AN ABSTRACT OF THE DISSERTATION OF

Thomas Patrick Ellen for the degree of Doctor of Philosophy in Biochemistry and Biophysics presented on June 27, 2003.

Title: The Mechanism of Interaction of the Linker Histone with DNA and Nucleosomes.

Redacted for privacy

Abstract approved: _____.
Kensal E. van Holde

This dissertation examines the interaction of the linker histone with DNA and with nucleosomes. The first goal of the project was to characterize the interaction of the linker histone with DNA. Three factors previously reported to influence the linker histone's interaction with DNA were examined: ratio of linker histone to DNA sites of binding, monovalent ions in the local environment, and conformation of the DNA molecules. Evidence obtained through gel mobility shift assays demonstrates the strong preference by the linker histone for DNA with superhelical torsion, i.e., supercoiling, and the negative cooperative mode of binding that the linker histone exhibits in association with supercoiled DNA.

The second part of the dissertation examines the location of linker histone binding on the nucleosome, and documents the pronounced tendency of the linker histone to bind to two DNA duplex strands. A preparation of homogeneous nucleosome core particles, consisting of a defined 238 base pair DNA fragment and the core histone octamer positioned precisely on this DNA, was used as a substrate for the UV-induced

crosslinking of the linker histone to the DNA of this nucleosome. By site-specific labeling of a single site on the DNA of the nucleosome, the linker histone was observed crosslinked at that labeled site, confirming that the linker histone binds at the pseudo-dyad axis of the nucleosome. This evidence was used to support a model of linker histone binding to the nucleosome that invokes the association of the linker histone with no fewer than two duplex strands of DNA of the nucleosome.

The Mechanism of Interaction of the Linker Histone with DNA and Nucleosomes

by

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

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Thomas Patrick Ellen, Author

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LIST OF ABBREVIATIONS

APS: ammonium persulfate

Arg: arginine

ATP: adenosine triphosphate

bp: base pair

cm: centimeter

CM-Sephadex: carboxymethyl Sephadex

dH_2O: distilled water

EDTA: ethylenediamine tetraacetic acid

EtBr: ethidium bromide

GH1: globular domain of histone H1

GH5: globular domain of histon H5

HaeIII: *HaeIII* restriction endonuclease

HNF-3: hepatocyte nuclear factor 3γ

HpaII: *HpaII* restriction endonuclease

hrs: hours

IPTG: isopropylthiogalactoside

L: liter

LH: linker histone

Lys: lysine

mg: milligram

mins: minutes

mJ: millijoule

ml: milliliter

mm: millimeter

mM: millimolar

MNase: micrococcal nuclease

mW: milliwatt

μCi: microcurie

μg: microgram

μl: microliter

nm: nanometer

PCR: polymerase chain reaction

PMSF: phenylmethylsulfonyl fluoride

PNK: polynucleotide kinase

rDNA: ribosomal DNA

rGH5: recombinant globular domain of chicken linker histone H5

rpm: revolutions per minute

rRNA: ribosomal RNA

SAP: shrimp alkaline phosphatase

SDS-PAGE: sodium dodecyl sulfate - polyacrylamide gel electrophoresis

TAE: Tris-acetate-EDTA buffer

TBE: Tris-borate-EDTA buffer

TE: Tris-HCl - EDTA buffer

TFA: trifluoroacetate

UV: ultraviolet

w/w: weight to weight ratio

DEDICATION

To my mom and dad, Bud and Catherine Ellen.

THE MECHANISM OF INTERACTION OF THE LINKER HISTONE WITH DNA AND NUCLEOSOMES

Chapter 1

Introduction

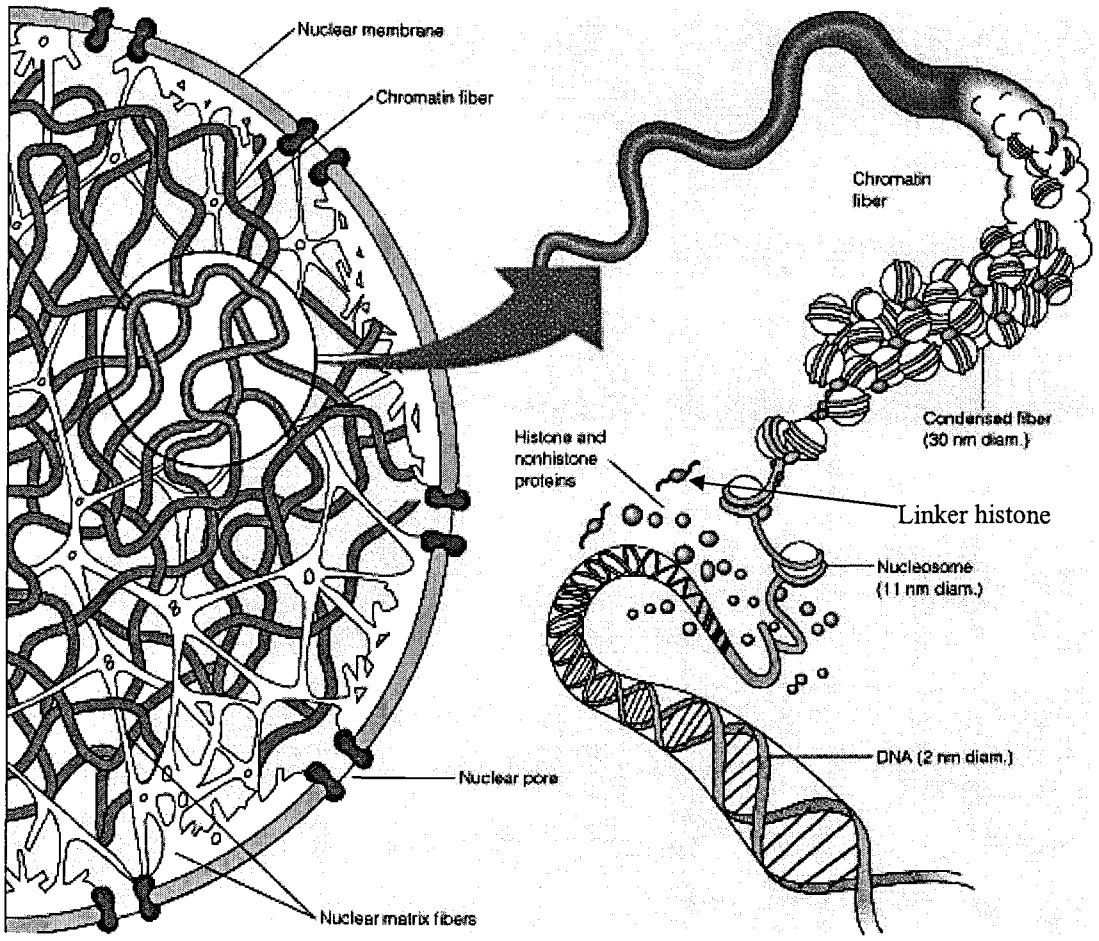
A. Chromatin Structure

DNA in all higher organisms, i.e., eukaryotes (those organisms with true cell nuclei), resides within the nucleus in a highly condensed state (Figure 1.1). DNA exists in virtually all somatic cells of eukaryotes in the form of a nucleoprotein complex called chromatin. In the electron microscope chromatin appears as a zig-zag fiber (Leuba et al., 1994) at low salt (0.2 mM EDTA, 1 mM triethanolamine chloride) and forms an irregular rodlike structure with a diameter of about 30 nm when observed at moderate, and physiological ionic strengths (ca. 100 mM NaCl).

The major breakthrough, that chromatin consisted of a repetitive fundamental nucleoprotein complex, came about through a combination of methodologies, including nuclease digestion, sedimentation analysis, electron microscopy and protein-protein crosslinking (Olins and Olins, 1974; Van Holde, et al., 1974; Kornberg, 1974; Kornberg and Thomas, 1974). Since the discovery of the nucleosome, the primary structural element of chromatin, it has become increasingly apparent that the proteins involved with the DNA in the chromatin are intimately connected to the regulatory processes controlling the metabolism of the DNA and expression of the genes carried within the DNA.

Figure 1.1. The structure of chromatin. DNA is wrapped around a core of histone octamers to form a nucleosome. This 11 nm subunit is bound by a single molecule of linker histone. Arrays of nucleosomes condense to form a 30-nm chromatin fiber. The chromatin fiber makes up the chromosomes of the cell. (Adapted from Mathews, Van Holde and Ahern, 2000.)

Figure 1.1


Nuclear membrane
Chromatin fiber
Chromatin fiber
Condensed fiber (30 nm diam.)
Histone and nonhistone proteins
Linker histone
Nucleosome (11 nm diam.)
DNA (2 nm diam.)
Nuclear pore
Nuclear matrix fibers

The nucleosome consists of a core: an octameric complex of protein subunits, known as histones, around which is wrapped 146 base pairs (bps) of DNA (Van Holde, 1989). Each nucleosome core is connected by virtue of its duplex DNA strands to another nucleosome core on either side of it. The connecting DNA strands are termed 'linker' DNA and are variable in length from species to species and tissue to tissue, from 8 bp to approximately 114 bp, with about 55 bps being a common linker length (Voet and Voet, 1995). The octameric protein core plus 146 bp of DNA wrapped around it is called the core particle. When the core particle is associated with a special histone termed the "linker histone", this complex is referred to as the nucleosome.

The original definition of the nucleosome core particle took into account the effect that an experimental analysis of the subunit had on it. Thus, it was defined as containing 146 bps remaining after cleavage by micrococcal nuclease. Because it is not necessary here to distinguish this exact amount of DNA, the nucleosome core particle or just nucleosome core shall be used to refer to the complex formed by the histone octamer and an inexact number of base pairs, but containing no linker histone.

The linker histone generally is known to confer nuclease protection on an additional 20 bps of DNA, in addition to the 146 bps of the core particle. The complex of the linker histone, plus the octameric protein core and a total of approximately 168 bps of DNA has been given the special name "chromatosome"

(Simpson, 1978). Here, the chromatosome will be used simply to designate the nucleosome core as defined above, but containing the linker histone.

B. The Histones

The nucleosome core protein composition includes four protein types, named histones H2A, H2B, H3 and H4. The octamer of histones that comprises the protein core of the nucleosome is made up of two each of the four types of histones. The histone proteins undergo protein-protein interactions between themselves and other chromosomal proteins, as well as protein-DNA interactions with the nucleosomal DNA; the latter interactions are predominantly electrostatic in nature. Our understanding of these interactions and relationships has been elucidated primarily by the analysis of the crystal structures of nucleosome core particles that have been regenerated from their components in the laboratory (Richmond et al., 1984; Arents et al., 1991; Arents and Moudrianakis, 1993; Luger et al., 1997; Richmond and Davey, 2003). All of the core histones are small basic proteins, 11 to 16 kilodaltons in mass. More than 20% of their amino acids are the positively-charged lysine and arginine.

The four core histones contain the histone fold domain (Arents et al., 1991) at their carboxyl (C-) terminal ends (Fig. 1.2a). The histone fold domain is highly conserved among the core histones, due to its central structural role in the nucleosome. The

Figure 1.2. The histone fold. The core histones – two each of H2A, H2B, H3 and H4 – make up the core octamer of the nucleosome. (a) The histone fold. The core histones contain the histone fold. (b) The handshake motif. The core histones form heterodimers through the handshake motif. (Adapted from Mathews, Van Holde and Ahern, 2000.)

Figure 1.2 (a)

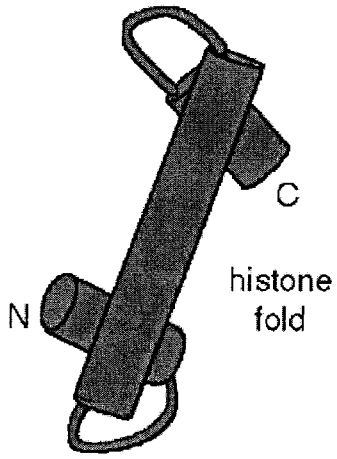
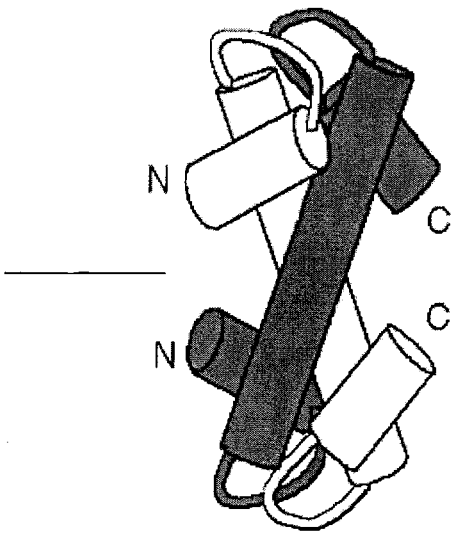


Figure 1.2 (b)



histone fold domain has been found in many other proteins, including some that have a regulatory function and bind DNA in a manner similar to that of the core histones (Gangloff, Y.G. et al., 2001). The histone fold is comprised of a long central α-helix bordered on each side by a loop segment and a shorter α-helix. The formation of dimers between H2A and H2B and between H3 and H4 monomers occurs, through the interaction of their respective histone folds, in what is known as a 'handshake motif' (Figure 1.2b; Arents et al., 1991).

Incorporation of DNA into a nucleosome begins with the formation of a handshake motif-type interaction between an H3 and an H4 molecule. This initial (H3,H4) heterodimer formation is responsible, through its precisely positioned, outward-facing arginine residues, for the nucleation of a DNA-binding reaction that, because of the regular spacing of the arginine residues, favors DNA having a helical periodicity of 10.7 bp/turn. Two H3/H4 heterodimers associate through H3-H3' α-helix interactions (Luger et al., 1997) to form the tetramer. A histone H3/H4 tetramer $(H3/H4)_2$ is capable of organizing DNA into a nucleosome-like particle (Camerini-Otero et al., 1976), and organizes a 120-bp stretch of DNA in a nucleosome identically to that of a full nucleosomal core particle (Hayes et al., 1991). The area of interface between these two heterodimers is less extensive than that between the (H3/H4) and (H2A/H2B) heterodimers. However, the interface between these latter is more accessible to solvent and is consequently less stable (Eickbusch and Moudrianakis, 1978; Karantza et al., 1996). There is evidence that tyrosines in the C-terminal α-helix

of histone H4 have a role in stabilizing the contacts between the (H2A/H2B) heterodimer and the (H3/H4) heterodimer (Santisteban et al., 1997; Zweidler, 1992).

The DNA makes its initial contact to protein by wrapping around the tetramer. After this initial nucleoprotein association each of two heterodimeric histone (H2A/H2B) pairs makes contact with the DNA and the $(H3/H4)_2$ tetramer to complete the formation of the nucleosomal core particle. The core octamers of histones form a wedge-shaped structure, shaped like a doorstop, but more rounded, with the $(H3/H4)_2$ tetramer forming the thinner, front end of the doorstop, and the two (H2A/H2B) heterodimers forming the back, thicker end of the doorstop, and around this 'rounded doorstop' wraps the DNA of the nucleosome (Figure 1.3).

The core histones also contain highly charged N-terminal tails (Figure 1.4). These charged tails are the sites of many post-translational modifications, and it has been established that transcriptional regulatory proteins target the core histone N-terminal tails as parts of signal transduction pathways, leading to the involvement of the histone tails in the modification of chromatin structure (Kuo et al., 1996; Hecht et al., 1995; Edmondson et al., 1996). These highly-charged N-terminal tails and their modes of covalent modification are as highly conserved as the histone fold structures among the core histones, attesting to their functional significance.

The second of the two types of histones is the linker histone. It is named in recognition of its binding to the linker DNA, i.e., the DNA linking adjacent nucleosomes together. The most common linker histone is called histone H1. Many

Figure 1.3. The nucleosome. Two each of H3, H4, H2A and H2B form the core octamer of histones around which is wrapped the DNA of the core particle. The linker histone associates with two duplex strands of DNA to complete the formation of the nucleosome. (Adapted from Mathews, Van Holde and Ahern, 2000.)

Figure 1.3

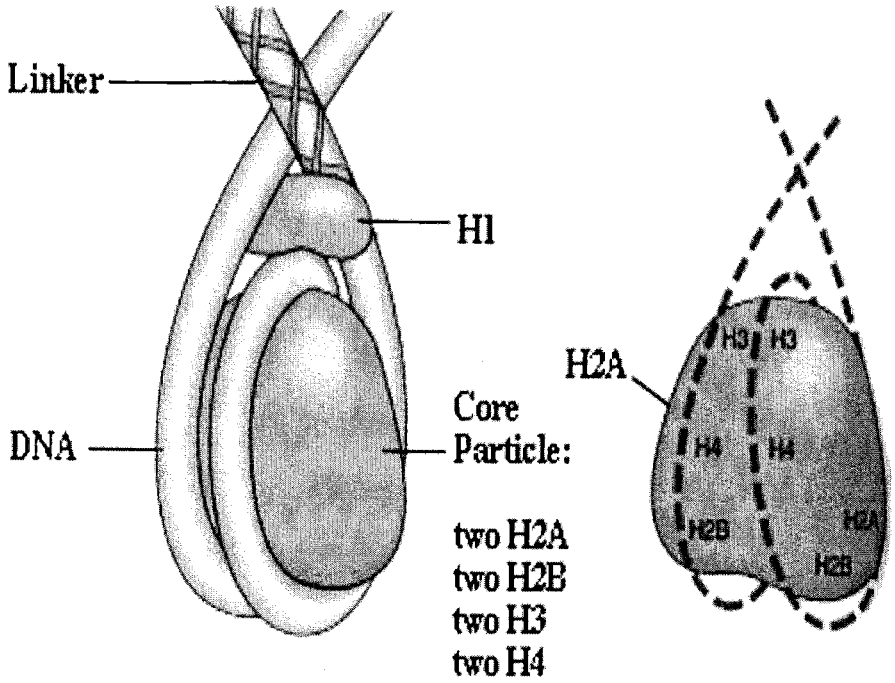
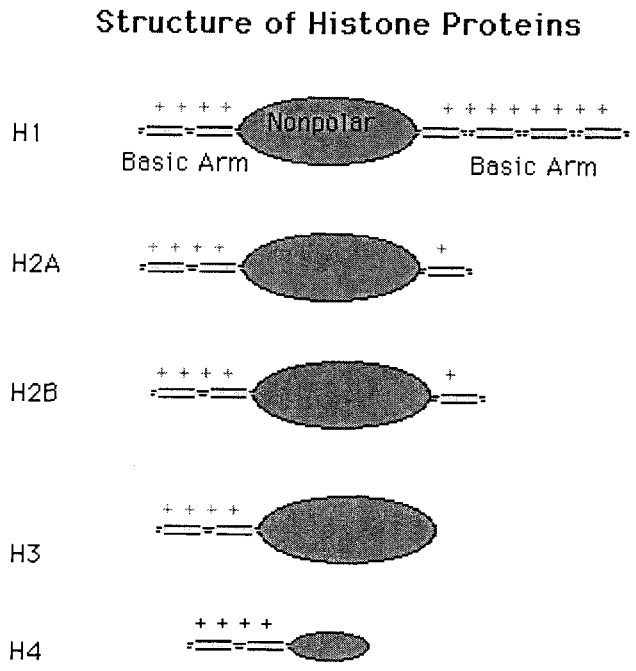

Linker
H1
DNA
Core
Particle:
two H2A
two H2B
two H3
two H4
H2A
H3
H3
H4
H4
H2B
H2A
H2B

Figure 1.4. Schematic diagram of the histone types. All of the histone types contain N-terminal tails which are positively charged and unstructured. The linker histones contain highly-charged C-terminal arms in addition to N-terminal ones. (Adapted from Mathews, Van Holde and Ahern, 2000.)

Figure 1.4

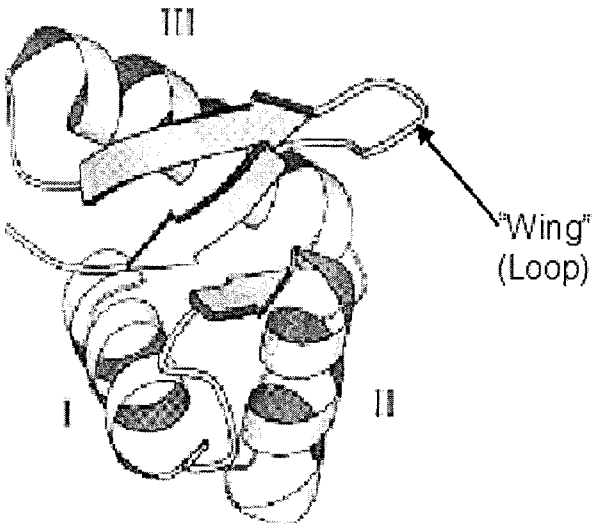

Structure of Histone Proteins
H1
Nonpolar
Basic Arm
Basic Arm
H2A
H2B
H3
H4

studies have utilized a specialized histone from chicken erythrocytes, called histone H5. There are many other linker histone variants (Wells and McBride, 1988). They differ from the core histones in architecture, evolutionary origin and function. They are biochemically similar to the core histones, having, as do the core histones, an abundance of lysine and arginine residues. The linker histones, however, do not contain the histone fold, the structural motif used in DNA compaction and protein dimerization (Arents and Moudrianakis, 1995). Linker histones do not function in the assembly of nucleosomes, and in fact, simple eukaryotes seem to survive without them, as has been demonstrated in lines of fungi and Protista having the genes encoding H1 completely knocked out (Shen et al., 1995; Patterton et al., 1998; Hellauer et al., 2001; Barra et al., 2000; Escher and Schaffner, 1997).

The linker histone contains a central, globular domain flanked on either side by unstructured, highly basic tails. The globular domain of H1 (GH1) is approximately 80 amino acids long and belongs to the 'winged helix' family of DNA-binding proteins (Ramakrishnan et al., 1993). The chicken erythrocyte linker histone variant, H5, has 79 amino acids in its central globular domain (Clore et al., 1987). It, too, has a central globular domain (GH5) of the winged helix family (Clark et al., 1993) and N- and C-terminal tails. GH5 is comprised of three α-helices (helices I-III) and three β-strands (S1-S3) in the order, from N- to C-termini, Helix I - S1 - Helix II - Helix III - S2 - S3 (Figure 1.5). Helix III intercalates into the major groove of DNA to form the primary DNA-binding interaction of GH5. S2 and S3 form antiparallel β-strands

Figure 1.5. Schematic ribbon diagram of GH5. The three broad arrows represent the three β-strands, S1 - S3. The alpha helices are represented here by ribbon helices, labeled I, II, and III. (Adapted from Ramakrishnan et al., 1993.)

Figure 1.5



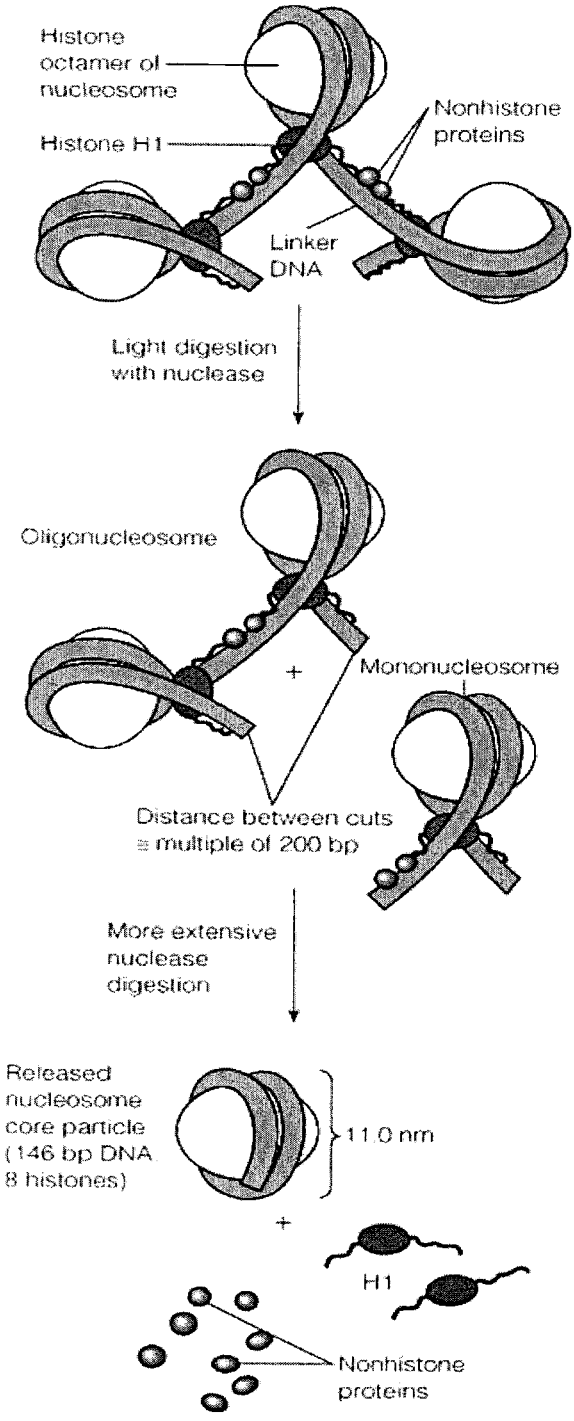
GH5

which, together with S1, form a three-stranded β-sheet. The characteristic winged structure of the winged helix motif is the loop which joins the S2 and S3 β-strands. The comparison of GH5 and GH1 in two-dimensional NMR studies shows that the 3D structure of the globular domain is conserved among linker histones (Cerf et al., 1994).

The linker histone occurs in higher eukaryotes on average at a frequency of one molecule per nucleosome. While the linker histone is probably involved in the stabilization of higher order chromatin structures, it is not known exactly what role it plays in DNA compaction. Early biochemical studies have shown H1 to associate with the entering and exiting DNA strands of the nucleosome, and its protection of these DNA strands from micrococcal nuclease (MNase) cleavage (Whitlock and Simpson, 1976; Noll and Kornberg, 1997; Allan et al., 1986, 1980). The chromatosome was originally defined as containing all of the core histones, one molecule of linker histone and 168 bps of DNA (Simpson, 1978). The implication is that H1 seals off the second superhelical turn of DNA as it wraps around the histone core octamer, and protects an additional 20 bps of DNA in the nucleosome from nuclease digestion (Figure 1.6). Because of the apparent symmetrical position of the pairs of identical histones comprising the core octamer, and the apparent pseudo-symmetry of the DNA as it winds nearly twice around the octamer, this postulated position of H1 binding is often referred to as being at "the dyad axis", in reference to a two-fold axis of (pseudo-) symmetry passing through the nucleosome core particle at this point.

Figure 1.6. Formation of the nucleosome and the nucleosomal core particle by micrococcal nuclease digestion of chromatin. The nucleosome, containing the linker histone, contains about 168 bps of DNA, while the core particle, devoid of linker histone, contains about 146 bps of DNA. (Adapted from Mathews, Van Holde and Ahern, 2000.)

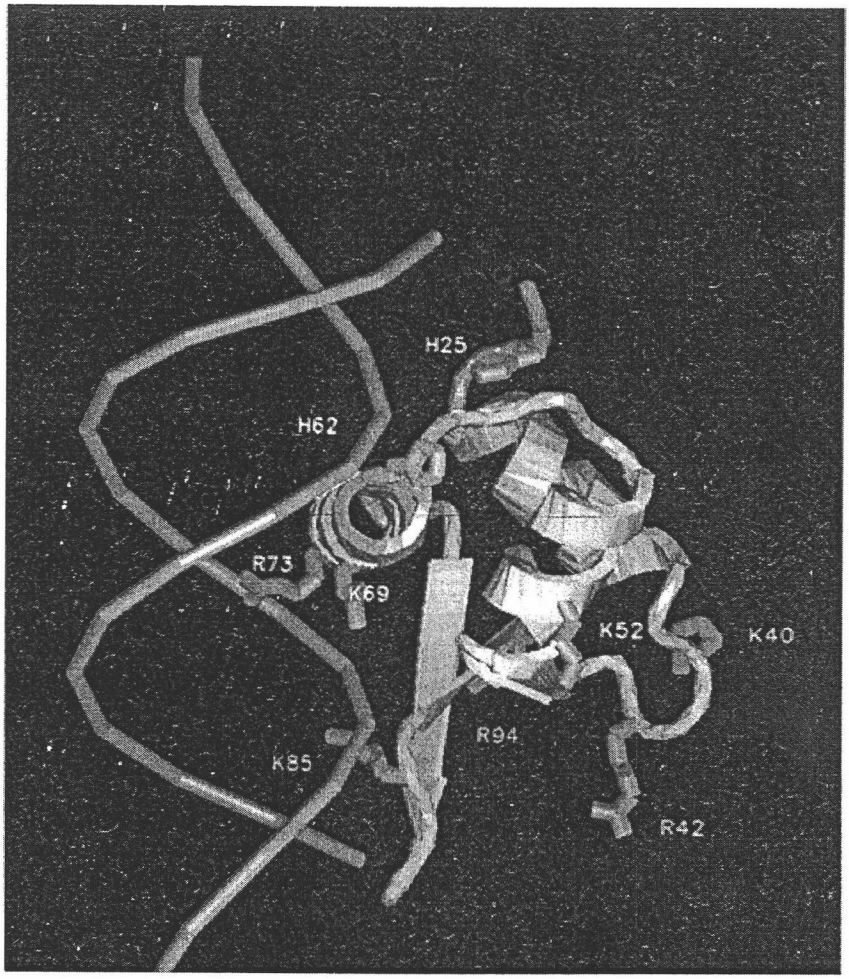
Figure 1.6


Histone
octamer of
nucleosome
Histone H1
Nonhistone
proteins
Linker
DNA
Light digestion
with nuclease
Oligonucleosome
+
Mononucleosome
Distance between cuts
= multiple of 200 bp
More extensive
nuclease
digestion
Released
nucleosome
core particle
(146 bp DNA
8 histones)
11.0 nm
+
H1
Nonhistone
proteins

The globular domain of the linker histone is sufficient to protect the extra 20 bps of DNA present in the chromatosome above that in the core particle (Allan et al., 1980). For the linker histone to bind at the dyad axis, the GH1 would have to have at least two DNA-binding sites (Allan et al., 1980). Investigations of the number of DNA-binding sites on the linker histone have revealed that there are, indeed, (at least) two DNA-binding domains (Singer and Singer, 1976; Thomas and Wilson, 1986; Goytisolo et al., 1996a; Duggan and Thomas, 2000). The same was found to hold true for the variant linker histone, found in transcriptionally repressed avian erythrocytes, known as H5 (Ramakrishnan et al., 1993). The X-ray crystal structure of the helix-turn-helix protein CAP (bacterial Catabolite Activator Protein) in complex with DNA provided the view that, because of GH5's structural similarity to CAP, helix III is the primary binding site (site I) on GH1 (Schultz et al., 1991; Ramakrishnan et al., 1993). This helix is referred to as the DNA recognition helix and is predicted to interact with the major groove of DNA via Lys 69 and Arg 73, with Lys 85 located at the base of the "wing" interacting with DNA backbone at an adjacent minor groove (Fig. 1.7). Replacement of Lys 85, by site-directed mutagenesis, with glutamine or glutamate (Buckle et al., 1992) helped to establish the necessity of this residue for protection of an additional 20 bps of nucleosomal DNA. These conclusions also gained strong support following solution of the co-crystal structure of the transcription factor, hepatocyte nuclear factor 3, HNF-3, with DNA (Clark et al., 1993). The DNA-recognition domain of HNF-3 is a typical winged-helix motif with strong structural

Figure 1.7. The GH5 - DNA complex. Helix three is shown end-on intercalated into a major groove of the DNA. The key residues of the proposed secondary binding site, confirmed by mutagenesis experiments (Goytisolo et al., 1996b), Lys 40, Arg 42, Lys 52 and Arg 94, are shown on the opposite side of the GH5 molecule from the primary site of DNA binding at helix three. (Adapted from Ramakrishnan et al., 1993.)

Figure 1.7



resemblance to GH5. The importance for correct binding of GH5 of Lys 69 and Arg 73 located in helix III was confirmed by mutagenesis (Goytisolo et al., 1996).

The crystal structure of GH5 also suggests the presence of a less defined secondary binding site (site II) which might interact with an adjacent duplex of nucleosomal DNA (Ramakrishnan et al., 1993). This second site is located on the opposite side of GH5, 25A away from the recognition helix III and involves two conserved residues which are part of a disordered loop between helices I and II (Lys 40 and Arg 42), Lys 52 in helix II, and Arg 94 in the S3 β strand (Fig. 1.7). The existence of this second binding site is consistent with earlier observations of H1/GH1 (and H5/GH5) DNA complexes which showed that both H1 and GH1 could bind cooperatively to two molecules of linear DNA and assemble them into 'tramline' complexes (Draves et al., 1992; Thomas et al., 1992). All four residues proposed to form DNA contacts in site II were confirmed by mutagenesis (Goytisolo et al., 1996b; Duggan and Thomas, 2000).

C. The Location of the Linker Histone on the Nucleosome

The location of the globular domain of H1 on the nucleosome has recently been the subject of intense debate. The paradigm had been that the globular domain alone had conferred specificity of binding of the linker histone to the DNA at the dyad axis, and thereby the full molecule interacted with equal lengths (about 10 bps) of both the

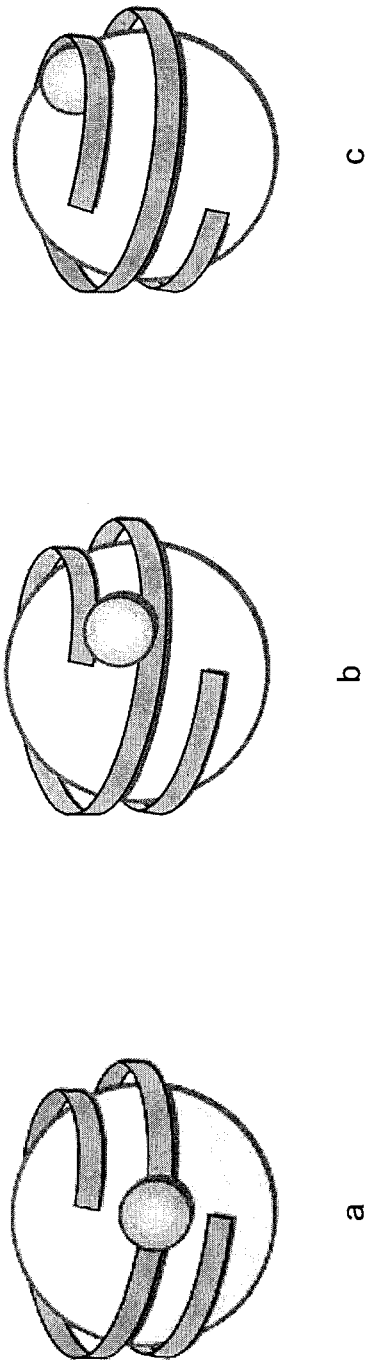
entering and exiting DNA of the core particle (Fig. 1.6). GH1 was positioned centrally, and the full molecule was thus thought to bind symmetrically (Allan et al., 1980).

The claim of a different location of binding by H1 to the nucleosome (Hayes and Wolffe, 1993; Hayes, 1996; Pruss et al., 1996) threw the consensus view of the linker histone's binding into disarray. *In vitro* experiments utilized the defined DNA fragment of 238 bps that incorporates the 120-bp *Xenopus laevis* 5S rRNA gene. Upon adding a full complement of the histone octamer proteins, a nucleosome core forms that is strongly positioned translationally along this 238-bp DNA fragment. The data from these experiments suggest that upon adding H1 to this reconstituted nucleosomal core particle, it binds to only one DNA gyre at a single internal site 65 bps away from the dyad, which is nearly on the opposite side of the nucleosome from where the DNA enters and exits (Figure 1.8). However, besides the different location, the model proposes that GH1 binds, not on the outside, but inside the DNA gyre, i.e., between the DNA and the core octamer of histones around which the DNA is wrapped.

This asymmetric model has been criticized because of the research methods used in coming to these conclusions. In one experiment (Hayes, 1996), the H1 molecule was altered by a rather bulky Fe(II)-EDTA conjugate, which may significantly disrupt H1's *in vivo* conformational state or its binding properties. In another experiment (Pruss et al., 1996), the DNA's bases were drastically covalently modified. Moreover, the data

Figure 1.8. The three proposed models of the binding location of the globular domain of the linker histone to the DNA of the chromatosome. (a) represents the model proposed by Allan et al., 1980. (b) shows the bridging model proposed by Zhou et al., 1998. (c) is a view of the model of Pruss et al., 1996. (Adapted from Travers, 1999.)

Figure 1.8



obtained from this latter investigation indicated significant binding by H1 all over the nucleosome, including at the dyad axis.

A third model of GH1 binding, based on data taken from site-specific protein-DNA crosslinking methods on mixed sequence chicken mononucleosomes, concludes that the globular domain of the linker histone spans DNA gyres between one terminus of the chromatosomal DNA and DNA close to the pseudo-dyad axis (Zhou et al., 1998). The terminus of DNA is thought to be bound by site I of GH5, while site II of GH5 is proposed to bind DNA near the pseudo-dyad axis (Figure 1.8). This asymmetric model of GH1 binding to the nucleosome is supported by the demonstration that reconstituted chromatosomes are afforded asymmetric protection by H1 with about 20 bps of DNA protected on one side of the core particle (An et al., 1998; Wong et al., 1997) and by the finding that the winged helix transcription factor HNF-3, which is structurally similar to GH5, also binds asymmetrically to nucleosomal DNA (Cirillo et al., 1998).

This model does not provide an explanation of how the GH1 selects one of the two locations available for it on the surface of the core particle. Both of the models (of symmetric and asymmetric binding of the linker histone at the pseudo-dyad axis) permit the C-terminal tail to bind the entering and/or exiting DNA duplexes. Nonetheless, the model of the asymmetric location of a naturally asymmetric H1 molecule provides a foundation for the concept of directionality in the folding of the

nucleosomal filament into higher order structure, and could provide clues to the question of the function of the linker histone.

The bridging of two DNA gyres by the globular domain of the linker histone assumes that GH5 binds to DNA in a very similar way as CAP (Ramakrishnan et al., 1993) and HNF-3 (Clark et al., 1993), with helix III interacting with the major groove of the DNA and the 'wing' of the winged helix motif lying alongside the DNA. The analysis of the RFX1 protein (Gajiwala et al., 2000), a winged helix protein very similar in structure to HNF-3, shows that this need not be the case. Helix III of RFX1 inserts its N-terminal end into the minor groove of DNA and makes a single base contact there. The reason for this entirely different mode of binding of HNF-3 and RFX1 lies in part in the difference in the electrostatic surface potential of the two proteins. In HNF-3, helix III is significantly more electropositive than the wing, and in RFX1 the opposite is true. The electrostatic properties of GH5 are much more similar to RFX1 than to HNF-3.

Linker histone sequences reveal considerable differences in the size of the wing among major taxa, and these differences could have important functional consequences.

In summary, determination of the mode and location of binding to DNA and to the nucleosome by the linker histone has remained an elusive goal, and to date, one that has generated some very perplexing data and contradictory conclusions. Since the linker histone associates to the nucleosome with much lower affinity than do the other

histones, and since much of the association in question must involve the unstructured N- and C-terminal tail regions of the LH, a method to determine its location on the nucleosome, other than X-ray crystallography, NMR, or other diffraction or light-scattering methods, would clearly be helpful.

D. The Higher Order Structure of Chromatin and Linker Histone Function

The search for an unambiguous function to the linker histone has paralleled the study of higher order structure in chromatin. Early studies used electron microscopy and neutron scattering to view chromatin (Thoma and Koller, 1977; Thoma et al., 1979; Olins and Olins, 1974; Olins et al., 1976). These studies were integral to the discovery of the basic subunit of chromatin, the nucleosome, and to another structure thought to make up the next higher level of organization of the eukaryotic chromosomal material, the "30-nm fiber". This 30-nm fiber is still thought to be the next level of chromosomal organization beyond the nucleosomal subunit (Figure 1.1).

Scientists have been trying for over a decade to clarify the nature and ascertain the extent of the 30-nm fiber in eukaryotes. The models best substantiated by experimental evidence fall into two classes: the solenoid- and the zig-zag-type models (McGhee et al., 1983; Woodcock et al., 1984). Both models propose that histone H1 has an important function in the compaction of chromatin and its folding into the 30-nm fiber (Graziano et al., 1988).

In the solenoid-type model, the nucleosomes lie with their long axes parallel and their dyad axes perpendicular to the fiber axis, while the linker DNA entry/exit site on each nucleosome faces the interior of the solenoid. Histone H1 has been observed in the interior of the chromatin 30-nm fiber, consistent with this model (Graziano et al., 1994). In the zig-zag-type model the zig-zag of consecutive nucleosomes condenses into a ribbon of two parallel rows, which, in turn, coils into a compact 30-nm fiber. In the solenoid-type model the linker DNA is bent, and follows the path induced by the nucleosome cores. In the zig-zag-type model the linker DNA is straight and the nucleosomes are not in close contact.

The available data show that the linker histone H1, when correctly positioned with the globular domain on the nucleosome and the C-terminal domain on the linker DNA, is involved in the induction of higher order structures (Thoma et al., 1979; Allan et al., 1986). However, H1-depleted chromatin has been shown to fold into structures that are as dense as the 30-nm fibers (Howe et al., 1998; Schwarz and Hansen, 1994; Clark and Kimura, 1990; Hansen et al. 1989; Yao et al., 1991). Therefore, although the functioning of the linker histone may be unnecessary in the compaction of DNA in higher-order structures of chromatin, perhaps the linker histone's asymmetric association with the nucleosome plays a role in determining how the higher-order structure forms. Interactions of H1 with naked linear DNA were reported to be cooperative (Clark and Thomas, 1979). In like manner, if H1 binds to the pseudo-dyad axis in a directional manner, i.e., with its C-terminal tail interacting preferentially

with one or the other DNA gyre of the entering or exiting DNA, then these H1-DNA interactions may be cooperative, either positively or negatively. Furthermore, this possible cooperative and asymmetric interaction of H1 with the nucleosomal DNA may be the mechanism that nucleates the formation of the 30-nm higher-order structure of chromatin. This model would be consistent with both the formation of the solenoidal helical structure implied by the solenoidal model, and with the formation of parallel rows, or ribbons, of nucleosomes, as envisioned by the zig-zag model.

The independent function of the N-termini of the core histones was unambiguously demonstrated by using core histones that have their N-terminal domains deleted. These experiments showed that without these N-terminal tails on the core histones, nucleosomal filaments are unable to condense into folded chromatin structures, despite the presence of properly bound H5 (Fletcher and Hansen, 1995; Caruthers and Hansen, 2000). Thus, it appears that the linker histone (LH) influences the folding of chromatin, but what roles the linker histone's properties of cooperativity of DNA binding, and asymmetric (or symmetric) interaction with the nucleosome and linker DNA may, or may not, play in the LH's influence is as yet unknown. Unknown too is the mechanism by which the linker histone has any influence in higher order structure.

Aside from physical considerations, the functional role of the LH in DNA metabolism is a subject of intense study and speculation. H1 can readily exchange between chromatin fragments at ionic strengths at which chromatin is folded into the compact 30-nm fiber (Caron and Thomas, 1981). This phenomenon is physiologically

relevant: *in vivo*, this migratory behavior of H1 has been observed during mitosis (Wu et al., 1986). During mitosis H1 is known to undergo massive phosphorylation (Van Holde, 1989). A larger fraction of H1 is stably bound to heterochromatin than to euchromatin in kinetic studies using fluorescence recovery after photobleaching (FRAP) (Misteli et al., 2000). Such behavior of H1, in steady-state rather than static binding to chromatin, is reminiscent of the behavior of transcription factors, and far removed from that of the core histones.

FRAP experiments have now confirmed that H1 molecules continuously exchange from chromatin, and suggest that the exchange is much more rapid than previously anticipated (Lever et al., 2000). This may be an explanation for the discordant results of the locations of the linker histone on the nucleosome, as shown in Figure 1.8. The residence time of H1 on chromatin is significantly reduced when core histones are hyperacetylated and chromatin is remodeled (Misteli et al., 2000). There was no mention of the acetylation state of the histones used in all of the experiments run to establish the location of the linker histone on the nucleosome as outlined in Figure 1.8. Actually, some of the data that were used in drawing the model of asymmetric binding used in Figure 1.8(c) employs pie charts to signify the percentage of H1 histones found in various locations of the nucleosomal DNA, and in no instance did these researchers find a "quorum", that is, a majority of instances of the H1 molecule located in one place. Perhaps what had been found is the clear reflection of the true nature of linker

histone binding to nucleosomes: a rapid exchange of proteins generating a dynamic, but stable, configuration of proteins on chromatin.

The picture painted is not unlike that of a transcriptional activator, such as glucocorticoid receptor (GR), and its response element. They, too, are continuously and rapidly in exchange, to provide a dynamic gene regulatory mechanism. In the case of LH, the importance of such behavior for understanding the processes of replication, transcription, recombination and repair lies within the specific protein-DNA interactions between the GH1 or GH5 and the nucleosome. The observation of such dynamic interchange indicates that a fundamental reinvestigation of the interaction of these proteins with DNA itself is in order. Part of this thesis is devoted to such a study.

Chapter 2

Linker Histone - DNA Interactions

A. Introduction

The Linker Histone is implicated in the formation and maintenance of higher-order structure in chromatin but its mode of action is unclear (Bradbury et al., 1973; Finch and Klug, 1976; Renz et al., 1977; Thoma et al., 1979). Aside from the structural role that the LH plays in the histone-DNA complex of the nucleosome, interest in the linker histone has accumulated in the past decade because it is thought to play a central regulatory role in DNA metabolism events, especially in transcription (Wolffe, 1994; Wolffe et al., 1995a; Ura et al., 1995; Shen and Gorovsky, 1996; Wong et al., 1997; Wu et al., 1995; Juan et al., 1997).

Access to genes, or the lack of it, has been shown to be influenced by histone H1 binding to the nucleosome (Ura et al., 1996). In the same study it was shown that transcriptional repression of a gene is directly correlated with structural transitions in chromatin that are induced by H1 binding to the nucleosome. Many studies have been done to assess the interrelation of nucleosome assembly on transcriptional elements of DNA and transcription factor binding on that element, required for proper initiation of transcription (Schild-Poulter et al., 1996). Through its role in restricting the mobility of nucleosomes on DNA (Ura et al., 1995), the linker histone is uniquely positioned to exert a major influence on the accessibility of chromatin to regulatory molecules.

Linker histones can act as either a positive or negative gene-specific regulator of transcription in vivo (Shen and Gorovsky, 1996). Extensive evidence demonstrates that the linker histone has an essential supporting role to the transcriptional machinery in regulating gene expression (Ura et al., 1995; Wolffe et al., 1995a; Wolffe et al., 1995b; Tsukiyama et al., 1994; Steger and Workman, 1996). Although it is still unclear whether the linker histone's primary role in regulating gene expression involves direct interaction with transcription factors, perhaps in direct competition, or a higher level of communication with such factors as histone acetylases and remodeling factors that affect the structures of which the linker histone is only a part, it is clear that linker histones provide regulation through their binding to the nucleosomal DNA.

In light of the linker histone's apparent role as a key player in the regulation of nucleosomal events leading to gene utilization of the underlying DNA, the question of exactly how the linker histone interacts with the DNA of the nucleosome has become all that much more important. The linker histone appears to be poised as a gate-keeper between functional factors that act upon the DNA, like the transcriptional machinery, and structural factors that modify the physical state of the chromatin that is largely the domain of the core histones. Structural factors include the nucleosome remodeling factors, which have been discovered only lately. The nucleosome remodeling factors' function appears to be solely to modify nucleosomal structures and positions of the histones on the DNA (Tsukiyama et al., 1994; Wu et al.,1995)

The linker histone's interactions with DNA are thought to predominate over its interactions with proteins (Clark and Thomas, 1986; Hayes et al., 1994). It is therefore likely that information about its binding to DNA is important to an understanding of its function in chromatin and other, more physiologically relevant states. As a first step to the study of the LH's interactions with the chromatosome, I studied the binding of linker histone H1 to free DNA. The current model of H1-DNA interactions defines three properties that affect the interaction of H1 to DNA: the ratio of H1 molecules to DNA sites of binding, the concentration of monovalent ions in the binding reaction's immediate environment, and the conformation of the DNA molecules (Clark and Thomas, 1986; Draves et al., 1992).

The conformational aspects of DNA, and its contribution to H1 - DNA interactions, would be useful in any inquiry into H1's mode of binding *in vivo*, while the ratio of H1 molecules to DNA sites of binding can shed light on affinity relative to other factors such as competing transcription factors, and perhaps on the role of DNA structure, by comparing apparent limiting numbers of preferred binding sites over nonpreferred sites, to allow LH to mediate specific regulatory mechanisms. The consideration of salt environment in studies of the binding of the linker histone to DNA may be related to the effect of salt concentration on the condensation level of chromatin *in vivo* (Hansen et al., 1989). There has been a lot of interest in salt-dependent changes in the structure of chromatin (Bashkin et al., 1993; Yager et al., 1989), and some of it has turned its focus toward the extent of the linker histone's involvement in the process of

condensation (Khadake and Rao, 1997). Thus, there is the possibility that relating salt's effect on condensation of chromatin to salt's effect on linker histone binding to chromatin may reveal more information about the LH's role in higher order structure.

As is shown below, monovalent salt's effect on the linker histone's binding to DNA is unremarkable, in that it appears no more than a general competitive inhibition of the reaction of interest. This could be easily expected, were one to assume that, with a protein as highly charged as the linker histone (the most highly charged of proteins), any binding reaction would be predominantly, if not exclusively, determined by ionic interactions with the phosphate backbone of the DNA molecule. However, earlier work has assigned a sharp, salt-dependent transition to the linker histone H1's mechanism of binding to linear DNA, from non-cooperative to cooperative (Clark and Thomas, 1986). A study of the effect of salt on the binding of DNA, both linear and supercoiled, by linker histone H1, is included here, to complete the survey of the three properties thought to affect the interaction of the LH to DNA.

B. Materials and Methods

DNA Preparations. Plasmid pML2αG contains the entire coding region plus 800 bp of the 5'- and 350 bp of the 3'- flanking regions of the mouse α-globin gene cloned into a pBR322 derivative (Nishioka and Leder, 1979). Plasmid pBR322 DNA was prepared from Qiagen purification kits (Chatworth, CA). The Qiagen plasmid

purification protocol is based on a modified alkaline lysis procedure, followed by binding of plasmid DNA to a proprietary type of anion-exchange resin under appropriate low-salt and pH conditions. RNA, proteins, dyes, and low-molecular-weight impurities are removed by a medium-salt wash. Plasmid DNA is eluted in a high-salt buffer and then concentrated and desalted by isopropanol precipitation.

Linear DNA was prepared from the plasmid pML2αG or pBR322 DNA by restriction digestions of the plasmids carried out with enzymes and buffers from New England Biolabs (Waltham, MA). The reactions were stopped by the addition of EDTA to 12.5 mM final concentration, and the DNA samples were extracted with phenol/chloroform (1:1) and precipitated with ethanol. The pellets were dissolved in 10 mM Tris-HCl, pH 7.5, 0.1 mM EDTA at a DNA concentration of 0.1 mg /ml. The concentration of DNA was determined spectrophotometrically by using an extinction coefficient of 20 $ml \cdot cm^{-1} \cdot mg^{-1}$ at 260 nm.

Isolation of Histone H1. Chicken histone H1 was isolated from erythrocytes under nondenaturing conditions as described (Garcia-Ramirez et al., 1990).

Gel Mobility Retardation Assays. Histone H1 was incubated with DNA in a total volume of 20 μl in a buffer composed of 50 mM Tris-HCl pH 8.0/ 1 mM EDTA/ 20 mM NaCl/ 0.1% Triton X-100 for 1 hr. at room temperature. The mixture was routinely electrophoresed through 1.0% agarose gels at 8-10 V/cm at room temperature in Tris acetate/ EDTA (TAE) buffer (40 mM Tris acetate, pH 7.5/ 1 mM

EDTA). Gels were stained with 0.1 μg of EtBr per ml for 20 min, briefly destained, and photographed on Polaroid 667 (Polaroid) film.

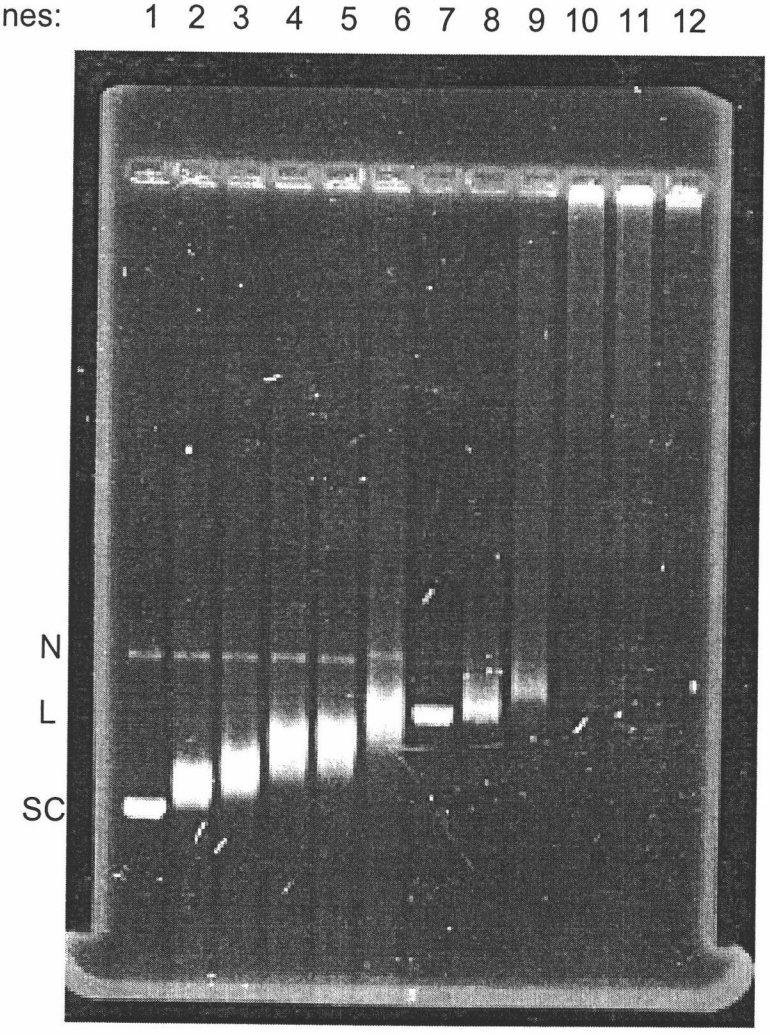
C. Results

The conformation of the DNA molecules was observed to play a major role in the mechanism of H1 binding to DNA. As shown in Figure 2.1, a comparison of gel electrophoretic patterns for H1-DNA complexes in which the DNA is either in the supercoiled or the relaxed (linear) states yields distinct variation. This variation can be extrapolated to incur distinct modes of H1 binding to DNA. Notice in the figure that precisely the same amounts of H1 are added in each corresponding lane for supercoiled and linear DNA. Yet the affect is qualitatively different. As the number of H1 molecules increases, the retardation of the DNA band increases, for all the lanes of supercoiled DNA. This is definitely not the case for those same titrations of H1 added to linear DNA (Figure 2.1, lanes 7-12). There is, at low amounts of H1, some lesser, but noticeable, retardation. Then, there is an abrupt transition to complete aggregation of the DNA into the wells of the gel.

Linker Histone Binding to Supercoiled DNA is Negatively Cooperative. Before drawing any further conclusions, let's consider one more observation concerning the binding of supercoiled DNA by H1 shown in Figure 2.1. In each lane where H1 is added, all of the DNA is migrating in a single, discrete band, retarded relative to the

Figure 2.1. Gel mobility retardation assay of H1 binding to plasmid pML2αG DNA. Titration of (Lanes 1 through 6) the supercoiled form of plasmid DNA, and (Lanes 7 through 12) the linear form of plasmid DNA with increasing amounts of histone H1 on a 1.0% agarose gel. N: nicked relaxed form of DNA; L: linear DNA; SC: supercoiled DNA. The protein to DNA ratios (designated on a weight-to-weight basis) are: 0, 0.05, 0.1, 0.15, 0.2, and 0.25, for lanes 1-6 and 7-12, respectively.

Figure 2.1



DNA in the corresponding (control) lane in which no H1 is added. There is no DNA, in those lanes where H1 is added, that is not bound by the H1. The H1 has distributed itself over the entire population of DNA molecules.

This result is qualitatively different from those of earlier published studies of the binding of H1 to DNA (Clark and Thomas, 1986). In those studies the authors concluded that the binding of H1 to DNA is cooperative. But in their experimental results, DNA migrated as two fractions, one portion bound by H1, and therefore retarded on the gel, and one portion migrating at the same rate as that of free DNA in a control lane run in parallel on the gel. The conclusion was obvious for those studies: the H1 molecules all bound to a fraction of the DNA, while other DNA was left completely unbound. That is absolutely positive cooperativity, and contrary to the results in this work. The basis for the discrepancy between the results shown by Clark and Thomas and those shown here is that here the DNA is supercoiled, while Clark and Thomas' DNA was short (146 bps), linear DNA fragments.

Thus far, two results have emerged from this experiment. First, H1 binds to DNA by two separate and distinct modes, dependent upon the conformation of the DNA, as exhibited, in this case, by binding experiments run in parallel on supercoiled DNA and on linear DNA. Linear DNA accepts only a limited number of H1 molecules before all of the linear DNA molecules are totally networked to one another, presumably by the dual binding of H1 molecules to two DNA molecules, and the "mega"-mer is aggregated and unable to penetrate the agarose gel matrix. The supercoiled DNA, in

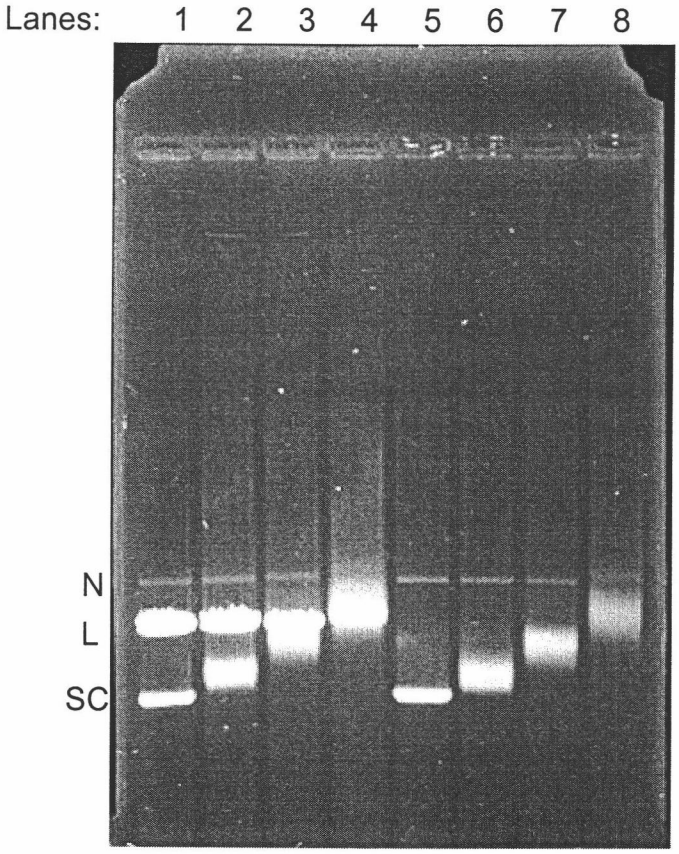
contrast, can accommodate a far greater number of H1 molecules and still retains independent, single DNA molecules, as reflected by its ability to associate the greater number of H1 molecules and still penetrate the gel matrix.

The second result is that H1 binding to DNA, in either case, supercoiled or linear, is distinctly not cooperative, at least not *positively* cooperative. We can reason that if H1 were to randomly bind to DNA molecules, then upon gel electrophoresis, there would simply be a smear of DNA extending from the migration position of the unbound DNA, with zero molecules of H1 bound, on up through every integer number of H1 molecules bound per DNA molecule. This would be an instance of noncooperative binding. Because this is not the case, but rather, there is only one band of DNA in each lane, all the protein-DNA complexes in that band are of approximately the same size, and the number of molecules of H1 bound on each DNA molecule in that band is approximately equivalent from one DNA molecule to the next, it must be concluded that H1 binding to DNA is not noncooperative either. In other words, binding of H1 somewhat inhibits further binding to the same DNA molecule, and the roughly equal distribution of H1 molecules per DNA molecule demonstrates what turns out to be an example of negative cooperativity.

Histone H1 Binds to Supercoiled DNA Preferentially over Linear DNA or Relaxed Circular DNA, in Direct Competition Experiments. A further comparison of the binding of H1 to different conformers of DNA by direct competition between

Figure 2.2. Gel mobility retardation assay of H1 binding to supercoiled and linear DNA. Lanes 1 to 4 contain a mixture of supercoiled and linear DNA titrated with increasing amounts of histone H1, in direct competition. This 1.0 % agarose gel shows that the linear DNA is not retarded by the binding of any histone H1, while the supercoiled DNA is significantly retarded. N: nicked, relaxed DNA; L: linear DNA; SC: supercoiled DNA. Lanes 5 to 8 show supercoiled DNA only, as a control. Histone H1 to DNA ratio on a weight to weight basis is: 0 in lanes 1 and 5; 0.2 in lanes 2 and 6; 0.5 in lanes 3 and 7; and 0.8 in lanes 4 and 8.

Figure 2.2



supercoiled and linear DNA for the binding of the linker histone H1 dramatically demonstrates the dependence of linker histone H1's mode of binding on the DNA's conformation (Figure 2.2). Linker histone H1, in the presence of both the supercoiled and linear DNA of the same sequence, will bind exclusively to the supercoiled DNA, leaving the other, relaxed conformer completely untouched. Note that there is a small amount of residual relaxed circular DNA contaminating the samples of supercoiled DNA (Figure 2.1, lanes 1-6), but this has no effect on the H1's binding to the supercoiled DNA. Thus, in the presence of all three conformations of DNA, supercoiled, linear, and relaxed circular, H1 binds exclusively to supercoiled DNA, proving that it is the supercoiled state itself that is of importance in H1's mechanism of binding to DNA.

As with the earlier experiment, two results have emerged from this experiment. First, there is some aspect of the supercoiling of DNA, not present in linear DNA, or relaxed circular DNA, that is the feature that the LH recognizes. Since the sequence of the DNA is exactly the same for all three forms of the DNA in this experiment, it is a structural aspect with which the H1 molecule preferentially interacts. Previous work in this laboratory has implicated the crossovers of duplex strands in superhelical DNA as the structural feature with which the H1 molecule preferentially interacts (Varga-Weisz et al., 1994). Crossovers of DNA in this context signify the duplex strands of DNA that overlap upon one another as a result of the writhe of the supercoiling in superhelical DNA.

Secondly, in the earlier experiment, H1 binding to linear DNA was easily observed, even at very small amounts of LH, by a retardation of the DNA, while in Figure 2.2 the linear DNA shows no retardation, suggesting that no H1 is bound to that linear DNA. Therefore, all the H1 is bound to the supercoiled DNA, and no H1 molecules are left free in solution. This gives us some information about the general range of saturation of the LH on DNA. With this information, and knowing the number of base pairs in the experimental DNA, we can begin to quantitatively estimate the number of H1 molecules bound per crossover, on average, of supercoiled DNA, in this experiment. From that calculation, in turn, a clearer picture of the preferential binding behavior of the LH to supercoiled DNA may be produced.

Ratio of H1 molecules to DNA molecules affects the mechanism of binding. The effect of the ratio of H1 molecules to DNA molecules was studied by titrating measured amounts of the DNA plasmid pML2αG with precise increasing amounts of chicken erythrocyte linker histone H1. As the amount of H1 relative to DNA increases, the migratory retardation of the electrophoresed samples increases, until, at a critical ratio of H1 to DNA, there is an abrupt transition wherein the samples no longer penetrate the gel matrix, suggesting that the molecules have aggregated into a single, polymeric complex (Figure 2.3). Note that this occurs even when the DNA is supercoiled (Figure 2.3), just as was shown in the earlier experiment for the linear DNA-containing samples (Figure 2.1, lanes 10 - 12). The amount of H1 necessary to create the transition to aggregation differs greatly for the two different forms of DNA,

Figure 2.3. Gel mobility retardation assay of supercoiled DNA titrated with histone H1. N: nicked, relaxed DNA; SC: supercoiled DNA. The ratio of histone H1 to DNA, on a weight to weight basis is: 0, 0.05, 0.1, 0.15, 0.2, 0.25, 0.5 and 0.7 for lanes 1 – 8, respectively.

Figure 2.3

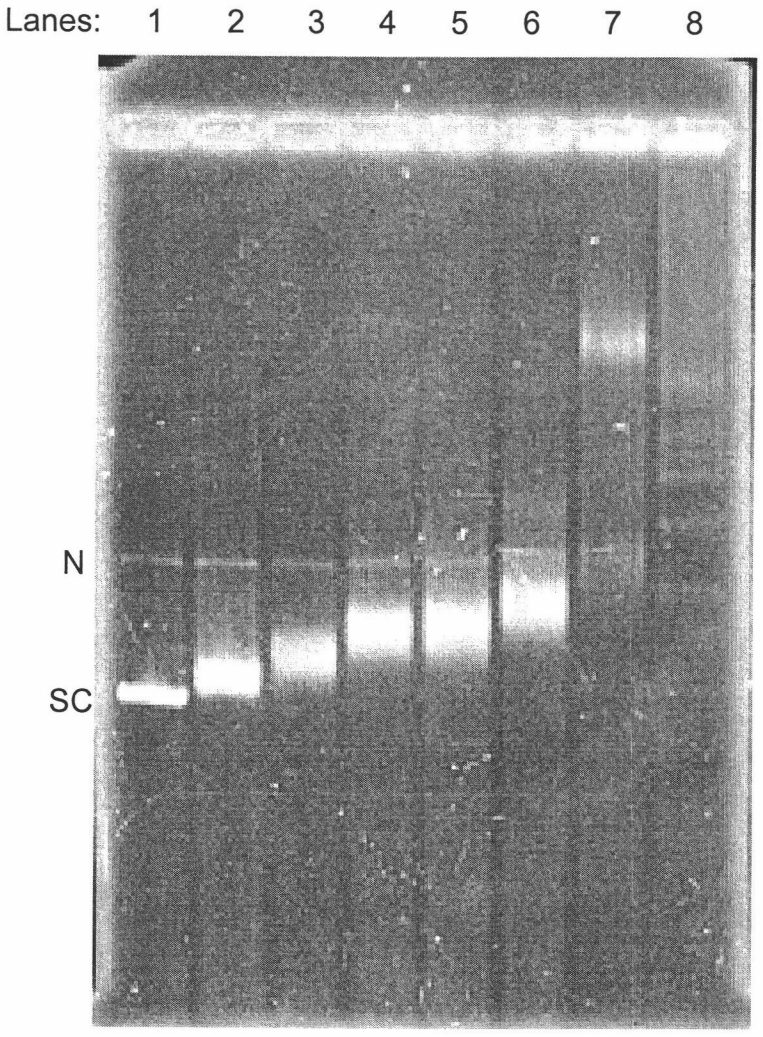
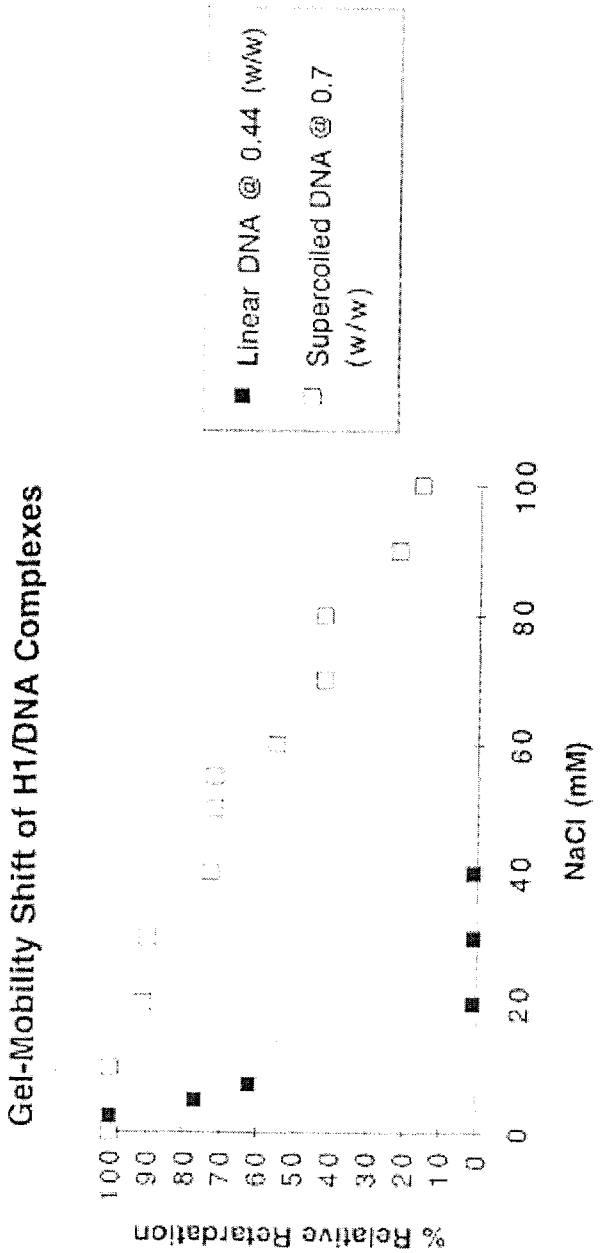


Figure 2.4. The effect of monovalent ions on the binding of histone H1 to supercoiled DNA and linear DNA. The graph shows percent relative retardation to the migration of free DNA as a function of NaCl concentration. Supercoiled DNA exhibits a steady decrease in retardation due to binding of the DNA by histone H1. Linear DNA retardation upon histone H1 binding is more sensitive to low salt concentrations. Given the greater resistance by the supercoiled DNA - H1 complex to the salt's inhibition, one might presume that the mechanism of binding of the supercoiled DNA - H1 complex involves electrostatic interactions less than does that of the linear DNA - H1 complex.

Figure 2.4


Gel-Mobility Shift of H1/DNA Complexes
Linear DNA @ 0.44 (w/w)
Supercoiled DNA @ 0.7 (w/w)
% Relative Retardation
100
90
80
70
60
50
40
30
20
10
0
0
20
40
60
80
100
NaCl (mM)

but the transition appears to be no different between the two. In either case the transition is made from individual molecules of DNA bound by numerous H1 molecules to the networking, or crosslinking, of molecules of DNA to one another through the mediation of the DNA-binding linker histone H1 molecules.

The Concentration of Monovalent Ions Affects the Binding of H1 to DNA in a Monotonic Manner, Suggesting Simple Competitive Inhibition. Further gel retardation assays were run, this time varying the amount of monovalent ions (as NaCl) present in the samples before and during electrophoresis. When no salt is added, samples of DNA in the presence of the H1 molecule will be retarded upon gel electrophoresis in agarose. As salt is added, the retardation of protein-DNA complexes decreases. Finally, with enough salt added, the effect of H1 binding to DNA in retarding electrophoretic migration is lost entirely (Figure 2.4). Many variations in the conditions were tried, from 0 to over 100 mM NaCl, with both linear and supercoiled DNA, with varying amounts of added H1 (data not shown). All that could be observed was a progressive diminution of the gel retardation effect on the DNA caused by the binding of H1. The only conclusion that can be drawn is that both putative mechanisms of binding of DNA, supercoiled on the one hand, and linear on the other, by H1, involve electrostatic interactions.

D. Discussion

As a first step to the study of the interaction of the linker histone with the nucleosome, the linker histone's interaction with free DNA was examined. The linker histone's interaction with the nucleosome is thought to be predominantly a protein-DNA interaction in nature. Although some protein-protein contacts have been documented between the linker histone and core histones (Boulikas et al., 1980; Maman et al., 1994; Baneres et al., 1994; Pruss et al., 1995; Hayes et al., 1996), the linker histone's interaction with nucleosomal DNA has come to be the standard starting point for any investigation seeking to determine the linker histone's biological role.

Gel electrophoresis is among the most commonly used biophysical techniques for studying protein-DNA interactions. Native gel electrophoresis mobility shift assays came into wide acceptance in the 1980's, beginning with studies of the nucleosome (Sandeen et al., 1980), and continuing with the study of the CAP protein (Garner and Revzin, 1981), and other regulatory protein-DNA complexes of the *lac* operon (Fried and Crothers, 1981). The gel mobility shift method has several advantages over other physical methods, including accuracy of determination of molecular weights, its ability to detect specific interactions in the presence of other interactions, and the ease and simplicity of its use as compared to methods such as mass spectrometry, X-ray crystallography and nuclear magnetic resonance (NMR).

The gel mobility retardation method was used here exclusively to provide information about the binding of the linker histone H1 to DNA. Previous work on the subject led to the proposal that three dynamic properties affected the interactions of the linker histone with free DNA: the ratio of LH molecules to DNA sites of binding, the concentration of monovalent ions in the immediate environment of the LH-DNA association, and the conformation of the DNA molecules. Each of these factors were systematically studied here.

The conclusions drawn by the authors of those earlier studies were that the LH binds to DNA cooperatively (Singer and Singer, 1976; Clark and Thomas, 1986; Rodriguez et al., 1991; Draves et al., 1992; Thomas et al., 1992), and that this cooperativity is salt dependent, and makes a sharp transition from noncooperative to cooperative at about 50 mM NaCl, depending on the size and conformation of the DNA and the ratio of number of H1 molecules to DNA sites of binding (Singer and Singer, 1976; Clark and Thomas, 1986; Rodriguez et al., 1991).

Gel mobility retardation assays in this present work showed that the nature of the complexes formed between histone H1 and DNA is highly dependent on the conformation of the participating DNA. This fact is dramatically demonstrated by the direct competition assay (Figure 2.2). When both linear and supercoiled DNA are present and available for binding by linker histone H1, there is an unmistakable preference of the LH for the supercoiled conformer.

This preference for supercoiled DNA over other more relaxed forms of DNA can be explained as a preference for the crossovers of duplex DNA found in supercoiled DNA that is absent in linear DNA and other relaxed conformations of DNA (Krylov et al., 1993). In a more general sense, the physical condensation of supercoiled DNA provides more possibilities for two-site interaction with DNA. In linear DNA, such interaction must involve either DNA bending or the aggregation of DNA molecules. The implication is that it is the tertiary structure of the DNA that is the recognition motif by which the linker histone negotiates its manner of association to its DNA substrate. Generally, a supercoiled DNA provides a specific structural "handle" that the LH prefers, which we are assuming for the moment is the crossover.

There is obviously some mechanism, arising from the H1-DNA interaction itself, that directs H1 molecules and/or DNA molecules to partition themselves in a regular and ordered fashion such that roughly equal numbers of H1 queue up, as it were, on each DNA molecule (there being multiple H1 molecules on each DNA molecule, and not vice versa), such that something signals when one DNA molecule has more H1 bound than another DNA, and thereby directs any new, incoming H1 molecule to locate itself elsewhere on some other, more H1-deficient DNA molecule. Thus, we see supercoiled DNA-H1 complexes migrating as a discrete band of apparently homogeneous nucleoprotein molecules. Cooperativity is defined as the alteration of binding of one molecule to its ligand by the addition of another molecule. In this case, the alteration is a decrease in the binding, which constitutes negative cooperativity.

If it is the crossovers of supercoiled DNA to which the H1 binds, then there is a convenient explanation for the preferential binding behavior that would be consistent with two phenomena observed in these experiments: 1) the *negative* cooperativity that took the appearance of an even distribution of H1 molecules across the population of DNA molecules such that no free DNA was observed in any gel lanes where the linker histone was present, and 2) the variation in migratory pattern between the supercoiled and linear DNA forms as a function of the increasing amounts of H1 added to the binding reaction, such that there is a low threshold of accommodation of linker histone binding to linear DNA before it aggregates, whereas there is accommodation of much greater amounts of H1, with significantly more retardation, on supercoiled DNA before it aggregates.

Recall that in Figure 2.1 the supercoiled DNA exhibits retardation when bound by H1 up through a ratio of H1 to DNA that was denoted as 0.25. This number translates to roughly 38 molecules of H1 per molecule of DNA. The pML2αG DNA molecule contains approximately 5300 bps. Therefore, there is an H1 molecule binding, on average, at every 139 bps. In supercoiled DNA, there can easily be one crossover in every 139 bps. Actually, we can estimate that the molecule has @30 superhelical writhes, using the equation:

$$\Delta L_K = \sigma L_0$$

where L_0 is the number of turns in the DNA (bps/10.5), ΔL is the linking number and σ the superhelical density.

With this information, we can calculate the stoichiometry, or at least the range, of the number of H1 molecules per crossover, on average, of the supercoiled DNA used in this experiment. The usual superhelical density for B-form DNA is given a σ-value of –0.06. L_0 is the number of bps divided by the bps per turn of the double helix, i.e., the twist. Using 5300 as the number of bps, and taking 10.5 as the number of base pairs per helical turn, we divide:

$5100 / 10.5 = 505 = L_0$

and multiply by the superhelical density, σ:

$-0.06 \times 505 = -30 = \Delta L_K$.

This estimate shows that the number of H1 molecules bound per molecule of supercoiled DNA approaches the number of crossovers per molecule of DNA, and that at a ratio of 2 molecules of H1 per crossover, the supercoiled DNA aggregates.

We have evidence (Ivanchenko et al., 1996) that H1 binding unwinds negatively supercoiled DNA. Now, suppose that, as H1 binds to supercoiled DNA it begins to unwind it. According to our conjecture, this would make that molecule a poorer

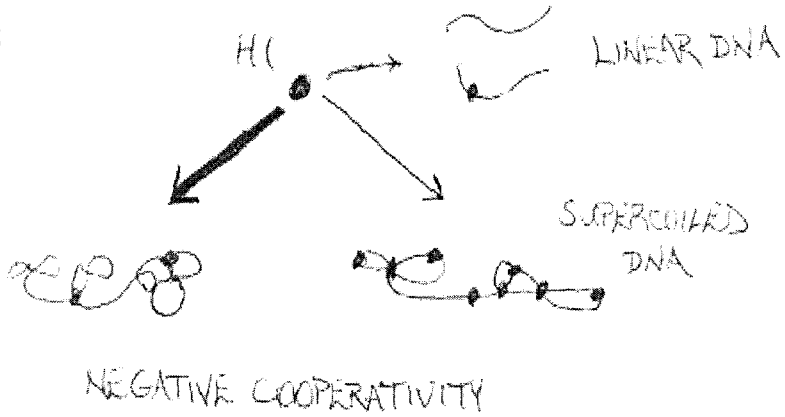
substrate for any subsequent H1-binding reaction; this is the requirement for negative cooperativity. Linear DNA would simply be the poorest substrate, as it is the most completely unwound (Figure 2.5).

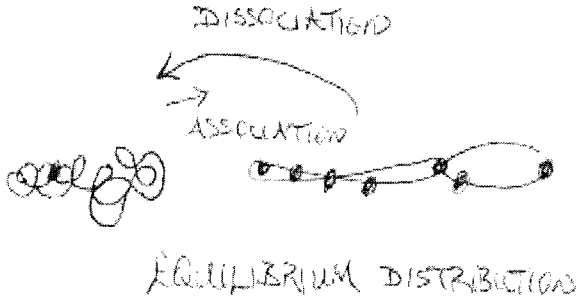
Statistically, as an H1 molecule free in solution confronts the two species of supercoiled DNA, one bound and the other devoid of LH, it would "choose" the DNA with the highest density of supercoils, or superhelical crossovers (Figure 2.5). Even if the whole population of H1 molecules is already bound, there will likely be association/dissociation equilibria such that the H1 molecules will become evenly distributed by virtue of their sensitivity to the state of superhelical density among the DNA molecules. Thus, there would be no band of free DNA molecules observable on the agarose gel, which is what we observe in all of the experiments run here.

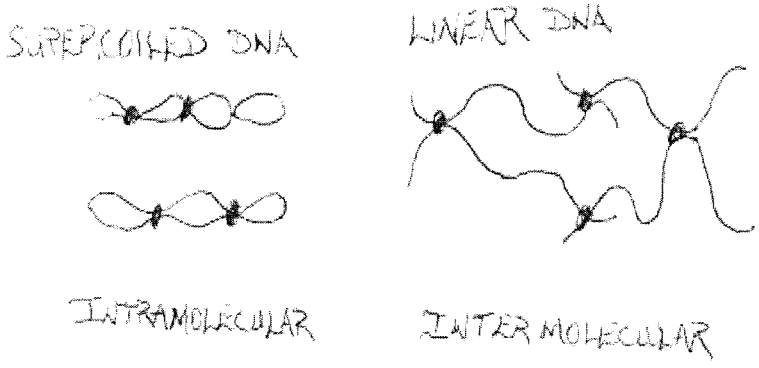
We also have evidence that linker histone H1 has at least two DNA-binding domains (Goytisolo et al., 1996; Ramakrishnan et al., 1993; Duggan and Thomas, 2000). If it were assumed that each linker histone H1 molecule in these experiments is bound to two DNA duplexes, then, not only could we easily envision the H1 molecule's preference for the crossovers on supercoiled DNA as an intramolecular bridging by H1 of the DNA molecule upon itself, but we could explain the aggregation of the linear DNA, which occurs upon the addition of much smaller amounts of LH than occurs upon the addition of LH to supercoiled DNA, as an intermolecular bridging by H1 (Figure 2.5).

Figure 2.5. Negative cooperativity of histone H1-binding to DNA. In the upper sketch, the H1 bound to DNA causes its unwinding, facilitating the next H1 binding event at a more highly supercoiled DNA molecule. In the middle sketch the unwinding facilitates dissociation of bound H1 histones. The bottom drawing compares the intramolecular associations of supercoiled DNA with histone H1, causing retardation in the gel electrophoresis assays to the intermolecular associations of linear DNA and histone H1 that cause the networked "mega" complexes that prevent penetration into the gel matrix.

Figure 2.5


HI
LINEAR DNA
SUPERCOILED DNA
NEGATIVE COOPERATIVITY


DISSOCIATION
ASSOCIATION
EQUILIBRIUM DISTRIBUTION


SUPERCOILED DNA
LINEAR DNA
INTRAMOLECULAR
INTERMOLECULAR

This is consistent with the data from the gel electrophoresis mobility shift assays. Linear DNA undergoes aggregation at a level of 0.1 H1 per DNA (w/w) (Figure 2.1, lane 9), which is equivalent to about 15 molecules of H1 per molecule of pML2αG DNA. In contrast, supercoiled DNA aggregates only after about 75 molecules of H1 have been added to each molecule of DNA (Figure 2.3, lane 6). It appears that if the saturation limit of H1 molecules per crossover in supercoiled DNA, here about 30 -40 H1 molecules per DNA molecule, is reached, then the H1 begins to bind intermolecularly, and the supercoiled DNA is aggregrated and cannot penetrate the gel matrix. Yet with only half of that critical, stoichiometric amount of H1 binding to linear DNA, the linear DNA aggregates. Obviously, in the absence of crossovers, H1 provides a compelling force by which to manipulate the DNA into a configuration by which it can bind two DNA duplex strands. In the case of linear DNA, this appears to be done by intermolecular bridging.

This is even more significant when one considers that if binding to the linear DNA in the absence of crossovers is by nonspecific, electrostatic association, as has been proposed (Manning, G.S., 1979), then there are many more sites available (as sites to be occupied rather than preferred sites of binding) on linear DNA, and yet with so few as 15 molecules per DNA molecule, the H1 associates to two DNA molecules simultaneously, giving an indication of just how strong this preference for dual DNA binding is in the LH molecule. So aggregation occurs at much lower protein-binding densities in linear DNA than in supercoiled DNA.

All of this behavior of the LH with supercoiled and linear DNA is in agreement with the aforementioned reported evidence that LH has two DNA-binding domains. For linear DNA this means that the DNA must either bend around on itself in a loop, which is difficult for it to do, or be linked by LH to a second DNA molecule. In the case of linear DNA, the latter is more favored. In the case of supercoiled DNA, the crossovers make two binding sites available in an intramolecular configuration.

That the linear DNA aggregates with so little H1 molecule present compared to supercoiled DNA, when there are many more sites of occupancy available on linear DNA, exemplifies how strongly the dual-binding mechanism of LH, binding as it does to two duplexes of DNA, is perpetuated. It also verifies at least one of the three dynamic properties earlier proposed to affect the interactions of the linker histone with free DNA. The conformation assuredly is one property that affects the interaction of LH with DNA. The affinity of H1 for crossover sites will probably be found to be an order of magnitude, or more, greater than that of any interaction on linear DNA, whether electrostatic, or by specific binding to nucleotide sequences of either the pML2αG or its parent, pBR322.

Clearly this demonstrates an absolutely different mechanism of association of H1 to linear DNA, as compared to the association of H1 to supercoiled DNA. It would probably be safest to say that the linker histone has a mechanism of binding that allows it to prefer supercoiled DNA. Thus, it would be more correct to say that H1's ligand is supercoiled DNA, or DNA crossovers, but not "DNA". Earlier research in

this laboratory implicated the crossovers of two strands of duplex DNA crossing over upon one another (Krylov et al., 1993), placing four single strands of DNA in close juxtaposition, as the preferential binding configuration of the linker histone (Varga-Weisz et al., 1993).

Except for the observation of aggregates caused by increasing the addition or loading of H1 molecules onto the DNA, linear or supercoiled, there were no other transitions as had been earlier reported by others in the literature. The ratio of H1 molecules to DNA sites of binding and the monovalent salt concentrations were examined extensively and failed to turn up any transitions in binding behavior. There was a clear linear inhibition of binding by the addition of NaCl in the case of both linear and supercoiled DNA (Figure 2.4). There was no apparent transition in the mode of binding in the range of 20 to 50 mM NaCl as has been previously reported by other groups (Clark and Thomas, 1986), nor was there evidence for positive cooperativity under any conditions in these gel electrophoresis mobility shift assays.

Further studies on the nature of the H1 - DNA interaction would likely need to address the point of contact at an atomic resolution, to determine the domain or domains on the protein molecule responsible for specific interactions with the DNA molecule. Any possible discoveries of this nature would need to be correlated with or contrasted to the case of H1 associating with DNA precomplexed with the core histones in the core particle.

Chapter 3

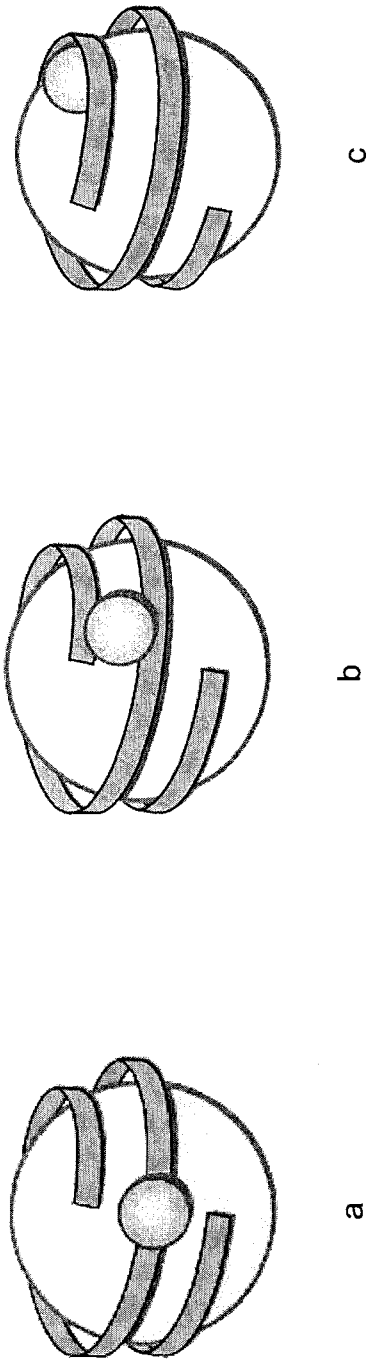
Linker Histone - Nucleosome Interactions

A. Introduction

Linker histones bind to DNA in the chromatin fiber and contribute to the compaction of chromatin and the formation of higher order chromatin structures. The nucleosomal core particle, discovered in the early 1970's, is the basic subunit of chromatin, and is defined as 146 bps of DNA wrapped around an octamer of histone proteins, two each of H2A, H2B, H3 and H4. Since the discovery of the chromatosome, defined as the nucleosomal particle containing about 168 bps of DNA wrapped around the histone octamer and one molecule of LH, research into understanding how the linker histone binds to the nucleosome has proliferated immensely. Despite all the effort on the part of biochemists, the issue is still clouded in uncertainty. Earlier data suggested that the LH or its globular domain bound near the dyad axis of the nucleosomal core particle (Figure 3.1a), with 10 bps of DNA protected against micrococcal nuclease (MNase) on each end of the DNA as it enters and exits the nucleosome core particle (Allan et al., 1980; Staynov and Crane-Robinson, 1988). This view is consistent with the evidence for two DNA-binding sites spread 2.5 nm apart on opposite sides of GH5 (Ramakrishnan et al., 1993), both of which are required for the formation of the chromatosome (Goytisolo et al., 1996a).

Figure 3.1. Schematic view of the location of GH5 in the nucleosome. (a) Symmetric model of Allan et al., 1980; (b) Asymmetric model of Zhou et al., 1998; (c) Model of Pruss et al., 1996.

Figure 3.1



More recently, alternative, asymmetric placements of the globular domain of the linker histone have been proposed (Hayes and Wolffe, 1993; Hayes et al., 1994; Ura et al., 1995). These recent proposals stem from studies of the location of the LH on chromatosomes reconstituted on the 5S rRNA gene from *Xenopus borealis*. On this sequence, it was reported that LH protects linker DNA asymmetrically distributed with respect to the nucleosome core. After protein-DNA crosslinking experiments, it was further reported that the globular domain of the linker histone H5 (GH5) contacted the DNA at a site 65 bps away from the dyad axis, on only one side of the nucleosomal particle (Figure 3.1c; Hayes et al., 1994). More recently, further experimental results from this same group of researchers led to their proposal of a model in which the globular domain of the linker histone is asymmetrically located inside the gyres of DNA that also wrap around the core histones in the nucleosomal particle (Pruss et al., 1996; Hayes, 1996).

A third model was proposed that placed the globular domain between one terminus of chromatosomal DNA and the DNA in the vicinity of the dyad axis, lying on the outside of the particle, bridging two adjacent DNA gyres (Figure 3.1b; Zhou et al., 1998; Crane-Robinson, 1997). Thus, although the conformation of the nucleosomal core particle is now known in exquisite detail (Richmond and Daley, 2003; Luger et al., 1997; Arents and Moudrianakis, 1993; Richmond et al., 1984), the structure of the next higher order structure of chromatin remains in debate.

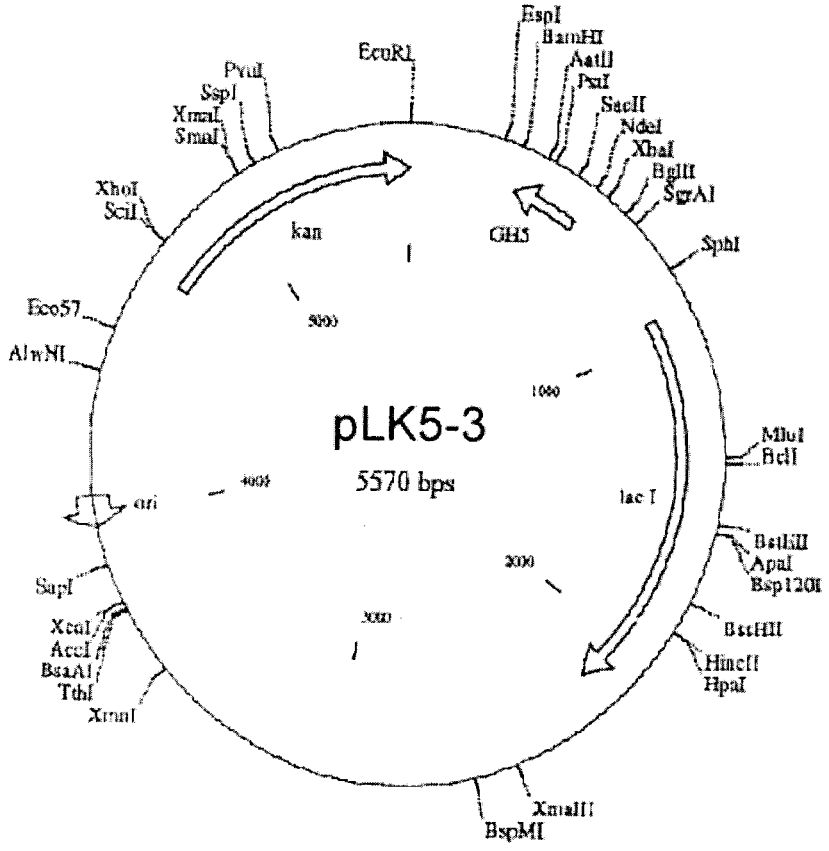
In an effort to reconcile the various purported binding locations of the linker histone onto the nucleosome, I used the DNA including the 5S rRNA gene from *Xenopus borealis* (known to strongly position histone octamers translationally along its sequence) in reconstitutions of nucleosomal particles with and without linker histones, and I subjected the resultant particles to UV irradiation-induced protein-DNA crosslinking, isolated the covalently crosslinked nucleoprotein complexes, and electrophoretically analyzed the whole and the proteolytically fragmented complexes. I discovered that this methodology produces covalently crosslinked nucleoprotein complexes that are discernible as complexes involving the globular domain of H5 (GH5), and that, in the absence of LH from these nucleosomal particles, no UV-induced protein-DNA crosslinks were detected.

B. Materials and Methods

Preparation of H5 Globular Domain (GH5). The plasmid p2.6 H5 (Kreig et al., 1983) was used as a starting point to clone the H5 gene into a T7 expression vector (Figure 3.2). Full length H5 cannot be expressed in *E. coli* (Gerchman et al., 1994). The gene fragment corresponding to the globular domain of the chicken erythrocyte linker histone variant H5 was ligated into a pET3c expression vector (Studier et al., 1990). For expression, the resulting plasmid, called pLK5-3 (Gerchman et al., 1994), was shuttled into the strain BL21(DE3). This strain contains on the host chromosome

Figure 3.2. Construct of the pLK5-3 clone of the gene for chicken erythrocyte histone variant, globular domain, GH5. The broad arrows indicate: kan: the kanamycin resistance gene; GH5: the gene for GH5; lac I: the gene for the *lacZ* repressor (IPTG temporarily inactivates the lacI repressor gene product, allowing the expression of the host lacZ gene.); ori: the origin of replication.

Figure 3.2


pLK5-3
5570 bps
EcoRI
EspI
BamHI
AatII
PstI
SacII
NdeI
XbaI
BglII
SgrAI
SphI
MluI
BclI
BstBII
ApaI
Bsp120I
BssHII
HincII
HpaI
XmaIII
BspMI
SapI
XcmI
AccI
BsaAI
TthI
XmnI
Eco57
AlwNI
XhoI
ScaI
PvuI
SspI
XmaI
SmaI
kan
GH5
lac I
ori
1000
2000
3000
4000
5000

a gene for T7 RNA polymerase under *lac* control. Induction is turned on by the addition of IPTG as described (Studier and Moffatt, 1986). The strain also lacks the outer membrane protease *ompT* (Grodberg and Dunn, 1988).

The pLK5-3 plasmid-containing BL21(DE3)-strain *E. coli* colony was selected on agar plates containing Luria-Miller media and kanamycin, and a positive colony was picked from the plate and incubated overnight in 5 ml L-Miller broth with kanamycin (30 µl/ml of a 10 mg/ml stock, or 0.03% w/v) at 37°C with shaking. Luria-Miller media is Luria-Bertani media with double the NaCl content. The 5-ml overnight was then added to 1 liter of L-Miller medium containing 0.03% kanamycin and incubated at 37°C with shaking until the O.D. at 600 nm wavelength reached 0.6 - 0.9, which takes approximately 4 to 6 hours. At that density, 5 ml of a 100 mM solution of IPTG was added to induce expression, and after 8 hrs. of further incubation at 37°C with shaking, PMSF to 0.5 mM was added to inhibit protease activity. At that point the medium can be stored at -20°C overnight. Stock PMSF is dissolved in isopropanol to 100 mM, and stored in the dark (brown glass bottles wrapped in foil) for up to 2 months.

The culture was centrifuged in four 250-ml Nalgene bottles at 7,000 rpm in a GSA rotor (Beckman) for 12 mins. and, after decanting off the supernatant, the pellet, comprised of the intact *E. coli* cells, was resuspended in 15 ml per each Nalgene bottle of a buffer containing 50 mM Tris-HCl, 2 mM EDTA, 0.5 M NaCl and 0.25 mM PMSF (Resuspension Buffer). This is a variation of the protocol of Buckle et al.

(1992), in which the salt is added after sonication. Experience had shown that the salt aided in lysis of the cells when present during sonication. The cell suspension was typically sonicated for 10 min. at a setting of 6 with a handheld sonicator (Branson Sonic Power, Model S75; Dansbury, CT.).

The viscous lysate was spun for 30 min. at 12,000 rpm in an SS34 rotor (Sorvall), and the supernatant decanted and transferred to new, 50-ml polypropylene centrifuge tubes on ice. 11.4 gm $(NH_4)_2SO_4$ was added to each 30 ml of cell supernate so that the final concentration of $(NH_4)_2SO_4$ was 380 mg/ml (Cerf et al., 1993). Tubes were inverted several times with parafilmed tops to mix, and the cloudy mixture was left on ice at 4°C for 16 hrs.

The sample preparations were next centrifuged at 12,000 rpm for 45 mins. in an SS34 rotor, and the supernatant transferred to SpectrPor 3 (MWCO = 3,500) dialysis membrane (Spectrum Laboratories, Inc.). The transferred sample preparations were dialyzed in 1.8 L dialysis buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA) + 0.25 mM PMSF per 30 ml sample preparation. Dialysis membrane must be prewetted in H_2O + 0.01% NaN_3 for at least 30 mins., and usually EDTA was added to the prewetting solution in order to chelate divalent cations and thereby protect against contaminating enzyme activity. Typically this was done the day before and the membrane was stored in a capped jar in the prewetting solution overnight at 4°C. Dialysis proceeded for 12-14 hrs. at 4°C, the dialysis buffer was replaced by the same volume of fresh buffer, and dialysis was repeated.

A gel filtration column was prepared by washing CM-Sephadex-25 resin extensively in 10 mM Tris-HCl buffer containing 0.5 M NaCl (CM-25 Sephadex Wash Buffer), decanting, and soaking the resin in ethanol, and finally replacing the ethanol with distilled deionized water containing 0.02% NaN_3, and soaking overnight. Through this procedure, 3 gms. of CM-Sephadex-25 swelled to ca. 15 ml of volume in the wash buffer. Fittings, glass column, and outlet tubes were washed in TE buffer, pH 7.5, and then equilibrated in the same buffer containing 0.3 M NaCl (Equilibration Buffer). After partial assembly of the column with the bed support frit, bottom cap with stop cock, outlet tube, fitting and clamp (but still without the lid, input tube and lid fitting) the column was filled up to the bed support with Equilibration Buffer by pouring it into the outlet tubing. Then, by using a funnel and glass rod, the column (2.5 cm. O.D. x 15 cm height) was packed with the resin slurry. Equilibration Buffer was pumped through the column using a peristaltic pump over a period of several days intermittently, in aliquots of 300 ml prior to use. Just before use, the Equilibration Buffer was replaced with CM-25 Sephadex Wash Buffer by peristaltic pumping.

Samples were run through the column, with the aid of a peristaltic pump, in Equilibration Buffer. After several bed volumes of Equilibration Buffer had been allowed to flow through the column, a gradient maker was washed in 95% EtOH, and then in Resuspension Buffer, and its two chambers were filled, one with Equilibration Buffer, and the other with Elution Buffer (40 mM Tris-HCl, pH 7.5, 1 M NaCl, 1.5 mM EDTA), and this was pumped through the gel filtration column, effectively

passing a linear gradient of NaCl from 0.3 M to 0.8 M through the column (Figure 3.3). Fractions of approximately 2 ml each were collected with an automated fraction collector, and each fraction was monitored by reading its absorption at 230 nm (deuterium lamp).

Peak UV-absorbing fractions were pooled and dialyzed in 2L of pH adjusted H_2O for 15 hrs. at 4°C, the H_2O was replaced, and the dialysis was repeated in the same way. The finished dialysates were stored in silanized tubes at -20°C.

The frozen protein dialysates were lyophilized with N_2 in a Speedvac for at least 15 hrs. The lyophilized pellets were resuspended in 25 µl of 1 mM sodium phosphate buffer, pH 7.7 (Goytisolo et al., 1996b). Each sample was assayed by gel electrophoresis on a denaturing discontinuous gel (Laemmli, 1970; Figure 3.4), and the successful preparations were stored at -80°C.

DNA Fragments. DNA encompassing the *Xenopus borealis* somatic 5S rRNA gene derived from the plasmid pXbs-1 (Peterson et al., 1980) was cloned into pUC19, a high copy number plasmid. The sequence of the 238-bp *Hpa* II-*Dde* I fragment bounded by PCR primer sequences containing the BamHI recognition sequence on either end was amplified by PCR, and then this PCR product (Figure 3.5) was cut with BamHI and ligated, using T4 DNA ligase (New England Biolabs), into the pUC19 vector's polylinker region at the BamHI site.

The cloned DNA was transformed into the DH5(*lacZ*Δ) strain of *E. coli*, after the cells were made competent by a variation of a recently reported 5-minute method

Figure 3.3. The salt elution profile of the rGH5 protein from the CM-Sephadex gel filtration column. The absorbance at 230 nm and the molar concentration of NaCl in the elution buffer are shown as a function of the fraction of elution, in milliliters.

Figure 3.3

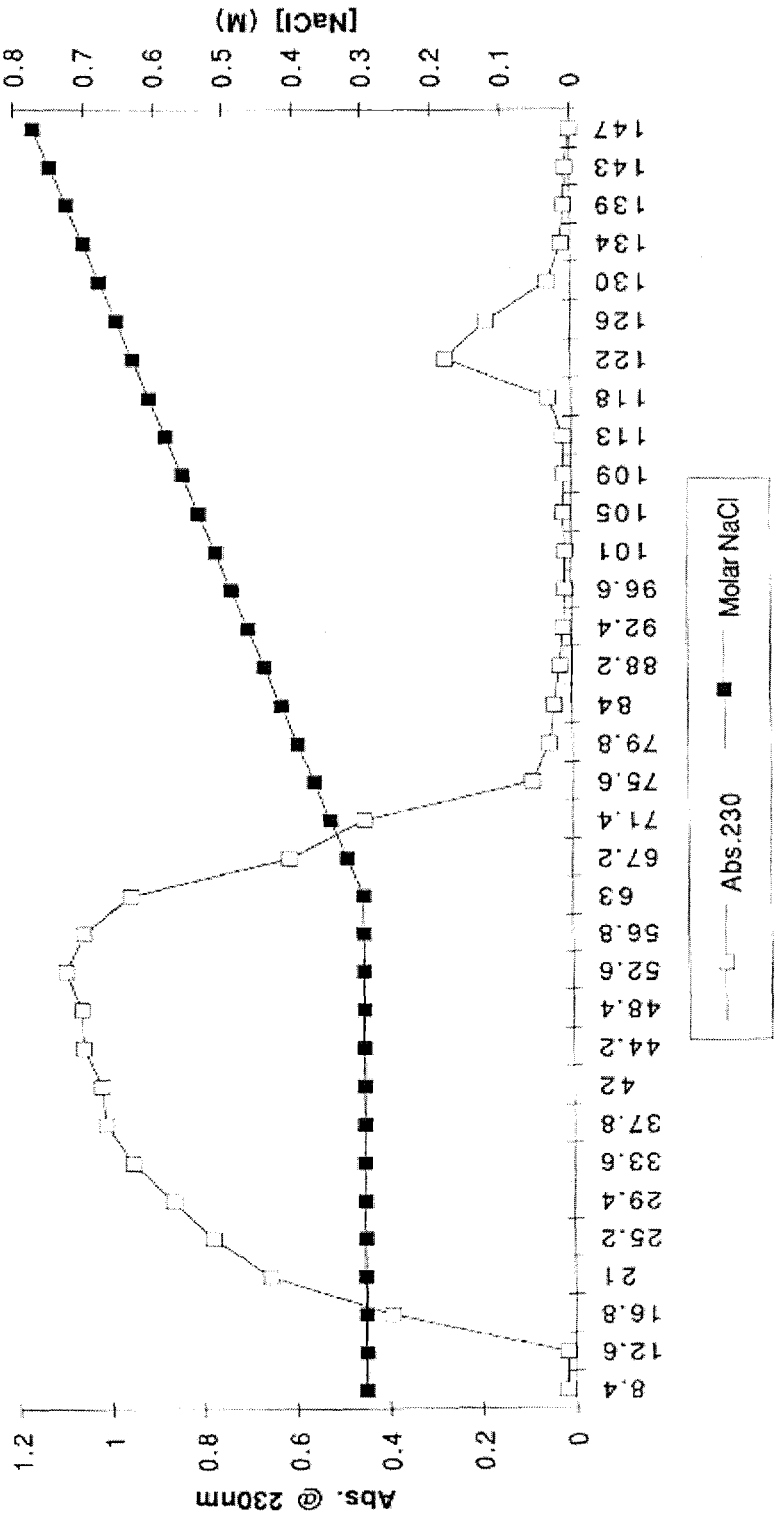

[NaCl] (M)
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
147
143
139
134
130
126
122
118
113
109
105
101
96.6
92.4
88.2
84
79.8
75.6
71.4
67.2
63
56.8
52.6
48.4
44.2
42
37.8
33.6
29.4
25.2
21
16.8
12.6
8.4
Abs. @ 230nm
1.2
1
0.8
0.6
0.4
0.2
0
Molar NaCl
Abs.230

Figure 3.4. Discontinuous SDS-polyacrylamide gel of the rGH5 protein isolated from the pLK5-3 clone expressed in the BL21(DE3) strain of *E. coli*. Lanes 1, 2, 5 and 10 show different fractions of the protein eluted from the CM-25 Sephadex column.

Figure 3.4

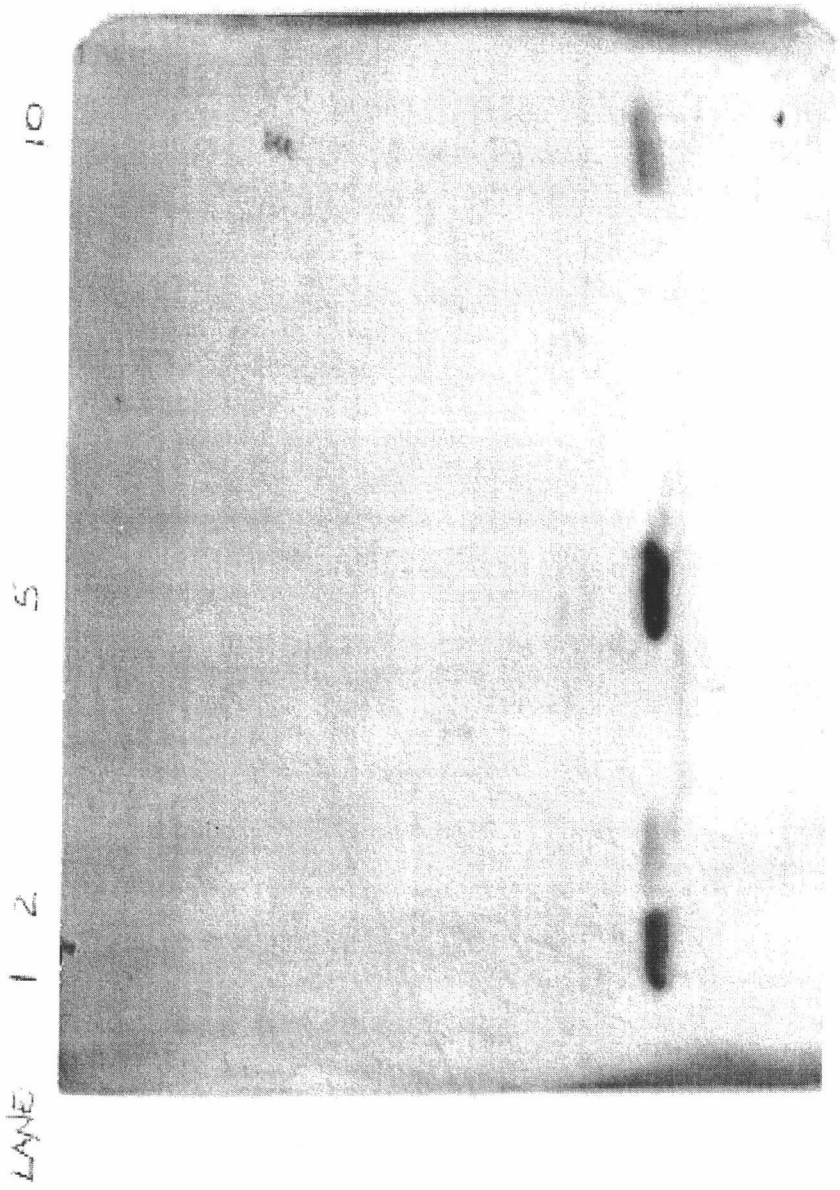
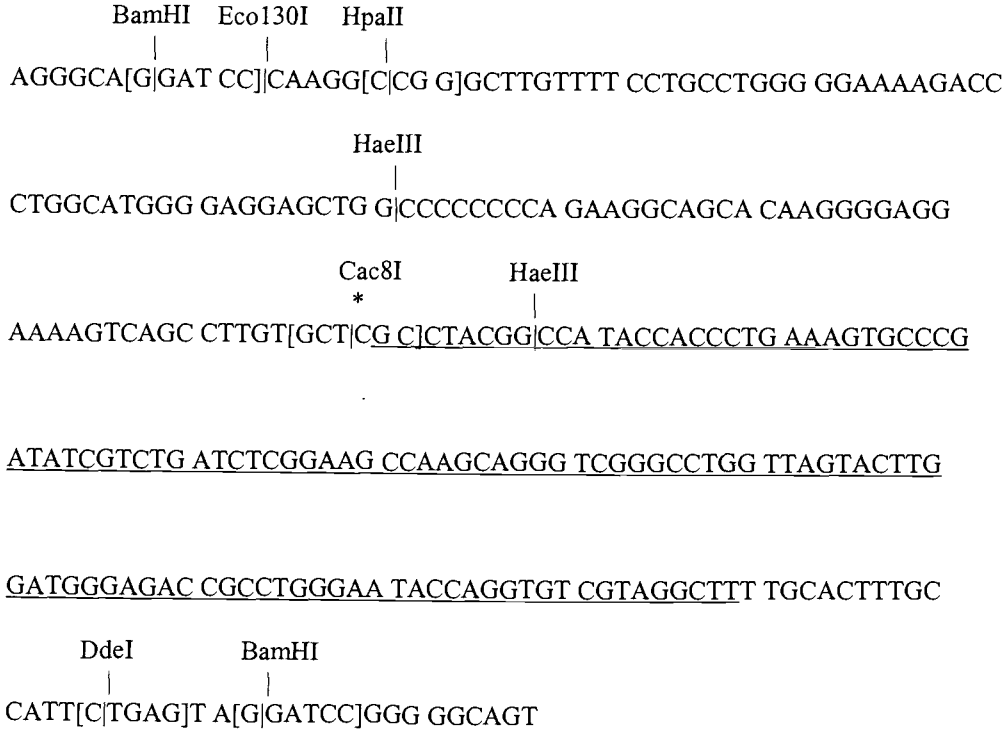


Figure 3.5. Nucleotide sequence of the plasmid pTPE1 *Bam*HI-site cloned insert, containing the 256-bp *Bam*HI DNA fragment used for the experiments in this chapter and in Chapter 4. The underscore line indicates the 5S rDNA gene of *Xenopus borealis*. Vertical lines indicate the cleavage sites used in these experiments as described in the text. Brackets indicate the recognition sequences of the restriction endonucleases indicated. The *Bam*HI and *Hae*III restriction endonucleases are used in the experiments in this chapter, and are described here in the text. The other restriction endonucleases shown are used in the experiments in Chapter 4 and are further described in that chapter. The asterisk refers to the site of ^{32}P labeling used in the experiments in Chapter 4, as described in that chapter.

Figure 3.5


BamHI Eco130I HpaII
AGGGCA[G|GAT CC]|CAAGG[C|CG G]GCTTGTTTT CCTGCCTGGG GGAAAAGACC
HaeIII
CTGGCATGGG GAGGAGCTG G|CCCCCCCCA GAAGGCAGCA CAAGGGGAGG
Cac8I
*
HaeIII
AAAAGTCAGC CTTGT[GCT|CG C]CTACGG|CCA TACCACCCTG AAAGTGCCCG
ATATCGTCTG ATCTCGGAAG CCAAGCAGGG TCGGGCCTGG TTAGTACTTG
GATGGGAGAC CGCCTGGGAA TACCAGGTGT CGTAGGCTTT TGCACTTTGC
DdeI
BamHI
CATT[C|TGAG]T A[G|GATCC]GGG GGCAGT

(Pope and Kent, 1996), employing RbCl in place of $CaCl_2$. This method of permeabilizing the cells is a variation of the methods employing $CaCl_2$, but is more effective, and easier and less time-consuming to do. Colonies were screened by X-gal, and after several unsuccessful attempts to retrieve the clone from white colonies, the blue colonies were tested and found to be positive for the clone. The explanation appears to be that the reporter gene, *lacZ*, was interrupted by the insertion of an exact multiple of three base pairs of the exogenous, target DNA. This apparently rendered the two parts of the *lacZ* gene on either side of the insert DNA in frame and readable. Lac Z protein was apparently produced and made active despite the insert, creating lacZ gene product, and cleaving X-gal to create blue colonies which, however, contained the target DNA in the plasmid.

Nucleosome Reconstitutions. Purified chicken erythrocyte core histones (a gift from Dr. Jeffrey Hansen, University of Texas Health Sciences Center, San Antonio) were reconstituted onto the *Bam*HI-terminal 256-bp *X. borealis* 5S rRNA gene-containing DNA, or the shorter fragment of the same sequence, the 238-bp *Hpa*II-*Dde*I fragment, by direct mixing of the components in high salt, followed by step dilution of NaCl concentration to low salt.

Spectrophotometrically determined concentrations of core histone octamers and DNA were mixed so that the weight-to-weight ratio of the protein to DNA was approximately 0.61 – 0.62. This ratio is chosen because experience shows that it will provide a reasonable amount of nucleosome without formation of dinucleosomes. The

concentration of NaCl was raised to 1.6 M at the initial mixing of the protein and DNA. The volume of the initial mixture was typically 25 μl, but this was varied to allow for the differing concentrations of starting stock solutions. The major concern was to start with as small a volume as possible containing the high concentrations necessary, since the final concentrations of NaCl needed to be greatly reduced by dilution.

The nucleoprotein mixture was incubated at 4°C for at least 5 hrs., whereupon a solution of TE buffer, pH 8.0, was added to double the volume of the reaction mixture, while at the same time halving the NaCl concentration from 1.6 M to 0.8 M. The reaction mixture was again incubated at 4°C for greater than 5 hrs. The dilution step was repeated, except that all further added buffer was 20 mM Tris-HCl, pH 8.5, and did not contain EDTA, until the concentration of NaCl was 100 mM. The integrity of reconstituted nucleosomal core particles was assayed by electrophoresis (Figure 3.6a).

The recombinant preparation of the globular domain of the linker histone H5 (rGH5) was added to the nucleosomes in a one-to-one stoichiometric ratio to each reconstituted preparation after the nucleosomal core particles had been diluted to 100 mM NaCl. The integrity of reconstituted chromatosomes was assayed by electrophoresis (Figure 3.6b). In each case, a considerable amount of "free" DNA remains. This is intentional, a result of using DNA-protein ratios that disfavor dimer formation. The presence of free DNA does not compromise later experiments.

Figure 3.6. Nucleosome core particle and chromatosome reconstitutions on the 5S rDNA fragment from *X. borealis*. In each case, the more rapidly migrating band represents free DNA, and the slower band is DNA of the reconstituted (a) nucleosome core particle and (b) chromatosome. (a) Reconstitution of DNA into core nucleosomes as visualized by the band shift assay. The 256-bp *Bam*HI fragment was reconstituted with core histone octamer. The resultant complex was resolved on 0.8% agarose gels (see Materials and Methods). Lane 1: 100-bp ladder. Lane 2: reconstituted nucleosomal core particle. Lane 3 and 4: aliquots of 256-bp DNA. (b) Reconstitution of DNA into chromatosomes as visualized by the band shift assay. The 256-bp *Bam*HI fragment was reconstituted into core histones, as before, and then the recombinant GH5 was added (LH/core histone ratios of 1.3 in lane 2). Lane 1: 100-bp DNA ladder. Lane 2: reconstituted chromatosome.

Figure 3.6 (a)

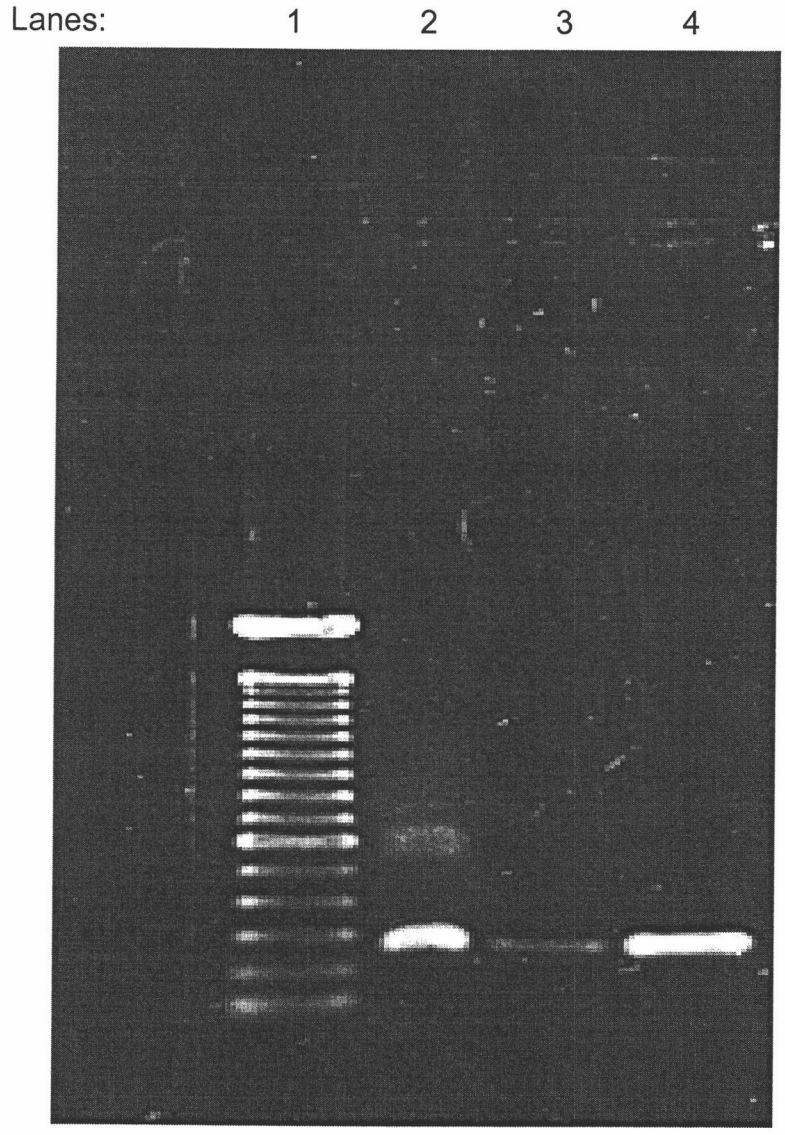
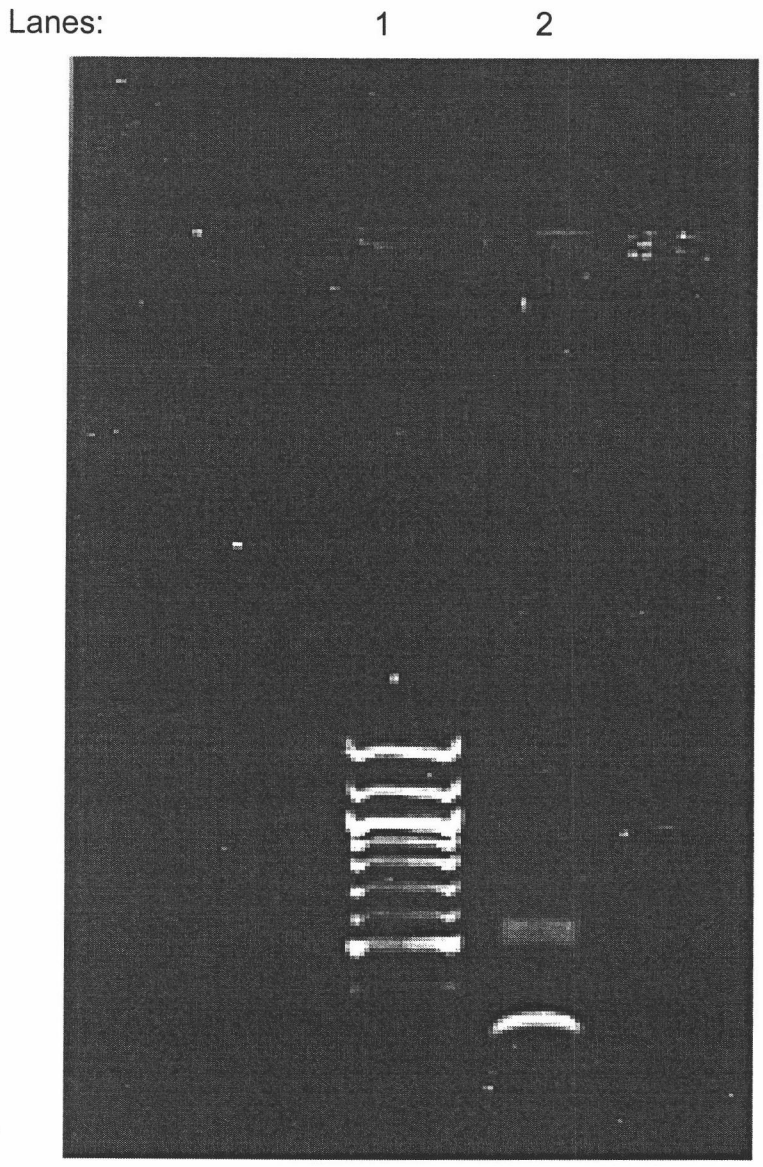


Figure 3.6 (b)



Photochemical crosslinking reaction of nucleoprotein complexes. Nucleosomes or chromatosomes were irradiated with ultraviolet light (λ_{max} = 254 nm), using the Stratalinker 1800 instrument (Stratagene; La Jolla, CA), for the purposes of inducing the covalent crosslinking of histone proteins to the nucleosomal DNA. The manufacturer's specification for this instrument is an output of 180,000 mJ x cm^{-2}, which corresponds to 3000 mW x cm^{-2} x s^{-1}. The instrument is designed to emit over 80% of this energy at a wavelength of 254 nm. The useful range of irradiation for the purposes of this set of experiments was determined empirically by exposing chromatosomal particles to the UV irradiation over a range of timed durations. The range of tested times was 5 seconds to 60 minutes. The effective range was 15 to 60 seconds, while longer exposures caused extensive DNA damage (data not shown).

Sample nucleosome solutions in low concentration Tris-HCl buffer (10 mM) were stored at 4^0C after reconstitution until UV-irradiations of the samples. Aliquots of 100 µl were routinely applied to a small, thin, flat, rectangular sheet of teflon composition manufactured by DuPont, supported in a tray of ice using a common disposable weigh tray. The teflon platform was protected from wetness by using a sheet of Saran wrap placed between the teflon support and the ice, and the teflon sheet was depressed slightly to create a shallow crater in the ice so that the chromatosomes, sitting on the teflon platform embedded in the ice, were maximally chilled during irradiation. The UV irradiation emission was generated at a distance of approximately 12 cm from the chromatosomes for the various times indicated.

On each experimental run, the Stratagene instrument was run for 1 minute prior to use. This allowed the UV-lamps to provide a more even output of energy from one experimental run to another. Whenever possible, multiple samples were irradiated simultaneously, or, for samples exposed for various lengths of time, the longer exposures overlapped the shorter ones, i.e., the samples were placed into the Stratalinker instrument at the same time, and the samples of shorter exposures were withdrawn from the instrument using a hand pipettor, while the samples of longer exposure remained in the instrument for additional irradiation. This helped to standardize the treatment to samples.

Post-irradiation treatment of crosslinked nucleosomal samples. After UV irradiation of chromatosomes, crosslinked nucleoprotein complexes were purified by phenol-chloroform extraction followed by isopropanol precipitation at -20°C. The samples were washed in chilled 100% EtOH, and again in 70% chilled EtOH. The samples were then dried in a Speedvac Concentrator (Savant), and the dried pellets, containing DNA and nucleoprotein complexes, but no free proteins, were resuspended in 20 mM Tris-HCl, pH 8.5 before being electrophoresed on agarose gels and on SDS-polyacrylamide gels.

Later experiments treated the nucleoprotein complexes, isolated as described above, with proteinase K, before being electrophoresed in parallel with control samples that had not been proteolyzed. Proteinase K solutions were made up from lyophilized powder (Sigma; St. Louis, MO), by dissolving in 20 mM Tris-HCl, pH 8.5,

to 20 mg/ml, with added $CaCl_2$ to 1 mM, and stored at -20°C in single use aliquots to minimize handling at ambient temperatures before use. The enzyme was tested on proteins, and the proteolyzed peptides were electrophoresed for confirmation of enzyme activity (data not shown).

Typically, proteinase K was added to a UV-crosslinked nucleosomal sample in Tris-buffer, pH 8.0 with added $CaCl_2$ to 1.25 mM. The proteolysis reaction was allowed to proceed for 8 hours at 37°C in most cases. Because a total digest was desired, it was not necessary to run timed digestions. The samples were then phenol-choloroform extracted and isopropanol precipitated, spun at 12,000 rpm in a benchtop microcentrifuge, washed with EtOH, and the washed pellets were dessicated in the Speedvac Concentrator to dryness (usually 5 hours). The pellets were resuspended in 40-50 µl of 20 mM Tris-HCl, pH 8.5.

Nucleoprotein Gel Electrophoresis. UV-crosslinked nucleosomal and chromatosomal particles were electrophoresed after purification by phenol-chloroform extraction and isopropanol precipitation. The 100-µl samples of the UV-irradiations were resuspended in smaller volumes, usually 50 µl, after the phenol-chloroform extractions. This enabled the loading of a great amount of sample onto one gel lane. It was important to allow the resuspended material to fully dissolve for at least one day before running the samples on gels. The histones and the crosslinked nucleoprotein complexes were extremely "sticky" to all known materials, and silanized vessels were used exclusively. Despite this precaution, redissolution in buffer was slow enough

that samples would not appear on gels very well if loaded within 24 hrs. of their resuspension in buffer.

Agarose gels at 1.8 % were used to assay the crosslinked nucleoproteins. TBE buffer was used exclusively both as the gel buffer and as the running buffer in both upper and lower buffer chambers. TAE running buffer gave inconsistent and unfavorable results (data not shown). Agarose gels were usually run at 100 V constant, which under these conditions ran at ca. 50 mA. EtBr was never put into the gel before the electrophoresis, as it is known to interfere with the proper association of proteins and DNA in uncrosslinked nucleosomes (McMurray et al., 1988), which were run on the gels as negative controls.

After running gels for 60 minutes, the gel was stained for DNA in a solution of 1: 10,000 dilution of 10 mg/ml EtBr in water for 5 minutes with gentle rocking, and destained for 1 hour in water with gentle rocking. Gels were recorded by digital scan on the UVP (Upland, CA) Model GDS7500 instrument.

High Performance Liquid Chromatography of Linker Histones, Core Histones and DNA. A reverse-phase C-18 HPLC column (4.6 x 250 mm) was washed with 50% isopropanol prior to use. The column was run with a 10-70% acetonitrile gradient, made by mixing two solutions, A (dH_2O + 0.1 % TFA) and B (acetonitrile + 0.08 % TFA), directly into the instrument, creating a linear gradient of the following design: 10% acetonitrile at 0 mins.; 10 – 40 % over 5 mins.; 40 – 50 % over 10 mins.; 50 – 70

% over 5 mins.; 70 % held over 3 mins.; 70 – 10 % acetonitrile (purge) over 2 mins. Total run time was 25 minutes.

Dual detectors allowed for the monitoring of protein and DNA content simultaneously. When histones alone were loaded and run on the HPLC instrument, detectors were set at 214 nm and 230 nm. The latter wavelength is distinctive for histones. When DNA and histones were run together the detectors were set for 260 nm and 214 nm.

C. Results

The cloned 238-bp DNA containing the somatic 5S rRNA gene from Xenopus borealis is a strongly translationally positioning sequence. Figure 3.5 presents the DNA sequence used in this series of experiments, which was taken from the pXbs1 plasmid (Peterson et al., 1980), and inserted into the BamHI site of the polylinker region of the pUC19 vector. The pUC19 plasmid is a high copy number plasmid which facilitated the harvest of abundant amounts of the DNA fragment. Originally, I had attempted to generate sufficient quantities of this DNA by PCR, using the pXbs1 plasmid as the template, along with appropriate, synthesized DNA primers. This proved unfeasible, as the DNA was somewhat intractable to replication with a polymerase enzyme. Perhaps this particular DNA sequence, in association with the primers chosen, spontaneously forms a secondary structure (a loop or hairpin) that

blocks an efficient polymerase reaction. At any rate, as the cost of polymerase enzyme became too great to expediently pool the required amount of PCR reaction products for a single experimental run, and the time it took to synthesize and purify the amount of DNA for just one experimental run approached about two weeks of running PCR reactions and the subsequent clean-up procedures necessary, I determined to clone the desired DNA sequence. I promptly abandoned the PCR methodology and used the described cloning procedure instead (see Materials and Methods).

I had originally followed the accepted procedure known as the exchange method (Hayes and Lee, 1997) to reconstitute nucleosomes. H1- and H5-depleted chicken erythrocyte chromatin was prepared for use as donor chromatin while performing the exchange method of reconstitution of nucleosomes. To this preparation, at high salt (2.0 M NaCl), was added the 238-bp 5S rDNA-containing fragment of interest. The mixture was dialyzed from high to low salt. Through a series of gel electrophoretic assays, it was shown that the histone proteins of the chromatin preparation bound both the DNA and the regenerated carboxycellulose dialysis membrane, and prevented the extraction of the DNA or nucleosomes from the dialysis tubing. An electrophoretic gel clearly shows that no DNA is released from the dialysis tubing at low salt. However, reintroducing salt to the dialysis solution causes release of the DNA, as judged again by gel electrophoresis of an aliquot taken from the dialysis tubing after dialyzing from high salt to low salt and back again to high salt (data not shown). Thus, the DNA had not been degraded in any way during the dialysis procedure nor

affected in any observable way by the chromatin solution into which it had been introduced (data not shown). When DNA alone was placed in the dialysis tubing, it could be recovered from the tubing at any salt concentration, including no salt, as assayed by gel electrophoresis (not shown). The exchange method of nucleosome reconstitution clearly failed in my hands. This was a rather puzzling phenomenon, made more so as the "stickiness" of the histones to the dialysis tubing has not been reported in the vast number of publications documenting the usefulness of this approach to making reconstituted nucleosomal particles. I adopted a newer protocol, which worked well. I shall use the term 'dilution method' when designating this protocol.

The dilution method provided a reliable means of obtaining reconstituted nucleosomes. The histone octamer associates at a particular translational orientation along the 5S rDNA-containing 238-bp DNA fragment used in these experiments to produce a strongly 'positioned' nucleosome (Hayes and Wolffe, 1993; Hayes et al., 1990). The apparent homogeneity of the product on gel analysis testifies to this positioning; nucleosomes spaced randomly on the DNA would produce a broad band. Subsequently, GH5 was added in a stoichiometrically appropriate amount. The resultant chromatosome is also stringently positioned and migrates slightly more slowly than the nucleosome, as judged by standards (Figure 3.6b). In both preparations, a considerable fraction of free DNA remains. This is a consequence of the low histone/DNA ratio chosen in order to eliminate the possible formation of

dimers of nucleosomes (dinucleosomes) on the long DNA. The presence of free DNA does not compromise any of these experiments.

UV Irradiation of Reconstituted Nucleosomes Creates Crosslinked Nucleoprotein Complexes. Early in the 1960s independent published experimental results indicated that the absorption of ultraviolet light could induce crosslinking of protein to DNA (Smith, 1962; Alexander and Moroson, 1962). Since these initial observations there have been an increasing variety of methods used to study photoinduced nucleic acid - protein crosslinking in UV-irradiated systems. Electron microscopy, mass spectrometry, filter-binding assays, ultracentrifugation, gel chromatography, gel electrophoresis and quantitative measurement of nucleic acid extracted from protein are just a few.

DNA can be separated from proteins by phenol-chloroform extraction. DNA covalently crosslinked to proteins will separate with the free DNA if the DNA is of substantially greater mass than the protein. If a protein is of substantially greater mass than a covalently crosslinked DNA, then the protein carries the DNA with it into the nonaqueous (phenol/chloroform) phase of the separation reagent in this protocol. The GH5 and the core histones are all under 20 kilodaltons, and the DNA is about 157 kilodaltons, so it is expected, and turns out to be the case, that the nucleoprotein complex is recovered in the aqueous phase. Thus, the UV-irradiated chromatosomes, and controls, were phenol-chloroform extracted prior to gel electrophoresis of the UV-treated samples.

The dosage of UV irradiation required for maximal crosslinking efficiency with minimal undesired side reactions is determined empirically. The Stratalinker 1800 instrument has been used extensively for the purposes of crosslinking DNA to proteins. The power output is constant with time (see Materials and Methods). The use of a range of dosages showed that degradation of the DNA ensued at the upper range (data not shown), while crosslinking was obtained at the very low range (Figure 3.7).

These preliminary results showed that chromatosomes form covalently crosslinked nucleoprotein complexes induced by UV-irradiation (Figures 3.7 - 3.10, 3.12). It is noteworthy that only chromatosomal complexes containing LH formed these crosslinks: in all of these experimental runs, reconstituted nucleosomal particles in which LH was absent were assayed in parallel, and these did not form the crosslinked products that were seen in the case of LH-containing particles (Figure 3.8).

Some Crosslinked Nucleoprotein Complexes are Multimers of DNA Covalently Associated by the Linker Histone. Two classes of crosslinked products were observed when chromatosome preparations were irradiated and then phenol extracted. A frequent product induced by UV exposure was the apparent 550-bp UV-induced crosslinked structure shown in Figure 3.7. But this was not the only product formed. Different high molecular weight products appeared in other attempts at UV-exposure of chromatosomes (data not shown). The 550-bp UV-induced crosslinked product was created more frequently than any other UV-crosslinked nucleotide-containing species.

Figure 3.7. Agarose gel electrophoresis of DNA and covalently crosslinked DNA-protein complexes phenol extracted from chromatosomes after irradiation for 0, 15, 30 and 60 seconds (lanes 2-5, respectively). Lane 1 contains 100-bp DNA ladder as a marker. The arrow points to the putative 550-bp photoadduct. The photoadduct does not occur in lane 2, where the chromatosomes had not been exposed to UV irradiation.

Figure 3.7

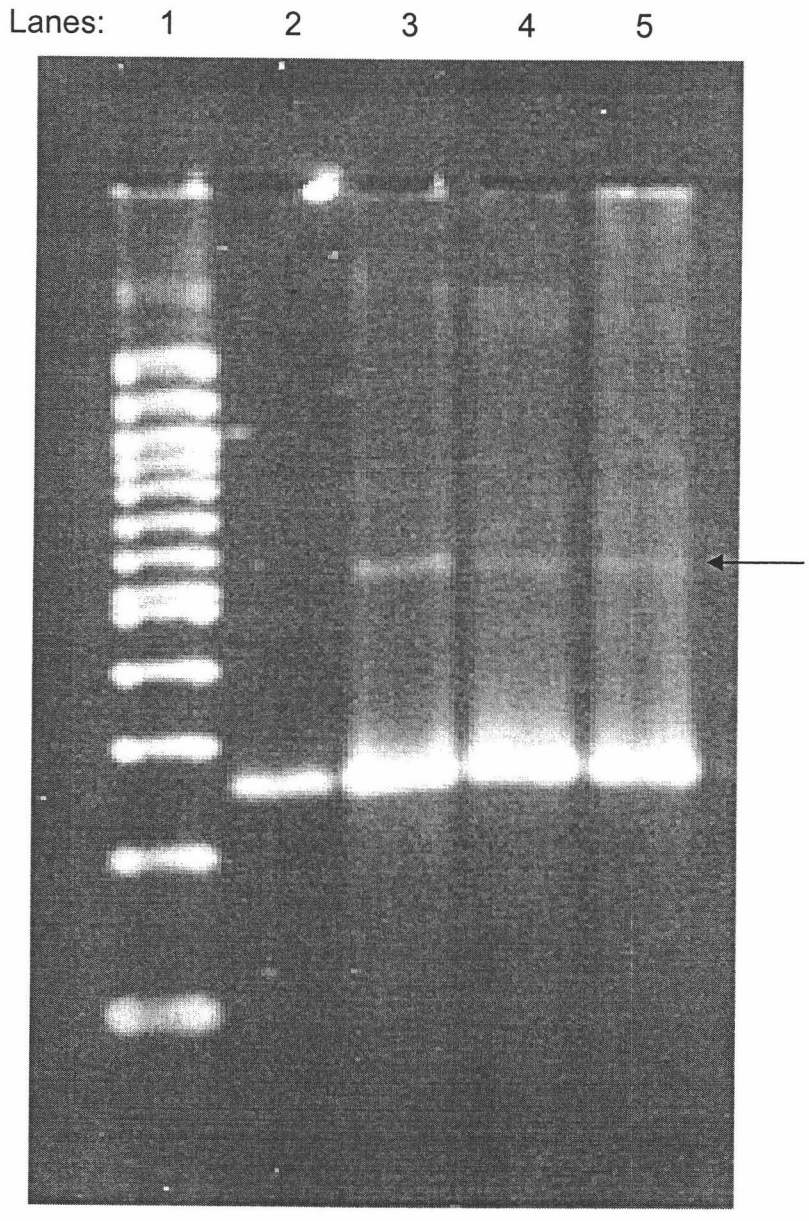
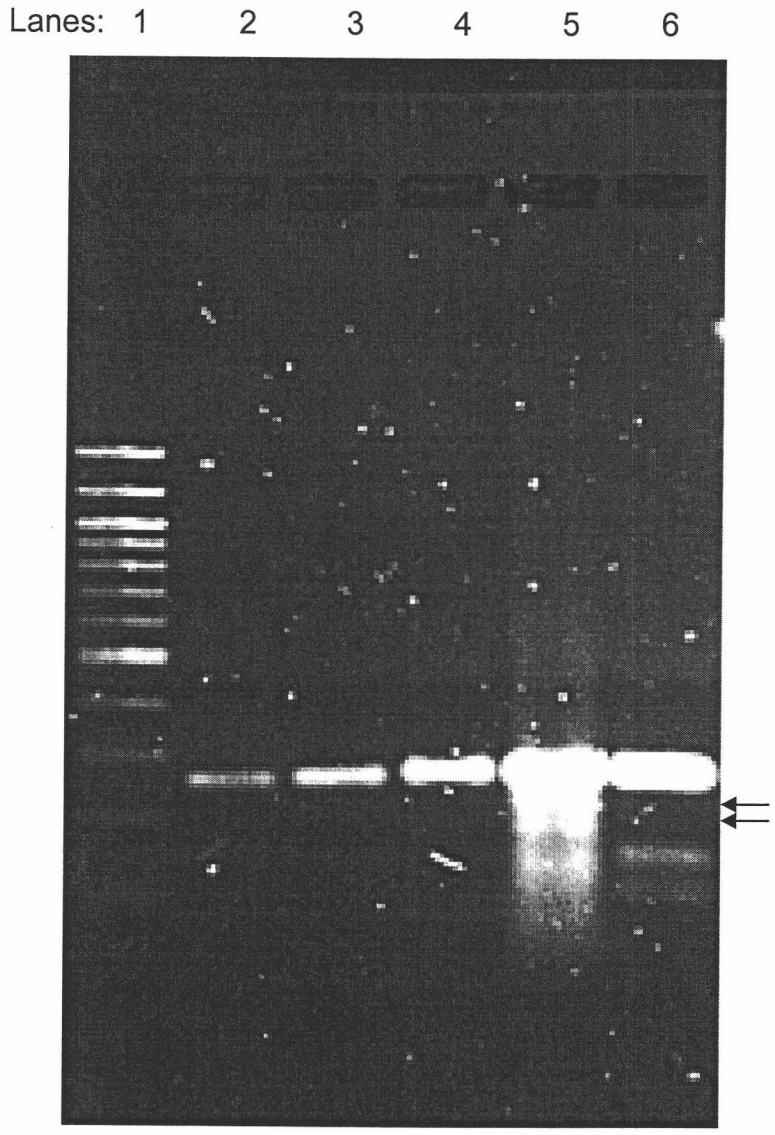


Figure 3.8. Agarose gel electrophoresis of DNA and covalently crosslinked protein-DNA complexes. Arrows indicate apparent low-molecular-weight DNA created by UV-irradiation. Lanes 2 and 3: nucleosomes (core particles with no LHs) after UV-irradiation for 15 seconds and phenol-chloroform extraction. The sample in lane 2 was treated with proteinase K before loading onto the gel. Lanes 4 and 5: chromatosomes (containing GH5) after UV-irradiation for 15 seconds and phenol-chloroform extraction. The sample in lane 4 was treated with proteinase K prior to loading onto the gel. Lane 1 contains 100-bp DNA ladder. Lane 6 contains DNA partially digested with *Cac*8I restriction enzyme.

Figure 3.8



However, the appearance of the 550-bp species and that of other crosslinked products, some of which migrated faster than the original DNA (Figure 3.9), were mutually exclusive: when the 550-bp product formed, the others did not; when the other products formed, the 550-bp product did not.

To study the nature of these putative crosslinked products, in each case a UV-irradiated chromatosomal sample was subjected to proteolysis by treatment with proteinase K. The result was clear: the "550-bp band" disappeared after proteolysis, leaving only the 256-bp DNA species (Figure 3.10). Numerous repetitions of this experiment yielded identical results. Similarly, when the rapidly migrating species formed, they were also treated with proteinase K, and they too disappeared, leaving only the 256-bp DNA fragment (Figures 3.8 and 3.9). The implication was that the 550-bp band was the product of two 256-bp DNA molecules covalently crosslinked to a linker histone molecule, induced by UV irradiation.

The pattern made clear that the various bands of DNA observed on the gels were in fact nucleoprotein complexes. Since DNA of no size other than the 256-bp DNA was present in these experiments, it would be safe to suggest that the proteins held together 256-bp DNA fragments, either inter- or intramolecularly. Once the proteins were cleaved by proteinase K, the multiple DNA fragments were no longer held together as multimers. The fact that only 256-bp DNA remained observable on the gel after proteolysis provides very strong evidence that the 550-bp species was a dimer of 256-bp DNA fragments held together by a protein. Since these dimers never occurred

Figure 3.9. Agarose gel electrophoresis of the DNA that was phenol-chloroform extracted from chromatosomes after irradiation for 60, 30, 15 and 15 seconds (lanes 2-5, respectively) and either treated with Proteinase K for 4 hours (lanes 2 - 4), or not treated with proteinase K (lane 5). The arrows indicate the low molecular-weight DNA species induced by UV irradiation and removed by proteolysis. Lane 1 contains 100-bp DNA ladder. Lane 6 contains 256-bp DNA run as a control.

Figure 3.9

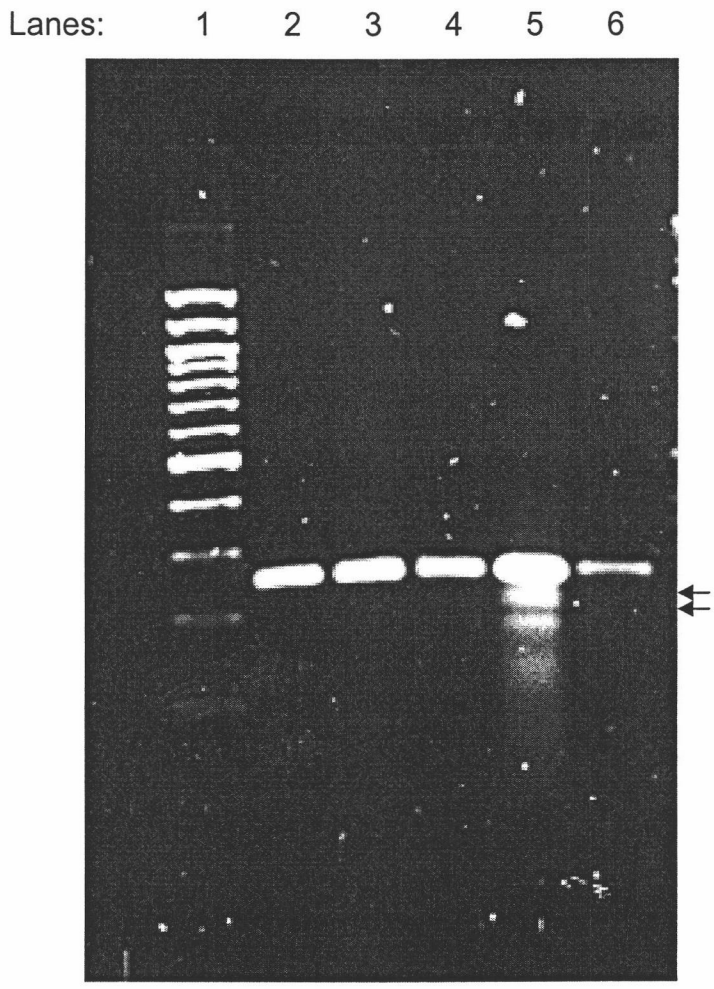
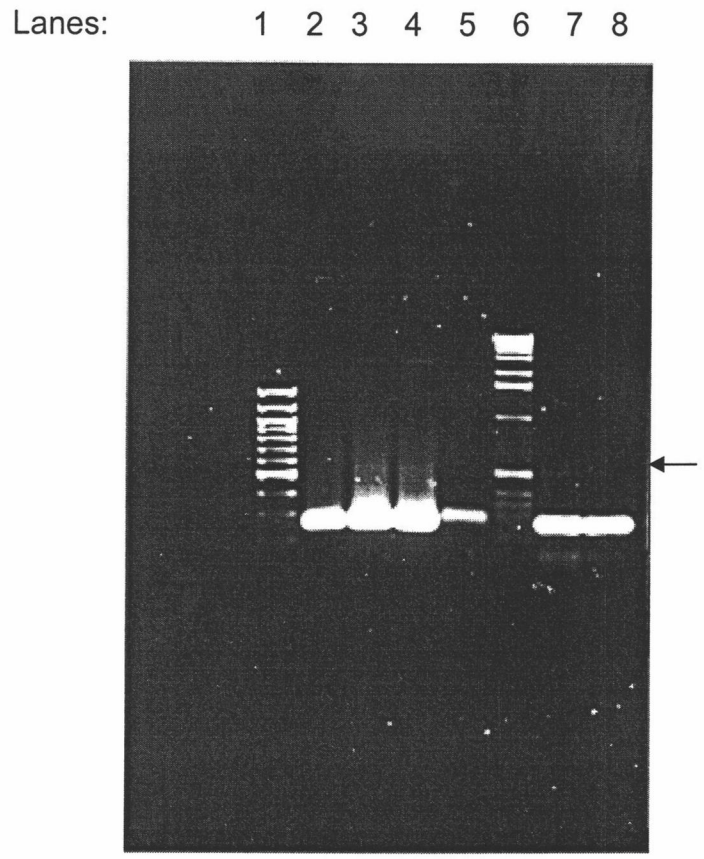


Figure 3.10. Agarose gel electrophoresis of DNA phenol-chloroform extracted from chromatosomes after irradiation for 60 seconds (lanes 2 and 3) or for 15 seconds (lanes 4 and 5), with (lanes 2 and 5) or without (lanes 3 and 4) proteinase K treatment. Arrow indicates the apparent 550-bp DNA species resulting from UV-induced crosslinking. Lane 1 contains 100-bp DNA ladder. Lane 6 contains 1 Kb ladder. Lane 7 and 8 contain 256-bp DNA preparations made for other experiments.

Figure 3.10



when the experimental substrate was a reconstituted nucleosomal particle in which the linker histone (GH5) had not been added (Figure 3.8), but the DNA dimers did occur cleaved by proteinase K, the multiple DNA fragments were no longer held together as multimers. The fact that only 256-bp DNA remained observable on the gel after proteolysis provides very strong evidence that the 550-bp species was a dimer of 256-bp DNA fragments held together by a protein. Since these dimers never occurred when the experimental substrate was a reconstituted nucleosomal particle in which the linker histone (GH5) had not been added (Figure 3.8), but the DNA dimers did occur nearly every time a reconstituted chromatosome (containing the linker histone (GH5)) was used as the experimental substrate, it was probable that the protein crosslinked to the putative 256-bp DNA dimer was GH5.

Other Crosslinked Nucleoprotein Complexes Involve Intramolecular Association with the Linker Histone. UV irradiation sometimes created a complex containing DNA that migrated at 550 bps, and sometimes it created a complex containing DNA that migrated to two positions, one at about 200 bps, and the other about 185 bps, as judged by the 100-bp DNA ladder run as a standard.

These faster migrating DNA species were puzzling because they appeared smaller than 256-bp DNA and therefore they could not be explained as an integral number of 256-bp DNA molecules. The DNA could have been degraded during the experimental protocol, but this is unequivocally ruled out by the observation that treatment with

proteinase K restored the 256-bp band, with no trace of the more rapidly migrating bands remaining (Figure 3.9).

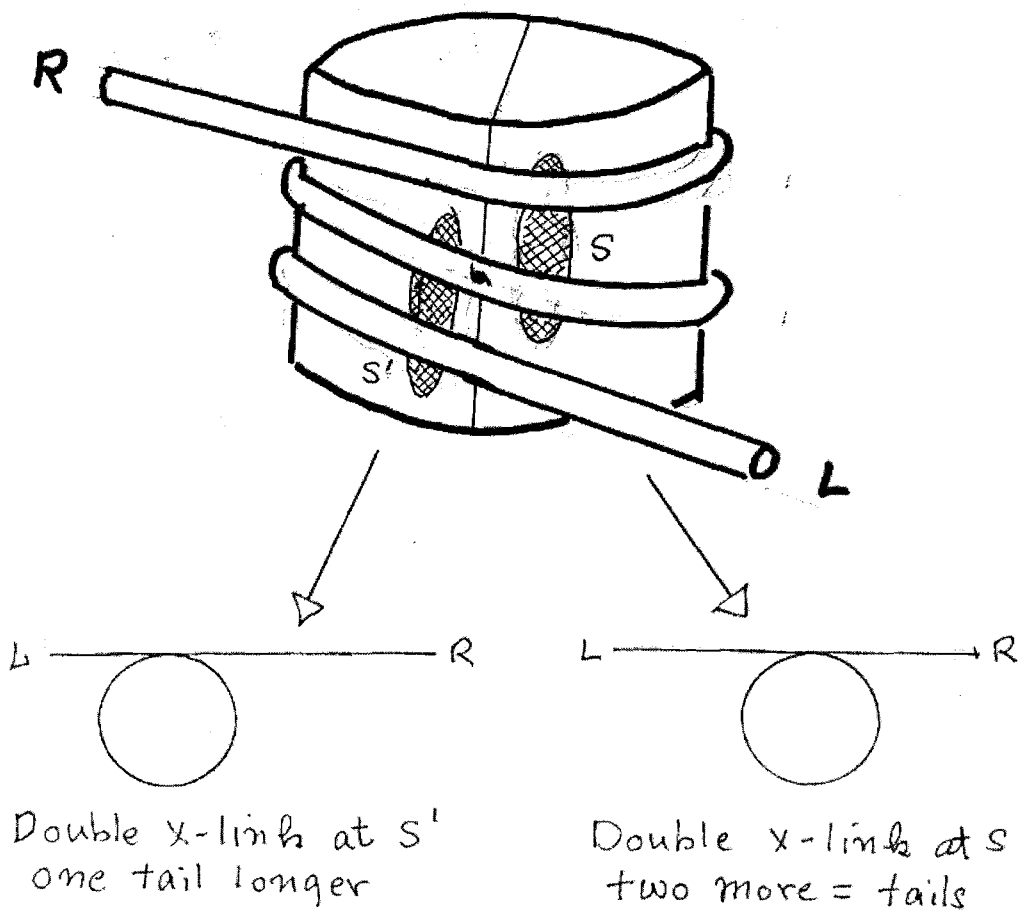
An obvious explanation for the more rapidly migrating species seen in Figures 3.8 and 3.9 is that intramolecular crosslinking of two gyres of the DNA on the chromatosome has occurred in this case (see Figure 3.11). It may be that an intermolecular crosslinking of two 256-bp DNA fragments, used to explain what is seen in Figures 3.7, and again in 3.10, has an intramolecular counterpart that is seen in Figures 3.8 and again in 3.9. Recall that in those experiments the 256-bp DNA is helically wrapped about the histone core. The GH5 could be binding twice to two locations on the same DNA fragment (Figure 3.11).

The appearance of two mobilities for these UV-induced DNA species might be explained as two alternate pairs of GH5-binding locations on the DNA. This would be most consistent with the model proposed in which the GH5 binds asymmetrically at the dyad axis, and bridges the DNA at one terminus to the DNA at the dyad axis (Zhou et al., 1998; Figure 3.1b).

Because of the sharpness of the two bands on the gel, it seems most reasonable that, if what is being observed in Figures 3.8 and 3.9 is indeed an intramolecular crosslinking of the DNA by GH5, that GH5 interacts with the nucleosome in two alternative positions, which, again, judging from the sharp bands on the gel, are both very well defined.

Figure 3.11. A sketch of the possible intramolecular crosslink of GH5 to the 256-bp DNA. Two models are shown, differing in the terminal strand to which the GH5 is crosslinked. The 256-bp DNA containing the 5S rRNA gene from *Xenopus borealis* is reported to strongly position the core histone octamer (Hayes et al., 1990). The DNA in the experiments migrates to two distinct positions on the gel, which would occur in the case of the two GH5-DNA complexes drawn in the figure.

Figure 3.11


R
S
S'
L
L
R
L
R
Double x-link at S'
one tail longer
Double x-link at S
two more = tails

The DNA that Becomes Crosslinked into 550-bp Structure Must Involve the Intermolecular Crosslinking. At this point it would be helpful to know if the DNA molecules associated with GH5 in the intermolecular crosslinking are strictly chromatosomal, or if free DNA also takes part in the UV-induced crosslinking. In an attempt to determine the origin of the DNA involved in the 550-bp dimerization, the UV-induced crosslinking procedure was repeated using chromatosomes to which radioisotopically end-labeled 256-bp DNA had been added after the reconstitution, so that it could be determined if it were the exogenous DNA or primarily chromatosomal DNA that was incorporated into the 550-bp dimer. The 550-bp dimer contained no label (Figure 3.12), suggesting strongly that the DNA of the 550-bp dimer originated from chromatosomal DNA, and not free DNA. Interestingly, the reaction products in this experiment included DNA of 1100 bps and of 1650 bps, corresponding to possible tetramers and hexamers of the 256-bp DNA. Only the 1650-bp species contained some labeled DNA (Figure 3.12b).

D. Discussion

While the study of crosslinking of proteins to nucleic acids by ultraviolet light has been studied extensively (Shetlar, 1980), the application of ultraviolet light to unmodified proteins and DNA of the nucleosome in an attempt to determine the location of LH-binding to the nucleosomal DNA has apparently never before been

Figure 3.12. Agarose gel electrophoresis of DNA phenol extracted from chromatosomes after UV irradiation in the presence of exogenous end-labeled 256-bp DNA. (a) The UV-irradiated DNA phenol extracted from chromatosomes was run on a 1.8% gel stained with EtBr (lane 4). The arrows indicate the 550-, 1100-, and 1650-bp DNA. Lane 1 is 100-bp DNA ladder. Lanes 2 and 3 are 256-bp DNA run as a control. (b) The autoradiogram showing the exogenous, end-labeled DNA running on the gel at an apparent 256 bps and at 1650 bps, while some signal is seen in the well. Arrows point to, in descending order, labeled DNA aggregated in the wells, as an apparent 1650-bp band, and as a 256-bp band.

Figure 3.12 (a)

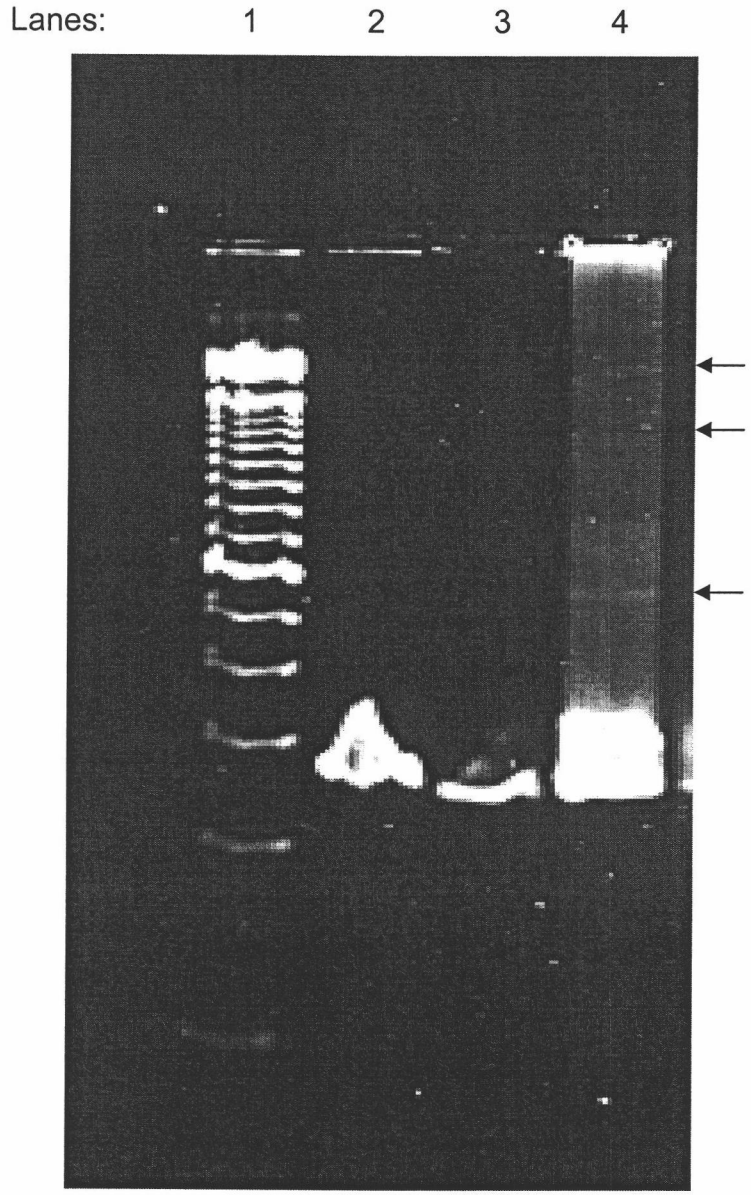
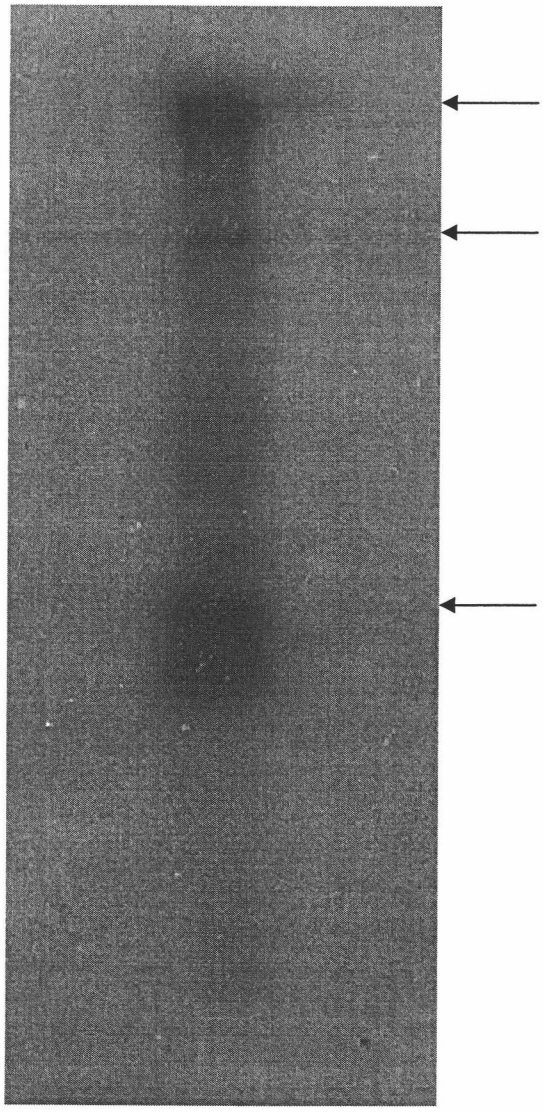


Figure 3.12 (b)



considered. UV-induced crosslinkings of the LH to free DNA have previously been studied in other laboratories (Kurochkina and Kolomijtseva, 1996). These other laboratories have, among other things, used the UV-induced crosslinking procedure to identify regions of the linker histone covalently crosslinked to DNA. This provides insight into the linker histone's DNA-binding domain. But the present work takes the study to the higher-order structure of the nucleus, the nucleosome.

In the present work, the purpose for the procedure was to find crosslinks between the linker histone and DNA that could reveal, by inference, the location of the linker histone in the nucleosome by locating the region, or point, in the nucleosomal DNA to which the linker histone was crosslinked. The inference could be made because the specific DNA of the nucleosome used in these experiments, the 5S rDNA-containing 256-bp fragment, precisely positions the histone octamer (Hayes and Wolffe, 1993; Hayes et al., 1990), along with the linker histone, as shown in this work, translationally along its sequence (Figure 3.6). Thus, any position on this DNA, when organized into a chromatosome, has one and only one corresponding position on the chromatosome. What has been shown by the results here is that the linker histone is unique among the histone proteins in conveying to the nucleosome the ability for histone proteins to be crosslinked to the nucleosomal DNA by exposure to UV irradiation in such a way that the crosslinked products can be observed by ordinary nucleoprotein agarose gel electrophoresis stained for DNA (Figure 3.7 - 3.10).

The protein-DNA complexes observed by nucleoprotein agarose gels in this work were formed exclusively by linker histone molecules complexed to DNA, either inter- or intramolecularly. The experiments in which proteinase K was added to the UV-irradiated crosslinked protein-DNA adducts (Figures 3.8 – 3.10) shows us that the anomalous migration of the 256-bp DNA fragment, as an apparent 550-, 200-, or 185-bp DNA fragment, is retained during phenol extraction, showing that DNA is the major constituent, yet cannot be maintained after proteolysis of the samples, showing that protein is present in these adducts. Since these UV-induced adducts are formed in the presence of linker histone and do not form in the absence of linker histone, they almost certainly are the product of covalent crosslinking of linker histone protein to DNA.

In the case of the first mentioned of these adducts, the apparent 550-bp DNA (Figures 3.7, 3.10), it is simplest to speculate that the linker histone is bound to the nucleosomal DNA prior to UV exposure, and that, upon UV irradiation of the chromatosome, the linker histone is crosslinked first to the chromatosomal DNA with which it is already associated, and then to a second duplex strand of DNA from another chromatosome. The data fits this model: two strands of the 256-bp DNA equals 512 bps total DNA, and the GH5 protein presumably attached would add apparent size, in terms of gel mobility shift, to that. We cannot expect this complex to run as though it were free DNA. The conformation would likely be restrained by perturbed, inflexible regions where the GH5 connects to one and/or both duplexes of

DNA. This could explain the migration to a position that makes it appear greater than simply the sum of two 256-bp oligonucleotides plus the relatively small contribution of the globular domain of the linker histone GH5 (approximately 9000 daltons).

The addition of exogenous labeled 256-bp DNA, and its failure to be incorporated into the 550-bp crosslinked adduct, shows that the crosslinked products observed are not the result of random interactions of GH5 with free DNA. If GH5 were binding nonpreferentially then we would surely see labelled DNA within the band of DNA migrating at 550 bps. Moreover, since the reconstitution products were shown to be homogeneous by gel electrophoresis (Figure 3.6b), it must be assumed that the first duplex of DNA crosslinked to GH5 in the UV-induced reaction is chromatosomal DNA and not free DNA. This experiment shows that GH5 constrained on a highly structured chromatosomal DNA strand prefers DNA from another chromatosome as its second binding ligand. This implies nucleosome - nucleosome interactions. No other conclusion can explain absolutely no radiolabel at 550 bps where EtBr was able to reveal a significant band of DNA (Figure 3.12).

In the case of the other UV-adducts, that is, the DNA adducts that migrate to a position that makes them appear smaller than 256 bps, the proteinase K treatments of these adducts show that they are, in fact, not really smaller than 256 bps (Figures 3.8 and 3.9). By reverse logic, if the DNA bands migrating at 200 and 185 bps were not 256-bp DNA complexed to proteins, then they would appear on the gel at a different

position than at a relative 256 bps after treatment with proteinase K. But there is no DNA found elsewhere on the gels after this treatment.

The model of intramolecular association (Figure 3.11) that echoes the one of Zhou and colleagues (Figure 3.1b), presented as an asymmetric positioning by GH5 that would occur in two places intramolecularly along a duplex 256-bp DNA fragment, has interesting correlations with the work of Chapter 2, which found that the same type of dual binding on supercoiled DNA, as well as linear DNA, as was observed here in chromatosomes, was the primary mode of action of the linker histone, in fact, the *exclusive* mode of action of LH.

These studies of the linker histone began with an interest in the controversy surrounding interpretation of the extant data on where the linker histone is located on the nucleosome (Crane-Robinson, 1997). The attempt was made to provide a clear, direct method to observe that location of LH binding to nucleosomal DNA. What often happens happened: the unexpected experimental results provided evidence that addressed the controversy mentioned above, but not in the manner that had been expected.

It's clear that the driving characteristic behind any of the possible explanations of linker histone - nucleosome interactions, at least as far as these experiments have shown, is the dual binding of the LH, and even the globular domain of such, to DNA. With that assumption, one of the three models presented in the introduction (Figure 3.1) is strikingly favored over the other two (Figure 3.1b). Moreover, one of the three

is distinctly disfavored. Based on the observations of GH5 crosslinking to two chromatosomal DNA sites, the preference of binding to supercoiled DNA over linear DNA, suggesting preference to binding at crossovers, a dual site of LH binding, and the differential aggregation on linear DNA over supercoiled DNA suggesting that in the paucity of conveniently apposed duplex DNA strands, the LH binds to two separate DNA molecules, all disfavors the model of the linker histone bound inside the DNA gyres such that it can only make a single protein-DNA association (Figure 3.1c).

The evidence that GH5 binds to two DNA duplexes corroborates other studies (Ramakrishnan et al., 1993; Goytisolo et al., 1996a; Duggan and Thomas, 2000) that suggest that the globular domain contains two potential DNA-binding domains, and that it alone confers specificity of action of the linker histone in binding to its physiologically relevant substrate, the nucleosome. The apparent binding to two duplexes *on the nucleosome* indicates that models invoking a single duplex-binding (Figure 3.1c) are unlikely.

The crosslinked nucleoprotein complexes were never observed in the absence of the linker histone. Earlier studies of the effect of irradiation with UV light have reported the crosslinking of core histones to the DNA (Martinson et al., 1976; Ptitsyn et al., 1981; Cao and Sung, 1982). Most of these studies have used wavelengths other than 254 nm. Some used bulk chromatin extracted from nuclei. Those studies that used chromatosomes as the experimental substrate used more highly modified

chromatosomes, to provide label, or photolabile substrates, than those used in the experiments of this thesis (Hayes, 1995).

Certainly the accessibility of GH5 to the exterior of the DNA of a chromatosome should be expected to provide a statistical advantage to its crosslinking to nucleotides in the DNA over that of the more buried, less accessible core histones. Recent studies on the dynamic behavior of LH in chromatin (Misteli et al., 2000) suggests other causes of higher exposures of the LH in interacting with the major and minor grooves of DNA facing the exterior of the chromatosome, where the UV light's effect would be most concentrated.

Chapter 4

The Location of the Linker Histone on the Nucleosome

A. Introduction

The location of the linker histone on the chromatosome is the focus of many recent studies. It is important for the understanding of the role that chromatin structure plays in transcription and other processes involving the underlying genes. With the postulation of multiple conflicting models of LH binding to the chromatosome, many who had been interested in higher order structures of chromatin joined the search for a conclusive demonstration of the LH's location on the chromatosome, as investigations of the mechanism of condensation of chromatin pointed to the key role that was played by the linker histone. To understand the process of condensation of chromatin, the mechanism and location of interaction of the LH with the nucleosome will need to be established. Thus, this perplexing issue stands in the direct path toward the elucidation of transcriptional regulation processes as well as one of the most fundamental processes of nuclear metabolism, the higher order structure of chromatin necessary for maintenance of eukaryotic DNA.

There is evidence that arrays of chromatosomes arrange themselves in the next higher level of organization, which is called the 30-nm fiber. The folding of arrays of chromatosomes is facilitated by the linker histone H1 and its variants, which include H5 (Thoma et al., 1979). How this folding occurs must include the clarification of the linker histone's location and mechanism of interaction with the chromatosome.

The globular domain of the linker histone has been shown to be sufficient for chromatosome formation (Ramakrishnan et al., 1993) and initiation of condensation (Thomas et al., 1979; Allan et al., 1986). But its position on the chromatosome has remained controversial and enigmatic.

This work began as an effort to differentiate among the three historic models of LH binding to the chromatosome described in Chapter 3 (Figure 3.1). The work described and discussed in that chapter gave evidence for the favoring and disfavoring of those models based on the linker histone's marked proclivity to binding two duplex strands of DNA. In the work done here, the linker histone appears to bind exclusively in this manner. A model (Figure 3.1c) placing the globular domain inside the DNA gyres, next to the core histones, and 65 bps away from the dyad axis (Pruss et al., 1996), where there is little likelihood of its binding to a second site on DNA is highly unfavored by the evidence presented in the previous chapters.

The work described in this chapter addresses the disparity in these models, and attempts to pinpoint the position of the globular domain of the linker histone on the chromatosome, using the photochemical crosslinking method as described in Chapter 3, and employing a site-specific DNA label to mark the position on the DNA where the protein-DNA crosslinking reaction occurs.

After an examination of the relevant literature on the chemical aspects of UV-induced crosslinking of proteins to nucleic acids, I had decided to use this method as a possible means of identifying the region of the nucleosome to which the globular

domain of the linker histone binds. The 5S rDNA-containing 238-bp DNA had been used in research that led to the model of a highly asymmetric placement of the linker histone on the chromatosome (Pruss et al., 1996; Hayes et al., 1994). It had been reported that the sequence strongly translationally positions the histone octamer (Pruss et al., 1996; Hayes et al., 1991; Hayes et al., 1994; Hayes and Wolffe, 1993; Hayes et al., 1990). Used in conjunction with this strongly positioning 238-bp 5S rRNA gene-containing DNA sequence, it was reasoned that one could crosslink the LH to the DNA, and then locate and isolate the LH-DNA nucleoprotein complex. The site of crosslinking of the LH on the DNA would, in the case of the strongly positioned chromatosome, correlate to the crosslinking site of the LH on the chromatosome.

By having researched the *X. borealis* DNA fragment and determined a feasible method of site-specifically labeling a site in a non-intrusive way at the dyad axis (site-specific ^{32}P-labeling), the dyad axis could be monitored for a crosslinked nucleoprotein complex, thus providing a means of determining if the LH (or any other histone) binds to the dyad axis of the chromatosome. Since the histone octamer is reported to be precisely positioned onto this DNA (Pruss et al., 1996) as mentioned above, to identify the DNA region of GH5-binding would, in essence, identify the location of GH5 binding on this positioned nucleosome. In addition, once a protein binding to the dyad axis was identified, it's analysis by, for example, trypsin digestion followed by amino acid sequencing of the ^{32}P-containing tryptic fragment, could provide another very important piece of information, i.e., the DNA-binding domain of the protein

bound at the dyad axis. All of this taken together would assuredly settle the debate over whether the globular domain has a location at or near the dyad axis or is located distinctly distal to this site (Figure 3.1).

The original experimental design was to isolate, and at the same time, identify UV-crosslinked nucleoprotein products, and then systematically fragment the complex by enzymatic and/or chemical means while analyzing the components by various biophysical means in conjunction with various biochemical manipulations. In this way the various oligonucleotidyl and peptidic components could be analyzed and identified. Any protein bound at the ^{32}P-labeled site on the DNA would be unambiguously identified.

Thus, the projected outcome of this experimental design was not only the aforementioned identification of the region of DNA bound by the LH while complexed to the tightly positioned nucleosomal core, but also the determination of the DNA-binding site, or sites, on the globular domain of the linker histone H5.

Gel electrophoresis of the site-specifically ^{32}P-labeled 238-bp DNA from the UV-treated chromatosomes (and nucleosomal cores as controls) was performed, and autoradiograms were inspected, to identify any successfully isolated UV-crosslinked nucleoprotein complexes containing the ^{32}P-labeled DNA.

This was originally expected to be a preliminary step in the full analysis. Mass spectrometric (MS) sequencing of proteins from silver-stained and Coomassie-stained gels after enzymatic cleavage of proteins into peptide fragments has been

voluminously reported in the literature over the past few years (Shevchenko et al., 1996). In addition, tryptic fragments of UV-crosslinked oligonucleoproteins have been successfully MS-sequenced while attached to oligonucleotides (Bennett et al., 1994). Thus, with a site-specific label on the DNA, MS analysis of the tryptic fragments bound to the label was hoped to potentially identify not only the protein bound to the labeled DNA site (the dyad axis of the chromatosome), but also the peptide region containing the DNA-binding domain of the protein bound.

This procedure had been reported to entail isolation of the protein-DNA complexes on acrylamide gels (Shevchencko et al., 1996). After separating protein-DNA complexes on a gel, the DNA would be trimmed away from the protein by enzymatic digestion, leaving only a short oligonucleotide attached to the protein. The only nucleoprotein complex of interest here would be that containing the ^{32}P label. Once the ^{32}P was isolated in association with a protein, the tryptic digest could be used to reveal both the protein's identity and DNA-binding domain by MS as well as conventional peptide sequencing (Edman degradation). An analysis of the amino acid sequences of the core and linker histones revealed that a fragment as small as 5 amino acids would suffice to distinguish the linker histone from any of the core histones.

Prior to complete enzymatic DNA degradation, it was desirable to cut away the DNA around the vicinity of the ^{32}P. *Hae*III cleaves at two sites on the 238-bp DNA, located 10 and 47 bps from the dyad axis and corresponding site of isotopic labelling.

B. Materials and Methods

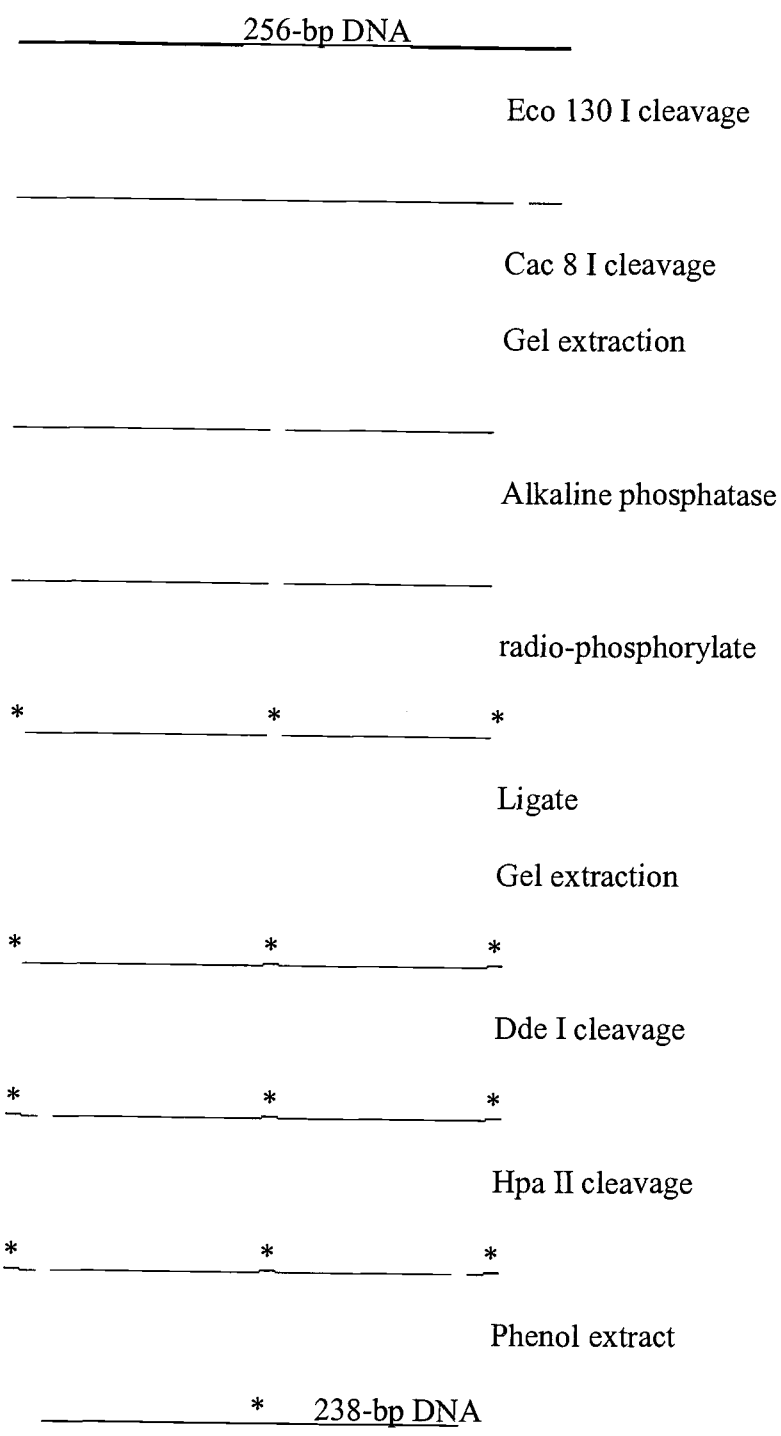
DNA Fragments Used for Reconstitution into Chromatosomes. The 238-bp *Hpa*II – *Msp*I fragment from the pXbs-1 plasmid (Peterson et al., 1980) was cloned into the pUC19 vector using *Bam*HI linkers embedded in primers used to amplify by PCR the fragment from the pXbs-1 template and ligate the *Bam*HI-cleaved PCR product into the *Bam*HI site of the polylinker region of the *Bam*HI-cleaved pUC19 vector. The clone was transformed, grown and harvested as previously described (Chapter 3, Materials and Methods).

Site-Specific ^{32}P-Labelling of the 238-bp DNA. The 256-bp *Bam*HI-fragment was first cleaved with *Eco*130I restriction endonuclease to create a 250-bp fragment that contained incompatible DNA ends (Figure 3.5). This was done as a necessary measure in this labeling procedure, because the DNA is to be cut and blunt-end religated downstream in this protocol (Figure 4.1). *Bam*HI cohesive ends would significantly out-compete the blunt ends for successful ligation, and these *Bam*HI cohesive end ligation products would be indistinguisable on a preparative agarose gel from the blunt end ligation products containing the same DNA fragments oriented the other way, since they would be of the same size. Therefore, this step was deemed essential and was taken.

A second restriction cut was made using *Cac*8I restriction endonuclease, for the purpose of kinasing the *Cac*8I ends with ^{32}P-containing phosphates. This enzyme, like

Figure 4.1. Flow diagram of the site-specific internal labeling of the 238-bp 5S rRNA gene-containing DNA fragment from pTPE1.

Figure 4.1


256-bp DNA
Eco 130 I cleavage
Cac 8 I cleavage
Gel extraction
Alkaline phosphatase
radio-phosphorylate
Ligate
Gel extraction
Dde I cleavage
Hpa II cleavage
Phenol extract
238-bp DNA

the previous one, cuts at a single site in the 256-bp DNA's sequence. This restriction enzyme does not cut any significant amount of the DNA to completion. It is therefore necessary after this reaction to isolate the two desired cleaved products from the uncleaved material.

A 2% preparative agarose gel (6 x 10 cm), run in TBE buffer, was used to separate the 144- and 106-bp *Cac*8I fragments from the uncut 250-bp DNA. Voltage was usually set to 120 V (about 10 V/cm), and corresponding amperage should be around 40 mA. The gels were run at this voltage for 75 minutes. EtBr was added to the agarose prior to making the gel so that the bands of DNA could be viewed and excised from the gel right away. Gel fragments containing the bands of interest were cut with a clean single-edged razor blade, and diced for placement into Eppendorf tubes. The agarose was melted by adding chaotropic reagents (Qiagen, Inc.; Chatsworth, CA) to the tubes and warming at 50°C, with occasional mixing by vortexing, for 30 minutes. Isopropanol was then added, and the DNA was extracted from the gel matrix using the proprietary spin columns and protocol as described in the Qiagen Company's handbook. DNA was recovered from the spin columns in a relevant volume (ca. 50 µl per 5 µg DNA) of 10 mM Tris-HCl, pH 8.5 (elution buffer).

The purified cleavage products of the *Cac*8I digest were subjected to three sequential enzymatic treatments. Experience has shown that this provides the greatest yield of internally-labeled DNA, and is a superior technique to that of cleaning the DNA of enzyme and buffer after each treatment. The first enzyme added is shrimp

alkaline phosphatase (SAP), which removes 5'-terminal phosphates. This has the advantage that the subsequent kinase reaction proceeds at an enhanced rate in the addition of phosphates, as compared to the substitution of phosphates. SAP is a heat-inactivatable alternative to the bacterial phosphatase, which often is not inactivated by heating. The SAP reaction was incubated at 37°C overnight. The reaction was stopped by heating to 65°C for 20 mins. The buffer systems for SAP and T4 polynucleotide kinase are similar enough that it was found unnecessary to change or add buffers when taking the next step. T4 polynucleotide kinase and 5 μl of 3000 μCi/mmol isotopically-labeled γ-[^{32}P]-ATP were added and the labeling of 5'-ends occured at 37°C. After 6-8 hours the reaction was cooled to room temperature. T4 DNA ligase was added, along with buffer that contains nonisotopic ATP. This reaction takes place optimally at room temperature, preferably below 22°C, and was left overnight.

These reactions were usually followed by a *Bam*HI restriction digest prior to gel extraction purification of the 250-bp DNA. The possible products of the ligase reaction are: two of the larger pieces (144-bp fragments) ligated at their blunt ends, two of the smaller pieces (106-bp fragments) ligated at their blunt ends, one large and one small ligated at their respective blunt ends (yielding the desired 250-bp product), and two of the larger pieces (144-bp fragments) ligated at their *Bam*HI cohesive ends. This latter is by far the most likely product, and treating the ligation reaction mixture with *Bam*HI restriction endonuclease significantly reduces the obstacle to gel

purification of the 250-bp DNA band that the presence of a copious quantity of 288-bp DNA on the gel would pose. The profusion of the much preferred 288-bp ligation product composed of two of the 144-bp fragments ligated by their cohesive *Bam*HI ends causes this 288-bp product to smear on a preparative agarose gel down toward the position of the desired 250-bp DNA band. 3% was as dense as is practical for these gel extraction procedures, so the separation afforded was as good as could likely be obtained. This overrunning of the major product of ligation obstructs a clean gel slicing of the 250-bp DNA ligate in the gel extraction procedure. The restriction reaction alleviates this problem, and was run for at least four hours. In some cases it was found useful to add further ligase enzyme to attempt to increase the amount of target product obtained, effectively running competing enzymatic reactions.

A 3% preparative agarose gel was used to separate the end products of the ligation and *Bam*HI cleavage reactions. After this second round of gel extraction purification, the 250-bp DNA isolated had four ^{32}P-labeled nucleotides, one on each 5' end of the earlier two *Cac*8I-fragments. Now, with the two *Cac*8I fragments religated, that leaves two labeled nucleotides on either DNA strand at the *Cac*8I site, and one labeled nucleotide on each terminus of the ligated 250-bp DNA fragment. Therefore, the ends were cleaved with *Hpa*II and *Dde*I restriction endonucleases in succession. This leaves a 238-bp DNA containing the label at the *Cac*8I site. Later work substituted the isoschizomer, *Msp*I, for *Hpa*II, as it could function more efficiently in the buffer recommended for the *Dde*I enzyme, and thus simplified the double digest reaction.

The *Hpa*II (*Msp*I) and *Dde*I restrictions were carried out in the presence of a large quantity of unlabeled 256-bp DNA. The amount of labeled DNA produced, despite the large amount of starting material (40 - 75 μg), was too little to allow for a full reconstitution, which required a minimum of 12 μg of the 238-bp DNA. A 12-μg yield corresponds to a yield of approximately 16 - 30 %, which is equivalent to the calculated expected yield for a ligation with four products. Factoring in yield losses from the earlier gel extraction of the *Cac*8I-digested fragments, and the subsequent gel extraction of the *Hpa*II-*Dde*I double digest products leaves significantly less DNA. In practice, the yield approached 16 % of starting DNA. In order to avoid risking loss of the entire labeled DNA on a potentially unsuccessful reconstitution procedure, unlabeled DNA was added to the labeled DNA. This did not compromise the results. The specific activity of labeled DNA was high enough to allow visualization of the results of the experiments with as little as a tenth of the DNA labeled. Adding unlabeled DNA at this double restriction step assured a homogeneous population of the 238-bp DNA sequence.

Nucleosome Reconstitutions. Nucleosomes were reconstituted from chicken erythrocyte core histones and the 238-bp internally-labeled DNA, in the same manner as was described in Chapter 3 of this work. Linker histone was added in the same way as described in Chapter 3, except that two different chromatosome preparations were made, one with the recombinant, truncated histone H5 (rGH5), prepared as described in Chapter 3, and one with the full length linker histone (H5) prepared from chicken

erythrocyte chromatin by the same method as used to prepare the histone H1 described in Chapter 2.

Photochemical Crosslinking Reaction of Nucleoprotein Complexes. Chromatosomes or nucleosome cores were irradiated with ultraviolet light (λ_{max} = 254 nm), in the same way as described in Chapter 3. All exposures for this set of experiments were of 60-second duration in the Stratalinker 1800 (Stratagene, La Jolla, CA).

HaeIII Restriction Digest of Chromatosomes. The solution containing UV-crosslinked histone-DNA complexes was made 10 mM with respect to $MgCl_2$, and 10 units of *Hae*III (New England Biolabs, Beverly, MA) was added per 5 μg of DNA. The samples were incubated at 37°C, and the reaction allowed to go to completion.

Nucleoprotein Acrylamide Gradient Gels. UV-crosslinked chromatosomal particles were electrophoresed after purification by phenol-chloroform extraction, isopropanol precipitation, drying by Speedvac, and resuspension in 20 mM Tris-HCl, pH 8.5, as described in Chapter 3.

Gradient acrylamide gels of various percentages were used in these experiments, ranging from 16 - 27 % to 4 - 20 %. The 16 - 27 % gradient gels provide clear separation of small peptide fragments. Use of these "peptide" gels was discontinued after it was discovered that the crosslinked nucleoprotein complexes could not be readily visualized on these gels. Plans to analyze tryptic peptides were later suspended pending further analysis of the oligonucleotide portions of the experimental samples.

A 4 - 24 % gradient SDS - PAGE was commonly used, and TBE was the running buffer of choice in all of the gels performed in these experiments. The upper chamber was made 0.1 % SDS, while the lower chamber contained only 1 x TBE. A 4 - 20 % Tris-HCl precast gel (BioRad, Hercules, CA) was occasionally used for comparison. Running buffer remained TBE with added SDS in the upper chamber only for all precast gels as well.

Stock solutions included 3 M Tris-HCl, pH 8.8, 1 M Tris-HCl, pH 6.8, an acrylamide stock solution made up of 50% acrylamide (w/v) and 1.34% bisacrylamide (w/v) in water, and commercial-grade TEMED, all stored at 4°C. TEMED was diluted 1:5 in water for each use just prior to pouring each gel. Stock APS was made 1.5% in single-use aliquots of 200µl and stored at -20°C. Stock SDS was 10% and stored at room temperature. A stock 40% (w/v) sucrose solution was stored at 4°C.

A small peristaltic pump was fitted by tubing to a small-sized gradient maker with side-by-side vessel design. A stir-bar was placed in the vessel that was directly proximal to the pump. This vessel was filled with the high-percentage solution while the bar stirred, the pump was off, and the gradient maker's stopcock was turned to the 'closed' position. Next, the far vessel was filled with the low percentage acrylamide solution with the cock still stopped, and then the stopcock was opened, and the peristaltic pump was turned on immediately.

For the 4% gel matrix, 200 µl stock acrylamide solution, 600 µl 3 M Tris-HCl, pH 8.8, 1500 µl water, 15 µl of the 1:5 TEMED dilution, 20 µl of 10% SDS were mixed,

and 90 µl of stock APS was added last. For the 24% gel matrix 1150 µl of acrylamide, 600 µl of 3 M Tris-HCl, 575 µl of the stock sucrose solution, 15 µl of the 1:5 TEMED solution, 20 µl of 10% SDS were mixed, and 60 µl of the APS stock was added last.

The gel was poured to within one centimeter of the top of a 10 x 7 cm plate with 0.75 mm spacers. The gel was topped with water and allowed to polymerize for at least 2 hours, more usually 12 hours. A 4% stacking gel was poured using the same reagents with the exception of the Tris-HCl, which was 1 M, pH 6.8.

C. Results

Experimental Approach. The DNA fragment of interest (Figure 3.5) was reconstituted with histone octamers, followed by the addition of LH according to the procedures outlined in Chapter 3, Materials and Methods. Reconstitution was monitored by band shift analysis like that described in Chapter 3 and illustrated in Figure 3.6.

Photochemical crosslinking was induced by irradiation of the nucleoprotein complexes, both chromatosomes and nucleosome cores, for 60 secs. UV-crosslinked samples were digested with *Hae*III and assayed on a 4 - 24 % gradient SDS-PAGE.

As a preliminary test, nucleosome cores that had been UV-crosslinked were assayed by treatment with *Hae*III. These UV-irradiated nucleosome cores were run on the gradient gel in parallel with a control nucleosomal core sample that had been UV-

irradiated but had not been exposed to the *Hae*III restriction enzyme (Figure 4.2). These samples were loaded onto the gel without having been phenol-chloroform extracted. By loading and assaying the entire nucleoprotein sample, it was intended that proteins, both crosslinked and not crosslinked, could be monitored and provide information about the extent of crosslinking of the various histones by staining for proteins. The results were unexpected and intriguing. First, Coomassie Brilliant Blue G-250 did not stain any proteins in the experimental samples (Figure 4.2, lanes 9 and 11). Second, after staining the protein with Coomassie, the gels were stained for DNA with EtBr, and, surprisingly, this dual-staining worked remarkably well (Figure 4.2). Third, the DNA showed anomalous migration, for its expected size.

To ascertain the reliability of the new dual-staining technique, and simultaneously investigate the cause of the apparent anomalous migration of the DNA, a 4 - 20 % gradient gel of standards, both protein and DNA, was run, showing that the technique of staining for protein and DNA was repeatable and reliable (Figure 4.3). The DNA in the standards ladder (Figure 4.3, lanes 5 and 7) migrates to the proper positions relative to the sizes of all other samples, regardless of the composition of the molecules, protein or DNA.

Next, a series of *Hae*III digestions of both UV-crosslinked chromatosomes and nucleosomal cores was performed, and the products were assayed in a series of gradient gels. Phenol-chloroform extractions prior to loading on gels were

Figure 4.2. UV-irradiated nucleosome core particles run on a gradient gel. Nucleosome cores were UV-irradiated for 60 seconds and then either treated with *Hae*III (lane 9) or not (lane 11) prior to running on a 4 - 24 % gradient SDS-PAGE. Arrows indicate the position on the gel of the *Hae*III-digested (lane 9) and nondigested (lane 11) nucleosomal core (no LH) samples stained for DNA with EtBr, and previously stained for proteins with Coomassie blue G-250. The protein standards (lanes 1 and 12) at the positions of the arrows are, in descending order, carbonic anhydrase (MW = 31,000 daltons) and lysozyme (MW = 14,400 daltons). Lane 1 and 12: Low-range SDS-PAGE standards (Bio-Rad, Hercules, CA). Lane 3: Histone H5. Lane 5: Polypeptide standards (Bio-Rad, Hercules, CA). Lane 7: core histones. Lane 9: Nucleosome cores that had been UV-irradiated and *Hae*III digested, but had not been phenol-chloroform extracted. Lane 11: Nucleosome cores that had been UV-irradiated but had neither been exposed to *Hae*III nor phenol-chloroform extracted. All other lanes were empty.

Figure 4.2

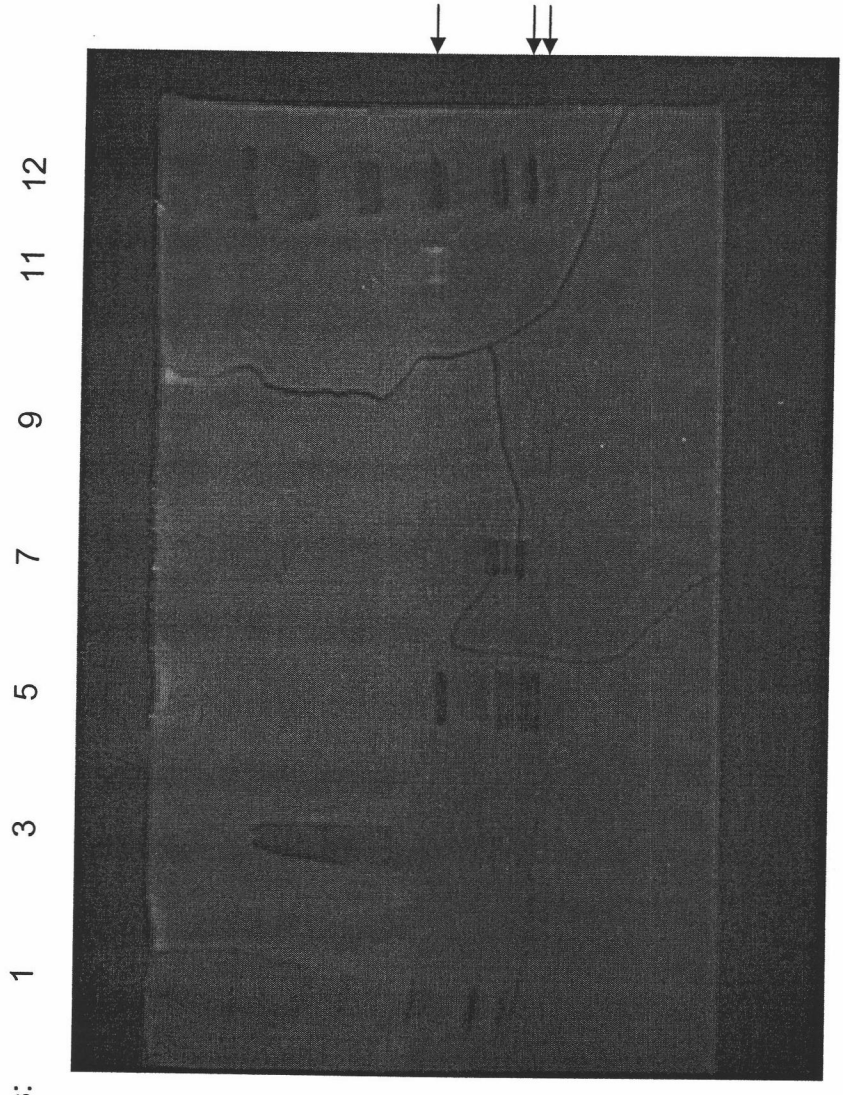
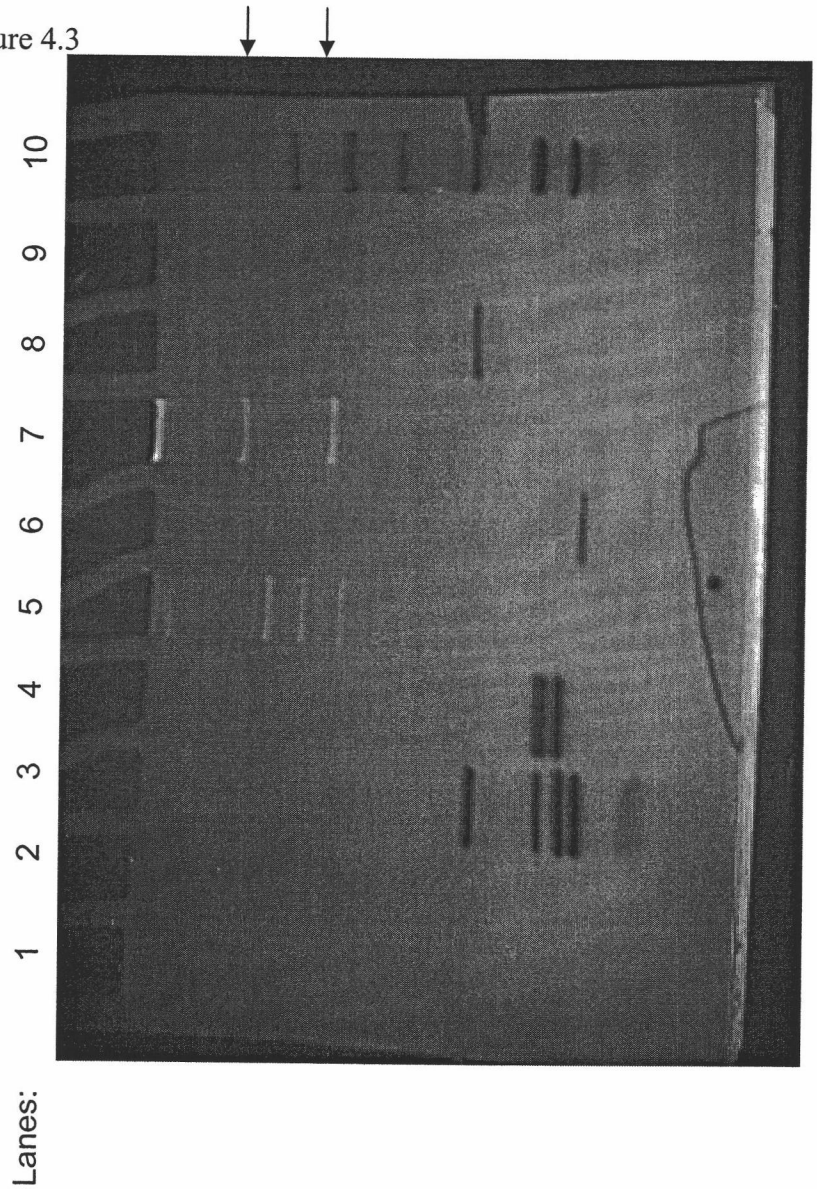


Figure 4.3. Standard protein and DNA samples run on a gradient gel and stained with both Coomassie Blue and EtBr. Standard protein and DNA samples were run on a 4 - 20 % gradient SDS-PAGE with TBE running buffer for 1 hr. 40 min. at 100 V using a Bio-Rad Mini Protean III apparatus and an Ocean power source (Edmonds, WA). Arrows indicate the 100- and 200-bp DNA from the 100-bp DNA ladder (Gibco BRL, Carlsbad, CA.). Lane 3: Polypeptide SDS-PAGE standards (Bio-Rad, Hercules, CA). Lane 4: Core Histones. Lane 5: pBR322-*Msp*I DNA size marker. Lane 6: Recombinant truncated H5 (rGH5). Lane 7: 100-bp DNA ladder. Lane 8: Full length linker histone H5. Lane 10: Low-range SDS-PAGE standards (Bio-Rad, Hercules, CA). All other lanes are empty.

Figure 4.3



consistently performed, and DNA of the chromatosomes and nucleosomes migrated on the gradient gels appropriately relative to size. Apparently, histone-DNA complexes rich in histones run in a very anomalous way on SDS-polyacrylamide gels. In like manner, *Hae*III-cleaved histone-DNA complexes rich in histone migrated to a position of about 20 bps (Figure 4.2, lane 9) although the smallest DNA fragment was 53 bps as indicated in the sequencing chart (Figure 3.5). This last migration pattern is in exact proportion to its DNA fragment sizes as is that of the full length 238-bp DNA-containing nucleosome core, migrating at an apparent 50 bps, to its size, suggesting that the cause for this anomaly is a compaction or "globularization" of the DNA around the histones, unaffected by the presence of SDS. One possible explanation for the anomalously fast migration of the DNA could be that the negative charges of the SDS molecules repel the negative charges of the DNA phosphate backbone, and thereby facilitate the DNA's tighter association with the highly-positively charged histones. The DNA may have no other possible avenue of movement away from the negative charges on the SDS, and could thus be compelled to more tightly associate with the positive charges on the lysine and arginine side-chains of the histones.

At any rate, by assaying the same chromatosomal samples on agarose gels, it was determined that the integrity of the DNA in these reconstituted chromatosomes had not been compromised, ruling out DNA damage or degradation as a cause of the anomalous migration.

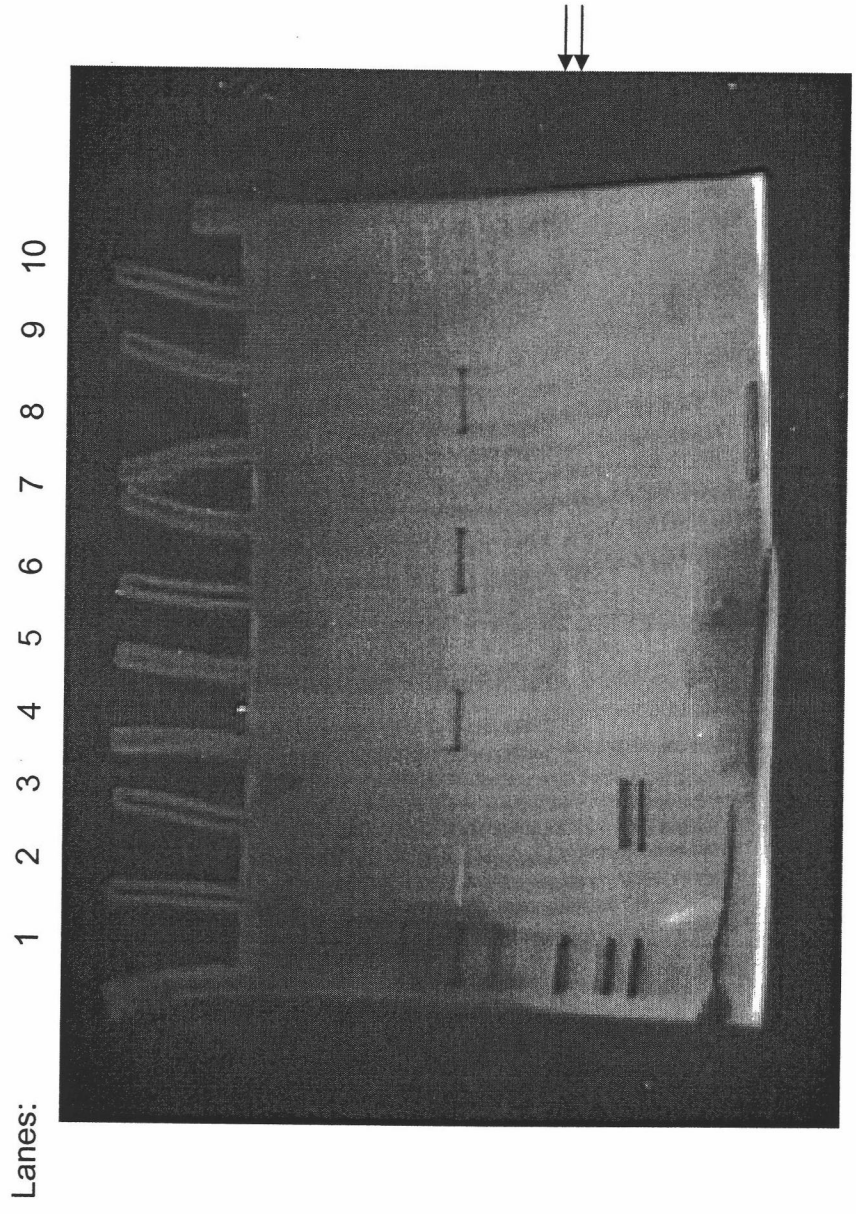
HaeIII Digestion of UV-Crosslinked Nucleoprotein Complexes Shows that DNA at the Dyad Axis is Crosslinked to the Linker Histone. In light of what had been revealed about the dual staining with Coomassie blue and EtBr, and the absence of visible proteins in the *Hae*III-digested and non-digested chromatosomes, the original effort to isolate crosslinked nucleoprotein complexes from silver-stained or Coomassie-stained acrylamide gels for sequencing was redirected to an attempt to show by gel mobility shift of the DNA on gradient acrylamide gels the presence of histones crosslinked to the mobility-shifted DNA. The aberrant migration of DNA in chromatosomes posed a difficulty which was removed by phenol-chloroform extraction of samples prior to gel electrophoresis. Whole, undigested chromatosomes run in parallel as controls were not phenol-chloroform extracted, as free 238-bp DNA was too large to penetrate the gels.

UV-irradiated chromatosomes with and without *Hae*III digestions were gel electrophoresed, along with ^{32}P end-labeled DNA standards (Figure 4.4). Only the HaeIII protein was visible by Coomassie staining. The chromatosome revealed, by EtBr staining, a DNA band at the 50-bp position, while the phenol-extracted *Hae*III fragments run slightly slower, to an apparent 56 bps, judging by both protein and DNA standards.

An autoradiogram of a 4 - 24 % gradient gel reveals that a *Hae*III digest of the UV-crosslinked chromatosome reconstituted on the *Cac*8I-site labeled 238-bp DNA, and with full-length linker histone H5, leaves multiple bands of DNA, migrating through

Figure 4.4. UV-irradiated chromatosomes were digested with *Hae*III and run on a gradient gel. Chromatosomes were UV-crosslinked and run on a 4 – 20% gradient gel either with (lanes 4,6,8) *Hae*III digestion or without it (lane 5). Arrows indicate the position of the DNA of the chromatosome in lane 5 and the 56- and 53 bp DNA from *Hae*III digestion in lanes 4 and 6. The protein stained in lanes 4,6 and 8 is HaeIII.

Figure 4.4



the gel to a variety of positions. One signal from a labeled DNA fragment is the 56-bp *Hae*III - *Hae*III internal fragment, containing the locale of the dyad axis, while the vast majority of the DNA is significantly retarded on the gel (Figure 4.5b). Once again, the chromatosome reveals DNA migrating as though it were 50 bps in length (Figure 4.5 (a) and (b), lane 4: "CHR"). To the opposite side of the experimental sample is control DNA, cleaved with *Hae*III into three pieces of 128, 56 and 53 bps, and end-labeled (Figure 4.5 (b), lane 8: "DNA"). None of the three pieces of control DNA correspond to any of the experimental DNA fragments, with the possible exception of the 56-bp fragment. Although the results are anything but clear, there is no doubt that the migration of the labeled DNA fragments from the *Hae*III-digested crosslinked chromatosome is greatly retarded on the gel. This suggests that the DNA has once again been crosslinked to histones, and again, only in the presence of the linker histone.

Further studies would begin with a proteolytic analysis of the *Hae*III digestion product assayed in Figure 4.5, followed by gel electrophoresis in parallel with an unproteolyzed sample. Evidence of an autoradiographically visualized DNA band missing from the proteolysis reaction would confirm the bridging of multiple fragments of DNA by protein.

Figure 4.5. *Cac*8I-site labeled chromatosomes were UV-irradiated and then *Hae*III digested before being electophoresed on a gradient gel. Chromatosomes labeled at the *Cac*8I site at the dyad axis were UV-crosslinked and *Hae*III-digested before being gel electrophoresed on a 4 – 24 % gradient SDS-polyacrylamide gel (a) and autoradiographed (b). (a) The arrow indicates the position of the chromatosomal DNA stained with EtBr. Lane 1: protein standards (Biorad; Hercules, CA). Lane 2: histone H5. Lane 3: nucleosomal core particles (no LH). Lane 4: chromatosomes. Lane 6: UV-irradiated chromatosomes (containing H5) *Hae*III digested. Lane 8: end-labeled 238-bp DNA *Hae*III-digested. Lane 10: pBR322-MspI DNA standards. Lane 12: 100-bp DNA ladder. All other lanes were empty. (b) The arrows indicate the position of 238-bp DNA *Hae*III-digestion fragments, end-labeled after complete digestion with *Hae*III (lane 8). The comparison with the *Hae*III-digested, UV-crosslinked, internally labeled chromatosome (lane 6) shows that most of the label is in complexes that migrate at an apparent size much larger than 56 bps. Lanes are the same as for (a).

Figure 4.5 (a)

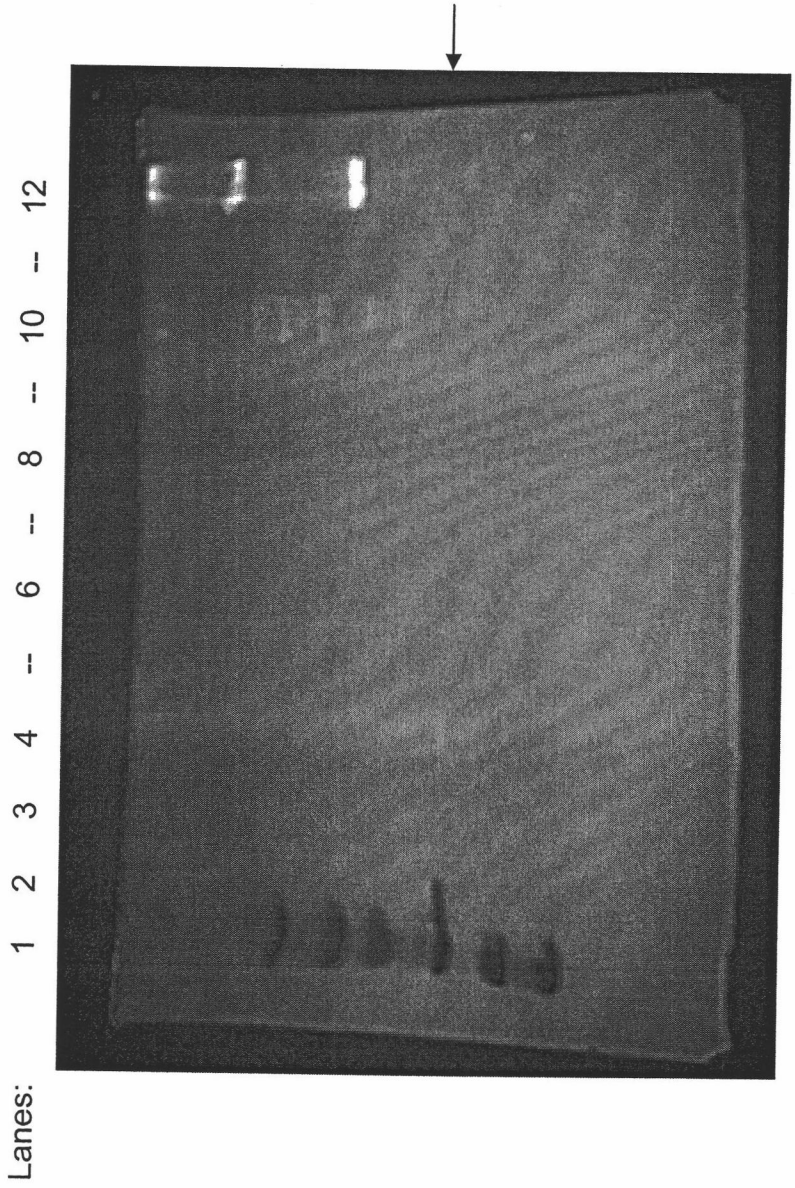
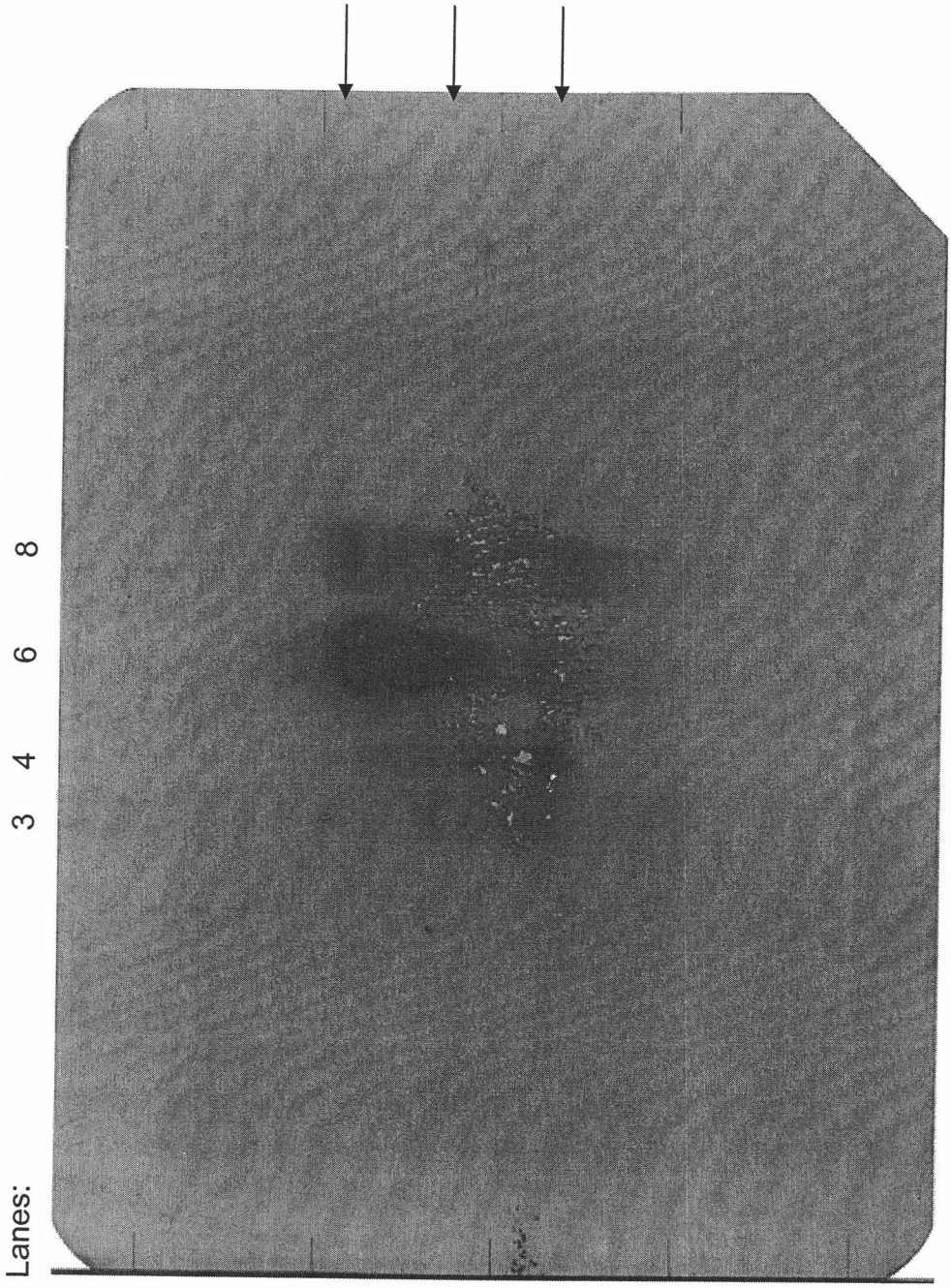


Figure 4.5 (b)



D. Discussion

The location of the linker histone on the chromatosome is so contentious that it will probably not be resolved by the present work. Nevertheless, the general approach applied here looks to be not only the best one to penetrate this issue, but also a fine method for investigating related issues about the nature of the binding of the linker histone to DNA in chromatin.

The evidence of the *Hae*III-digest of UV-crosslinked chromatosomes, while preliminary, does clearly show that the linker histone binds to a 56-bp fragment of DNA surrounding the dyad axis. This has been inferred from the high-molecular weight site-specifically labeled DNA, well above, and separated from, the band of DNA seen at a position corresponding to 56 bps in lane 6 on the autoradiogram. The positions are calibrated to sizes based on the inspection of the autoradiogram as it is positioned over the original gel containing clearly visible DNA size markers (Figure 4.5a, lanes 10 and 12) and protein size markers (same Figure, lane 1). The DNA bands in the experimental lane are also compared relative to the end-labeled *Hae*III-digested 238-bp DNA (Figure 4.5a, lane 8) run as a control.

Since there is a band of DNA visible that corresponds to the free 56-bp *Hae*III-*Hae*III fragment , the bands above that DNA are distinguishable from the dyad axis-containing DNA fragment and large enough to be considered a complex of at least two

DNA duplexes, one of which must be the 56-bp labeled fragment, crosslinked by the linker histone.

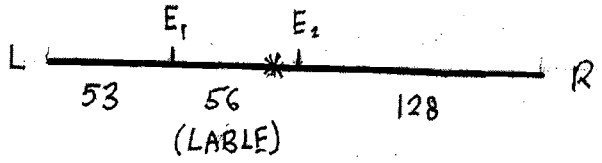
The appearance of multiple bands can be interpreted, facilitated by a diagram of the three *Hae*III-digestion fragments as they appear in association with the histones in the chromatosome (Figure 4.6). There are three combinations of LH-DNA association within one chromatosome. Keep in mind that the chromatosome was dissociated by phenol-chloroform extraction prior to the digestion with *Hae*III enzyme. Therefore, there is no risk of incomplete cleavage. As with the control DNA, the *Hae*III enzyme cut the UV-induced crosslinked protein-DNA complex completely into the three fragments outlined in Figure 4.6. Two of the three "intramolecular" combinations would be labeled by dyad axis ^{32}P, consisting of 184 bps and 109 bps total DNA, comprised of two fragments connected by one, or more, linker histone(s). A third band on the autoradiogram could be created by intermolecular LH-DNA interactions, as the evidence in Chapter 3 indicates (Figure 3.7, 3.10 and 3.12).

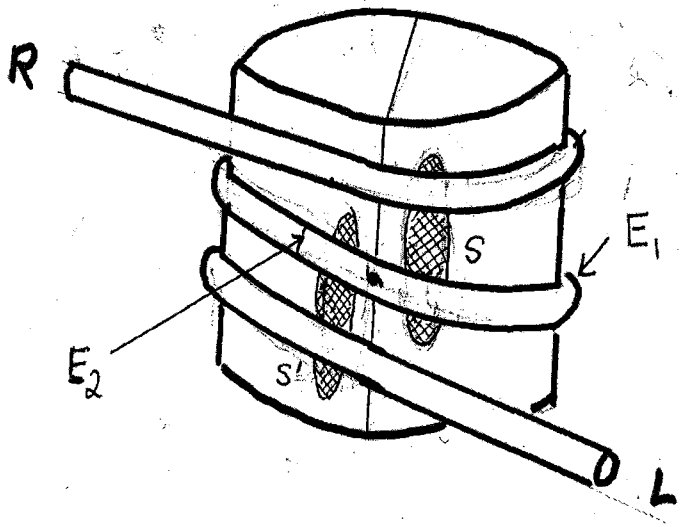
The results here indicate that the linker histone binds to a chromatosome with two distinct DNA-binding domains, oriented on the linker histone protein such that its interaction necessarily includes two DNA duplexes. This is in agreement with all of the data presented in Chapter 3.

The preponderance here of high molecular weight crosslinked DNA species to the near exclusion of smaller DNA migrating at or near 56 bps (Figure 4.5b) suggests that the linker histone is very unlikely to leave one of its DNA-binding domains

Figure 4.6. A schematic drawing of the chromatosome from Figure 4.5. The hatched ovals represent the possible positions of LH-binding that could reconcile the appearance of autoradiographic signals in Figure 4.5b much larger than the 56-bp *Hae*III-*Hae*III fragment produced by digestion of the 238-bp *Cac*8I-site ^{32}P-labeled chromatosomal DNA. The asterisk denotes the site of the ^{32}P at the dyad axis of the chromatosome. L and R signify the left and right ends of the 238-bp *Hpa*II-*Dde*I DNA fragment. E_1 and E_2 mark the points of *Hae*III cutting.

Figure 4.6





2 crosslinks @ S → 56 + 128 = 184 bp

2 crosslinks @ S' → 56 + 53 = 109 bp

Both will be labeled.

unassociated. Considered together, with the evidence from the earlier chapters, the persistence with which the LH binds to two DNA duplexes rules out certain orientations for the two DNA-binding strands bound by LH in the chromatosome. The two DNA duplexes bound must be, to a certain extent, oriented on opposite sides of the globular domain of the linker histone. This can be inferred from the data in Chapter 2 showing that binding to two separate DNA molecules does not occur as readily for linear DNA as for supercoiled DNA. The marked difference in the two is that the possible orientation of DNA-binding sites in the supercoiled DNA includes crossovers, while that of the linear DNA does not without extreme bending or the reliance on other DNA molecules for intermolecular associations.

We know intramolecular binding does occur; otherwise the supercoiled DNA in Chapter 2 would aggregate as readily as did the linear DNA. The linear DNA in those same studies showed a small, but observable, ability to retard upon LH binding. This could be explained by the DNA bending in upon itself, forming a coil, so that an intramolecular interaction with LH at the site of two DNA duplexes crossing over upon one another occurs. These data support the conclusion here that the high-molecular weight species are intramolecular crosslinks of LH and DNA which has been subsequently cleaved into two fragments of DNA by the *Hae*III digestion. The binding by the LH inside the DNA gyres 65 bps away from the dyad axis would make it impossible for the linker histone to bind to two DNA duplexes, as it is observed to be doing here.

In Chapter 2, the only possible means of aggregation of the linear DNA was through the intermolecular association of the LH, by its two DNA-binding domains, to two separate molecules of linear DNA. The supercoiled DNA seems to exhibit, on the other hand, far more intramolecular interaction with the LH, and aggregates far less readily than linear DNA. The predominance of intramolecular binding of LH to supercoiled DNA as compared to that of linear suggests that the mode of LH binding in the chromatosome will be heavily influenced by the higher torsion, as a result of the association of the core histone octamer, in chromatosomal DNA over that of free DNA in solution, and will likely interact with chromatosomal DNA in a manner more similar to that observed in LH interactions with the supercoiled form of DNA than that of the linear form in Chapter 2.

The negative cooperative mode of binding by LH to DNA, unreported in the literature until now (Chapter 2), is simply another perspective of the same behavior that exhibits itself as a distinct preference of the LH for binding to two DNA duplexes. The purported unwinding of superhelical DNA upon LH binding (Ivanchenko et al., 1996) causes the distribution of LH molecules across the population of superhelical DNA molecules. This is dependent on the initial preference by LH for binding to two DNA duplexes. This dependency is most dramatically illustrated by the direct competition of supercoiled and linear DNA for binding by LH (Figure 2.2). The exclusive binding to supercoiled DNA in the presence of completely unbound linear DNA in that experiment provides strong evidence by which the current controversy

surrounding the precise position of the LH on the chromatosome can be resolved (Figure 3.1). The binding of LH to supercoiled DNA in the experiments of Chapter 2 shows that the LH distributes itself evenly over the entire population of DNA crossovers, with absolutely no recognition of linear duplex strands of DNA by any other means. In the absence of DNA crossovers, we still see in the aggregation of linear DNA that the LH exhibits one and only one pattern of binding, namely, dual binding, to two DNA duplexes.

How can this evidence be reconciled with the three models of LH binding to the chromatosome (Figure 3.1)? The orientation in which each of the two DNA-binding domains of the LH can interact individually with an independent DNA duplex strand will be preferred over a position in which one of LH's DNA-binding domains associates with a DNA duplex and the other is unable to interact with DNA. Clearly, the model in which the LH binds inside the DNA gyre, 65 bps away from the dyad axis, where there is no possibility of the interaction of the LH with a second DNA duplex, is highly disfavored (Pruss et al., 1996; Figure 3.1c), if not entirely discounted, by the evidence shown here.

The crosslinking of two DNA duplex strands by UV irradiation, held together by the LH, as verified by the subsequent release of the two strands though proteinase K digestion, establishes that the identical preference for two DNA duplexes, exhibited in Chapter 2 in the case of DNA, occurs, in Chapter 3, in the case of nucleosomes. The other UV-adducts formed in those crosslinking experiments involving nucleosomes,

which migrate faster than the 256-bp DNA, are also the result of a crosslinking induced by UV irradiation, and involving the binding of two DNA duplexes by LH, as verified by their proteinase K cleavage into the 256-bp DNA species. The experiments of Chapter 3 agree with those of Chapter 2 in their corroboration of a model of LH binding to the chromatosome in which two DNA duplex strands are bound, further raising doubts that any model invoking a single DNA duplex strand-binding event of the LH on the chromatosome could be a legitimate, biochemically relevant structure.

Although what we have been seeing indicates a shift in the Thesis emphasis, what reappears throughout the work is the major importance of the capability of the LH to bind to *two* DNA sites - in fact, its insistence on doing so. As shown in Chapter 2 by the preference of supercoiled DNA over linear DNA, and the formation of networks of linear DNA, in Chapter 3 by the formations of crosslinked dimers of the 256-bp DNA and intramolecular crosslinks which dissolve into 256-bp DNA upon proteinase K treatment, and in this chapter by the crosslinking of two independent fragments of *Hae*III-digested DNA, despite the obstacle to the realization of the original aim of the experiments of Chapter 4 that the unexpected results generated, the conclusions drawn by these results, in agreement with those of the earlier chapters, are substantially supported by the evidence. The arguments made, supported by these data, are as compelling as, or more compelling than, those that could be reached by the original aim of this work in isolating a linker histone bound to a chromatosome at the dyad axis. That argument, to recap, is that a model of the LH positioned on the

chromatosome, binding two DNA duplexes (Figure 3.1b) is favored, by the evidence shown here, and the model invoking a single DNA-binding site on the chromatosome with which the LH interacts Figure 3.1c is highly disfavored.

The interpretation of the data centers upon linker histone binding to DNA through two domains. This dual binding has importance of its own, beyond the question of the location of the linker histone on the nucleosome subunit of chromatin. Dual binding of LH to the chromatosome, as observed in the experiments of this chapter and in Chapter 3, must be functionally relevant, and that relevance makes the methods employed in this work applicable to progress in the understanding of any processes involving chromatin structure. These methods seem to be adaptable to other considerations of chromatin function, such as chromatin remodeling, transcription factor binding, and gene metabolic regulatory processes.

In future work, one might begin to assess the affect of competing protein factors on formation of UV-inducible linker histone – DNA crosslinks. A lot of effort is being put into the mass spectrometric analysis of tryptic peptide fragments of DNA-bound proteins lately (von Hippel et al., 1980; Jensen et al., 1994). This could be a fruitful next step in the analysis of linker histone crosslinked to the chromatosomal DNA. Had there been more time, the HPLC reversed phase column could have been used for isolation of purified nucleoprotein fragments. The HPLC investigations started, and described in Chapter 3, could be continued to provide isolated and purified

nucleoprotein and tryptic nucleopeptide samples for sequencing analysis. It has also become common recently to run HPLC and mass spec in a combined procedure.

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