1/30 molecule of H1 per bp, respectively, for each consecutive lane) (A) or GH1 (0, 1/50, 1/33, 1/20 molecule of GH1 per bp) (B), nicked pBR322 (prepared by mild DNase I treatment) was ligated with T4 ligase. Using two-dimensional agarose gel electrophoresis (in Tris acetate/EDTA buffer plus 20 mM sodium acetate) (Nissen and Reeves, 1995) the topoisomers were resolved and analyzed. The concentration of chloroquine in the second dimension was 2.5 μg/ml. Electrophoresis was carried out at 1 V/cm at room temperature. Since the globular domain inhibited T4 ligase less than the intact protein, higher protein/DNA ratios could be tested. Schemes to the right of the gels show the topoisomers produced in the ligase-mediated reaction. (C) Quantitation of the topoisomer distributions obtained in the GH1 titration. The histone H1/DNA ratios in a, b, c, and d are 0, 1/50, 1/33, and 1/20, respectively. The sum of topoisomers -1, 0, and +1 is indicated by a bar at zero, since these topoisomers are not well resolved on the gels. This is why this bar is large. The centers of the distributions are shown by arrows.

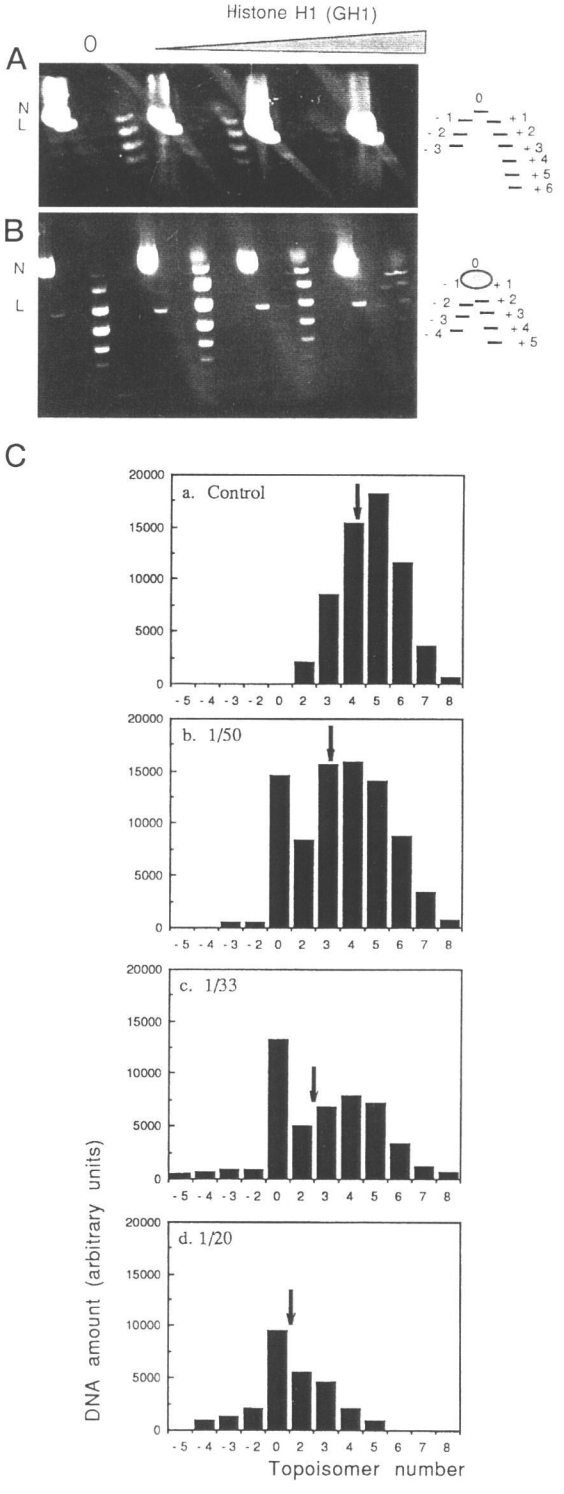

Histone H1 (GH1)
0
A
N
L
0
- 1
+ 1
- 2
+ 2
- 3
+ 3
+ 4
+ 5
+ 6
B
N
L
0
- 1
+ 1
- 2
+ 2
- 3
+ 3
- 4
+ 4
+ 5
C
a. Control
b. 1/50
c. 1/33
d. 1/20
20000
15000
10000
5000
0
-5 -4 -3 -2 0 2 3 4 5 6 7 8
DNA amount (arbitrary units)
Topoisomer number

Figure 4.4

To this end, H1/DNA complexes were transferred to nitrocellulose membrane as described by Ivanchenko and Zlatanova (1996) and visualized by immunostaining using anti-H1 antiserum. Figure 4.5 shows no difference in the H1 level, when H1 in a complex with DNA (under ligase conditions) was visualized by western blotting and compared to the same amount of H1 directly applied to nitrocellulose membrane.

Figure 4.6 graphs the topoisomer number at the center of the distribution produced by topoisomerase I relaxation (Figure 4.3B) or ligase supercoiling (Figure 4.4C) as a function of H1/DNA ratio. Using the slope of the lines from this graph in the below equation, we can estimate the degree of rotation corresponding to one H1 molecule.

Unwinding angle produced by binding = Slope (360°/4363), where 4363 is the length of the plasmid in bp.

of one H1 molecule to DNA

The slope gives the change in the number of superhelical turns per H1 molecule per bp. Using this equation, an unwinding angle of 8.0°/H1 molecule bound to superhelical DNA is obtained. For GH1, an unwinding angle of 5.0°/ protein molecule bound to nicked DNA is calculated, suggesting that the tails of linker histones may contribute to the unwinding of DNA by the protein.

The unwinding of DNA by linker histones is relatively small compared to the unwinding angle obtained for a non-histone protein (HMG 1/2); the unwinding of the negatively supercoiled DNA by HMG 1/2 is 58° (Sheflin et al., 1993). However, although small, the unwinding be H1 is real, since it has been shown by two different assays. It is crucial to consider this effect in the studies of chromatin fiber structure. More studies, however, are needed to reveal the mechanism behind this unwinding of DNA by linker histones.

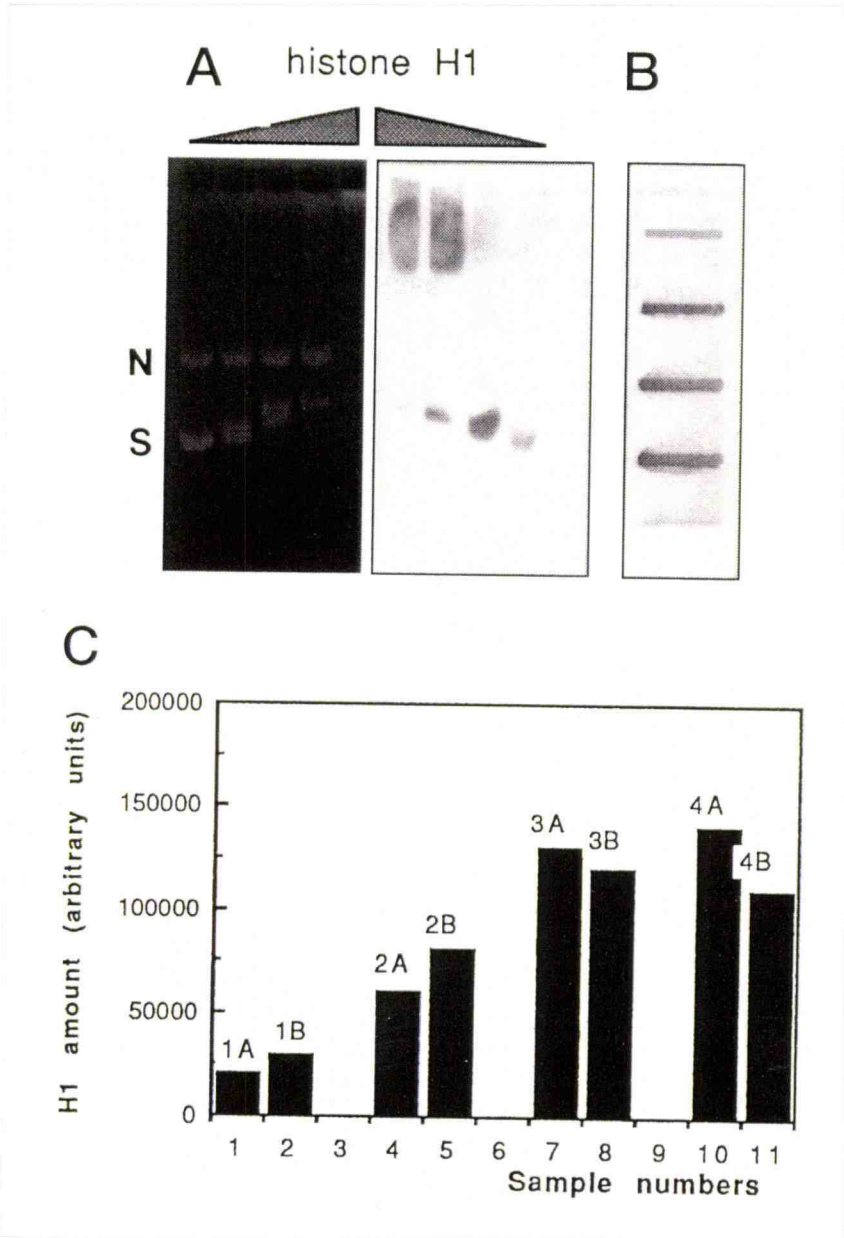


Figure 4.5. (A) Agarose gel nitrocellulose replica stained with anti-H1 antibody. N and S show the positions of the nicked and the supercoiled pBR322 bands, respectively. (B) The same amount of histone H1 as in (A) directly applied to nitrocellulose membrane by slot-blotting. (C) Graph comparing the intensity of histone H1 spots in (A) (nitrocellulose membrane) and with the same in (B).

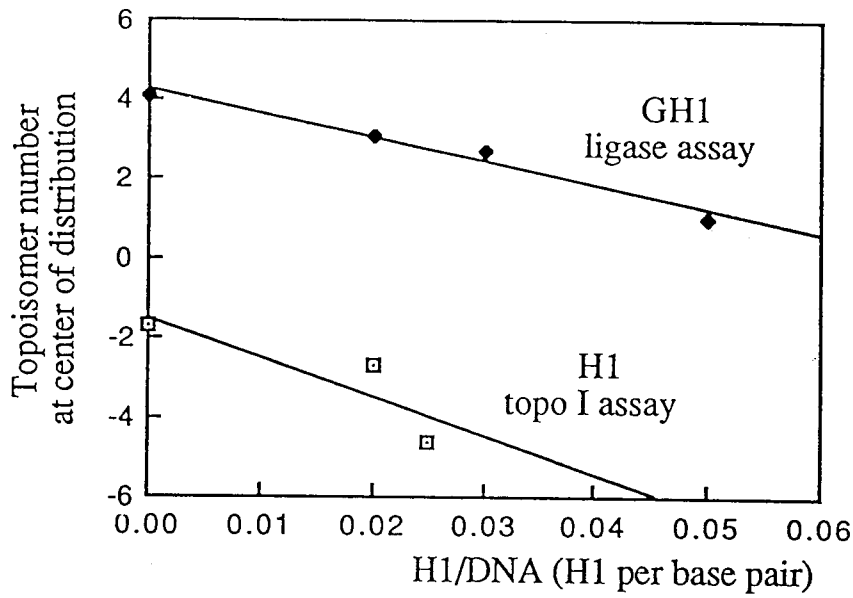


Figure 4.6. Graph of the topoisomer number at the center of distribution by topoisomerase I relaxation (Figure 4.3B) or ligase supercoiling (Figure 4.4C) as a function of the histone H1/DNA ratio. The lower and the upper lines correspond to the unwinding produced by H1 and GH1, respectively. The slope of the lines give the change in the number of superhelical turns per H1 molecule per bp.

What is the significance of this unwinding of supercoiled DNA by linker histones? Some very recent studies have indicated that H1 may modulate the DNA-binding properties of other proteins (such as transcription factors). Spiker et al. (1996) showed that in wheat H1 enhances the DNA-binding activity of a transcription factor (EmBP-1). In addition, Zaret et al. (personal communication to J. Zlatanova) found that the affinity of HNF3 for the core particle increases in the presence of H1 on the nucleosome. Both of these studies suggest that the presence of histone H1 is important in the effective binding ability of other proteins. Thus, it appears that the linker histones by changing the conformation of the DNA, perhaps, expose regions of DNA that otherwise are inaccessible to some transcription factors. Furthermore, it is possible that the generation of positive superhelical torsion ahead of a moving RNA polymerase during transcription helps release H1 (which in view of its unwinding activity would be expected to have a reduced affinity to overwound DNA) and permit this large complex to move on.

Bibliography

Adrian, M., B. ten Heggeler-Bordier, W. Wahli, A. Z. Stasiak, A. Stasiak and J. Dubochet (1990). *EMBO J.* 9, 4551.

Allan, J., P. J. Hartman, C. Crane-Robinson and F. X. Aviles (1980). *Nature* 288, 675.

Allan, J., T. Mitchell, N. Harborne, L. Böhm and H. Gould (1986). *J. Mol. Biol.* 187, 591.

Banchev, T., L. Srebreva and J. Zlatanova (1991). *Biochem. Biophys. Acta* 1073, 230.

Bednar, J., P. Furrer, A. Stasiak and J. Dubochet (1994). *J. Mol. Biol.* 235, 825.

Belyavsky, A. V., S. G. Bavykin, E. G. Goguadze and A. D. Mirzabekov (1980). *J. mol. Biol.* 139, 519.

Bina-Stein, M. and M. F. Singer (1977). *Nucleic Acids Res.* 4, 117.

Bina-Stein, M., T. Vogel, D. S. Singer and M. F. Singer (1976). *J. Biol. Chem.* 251, 7363.

Boulikas, T., J. M. Wiseman and W. T. Garrard (1980). *Proc. Natl. Acad. Sci.* USA 77, 127.

Bowater, R., F. Aboul-ela and D. M. J. Lilley (1992). *Methods Enzymol.* 212, 105.

Braddock, G. W., J. P. Baldwin and E. M. Bradbury (1981). *Biopolymers* 20, 327.

Buckle, R. S., J. D. Maman and J. Allan (1992). *J. Mol. Biol.* 223, 651.

Bustin, M. and R. Reeves (1996). *Prog. in Nucleic Acid Res. and mol. Biol.* 54, 35.

Camerini-Otero, R. D., B. Sollner-Webb and G. Felsenfeld (1976). *Cell* 8, 333.

Carballo, M., P. Puigdomenech and J. Palau (1983). *EMBO J.* 2, 1759.

Cerf, C., G. Lippens, V. Ramakrishnan, S. Muyldermans, A. Segers, L. Wyne, S. J. Wodak and K. Hallenga (1994). *Biochemistry* 33, 11079.

Clark, D. J. and G. Felsenfeld (1991). *EMBO J.* 10, 387.

Clark, D. J. and J. O. Thomas (1986). *J. Mol. Biol.* 187, 569.

Clark, D. J. and J. O. Thomas (1988). *Eur. J. Biochem.* 178, 225.

Clark, D. J., C. S. Hill, S. R. Martin and J. O. Thomas (1988). *EMBO J.* 7, 69.

Clark, K. L., E. D. Halay, E. Lai and S. K. Burley (1993). *Nature* 364, 412.

Clore, G. M., A. M. Gronenborn, M. Nilges, D. K. Sukumaran and J. Zarbock (1987). *EMBO J.* 6, 1833.

Cole, R. D. (1987). *Int. J. Peptide Protein Res.* 30, 433.

De Bernardin, W., R. Losa and T. Koller (1986). *J. Mol. Biol.* 189, 503.

Delange, R. J., D. M. Farnbrough, E. L. Smith and J. Bonner (1969a). *J. Biol. Chem.* 244, 319.

Delange, R. J., D. M. Farnbrough, E. L. Smith and J. Bonner (1969b). *J. Biol. Chem.* 244, 5669.

Depew, R. E. and J. C. Wang (1975). *Proc. Natl. Acad. Sci.* USA 72, 4275.

Diez-Caballero, T., F. X. Aviles and A. Albert (1981). *Nucleic Acids Res.* 9, 1383.

Dimov, S. I., E. A. Alexandrova and B. G. Beltchev (1990). *Biochem. Biophys. Res. Commun.* 166, 819.

Dravers, P. H., P. T. Lowary and J. Widom (1992). *J. Mol. Biol.* 225, 1105.

Espel, E., J. Bernues, J. A. Perez-Pons and E. Querol (1985). *BBRC* 132, 1031.

Fenley, M. O., W. K. Olson, I. Tobias and G. S. Manning (1994). *Biophys. Chem.* 50, 255.

Finch, J. T. and A. Klug (1976). *Proc. Natl. Acad. Sci.* USA 73, 1897.

Freeman, L. A. and W. T. Garrard (1992). *Eukaryotic Gene Expr.* 2, 165.

Garcia-Ramirez, M., F. Dong and J. Ausio (1992). *J. Biol. Chem.* 267, 19587.

Gebe, J. A., J. J. Delrow, P. J. Heath, B. S., Fujimoto, D. W. Stewart and J. M. Schurr (1996). *J. Mol. Biol.* 262, 105.

Goytisolo, F. A., S. E. Gerchman, X. Yu, C. Rees, V. Graziano, V. Ramakrishnan and J. Thomas (1996). *EMBO J.* 15, 3421.

Hamiche, A., P. Schultz, V. Ramakrishnan, P. Oudet and A. Prunell (1996). *J. Mol. Biol.* 257, 30.

Hayes, J. J. and A. P. Wolffe (1993). *Proc. Natl. Acad. Sci.* USA 90, 6415.

Hayes, J. J., D. Pruss and A. P. Wolffe (1994). *Proc. Natl. Acad. Sci.* USA 91, 7817.

Ivanchenko, M., A. Hassan, K. van Holde and J. Zlatanova (1996a). *J. Biol. Chem.* 271, 32580.

Ivanchenko, M. and J. Zlatanova (1997). *Electrophoresis* 18, 72.

Ivanchenko, M., J. Zlatanova and K. van Holde (1997).*Biophysical J.* 72, 1388.

Ivanchenko, M., J. Zlatanova, P. Varga-Weisz, A. Hassan and K. van Holde (1996b). *Proc. Natl. Acad. Sci.* USA 93, 6970.

Javaherian, K. and L. F. Liu (1983). *Nucleic Acids Res.* 11, 461.

Kordiyak, G. T., S. Jakes, T. S. Ingebritsen and R. M. Benbow (1994). *Biochemistry* 33, 13484.

Krylov, D., S. Leuba, K. van Holde and J. Zlatanova (1993). *Proc. Natl. Acad. Sci.* USA 90, 5052.

Laemmli, U. K. (1970). *Nature* 227, 680.

Lilley, D. M. J. (1980). *Proc. Natl. Acad. Sci.* USA 77, 6468.

Maniatis, T., E. F. Fritsch and J. Sambrook (1982). *Molecular cloning: a laboratory manual*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Mathews, C. K. and K. van Holde (1995). *Biochemistry*, second edition. The Benjamin/Cummings publishing company, inc.

Mc Ghee, J. D., J. M. Nickol, G. Felsenfeld and D. C. Rau (1983). *Cell* 33, 831.

Mc Ghee, J., D. C. Rau, E. Charney and G. Felsenfeld (1980). *Cell* 22, 87.

Miki, B. L. and J. M. Neelin (1975). *Can. J. Biochem.* 53, 1158.

Mirzabekov, A., D. V. Prus and K. K. Ebralidse (1989). *J. Mol. Biol.* 211, 479.

Morse, R. H. and C. R. Cantor (1986). *Nucleic Acids Res.* 14, 3293.

Neelin, J. M., P. X. Callahan, D. C. Lamb and K. Murray (1964). *Can. J. Biochem.* 42, 1743.

Newrock, K. M., C. R. Alfageme, R. V. Nardi and L. H. Cohen (1978). *CSHSQB* 42, 421.

Nissen, M. S. and R. Reeves (1995). *J. Biol. Chem.* 270, 4355.

Pruss, D., J. J. Hayes and A. P. Wolffe (1995). *BioEssays* 17, 161.

Pulleyblank, D. E., M. Shure, D. Tang, J. Vinograd and H. P. Vosberg (1975). *Proc. Natl. Acad. Sci.* USA 72, 4280.

Ramakrishnan, V., J. T. Finch, V. Graziano, P. L. Lee and R. M. Sweet (1993). *Nature* 362, 219.

Ray, E., J. Yaneva, M. Ivanchenko, K. van Holde and J. Zlatanova (1996). *Biochem. Biophys. Res. Commun.* 222, 512.

Richmond, T. J., J. T. Finch, B. Rushton, D. Rhodes and A. Klug (1984). *Nature* 311, 532.

Richter, A. and M. Kapitza (1991). FEBS *Lett.* 294, 125.

Ring, D. and R. D. Cole (1983). *J. Biol. Chem.* 258, 15361.

Rodriguez, A. T., L. Perez, F. Moran, F. Montero and P. Suau (1991). *Biophysical Chemistry* 39, 145.

Rybenkov, V. V., N. R. Gozzarelli and A. V. Vologodskii (1993). *Proc. Natl. Acad. Sci* USA 90, 5307.

Schlick, T., B. Li and W. K. Olson (1994). *Biophys. J.* 67, 2146.

Schultz, S. C., G. C. Shields and T. A. Steitz (1991). *Science* 253, 1001.

Segers, A., L. Wyns and I. Lasters (1991). *Biochem. Biophys. Res. Commun.* 174, 898.

Seyedin, S. M. and W. S. Kistler (1980). *J. Biol. Chem.* 255, 5949.

Shaw, S. Y. and J. Wang (1993). *Science* 260, 533.

Sheflin, L. G. and D. Kowalski (1985). *Nucleic Acids Res.* 13, 6137.

Sheflin, L. G., N. W. Fucile and S. W. Spaulding (1989). *Biochemistry* 28, 5658.

Sheflin, L. G., N. W. Fucile and S. W. Spaulding (1993). *Biochemistry* 32, 3238.

Simpson, R. T. (1978). *Biochemistry* 17, 5524.

Singleton, C. K. and R. D. Wells (1982). *Anal. Biochem.* 122, 253.

Staynov, D. Z. and C. Crane-Robinson (1988). *EMBO J.* 7, 3685.

Stein, A. (1980). *Nucleic Acids Res.* 8, 4803.

Strauss, J. K. and L. J. Maher (1994). *Science* 266, 1829.

Suggs, J. W. and R. W. Wagner (1986). *Nucleic Acids Res.* 14, 3703.

Takeuchi, H. and J. Sasamori (1994). *Biopolymers* 35, 359.

Thoma, F., Th. Koller and A. Klug (1979). *J. Cell. Biol.* 83, 403.

Thomas, J. O. and A. J. A. Khabaza (1980). *Eur. J. Biochem.* 112, 501.

Thomas, J. O., C. Rees and J. T. Finch (1992). *Nucleic Acids Res.* 20, 187.

Tsanev, R., G. Russev, I. Pashev and J. Zlatanova (1992). *Replication and transcription of chromatin.* (Boca Raton: CRC Press).

Turna, J., A. Pudzisova, M. Osusky, L. Supekova and T. Kuchta (1994). *Folia Microbiol.* 39, 105.

van Holde, K. E. (1988). *Chromatin.* Springer-Verlag, Berlin and New York.

Varga-Weisz, P., K. van Holde and J. Zlatanova (1993). *J. Biol. Chem.* 268, 20699.

Varga-Weisz, P., J. Zlatanova, S. H. Leuba, G. P. Schroth and K. van Holde (1994). *Proc. Natl. Acad. Sci.* USA 91, 3525.

Vogel, T. and M. F. Singer (1975). *Proc. Natl. Acad. Sci.* USA 72, 2597.

Vogel, T. and M. F. Singer (1976). *J. Biol. Chem.* 251, 2334.

Vologodskii, A. V. and N. R. Cozzarelli (1994). *Annu. Rev. Biophys. Biomol. Struct.* 23, 609.

Vologodskii, A. V. and N. R. Cozzarelli (1996). *Biophys. J.* 70, 2548.

Vologodskii, A. V., S. D. Levene, K. V. Klenin, M. Frank-Kamenetskii and N. R. Cozzarelli (1992). *J. Mol. Biol.* 227, 1224.

Watanabe, F. (1986) *Nucleic Acids Res.* 14, 3573.

Wells, D. and D. Brown (1991). *Nucleic acids Res.* 19 (Suppl.), 2173.

Woodcock, C. L. F., L. L. Y. Frado and J. B. Rattner (1984). *J. Cell. Biol.* 99, 42.

Yaneva, J., G. P. Schroth, K. van Holde and J. Zlatanova (1995). *Proc. Natl. Acad. Sci.* USA 92, 7060.

Yang, G., S. H. Leuba, C. Bustamante, J. Zlatanova and K. van Holde (1994). *Nature Struct. Biol.* 1, 761.

Zlatanova, J. and J. Yaneva (1991). *DNA Cell Biol.* 10, 239.

Zlatanova, J., J. Yaneva, M. Ivanchenko, P. Varga-Weisz, G. P. Schroth and K. van Holde (1996). *Biological Structure and Dynamics* (Sarma, R. H. and M. H. Sarma eds.) 1, 173, Adenine Press, Guilderland, NY.