To the Graduate Council:

I am submitting herewith a dissertation written by Arthur Elliot Paton entitled "Structure of Nucleosome - HMG Complexes." I have examined the final copy of this dissertation for form and content and I recommend that it be accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy, with a major in Biomedical Sciences.

Donald E. Olins, Major Professor

We have read this dissertation
and recommend its acceptance:

Accepted for the Council:

Vice Chancellor
Graduate Studies and Research

STRUCTURE OF NUCLEOSOME-HMG COMPLEXES

A Dissertation

Presented for the

Doctor of Philosophy

Degree

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Arthur Elliot Paton

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DEDICATION

This work is dedicated to my wife, Carol, and to my son, David, for their love and encouragement; and for making everything seem easier than it was.

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ABSTRACT

This dissertation contains results from the study of nucleosome-HMG protein complexes. The complex was studied by thermal denaturation, electrophoresis, circular dichroism, sedimentation and electron microscopy. Initial studies focused on the formation of complexes when HMG 14 or 17 was added to nucleosome core particles. Discrete complexes were observed by electrophoresis on non-denaturing gels. Titration of nucleosome core particles with HMG 14 or 17 caused the cooperative formation of complexes containing 2 HMG proteins per nucleosome. The cooperative binding was dependent on ionic strength. Further studies, employing nuclease digestion and high resolution gel electrophoresis, located the binding sites of HMG proteins along the nucleosomal DNA. These results, coupled with the titration data, resulted in a model for the binding of HMG proteins to the nucleosome.

The preceding work was followed by extensive characterization of the complex. Thermal denaturation and circular dichroism showed HMG 14 and 17 stabilized the nucleosome core particle. These results argued against a role for HMG protein as a structural protein that loosened chromatin structure prior to transcription. Sedimentation in the analytical ultracentrifuge and electron microscopy both showed no significant structural changes in the nucleosome after HMG binding.

Later studies employed urea as an outside perturbant to see if HMG protein could stabilize nucleosomes against unfolding and to see if partially unfolded nucleosomes bound HMG protein. Thermal denaturation, circular dichroism and electrophoresis showed HMG 14 and 17 did stabilize

the nucleosome against unfolding by urea. In addition, it was discovered that compact nucleosome structure is required for specific binding of HMG 14 and 17.

Nucleosomes were crosslinked extensively with formaldehyde to test whether the cooperative binding of HMG proteins required a nucleosome conformational change. This experiment also revealed whether the histone basic tails were required for HMG binding. The results showed a significant conformational change was not required for a shift from cooperative to noncooperative HMG binding and that crosslinking the histone tails did not prevent specific binding of HMG 14 or 17. Nucleosomes were trypsinized to remove histone basic tails then complexed with HMG proteins. Electrophoresis showed that removal of histone basic tails destroyed specific binding of HMG proteins.

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CHAPTER I

INTRODUCTION

Overview

The DNA of eucaryotic cells, including the specialized cells of animals and higher plants, is the subject of intense study because of its central role in determining the structure and function of a cell. The DNA in the nucleus of a typical vertebrate cell is a faithful copy of the DNA contained in the fertilized egg from which the organism developed. It follows that the cellular DNA contains all the information necessary to guide the growth and development of the entire organism. However, within the nucleus of any given cell, only a small fraction of the information available is used for the growth and maintenance of that cell. Within the nucleus of each cell, the DNA must be stored in a fashion that allows selective retrieval from the vast array of information available.

The packaging of the DNA is constrained to allow a large amount of DNA to be stored in a small volume. The typical mammalian cell has a DNA complement of 5.5×10^9 base pairs. At a spacing of 3.4×10^{-10} meters between bases along the DNA, each cell nucleus contains about 1.87 meters of DNA. This length of DNA must be folded or coiled into a nucleus typically 10×10^{-6} meters in diameter. The folded or coiled form of the DNA is further required to perform an astonishing array of dynamic activities. One need only view a film of the nucleus during a typical cell cycle to acquire a lasting curiousity about how such

dynamics are accomplished. There are two things, then, that are characteristic of eucaryotic DNA. It must be accessible for replication and normal cell function; and it must be packaged into a very small volume.

In order to deal with information retrieval and DNA folding, eucaryotic cells use an elegant packaging system remarkably conserved over evolutionary time. The DNA of eucaryotic cells, is complexed with specific proteins to form an ordered structure known as chromatin. In all eucaryotic organisms investigated, including pond water protozoans, garden peas, fish and humans, the vast array of genetic information in each cell is stored, and functions, as chromatin.

Knowledge of the structure of chromatin emerged slowly until 1973-1974 when techniques were developed for spreading out the chromatin for electron microscopy. The resulting micrographs, combined with nuclease digestion patterns of chromatin as seen on electrophoretic gels, crystallized the concept of a repeating subunit structure for chromatin. As more laboratories joined the study of chromatin, the subunits were embraced as a model structure for all chromatin. The subunits became known as nucleosomes; the most commonly used term of several initially proposed. With the general structure of chromatin and nucleosomes more clearly defined, attention turned toward what structural differences might exist between transcribed chromatin (active) and non-transcribed chromatin (inactive). Results from several laboratories indicated digestion of chromatin by pancreatic deoxyribonuclease I (DNase I) (E.C.3.1.4.5) occurred in two phases. The initial phase was rapid, then digestion slowed, indicating two or more domains of chromatin accessible to the nuclease. When chromatin was digested in this manner and the DNA was isolated and hybridized to cloned DNA from known active or inactive

genes, the results were startling. Chromatin from actively transcribing genes was digested much more rapidly than chromatin from inactive genes, as indicated by the ability to hybridize to the cloned DNA before, but not after, digestion. This result was subsequently confirmed by several laboratories and work was begun to isolate a factor or structural difference conferring the preferential DNase I sensitivity on chromatin from active genes.

Between 1964 and 1973, a class of nuclear non-histone proteins, known as high mobility group or HMG proteins, was discovered and the proteins were purified. The proteins were smaller than the histones but were rich in charged amino acids, asymmetrically distributed within the sequence, similar to histones. The HMG proteins were associated with chromatin and could be eluted with moderately high salt. Finally, it was shown that removing the HMG proteins from active chromatin destroyed the preferential DNase I sensitivity of active chromatin. Adding back purified HMG protein restored the DNase I sensitivity. Further work revealed the presence of HMG proteins in active chromatin isolated by selective fractionation. It became clear that experiments to study the structure of chromatin in the presence and absence of HMG proteins could provide information on the structure of active chromatin and how it might differ from inactive chromatin. The thrust of all the work described was to uncover structural differences that would allow a cell to selectively transcribe its complement of DNA.

There is now compelling evidence that the difference between an active gene and an inactive gene resides at the level of chromatin structure.

This dissertation will concentrate on the structure of HMG-nucleosome complexes, and how they differ from nucleosomes alone. The first chapter provides an introduction to chromatin and an overview of the field.

The second and third chapters describe what kinds of nucleosome-HMG protein complexes form in solution, and where the HMG proteins may bind on the nucleosome. A model is proposed that locates the HMG binding sites on the nucleosome core particle.

The fourth chapter describes the biophysical characterization of the complex. The methods include thermal denaturation, circular dichroism and sedimentation velocity, all done under variety of solvent conditions. These methods reveal a great deal of information on the stability and interactions of the complex.

The fifth chapter will describe conformational probes of the complex. These results reveal the structural transitions that occur when HMG protein binds to the nucleosome as well as the parts of the nucleosome essential for the binding reaction.

The Nucleosome

The concept of the nucleosome as a discrete particle, serving as the subunit for chromatin structure was synthesized from several independent lines of evidence. Digestion of chromatin with an endogenous nuclease (1), or with micrococcal nuclease (2), produced a series of discrete bands on electrophoretic gels with molecular weights an integral multiple of the smallest band, and suggested a repeating structure of 200 base pairs of DNA (2). Electron microscopy provided convincing visual evidence for a repeating structure by showing chromatin as a series of discrete spherical particles linked by short

stretches of DNA (3, 4) [Figure 1]. The string of beads appearance and the size of the particle led to a proposal for a repeating subunit structure composed of the four histone proteins and DNA (3).

Chromatin subunits were isolated and sedimented in the ultracentrifuge, giving sedimentation values characteristic of a compact structure (2, 6). The isolated particles appeared identical, in the electron microscope, to the particles linked together in chromatin (7-9). Histone association studies provided information suggesting that only the four histones H2A, H2B, H3 and H4 were involved in forming the particle (10-12). Models were proposed for a subunit of chromatin structure composed of 200 base pairs of DNA and two each of the four histones H2A, H2B, H3 and H4 (3, 5, 13). Subsequent experiments provided support for these models of compact structure composed of only histones and DNA. Reconstitution of chromatin from purified histones and λ phage or calf thymus DNA was sufficient to give complexes identical to native chromatin as seen in the electron microscope (8). In addition, the name nucleosome was proposed for the particle (8). Neutron scattering studies provided evidence that the histones formed a protein core with the DNA wound on the outside (14). The model presented from the neutron scattering results suggested a globular histone core surrounded by 1.75 to 2 supercoils of DNA [Figure 2]. Further studies of chromatin using electron microscopy (15-17), nuclease digestion (17-21) and reconstitution (8, 22-24), gave broad support for the nucleosome as the subunit of chromatin (25-27).

When appropriate models for nucleosome structure emerged, many laboratories began to characterize the nucleosome to test the models, and to study the structure and dynamics of the chromatin subunit. One

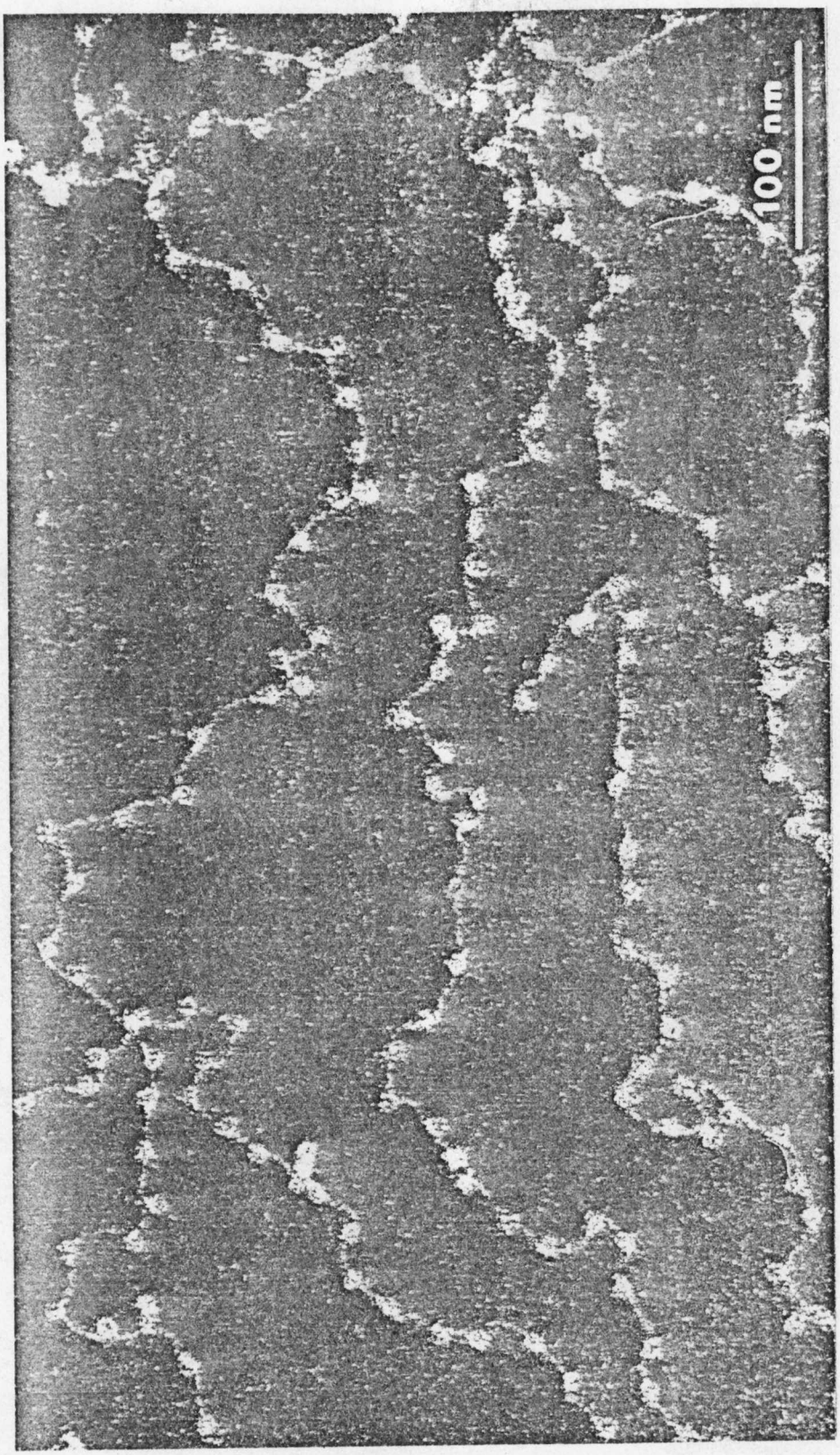

Figure 1

Dark field electron micrograph of chicken erythrocyte chromatin. Negatively stained with uranyl acetate.

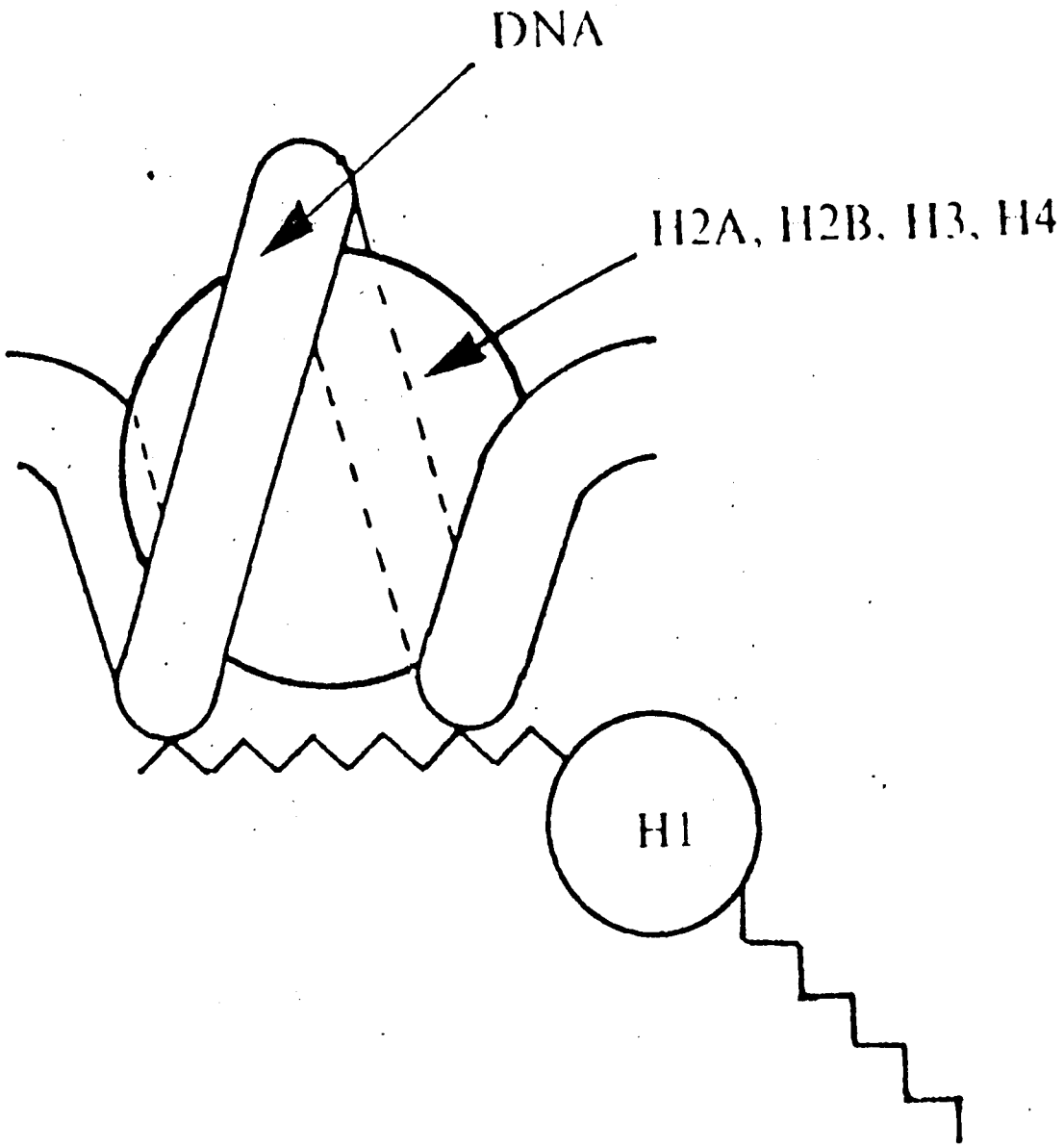

Figure 2

Model of the nucleosome from neutron scattering studies of Baldwin et al. (14).

of the first characteristics studied was the length of DNA associated with the nucleosome.

The digestion of chromatin with micrococcal nuclease occurs in two phases (4, 28). First, the nuclease cuts the DNA within the linker region between nucleosomes. This produces nucleosome oligomers containing DNA lengths that are multiples of 180 to 205 base pairs. Second, micrococcal nuclease preferentially degrades the remaining linker DNA. This produces nucleosomes containing the four nucleosomal or "inner" histones, histone H1, and 160 to 168 base pairs of DNA (29). Simpson has termed this the "chromatosome" (30). Further digestion frees H1 and yields a nucleosome core particle with 146 base pairs of DNA and the inner histones (28) [Figure 3].

The initial digestion of chromatin, that produces a ladder of fragments, is used to find the DNA length of the nucleosome repeat. When the digestion is done to minimize further degradation of linker DNA, and is extrapolated to zero time (26), one finds the DNA length of the nucleosome repeat differs among species (26, 31) [Table I]. In contrast, extensive digestion of chromatin, or chromatin that has the H1 histone removed prior to digestion, yields the nucleosome core particle (32). The DNA length, 146 base pairs, associated with the core particle appears to be invariant among species (33), and is identical to the DNA length of nucleosome cores reconstructed from purified DNA and inner histones (24, 34, 35).

The invariant DNA length and uniform histone content of core nucleosomes implies a uniform structure. A number of different experimental approaches yield remarkably similar results for the size and shape of the nucleosome core particle. Electron microscopy of

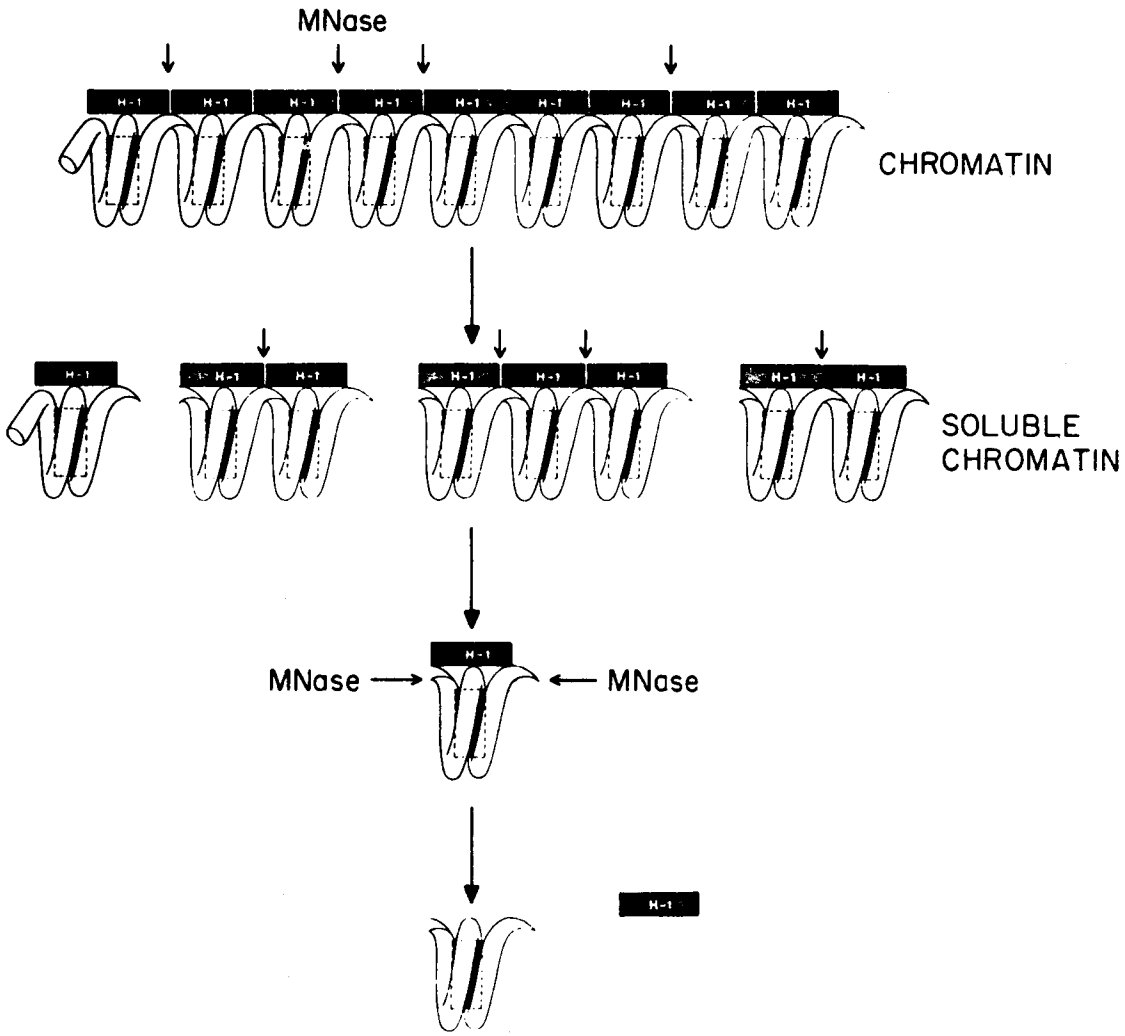

Figure 3

Digestion products of chromatin treated with micrococcal nuclease.

Table I

NUCLEOSOME REPEAT LENGTHS FROM VARIOUS CELL TYPES

Cell Types	DNA Content of Nucleosome (base pairs)
Aspergillus	154
Rabbit cortical neuron	162
Yeast	163-165
Neurospora	170
Physarum	171,190
Tissue culture cells:	
CHO	177
HeLa	183,188
Hepatoma	188
Teratoma	188
P815	188
Myoblast	189
CV-1	189
BHK	190
Rat Kidney, primary	191
Myotube	193
C-6	198
Rat bone marrow	192
Rat, fetal liver	193
Rat, adult liver	196,198
Rat kidney	196
Syrian hamster liver	196
Syrian hamster kidney	196
Chicken oviduct	196
Rabbit cortical ganglia	197
Tetrahymena, micro and macro nuclei	199
Rabbit cerebellar neuron	200
Stylonichia, micronucleus	202
Chicken erythrocyte	198,207
Sea urchin, gastrula	218
Stylonichia, macronucleus	220
Sea urchin, sperm	241

Compiled from references 26 and 31

isolated nucleosome cores shows a flattened sphere or disk with a diameter of 7.0 to 13.5 nm (16, 36) and a height of 5.0 nm (16). Neutron and X-ray diffraction give dimensions of 11 nm by 5.7 nm for a flattened disk or wedge (37, 38). Data from sedimentation velocity (2, 6), and electric dichroism (39) also support the idea of a compact structure. Recent x-ray diffraction studies on histone octamers, combined with image reconstruction techniques, have led to the proposal of a wedge-shaped helical ramp, upon which 1.75 left-hand supercoils of DNA are wound (40). The dimensions of the central histone wedge are 7.0 nm in diameter and 5.6 nm thick. This corresponds well with previously reported dimensions of 11 nm by 5.5 nm, when 1.75 supercoils of 2.0 nm wide DNA are added to the circumference [Figure 4].

It has been suggested that the arrangement of the histones in the nucleosome determines the path of the DNA supercoils around the histone core (22, 23, 40). Information about histone arrangement comes from studies using crosslinking reagents and studies of histone association in solution (41, 42). The most useful crosslinking data come from experiments using "zero-length" or short-length crosslinkers to probe histone-histone contact sites within the nucleosome (43-47). NMR experiments show that histone-histone contacts occur near the globular carboxy-terminal ends of the histones, not the lysine rich amino-terminal tails (48). Removal of the lysine rich "tails" with trypsin does not destroy histone-histone interactions (49-50). Thus the important crosslinking results employ zero-length crosslinkers specific for the globular, carboxy-terminal portion of the histones. The crosslinking results appear to show close contacts between H2A-H2B,

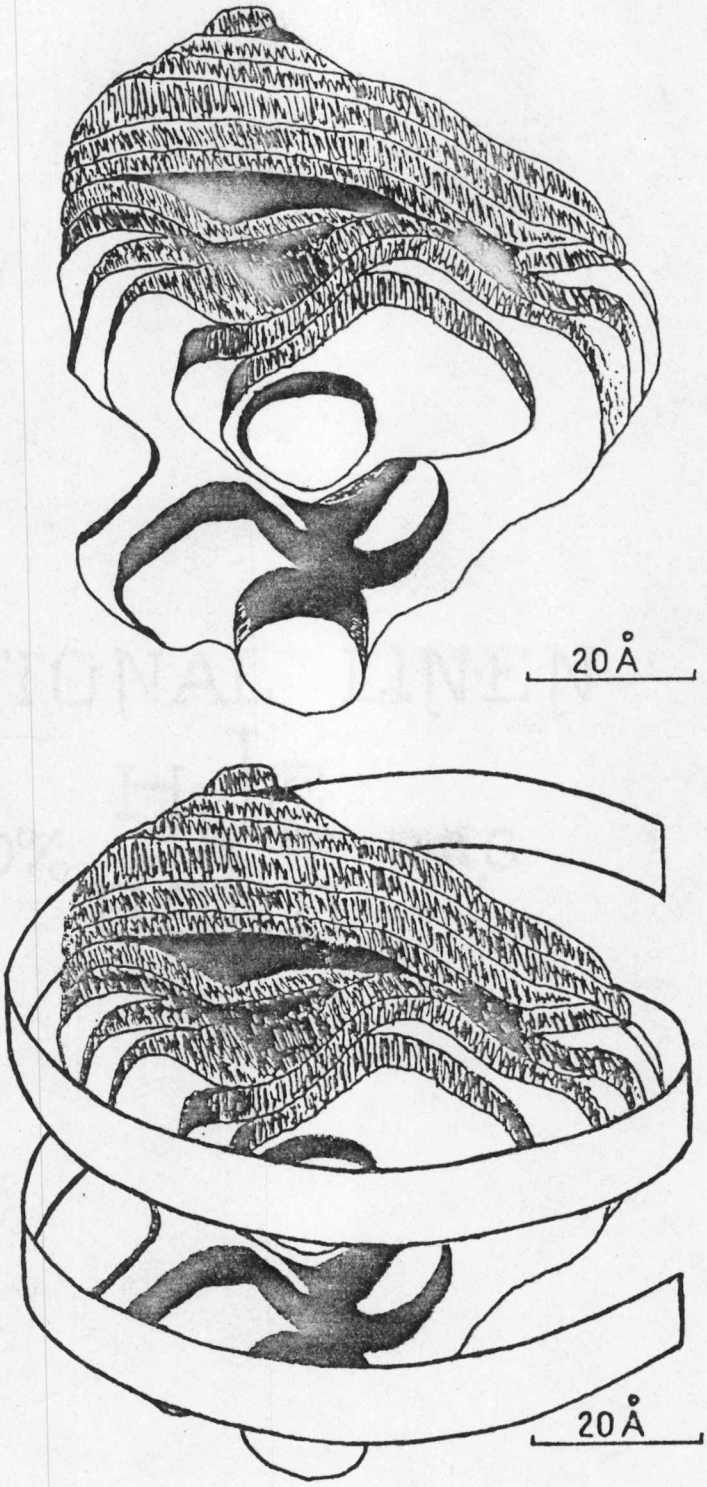

Figure 4

Model of the histone octamer, and nucleosome core particle to 20Å resolution. Redrawn from Klug et al (40).

H3-H4 and H2B-H4. These results closely agree with data on the association of histones in solution (10-12, 41).

The crosslinking and association of histones in solution gives information on which histones are close together, but not how they are arranged relative to the DNA. Studies using specific crosslinks between histones and DNA provide insight into how the histones are arranged along the DNA supercoil and thus provide positional information within the nucleosome (51, 52). A model for histone arrangement has been proposed by Klug et al. (40). In this model, the three-dimensional structure of the histone core, obtained by image reconstruction to a resolution of 2.0 nm, is combined with the results of the DNA-histone crosslinking studies (40). The positions of the individual histones within the nucleosome are assigned relative to 1.75 left-hand supercoils of DNA.

The path of the DNA around the histone core is not known precisely. However, several lines of evidence led to the concept of 1.75 - 2 left-handed supercoils around the histone core. Neutron scattering gave the first good evidence for the DNA forming a coil or shell outside the histone core (14). The results showed the radius of gyration for the DNA was larger than that for protein. This put the DNA on the outside of the protein and suggested the supercoiling of DNA around a central protein core (14, 53, 54). Other evidence comes from nuclease digestion studies that show the DNA is freely accessible to digestion (22) and from work with viral chromatin that shows binding of ethidium bromide and migration on agarose gels characteristic of nucleosomes formed with left-hand supercoils of DNA (55).

Histones

The histone proteins are generally divided into two classes (41). The first class includes the four inner histones, H2A, H2B, H3 and H4. Two copies of each inner histone make up the central protein octamer of the nucleosome. The second class includes histones H1 and H5. This class of histone is found on the outside of the nucleosome and is required for higher-order folding of chromatin. Table II summarizes a number of histone characteristics from references 41 and 42. This dissertation is mainly concerned with the nucleosome core particle and higher-order folding will not be discussed. As a result, the H1/H5 class of histones will be described only in comparison to the inner histones.

The primary structure is known for the histones of several species (41). Figure 5 shows the sequences of histones from calf thymus in schematic form. Inspection of these sequences reveals three major points:

First, the histones are highly charged proteins, enriched in both acidic and basic amino acids. The basic residues were originally used as a classification for the histones, with the H1 class known as very lysine rich, H2A-H2B as slightly lysine rich, and H3-H4 as arginine rich histones (41, 42).

Second, the charged residues of the histones are distributed asymmetrically. The inner histones each have amino-terminal sections that are highly basic. The remainder of each sequence shows a rough balance of charged residues and is characteristic of globular proteins (41, 42). Histone H1 is larger and has a central region similar to

TABLE II

PROPERTIES OF CALF THYMUS AND CHICKEN ERYTHROCYTE HISTONES

Histone	Molecular Weight	#Amino Acids	Basic Residues	Acidic Residues	Regions of 2° Structure
H2A	14,000	129	30	9	25-109
H2B	13,800	125	31	9	45-115
H3	15,300	135	33	11	42-110
H4	11,300	102	27	7	33-102
H1	21,000	200-215	-	9	42-112
Chicken H5	20,580	189	68	5	22-100

sequences of globular proteins with the amino-terminal and carboxy-terminal ends forming highly basic "tails". The carboxy-terminal residues are nearly a copolymer of lysine, alanine and proline (41, 42). Histone H5 is closely related to histone H1 and is found in the nucleated erythrocytes of birds and fish (41). It is thought to represent an H1 variant specific for this cell type (41).

Third, the histones appear to be conserved over evolutionary time. Histones H3 and H4 are among the most highly conserved proteins known. Histones H2A and H2B show significant sequence conservaton in the carboxy-terminal region, known to form globular structure, while the amino-terminal portions are less conserved (41). Histones H1 and H5 are quite variable between species and variants within tissues of a species are known (41). The inner histones of sea urchins have been studied during the course of development. Stage specific histone variants have been described that may alter chromatin structure as part of the

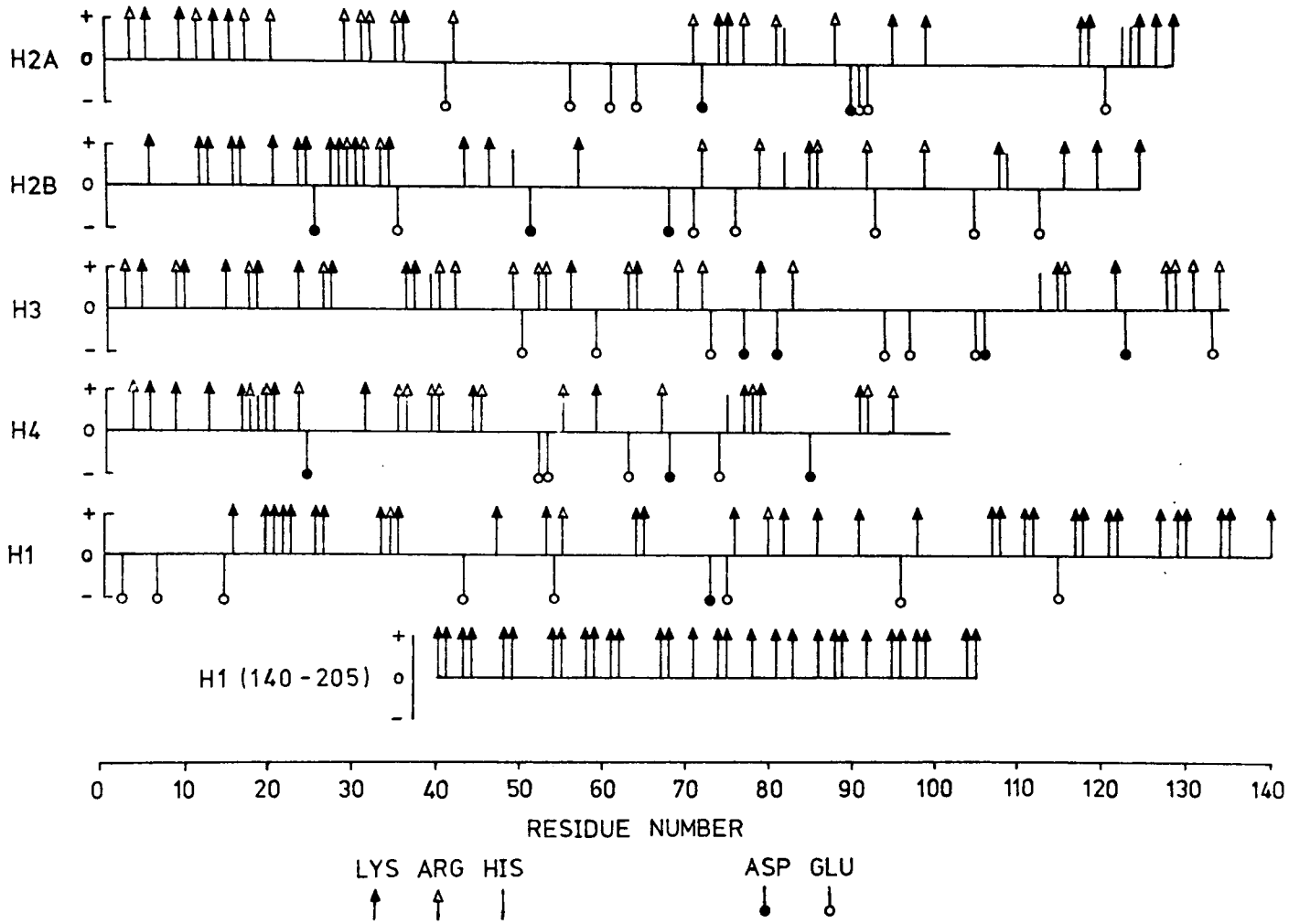

Figure 5

Schematic diagram of histone sequences from calf thymus. Sequences from ref. 41.

developmental process (56). The amino acid substitutions occur within regions known to be involved in histone-histone interactions and could alter nucleosome conformation or structural transitions.

Information on the interactions between histones within the nucleosome comes from studies on the association of histone pairs in solution and from crosslinking studies on nucleosomes and chromatin [Figure 6]. In solution, the inner histones interact specifically to form complexes with strong, intermediate or weak binding (12). Strong complexes are formed between H2A-H2B, H2B-H4 and H3-H4. H3 and H4 also form a tetramer (56). A complex of intermediate strength is formed between H2A-H3 and there are two weak complexes formed between H2A-H4 and H2B-H3. The relative strength of the interactions is indicated by the width of the line between histones in Figure 6. Mixed histone pairs from peas and calf, yeast and calf, and tetrahymena and calf, interact with nearly identical strength and specificity to those from a single species (57). This is striking evidence for evolutionary conservation of histone and nucleosome structure.

Crosslinking of histones in chromatin or nucleosomes, using "zero-length" crosslinkers, reveals probable histone-histone contacts in the native structure (41, 42, 58). Irradiation with ultraviolet light crosslinks H2A-H2B and H2B-H4 (45). Treatment with tetranitromethane or formaldehyde crosslinks H2B-H4 (44). Crosslinking with carbodimide results in H3-H4 dimers (43). The crosslinking results are shown schematically in Figure 6. It is interesting to note that the crosslinking data agree with the pattern of strong histone interactions. It has also been shown that the H2B-H4 crosslinks occur in the region of

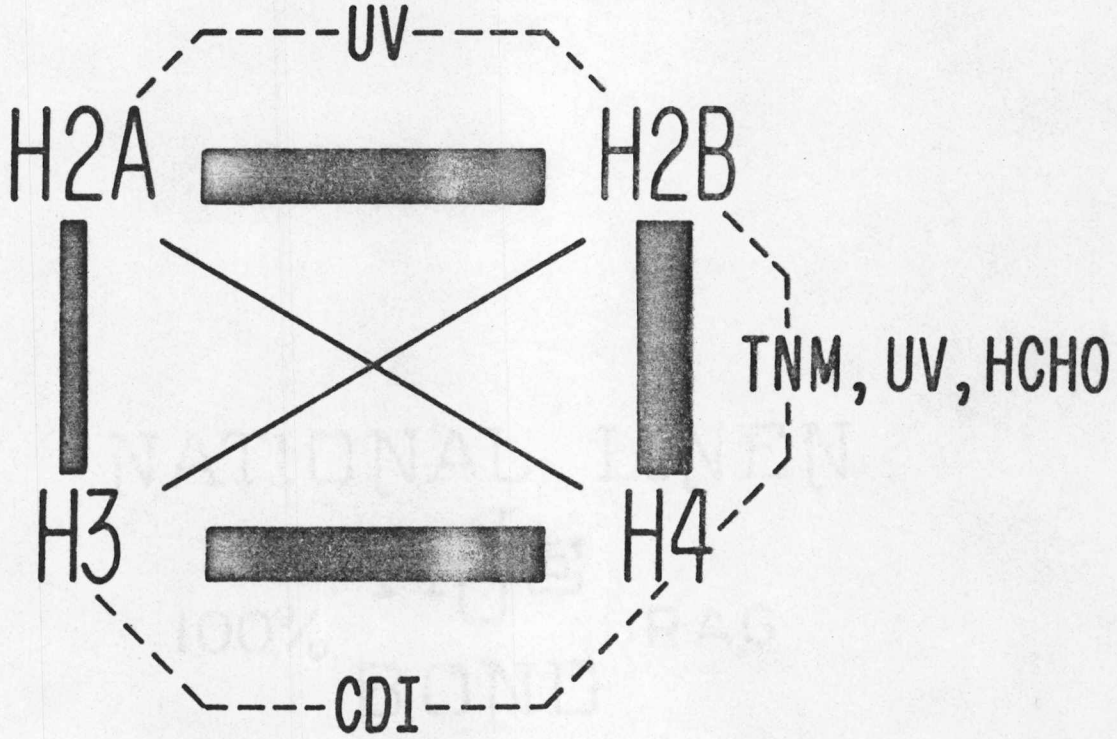

Figure 6

Diagram of inner histone interactions and crosslinking. Thickness of connecting bar is proportional to strength of association. TNM: tetranitromethane; HCHO: formaldehyde; UV: ultraviolet light; CDI: carbodiimide; ---- crosslinks.

the histones known to form globular structure and known to be most conserved between species (47).

The crosslinking and histone association studies reveal an essential role for H3 and H4 in the structure of the nucleosome. Further evidence comes from reconstruction experiments using purified histones and DNA. H3 and H4, as a tetramer, have been shown to organize DNA into nucleosome like structures in the absence of other histones (23, 59). Combinations of histones that do not include both H3 and H4 fail to organize DNA into a spherical repeating structure (23, 59). Successful reconstructions using a mixture of histones from different species demonstrate the conservation of histone sequences and conformations essential for proper histone interaction and DNA folding (57). Reconstruction experiments using all four inner histones and a variety of natural and synthetic DNAs have shown that histones can organize double-stranded DNA into nucleosomes regardless of sequence or base composition (35, 60). The folding of DNA around the histones to form a discrete repeating structure implies specific contact sites for histone-DNA interactions. These contact sites have been explored in detail by crosslinking DNA to lysine groups of the histones with dimethylsulfate (51, 52, 61, 62). The crosslinking reaction subsequently breaks the DNA at the crosslink site, leaving the histone covalently bound to the end of the 5' fragment of DNA. Two-dimensional electrophoresis is used to separate the DNA fragments and to identify the histones bound to each fragment. The results of such experiments provide a linear map of histone contact sites along the DNA (62). Figure 7 is a diagram showing the histone-DNA contacts in both the linear and supercoiled forms, modified from Schick et al. (51). Some

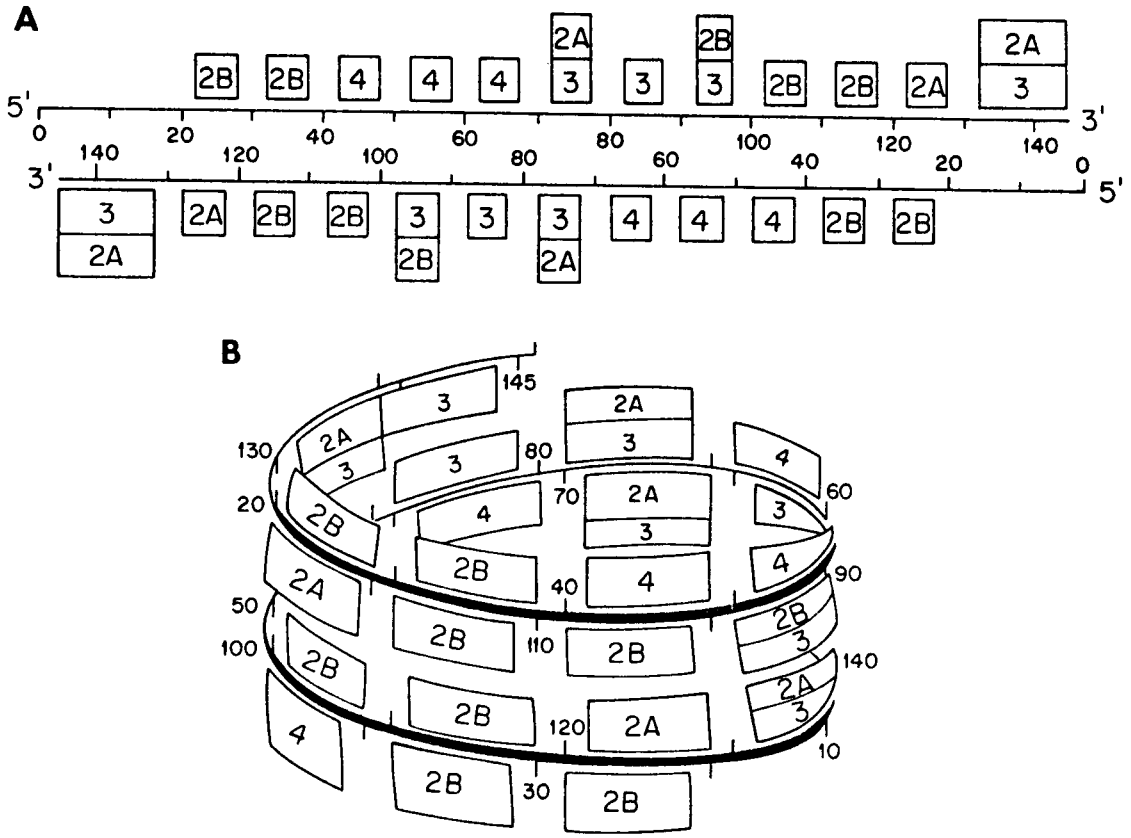

Figure 7

Histone-DNA contact sites deduced from crosslinking studies (51).
A: linear map. B: supercoiled form. In B the thick line represents the DNA helix.

care is needed in the interpretation of experiments that crosslink DNA to lysine residues of the histones. All of the inner histones contain more lysine residues in the amino-terminal tails than in the regions of globular structure. Yet removal of the histone tails with trypsin does not substantially alter nucleosome structure. This is an important consideration when trying to determine if a lysine-DNA crosslink reflects a contact site essential for DNA folding. The lysine residues in the tails may contact the DNA at sites far removed from those between DNA and histone globular regions and give a misleading picture of histone arrangement within the core particle. This situation may be partially resolved by studies on DNAse I digestion of trypsin-treated nucleosomes. Whitlock and Simpson have shown that removal of the amino-terminal tails with trypsin, permits the nuclease to attack previously protected sites at specific points along the DNA (63). The results suggest contacts between histone tails and DNA at 20-35, 60-80, and possibly 105-120 bases.

Another important aspect of histone-DNA contacts is histone modification. The inner histones are modified by phosphorylation, methylation, ADP-ribosylation, covalent linkage and acetylation (41, 42). Of these modifications, the one most important to this dissertation is acetylation of the amino-terminal lysine residues in each of the four inner histones. The sites of acetylation, taken from reference 41, are diagrammed in Figure 8. Acetylation of the inner histones is a highly dynamic post-translational event under the control of both the acetylase and deacetylase (64). Lysine acetyl-groups turnover rapidly with a half-life of 10-15 minutes (65, 66). Inhibition of the deacetylase reaction by sodium butyrate saturates the acetylation sites producing

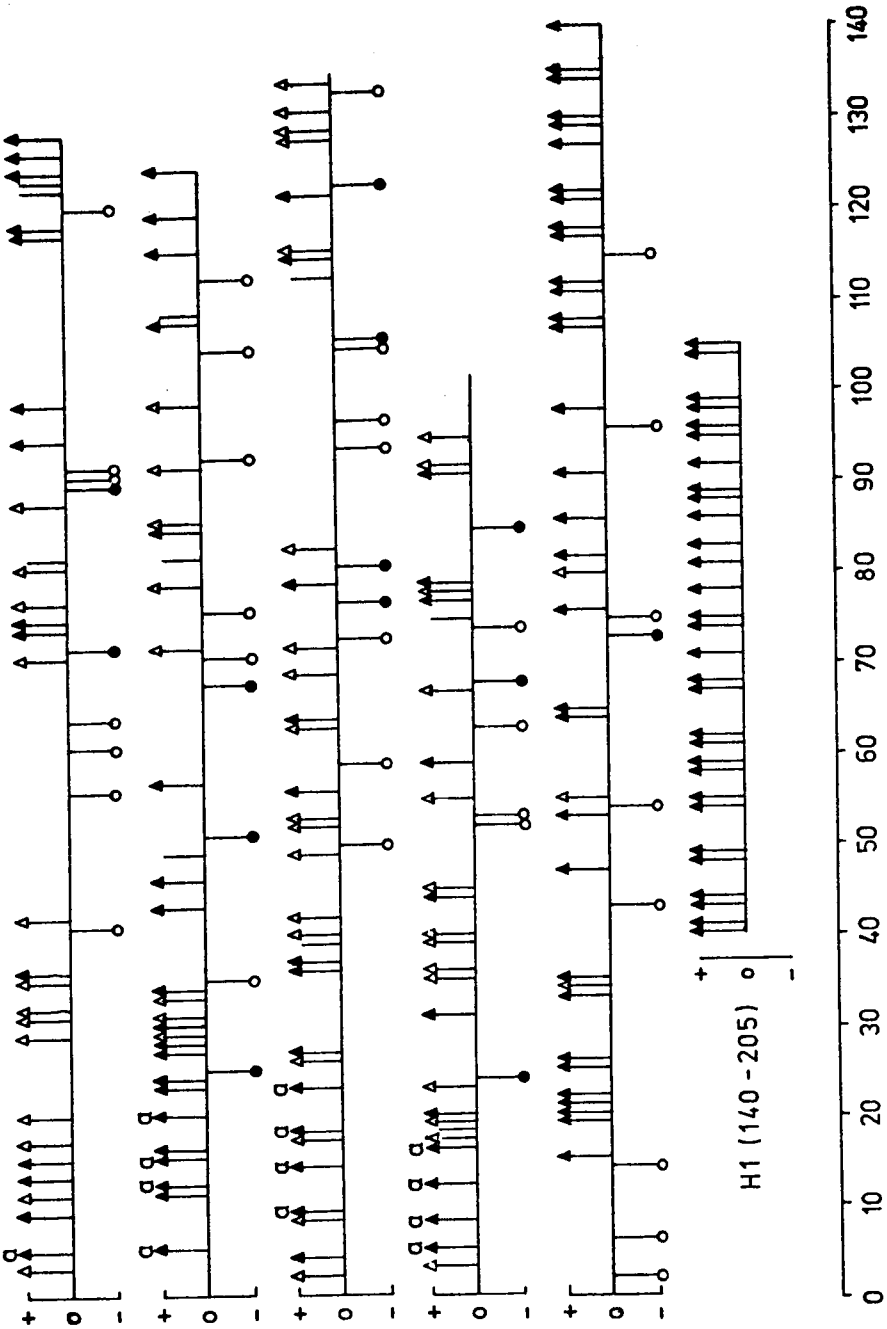

Figure 8

Schematic diagram of calf thymus histone sequences showing sites of acetylation.

hyperacetylated histones (67, 68). Hyperacetylation also occurs *in vivo* and is reported to be characteristic of actively transcribing chromatin (69, 72). Acetylation of the ϵ-amino group of lysine neutralizes the normal positive charge and may reduce or eliminate the binding of histone tails to DNA. This could result in loosening histone-DNA interactions and altering the binding of other chromatin associated proteins.

DNA Conformation

DNA conformation in the nucleosome addresses both the supercoiling of double-stranded DNA around the histone core and the pitch or twist of the DNA helix. The DNA appears to be smoothly coiled around the central histone core. Nuclease digestion results (58) and chemical modification (73) suggest that the DNA is freely accessible to small molecules and sizeable enzymes. NMR studies comparing nucleosomes to extracted DNA show no alterations in phosphate backbone structure of the DNA when it is complexed with the histone core (74, 75). Theoretical calculations using the known restrictions on bond angles show the DNA may be smoothly supercoiled to a radius of 4.5–5.5 nm without violating interatomic distances (76, 77). Calculations show the energy required to bind DNA into a supercoil of nucleosome dimensions is only 2% above that of free DNA in solution, about 25 Kcal per mole of nucleosomes or 0.086 Kcal per mole of DNA phosphate (76). While the majority of evidence supports a smoothly bent supercoil, an alternative model has been suggested for anisotropic or kinked DNA folding, that requires only a small change in the pucker of the ribose ring (78). This change would not affect the

phosphate backbone and would be undetected by ^{31}P-NMR. However, there is no positive evidence to support a kinked structure (58, 79). The evidence for a left-hand supercoil comes from nuclease digestion studies, and from the supercoiling of closed circular viral DNA into nucleosomes (32, 55). Digestion of nucleosomes with DNAse I shows rapid cleavage of sites near the 5' ends of nucleosomal DNA, while distal sites are digested more slowly (32). Lutter has suggested that this is evidence for a left-handed supercoil (32). The frequently cut sites are thought to occur on the exposed side of the DNA helix away from the adjacent supercoil, and are assumed to offer less stearic hindrance to the enzyme.

The supercoiling of closed circular DNA on nucleosomes in certain viruses confers, on the DNA, a topological property known as the linking number (80, 81). With respect to chromatin, the linking number, is the algebraic sum of helix twist and supercoiling. The linking number is the value obtained from SV-40 viral chromatin incubated with nicking-closing enzyme and run on agarose gels. From a knowledge of the linking number, and the pitch or twist of the DNA from X-ray measurements, the number and polarity of supercoiling can be calculated. The results suggest about 1.2 left-handed supercoils for each nucleosome in SV-40 chromatin. To reconcile this number with the two left-hand supercoils proposed from X-ray and nuclease digestion results (32, 37), it is necessary to invoke a change in helix twist from 10.6 base pairs per turn in chromatin, to 10.0 base pairs per turn in protein-free DNA. However, recent measurements of DNA in solution argue for helix twists of greater than 10.0 (82-85).

The uncertainty about the twist of the DNA helix in solution does not allow a precise calculation to be made for the number of supercoils in the nucleosome. However, the evidence from SV-40 does support the idea of a left-handed supercoil.

High Mobility Group Proteins

The High Mobility Group or HMG proteins are a class of nuclear, non-histone proteins extracted from nuclei at 0.35 M salt and soluble in 2% trichloroacetic acid (TCA) (86). The name HMG comes from their low molecular weight and corresponding high mobility on electrophoretic gels (86). They have been found in a wide variety of species including mammals, birds, insects, fish, fungi, and plants (86-88). The HMG's can be subdivided further into two groups; the first containing HMG 1 and 2, and the second, HMG 14 and 17. The subdivision is made on the basis of size, differential solubility in high concentrations of TCA and chromatography on CM-Sephadex (86). Sequences are known for HMG's of both classes from calf thymus and for HMG-17 from chicken erythrocytes. Amino acid compositions are known for the HMG proteins from several other species and are very similar to those of calf thymus (86-88). Table III shows the amino acid composition of calf thymus HMGs (86). Amino acid compositions for each HMG are also known for several tissues of the calf and are nearly identical among the different tissues. These compositions show the HMG's are not tissue specific, and suggest the HMG's may be a common structural protein of chromatin. This is supported by their high concentration in nuclei, up to 10^6 molecules of each HMG protein per nucleus (86). Table IV summarizes the properties of the HMG proteins from calf thymus. The HMG proteins are highly

TABLE III

AMINO ACID ANALYSIS OF CALF THYMUS HMG PROTEINS (moles %)

Residue	HMG 1	HMG 2	HMG 14	HMG 17
ala	9.0	8.1	14.5	18.4
arg	3.9	4.7	5.6	4.1
asp	10.7	9.3	8.1	12.0
cys	trace	trace	0.7	-
glu	18.1	17.5	17.1	10.5
his	1.7	2.0	0.3	-
ile	1.8	1.3	0.5	-
leu	2.2	2.0	2.0	1.0
lys	21.3	19.4	19.0	24.3
met	1.5	0.4	-	-
phe	3.6	3.0	0.6	-
pro	7.0	8.9	8.5	12.9
ser	5.0	7.4	7.8	2.3
thr	2.5	2.7	4.2	1.2
tyr	2.9	2.0	0.4	-
val	1.9	2.3	4.2	2.0

TABLE IV

PROPERTIES OF CALF THYMUS AND CHICKEN ERYTHROCYTE HMG PROTEINS

	Mol. Wt.	Number Residues	Basic(+) residues	Acidic(-) residues	Total % charged residues	Regions of 2° structure
Calf						
HMG 1	29,883	259	64	72	53%	Yes
HMG 2	28,769	250	62	62	50%	Yes
HMG 14	10,699	100	26	20	46%	None
HMG 17	9,247	89	26	14	45%	None
Chicken						
HMG 14	12,950	121	37	31	56%	None
HMG 17	9,321	89	26	14	45%	None

Compiled from refs. 89-96.

charged. Up to 50% of the sequence is basic or acidic residues. The charged residues are asymmetrically distributed, like the histones, with highly basic amino-terminal regions and acidic carboxy-terminal regions. A notable feature of HMG 1 and 2 are the carboxy-terminal regions that contain 41 and 32 consecutive acidic residues, respectively. HMG 1 and 2 are highly homologous proteins with most of the differences between them being conservative amino acid replacements. Biophysical studies have revealed 40 to 50% alpha helix in HMG 1 and 2 and fluorescence studies show the tertiary structure is stable from pH 4.5 to 10. Thus, HMG 1 and 2 have structures like typical globular proteins.

In contrast to HMG 1 and 2, HMG 14 and 17 are smaller proteins with less extensive homology. HMG 14 and 17 contain a region near the amino-terminus enriched in proline residues. This region is highly conserved between HMG 14 and 17 and has been identified as the DNA binding region of the molecule in HMG 17 (86, 89).

Considering the homology between HMG 14 and 17 in this region, it is reasonable to assume the same DNA binding properties for this portion of HMG 14. Again in contrast to HMG 1 and 2, HMG 14 and 17 show no tertiary structure in solution (86, 89). The random coil structure of HMG 14 and 17 may be the result of an enrichment for proline in the N-terminal region and glycine in the C-terminal region of the molecule (86).

Figure 9 summarizes the structure of calf thymus HMG proteins in schematic form (90-96). HMG proteins are also known to be modified, in the same way the histones are, by phosphorylation (97-99), acetylation (100, 101), and ADP-ribosylation (102). HMG 14 and 17 have also been reported to be glycosylated (102). These modifications suggest the HMG

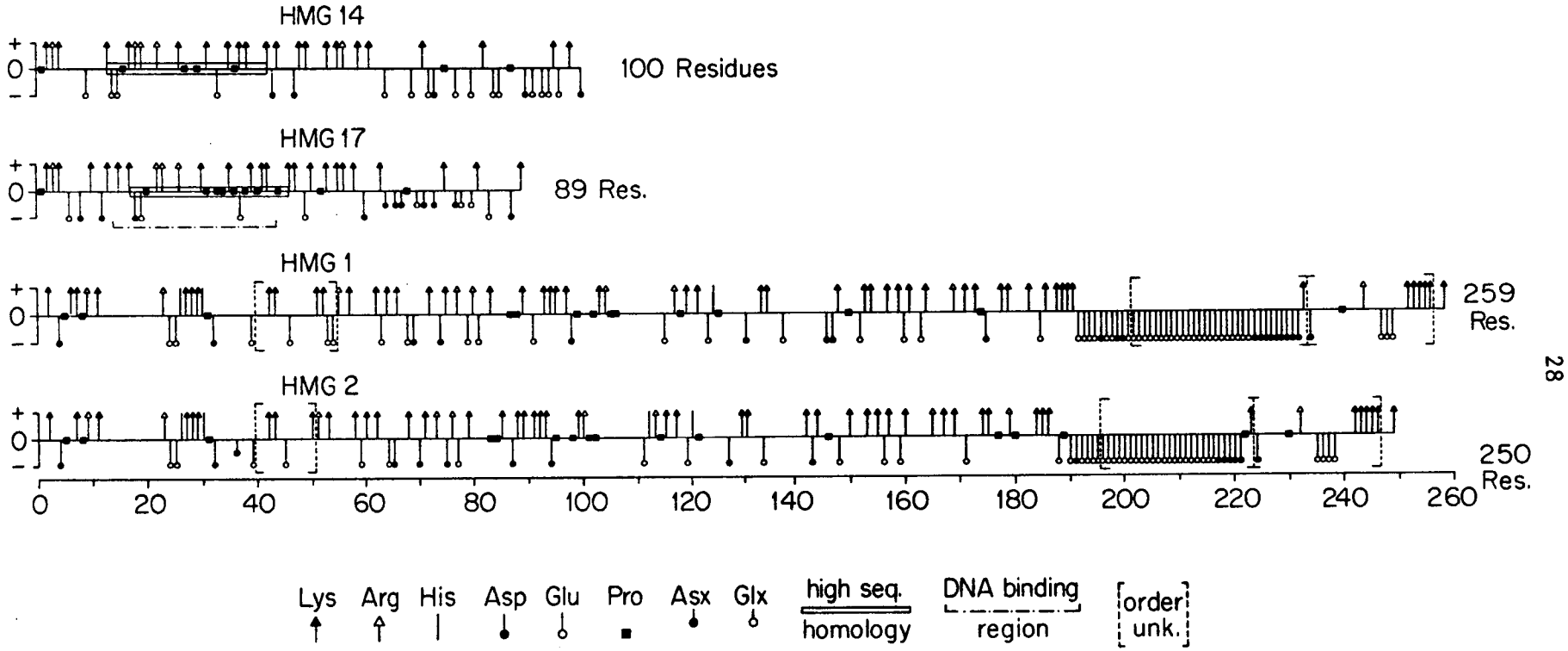

Figure 9

Schematic diagram of HMG protein sequences from calf thymus.

proteins are subject to the same cellular controls as the histones, and may have their binding to chromatin altered by these modifications. While much is known about the structure of HMG proteins, they have not yet been assigned a function.

HMG 1 and 2 are known to bind both single and double-stranded DNA (103-106). At low ionic strength they appear to stabilize DNA, against thermal denaturation, while at higher ionic-strength the DNA is destabilized (107, 108). HMG 1 and 2 also co-isolate with nucleosomes suggesting that they interact with histones and DNA to alter chromatin structure (109-111). While the evidence is suggestive, there is still no clear role for HMG 1 and 2.

HMG 14 and 17 have received more attention than HMG 1 and 2 because of their association with active regions of chromatin (112-114). They have been reported to be required for the DNAse I sensitivity of active genes (112-115), and when added back to chromatin stripped of HMG's, they preferentially bound to active chromatin (114).

It seemed clear that HMG 14 and 17 must be altering chromatin structure in active genes to cause their increased sensitivity to DNAse I. Suggestions were made about HMG 14 and 17 altering the binding of DNA to histones, loosening the complex, in preparation for transcription (116, 117). It became essential to obtain information on the structure of chromatin in the presence of HMG proteins.

It has been shown that HMG 14 and 17 bind to two equivalent sites located near, but not at, the ends of the DNA in nucleosome core particles (118, 119). The binding sites appear to be a feature of all nucleosomes not just those from active regions. However, a recent report shows that nucleosomes binding with highest affinity to a column

containing an HMG 14 and 17 affinity matrix, are enriched in the structural features of active chromatin (120). While this argues for specific binding to active chromatin it provides no information on how the specificity is obtained. The majority of evidence supports a general structural role for HMG 14 and 17. They are not tissue specific and are present in too great a quantity to be specific activators of transcription (86, 114, 115). Thus, a clear role for HMG 14 and 17 is still not defined. Evidence on the nature of the HMG-chromatin interaction is necessary to begin defining the role of HMG's in active chromatin. The binding of HMG 14 and 17 to nucleosome core particles is an appropriate model system for this study because of the vast amount of information on chromatin structure obtained by the study of nucleosomes (58, 79). Extensive characterization of HMG-nucleosome complexes is reported in the remainder of this dissertation.

CHAPTER 2

FORMATION OF HMG-NUCLEOSOME COMPLEXES

Introduction

The high mobility group (HMG) proteins are isolated from chromatin by extraction of nuclei, or isolated chromatin, with 0.35 M salt or 3% perchloric acid (PCA) (86). HMG 14 and 17 are reported to be associated with actively transcribing chromatin and to confer preferential DNAse I sensitivity on active genes (112-115). As nuclear non-histone proteins, bound to chromatin, it is likely the HMG proteins interact specifically with chromatin to alter structure and function.

Nucleosome core particles are the basic subunit of chromatin structure (25, 27). They serve as a more easily studied model for chromatin and its interactions with other macromolecules. A great deal has been learned about chromatin through studies of the nucleosome (58, 79).

In order to study the interaction of HMG proteins with chromatin, complexes were formed between nucleosome core particles and various HMG proteins. The complexes were assayed for discrete binding by electrophoresis on non-denaturing particle gels. The effect of ionic strength on the complex was explored on gels made up in buffers of varying ionic strength.

The results presented, show that the binding of HMG 14 and 17 is specific and cooperative, and the cooperativity of binding is sharply determined by ionic strength. The binding of HMG 1 and 2 is non-specific.

Materials and Methods

HMG Proteins

HMG proteins were extracted from chicken erythrocyte nuclei by a modification of published methods (86, 121, 122). 35 liters of chicken blood was collected into EDTA to prevent clotting. Blood was caught in large polycarbonate containers preloaded with 200 µl of 5% w/v Na_2 EDTA pH 7.0. Containers were removed and contents pooled when 4 liters of blood had accumulated. Blood was pooled into four 10 l carboys by pouring through 4 layers of cheesecloth to remove feathers. The carboys were packed in ice for transport from the site of collection to the laboratory. All subsequent operations were carried out at 4°C unless stated otherwise. Separated plasma was aspirated off and 35 liters of blood was suspended in 155 liters of 10 mM Tris-HCl, 10 mM NaCl, 3 mM $MgCl_2$ pH 7.4 (RSB buffer), using a 200 l carboy and a paddle. Phenyl methyl sulfonyl fluoride (PMSF), a potent protease inhibitor, was added to a final concentration of 0.1 mM from a 50 mM stock made up in isopropyl alcohol (PMSF is only slightly soluble in water). Erythrocytes were pelleted at room temperature in a Sharples continuous flow centrifuge. The 6 liter pellet was resuspended in 190 liters of RSBN (RSB + 0.5% NP-40) + 0.1 mM PMSF and allowed to stand for 20 minutes to lyse cells. Nuclei were pelleted at room temperature, using the K-2 zonal centrifuge with a 3.6 liter rotor at the pilot plant, Biology Division, Oak Ridge National Laboratory. The cold suspension was pumped in at a flow rate of 1.25 l/min, at a rotor speed of 6000 rpm (2400 x g). The 3 liter nuclear pellet was resuspended in 10 liters of 0.35 M NaCl + 0.1 mM PMSF and stirred for 30 minutes in the cold. At this point, it

was noted that the use of EDTA to prevent clotting also trapped hemoglobin in the erythrocyte nuclei during nuclear isolation. The nuclei appeared salmon colored. This was not a problem because the hemoglobin was totally removed during the later steps of HMG purfication. The use of sodium heparin to prevent clotting did not trap hemoglobin in the nucleus during smaller scale preparations but was too expensive to use in a large scale preparation.

After stirring in 0.35 M salt, to extract HMG proteins, the nuclei were pelleted in a Sorvall GSA rotor (6 × 250 ml bottles) at 6000 x g for 5 minutes. The supernatant was pooled into 6 l Erlenmeyer flasks and brought to a final concentration of 3% trichloroacetic acid (TCA) by adding, 0.031 X starting volume, of 100% w/v TCA. The TCA was added slowly with stirring. An immediate brown precipitate was observed. This represented the cytoplasmic and nuclear fractions of proteins, other than HMGs, which are not soluble in TCA. HMG proteins are soluble in 3% TCA. The precipitation was allowed to stand overnight, in the cold, to develop. The supernatant containing HMG proteins was filtered successively through Whatman #2 paper, then through 3μ Millipore with two prefilters. The settled precipitate slurry was spun at 4,000 x g for 10 minutes, the supernatant was collected, filtered through 3μ Millipore and pooled with the previous filtered supernatant. To the clear, pooled supernatant, 7/90 × volume of 100% TCA was added for a final concentration of 10% TCA. The precipitation was allowed to stand overnight. The supernatant was collected by aspiration. The pellet slurry, containing crude HMG 1 and 2, was spun at 8,000 x g and the supernatant pooled with the aspirated fraction. The remaining pellet was washed in acidified acetone (1 ml constant boiling HCl per 200 ml

acetone), then washed three times in acetone. The washed pellet was dried under vacuum and stored, dessicated, at -20°C as the crude HMG 1 and 2 fraction. The 10% TCA supernatant was filtered through prewashed 3μ Millipore filters, with 2 prefilters, and the clear filtrate was brought to 18% TCA by adding 8/82 × volume of 100% (w/v) TCA. The precipitate was allowed to form for 3 days in the cold. The supernatant was aspirated off and the pellet slurry was spun in Corex tubes at 10,000 x g for 10 min. The pellet containing crude HMG 14 and 17 was washed in acidified acetone, then 3 times in acetone dried under vacuum and stored dessicated at -20°C. The yield of crude HMG 14 and 17 was 600 mg.

HMG Purification

HMG 14 and 17 were purified using a modification of published methods (86). Crude HMG 14 and 17 were isolated as described above then dissolved in 7.5 mM Borate buffer pH 8.8 (91 mg in 6 ml). The sample was dialyzed against 7.5 mM Na-borate for 4 hours and spun at 12,000 x g for 5 minutes to remove particulates. The sample was loaded directly onto a 2.5 cm × 120 cm column of CM-Sephadex equilibrated in 7.5 mM Na-borate pH 8.8. The sample was run into the gel and overlayered with 7.5 mM borate. The elute was monitored at 225 nM and the sample was eluted from the column using a 0.1 M to 0.6 M gradient of sodium chloride in 7.5 mM Na-borate buffer. Flow rate was 25 drops per minute initially and slowed considerably after 2 hours. Separation between HMG 14 and HMG 17 was 20 fractions. Purity of the fractions was monitored on an SDS microslab gel (123). Pools of HMG 14 and 17 showed greater than 99% purity for each protein. 2 μg loads of each protein

showed no contaminants at a detection limit of 20–25 ng. Pooled fractions of HMG 14 or HMG 17 were brought to a final concentration of 25% (w/v) TCA and stored at 5°C for 72 hours. The precipitates were pelleted at 2500 x g for 30 minutes and the pellets resuspended in 0.01 N HCl. Samples of HMG 14 or HMG 17 were then desalted on Sephadex G-25 equilibrated in 0.01 N HCl. The final pools of each protein were lyophilized and stored at -20°C. Yields were 20 mg HMG 14 and 15 mg HMG 17. Stock solutions of HMG 14 and 17 were made by dissolving HMG 14 or 17 in distilled water at a concentration of 4 mg/ml. These stocks were stored frozen at -20°C and were not degraded by freeze-thaw for over one year. Stocks of HMG 1 and 2 were made by dissolving the crude mixture in distilled water.

Nucleosome Core Particles

Nucleosome core particles used in these studies were isolated using a modification of the method of Lutter (32). Briefly, nuclei are isolated from chicken erythrocytes contained in 150 ml of chicken blood. Nuclei at a concentration of 65 A260 units/ml are digested for 2 minutes at 37°C in the presence of 45 units/ml of micrococcal nuclease to solubilize the chromatin. The digestion is terminated with excess EDTA and cooled on ice. The nuclei are pelleted at 8,000 x g for 2 minutes and resuspended in 0.2 mM EDTA pH 7.0 for lysis. Gentle lysis is accomplished by pipetting up and down with a 25 ml pipette or by a very low speed setting on a Tissuemizer (Tekmar Corp.). Insoluble chromatin and nuclear debris are removed with a spin at 8,000 x g for 2 minutes. The long soluble chromatin is brought to a final salt concentration of 0.6 M NaCl by extremely slow dropwise addition of 4 M NaCl. The

solution shows a white filmy precipitate initially which disappears near the end of the titration. This step dissociates histone H 1 and other non-histone proteins from the long soluble chromatin. Aliquots containing 450 mg chromatin are made, and sucrose is added to a final concentration of 3%. The sample is then underlayered onto a Sepharose 4B column 5 cm × 150 cm equilibrated in 0.65 M NaCl, 5 mM Tris-HCl, 0.2 mM 2-mercaptoethanol, 0.02% NaAzide pH 7.5. Fractions containing oligonucleosome lengths greater than trimers are pooled and dialyzed into 5 mM Tris-HCl, 20 mM NH_4-acetate, 0.2 mM EDTA, 2 mM 2-mercaptoethanol. Removal of H-1 is monitored on SDS microslab gels (123). The pool is concentrated to 5 mg/ml in an Amicon apparatus using an XM-50 membrane. The concentrated sample is diluted to 5 mg/ml for a total of 200–250 mg of chromatin and digested with micrococcal nuclease for an amount of time determined by a previously run test digestion as suggested by Lutter (32). The test digestion is essential for high yields of nucleosome core particles. The digestion is stopped with EDTA, cooled on ice, and immediately brought to a final concentration of 3% sucrose. The sample is underlayered onto a 7 cm × 250 cm column of Sephacryl S-300 equilibrated in 5 mM Tris-HCl, 20 mM NaCl, 2 mM 2-mercaptoethanol, 0.2 mM EDTA and 0.02% NaAzide pH 7.5. PMSF is added to 0.1 mM in both sample and column buffers. Fractions containing nucleosome monomers are detected on denaturing DNA gels. Single strand gels show DNA length at higher resolution, and will detect breaks in one strand of the DNA if they are present. Nucleosome monomer fractions are pooled and dialyzed against buffer containing 12 mM $MgCl_2$, 100 mM KCl, 1 mM PIPES pH 7.2 to precipitate any residual dimer contamination. Any precipitate is removed with a spin at 8,000 x g for 20 minutes. The supernatant

fraction containing nucleosome core particles is exhaustively dialyzed against a storage buffer containing 5 mM TES, 20 mM NaCl, 0.2 mM EDTA, 3 mM NaAzide and 0.1 mM PMSF pH 7.4. The sample is concentrated, using collodian bags, to a concentration of 20 mg/ml and stored at 4°C. Freezing is avoided. Nucleosome core particles stored in this fashion are stable for over a year.

Electrophoresis

Native particle gels used to analyze nucleosome-HMG complexes were modified from previous methods (124). Gels contained 6% acrylamide, 0.2% Bis-acrylamide, diluted from concentrated stock, made up in the Tris-Borate EDTA (TBE) buffer of Peacock and Dingman (125). Gels were polymerized using ammonium persulfate as the initiator and tetramethylethylenediamine (TEMED) as the catalyst. 30 ml of gel contained 6 ml of 29% acrylamide-1% Bis-acrylamide, 0.4 ml of 10% ammonium persulfate in water, 0.6 ml buffer, 23 ml water, and 25 µl of TEMED. The buffer concentration in the gels was varied to raise or lower the ionic strength and is expressed as a fraction of TBE. 1/10 or 3/10 strength TBE buffer, expressed as 0.1 × or 0.3 × TBE, were typical values. TBE is 90 mM Tris base, 90 mM boric acid, 2.5 mM EDTA pH 8.3. Gels (10 cm × 16 cm × 1.5 mm) were formed in a vertical apparatus made by Hoefer Instruments or from Biorad. Sample preparations will be described in the text for the appropriate figures but typically involved adding to the sample 1/5 volume from a 5 fold stock of sample buffer. Sample buffer contained the same buffer components and ionic strength as the gel, plus glycerol for density and bromophenol blue as tracking dye. Electrophoresis was typically done at 200 volts with cooling of the

apparatus provided by 10°C tap water. Time of the run varied according to the ionic strength of the gel. Some gels contained urea to denature the nucleosome. Urea used in these gels was added from a 10 M stock of urea that had been deionized on mixed bed resin and filtered through 0.2 μ Duropore filters (Millipore Corp.). Conductivity was checked before each use and urea was deionized again if conductivity readings rose above 20 μMHO. True molarity of the urea was checked by refractive index readings (126). Gels were stained with Coomassie Blue in a solution of 5:5:1 methanol:water:acetic acid. Destaining was done in the same solution minus stain for 2-4 hours. Final destaining and storage was done in 10% acetic acid, 7.5% methanol. Gels stained for DNA with Toluidine Blue or ethidium bromide gave identical patterns.

Reconstruction of Nucleosomes

Nucleosome core particles were reconstituted from purified chicken inner histones and synthetic poly dA·dT according to Bryan et al. (60). Purified chicken inner histones were obtained from chicken erythrocyte nuclei by the method of Butler et al. (127). This method dissociates histones from DNA using 2 M NaCl and pellets the DNA with high speed centrifugation. The histone supernatant is concentrated in a collodian bag and run on 5–20% sucrose gradients in 2 M NaCl to separate inner histone octamers from histone H 1 and H 5. The fractions containing inner histone are pooled and dialyzed into storage buffer containing 2 M NaCl to preserve the octameric complex and frozen at -20°C. Synthetic poly dA·dT was purchased from P-L Biochemicals, checked for size by bouyant density centrifugation and used without further purification.

For reconstruction of nucleosome core particles, histones and DNA were mixed together at a ratio of 0.68 mg inner histones per mg synthetic DNA, in 10 mM Tris-HCl containing 2 M NaCl. The final concentration of DNA, before dialysis, was 0.5 mg/ml. Samples were step-dialyzed according to the following schedule:

1.4 M NaCl, 10 mM Tris-HCl, 0.2 mM EDTA, 0.1 mM DTT, pH 7.0, 4–6 hours.

1.0 M NaCl, 10 mM Tris-HCl, 0.2 mM EDTA, pH 7.0, 4–6 hours.

0.4 M NaCl, 10 mM Tris-HCl, 0.2 mM EDTA, pH 7.0, 4–6 hours.

0.1 M NaCl, 10 mM Tris-HCl, 0.2 mM EDTA, pH 7.0, 4–6 hours.

10 mM Tris-HCl, pH 7.0, 6–8 hours.

All samples were dialyzed in the presence of 0.1 mM PMSF. After dialysis, the sample was adjusted to 8–10 A260 units per ml and digested with 50 units per ml of micrococcal nuclease for 7 minutes at 37°C. The digestion was stopped with EDTA and cooled on ice. The samples were then centrifuged on 5–20% sucrose gradients in 20 mM NaCl and 0.2 mM EDTA pH 7.0 to isolate monomer nucleosomes. Centrifugation time was 12 hours at 40,000 rpm in a Beckman SW-41 rotor. Fractions containing nucleosome core particles were monitored on single-strand DNA gels and pooled, dialyzed into storage buffer, concentrated, and stored at 4°C. Digestion time of the reconstructed complex was determined by test digestions that were analyzed either on sucrose gradients or directly on single-strand DNA gels and particle gels. This insured a homogeneous yield of particles containing 146 base pairs of DNA.

Results

Chicken nucleosome core particles were mixed with chicken HMG 14 or HMG 17 at molar ratios of 0, 1, 2, or 3 HMG proteins per nucleosome. The resulting complexes were electrophoresed on 6% acrylamide gels, and stained with Coomassie Blue. The pattern of complexes formed in 0.1 × TBE buffer is shown in Figure 10. The staining pattern reveals that HMG 14 or HMG 17 can form discrete complexes with nucleosome core particles. The titration of nucleosome core particles with HMG 14 or HMG 17 initially produces a discrete and slower migrating band of material in addition to the core particle band. Titration with higher amounts of HMG protein produces a second discrete band of even slower migration. The presence of these two discrete bands suggests there are nucleosomes with one and two HMG proteins bound, respectively. Titration of nucleosomes with greater than three HMG proteins per nucleosome begins to yield diffuse staining in regions trailing the second discrete band of the complex. The diffusely staining region appears to result from non-specific binding of additional HMG proteins to the nucleosome core, since no further formation of discrete complexes is seen at high molar ratios of HMG protein to nucleosome cores. It should be noted that nucleosome core particles are reported to bind up to an additional octamer equivalent of inner histones, non-specifically (128). It is likely that the additional binding of HMG proteins occurs in the same way considering the similarity between histone and HMG on the basis of overall charge and charge asymmetry. The two discrete bands formed at lower input ratios of HMG proteins were further investigated by electrophoresing a lane from the particle gel in the

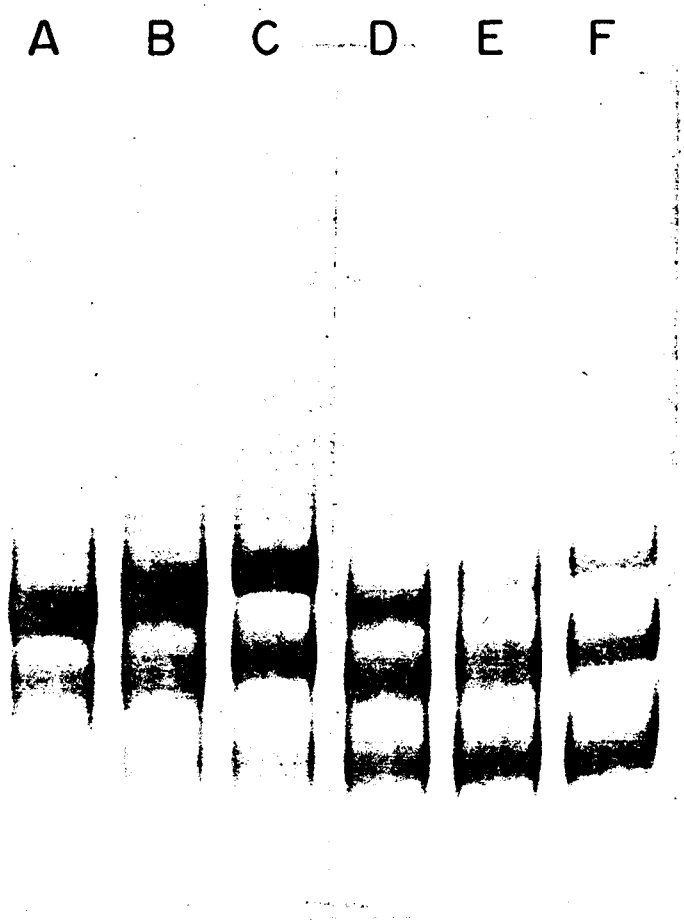

Figure 10

Titration of nucleosome core particles with HMG 14 and 17. Molar ratios of HMG proteins to nucleosome core particles: A, HMG 17, 1.72; B, HMG 14 and 17, 0.86 each; C, HMG 14, 1.72; D, HMG 17, 0.86; E, HMG 14 and 17, 0.43 each; F, HMG 14, 0.86.

second dimension, on SDS polyacrylamide gels (129). The protein staining revealed HMG protein in the first slower migrating band. The HMGs stained with about half the intensity of the individual histone bands. The HMG protein in the second slower migrating band stained with an intensity equivalent to the individual histones. This strongly suggests that the first and second slower migrating bands correspond to nucleosome cores with one or two HMGs bound, respectively. Figure 10 also shows the results of titrating nucleosome cores with a 1:1 mixture of HMG 14 and 17. The band migrating in the position of 2 HMG proteins per nucleosome clearly spans the area between the position of HMG 14 and HMG 17 containing nucleosomes. This shows that nucleosomes containing both HMG 14 and 17 exist along with nucleosomes containing exclusively HMG 14 or 17. The simplest interpretation is that HMG 14 or 17 each bind to the same site on the nucleosome core. The gel patterns further suggest there are two such equivalent sites for the binding of HMG 14 or 17 on each nucleosome core particle (118, 119).

Nucleosome core particles reconstructed from poly dA·dT and purified histones are known to have a structure very similar to native nucleosomes (60). Figure 11 shows the results of titrating nucleosome core particles, reconstructed from poly dA·dT and chicken histones, with HMG 14. This shows that HMG binding sites require only histone and DNA and exist as a result of DNA folding around the histone core. Normal nucleosome structure is sufficient for specific binding of HMG 14 and 17.

Complexes formed between chicken nucleosome core particles and a mixture of HMG 1 and 2 do not give a pattern of discrete bands when run on a 6% particle gel. Instead the pattern is smeared and diffuse indicating a weak and non-specific mode of binding to nucleosomes.

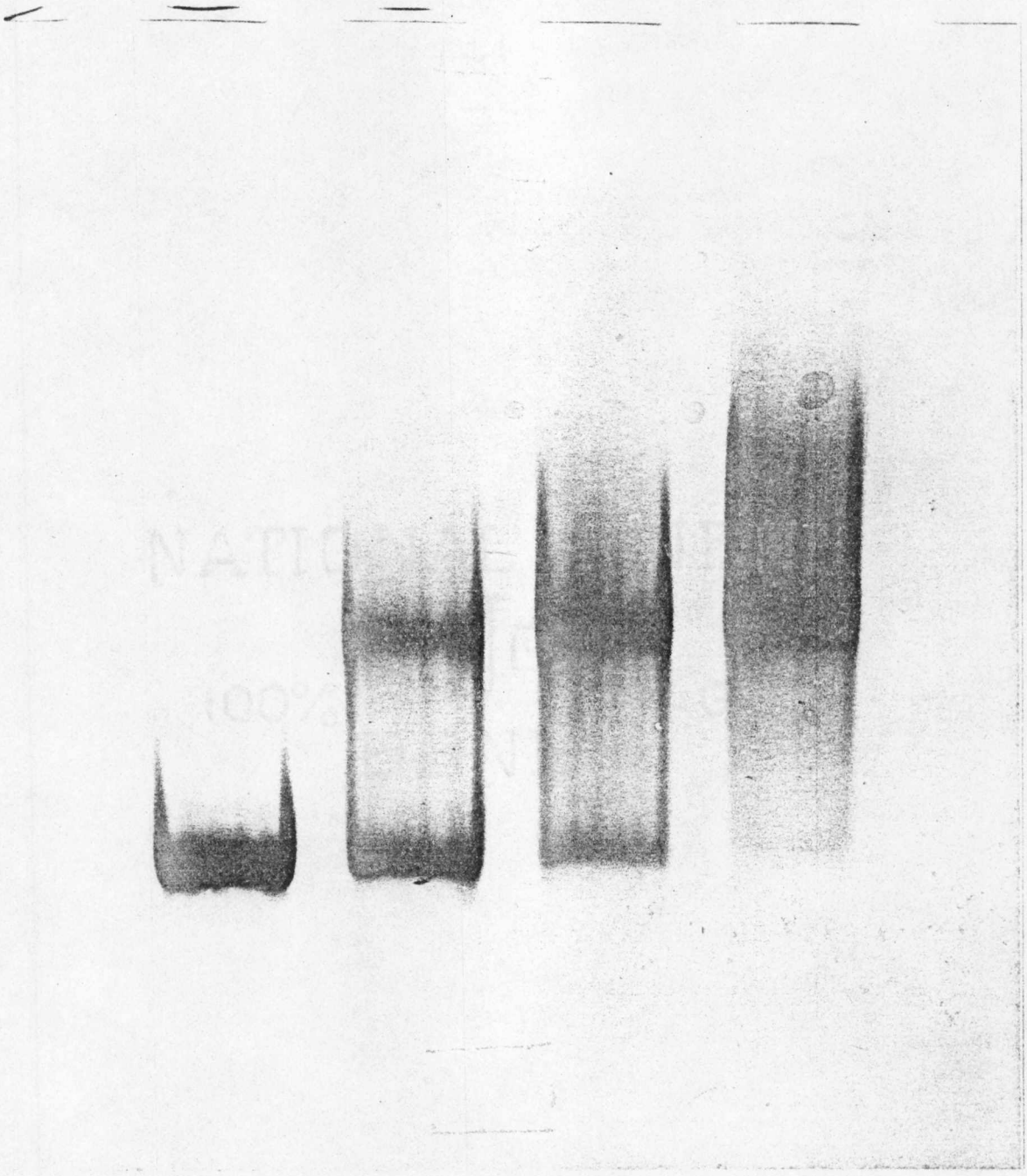

Figure 11

Titration of reconstructed nucleosomes with HMG 14. Left to right, 0, 1, 2, 3 moles HMG 14 per mole nucleosomes.

As this work was in progress, personal communication from Gary Felsenfeld revealed that gels run at higher ionic strength altered the pattern of complexes. Figure 12 shows the results of titrating nucleosome core particles with HMG 14 followed by electrophoresis in 1 × TBE. The banding patterns show only two kinds of complexes; those migrating as nucleosomes alone, and those migrating in the position of 2 HMG proteins per nucleosome. The intermediate band, corresponding to one HMG protein per nucleosome is missing. This strongly suggests a cooperative mode of binding such that nucleosomes, mixed with HMG at a ratio of 1 HMG protein per nucleosome, yield two species: nucleosomes with 2 HMG proteins bound, and nucleosomes without HMG bound. In addition, Figure 12 shows the results of various complex formation methods. Nucleosomes mixed with HMG 14 in 0.35 molar salt or 0.1 M salt and dialyzed down to 10 mM Tris-HCl pH 7.0 give the same electrophoretic pattern as nucleosomes mixed with HMG 14 in 10 mM Tris-HCl pH 7.0 and immediately loaded on the gel. This shows the binding is very rapid and supports the concept of specific, high affinity sites, on the nucleosome, for HMG proteins. Since the non-cooperative and cooperative binding modes were determined by ionic strength, it seemed appropriate to study the transition, from one mode to another, more carefully. Nucleosome core particles are known to change conformation when shifted from ionic strengths above 1–2 mM, to ionic strengths below 1–2 mM (130-135). It appeared that the observed cooperative change in HMG binding could be the result of an ionic strength-induced conformational change in the nucleosome. To investigate this idea further, particle gels were made up at various concentrations of TBE buffer between 0.1 × TBE and 1 × TBE. The results are shown in Figure 13. Particle

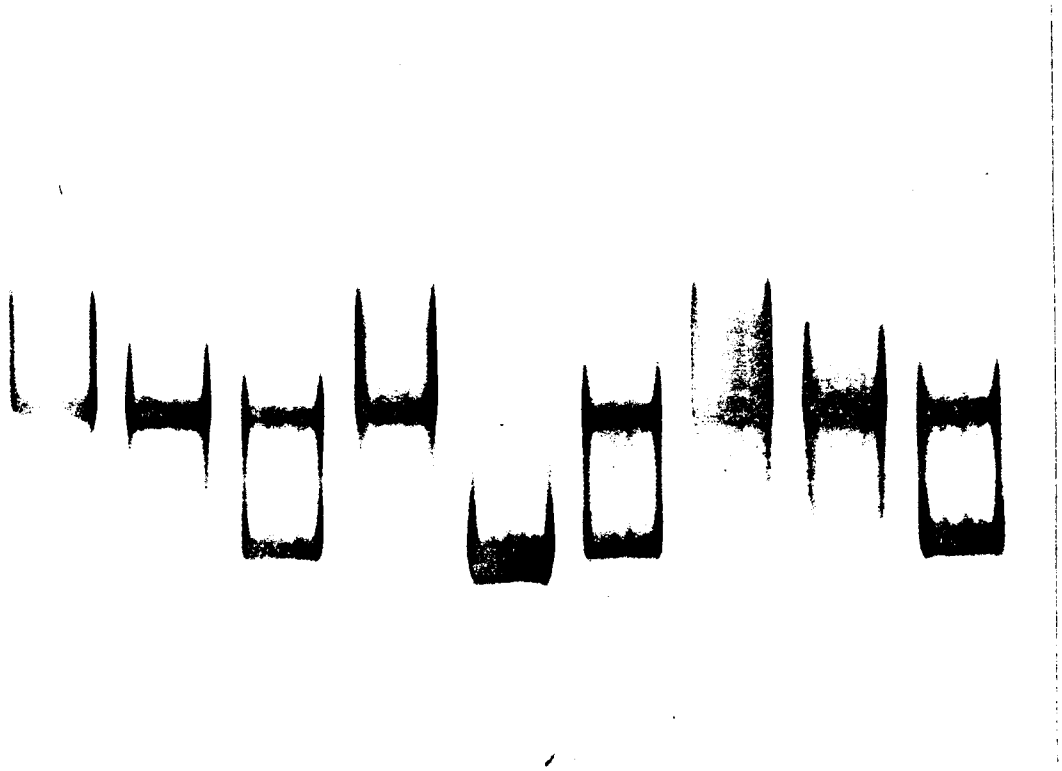

Figure 12

Nucleosome-HMG 14 complexes formed by dialysis or mixing. Left to right: 3, 2, 1 moles HMG 14 per mole nucleosomes mixed in 0.35 M NaCl and dialyzed to 10 mM Tris pH 7; 3, 0, 1 moles HMG 14 per mole nucleosomes mixed in 0.1 mM NaCl and dialyzed to 10 mM Tris pH 7; 3, 2, 1 moles HMG 14 per mole nucleosomes mixed directly in 10 mM Tris pH 7. Gel contains 1 × TBE. Tris was titrated with HCl for pH 7.

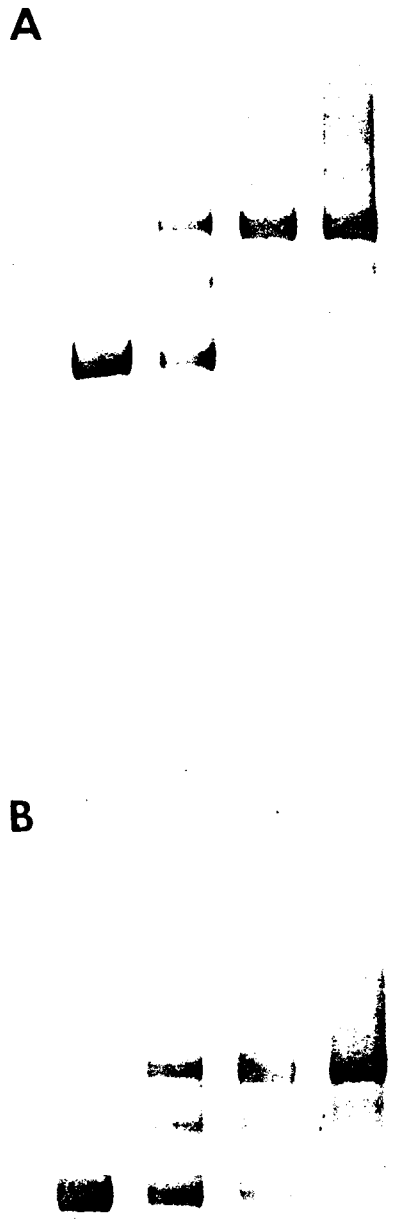

Figure 13

Electrophoresis of nucleosome-HMG 14 complexes in various concentrations of TBE buffer. A: 0.1 × TBE; B: 0.15 × TBE. All samples, left to right, 0, 1, 2, 3 moles HMG 14 per mole nucleosomes.

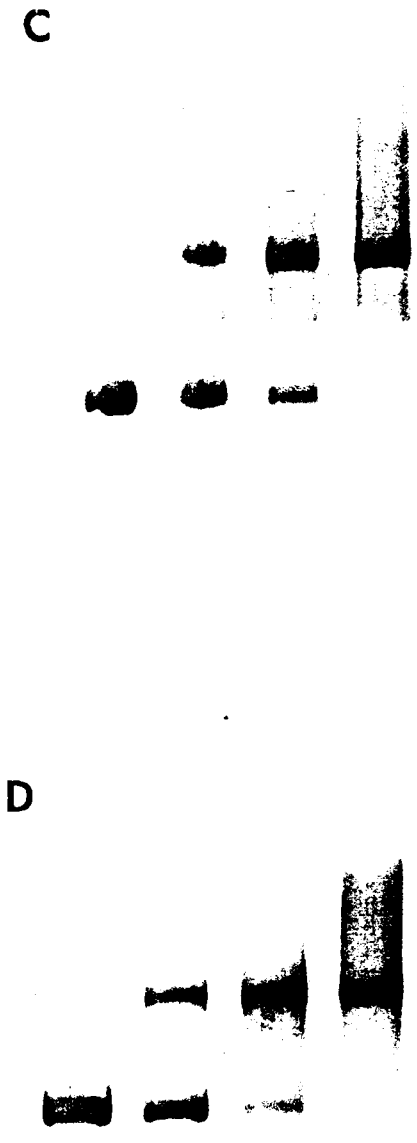

Figure 13 (continued)

C: 0.2 × TBE; D: 0.25 × TBE.

gels in 0.1 ×, 0.15 ×, 0.2 × and 0.25 × TBE clearly show a sharp transition from the non-cooperative HMG binding pattern to the cooperative binding pattern between 0.2 × and 0.25 × TBE. The concentration of 0.2 × TBE is 18 mM TRIS, 18 mM boric acid, and 0.5 mM EDTA pH 8.3. This is much higher than the concentration of 1.2 mM found at the midpoint of the nucleosome structural transition. However, preliminary sedimentation runs in the analytical ultracentrifuge showed sedimentation coefficients that were consistent with a more open nucleosome structure in 0.1 × TBE than found at 0.3 × TBE. Further, conductivity readings taken on various dilutions of TBE buffer showed unusually low values compared to a KCl standard (Table V). These results suggest an effective ionic strength of TBE buffer, far below the expected value, that is inducing a conformational change in the nucleosome core particle.

TABLE V

CONDUCTIVITY TABLE

KCl Standards		TBE Buffer		Equivalent
Molarity KCl	mMHO	Dilution	mMHO	Molarity KCl
1 mM	0.123	0.1 ×	0.141	1.2 mM
5 mM	0.620	0.15 ×	0.202	1.7 mM
10 mM	1.11	0.25 ×	0.320	2.6 mM
25 mM	2.85	0.5 ×	0.550	4.5 mM
50 mM	5.60	1 ×	0.98	8.7 mM
100 mM	11.2	5 ×	3.07	27 mM
Water Blank	0.08- 0.085			

To show this conformational event was ionic, 0.1 × TBE particle gels were made up containing 5 mM NaCl. Nucleosomes were titrated with HMG 14 and run on the gel. The pattern is shown in Figure 14. The absence of the central band in the presence of 5 mM additional salt strongly suggests the change from non-cooperative to cooperative binding is ionically induced. Sandeen et al. (119), have also observed the shift in banding pattern on gels run in high and low concentrations of TBE buffer.

Discussion

The results clearly show that HMG 14 and 17 form discrete complexes with nucleosome core particles. The number of specific sites for HMG binding appears to be two per nucleosome. The binding occurs equally well on nucleosomes reconstructed from synthetic poly dA·dT and purified chicken inner histones. The binding of HMG 14 and 17 to nucleosome core particles occurs cooperatively at ionic strengths where the nucleosome is known to be in its compact, native form, and non-cooperatively at very low ionic strength where the nucleosome is more open or swollen. The binding of HMG 1 and 2 occurs non-specifically and does not form discrete complexes with nucleosome cores.

The fact that HMG 14 and 17 bind to nucleosomes is not extraordinary since they are isolated by elution from chromatin. In addition, a region of the HMG 17 sequence is known, from NMR results, to bind to DNA (89). What is surprising is the indication of specific binding sites for two HMG proteins on each nucleosome, and the high level of cooperativity displayed by binding at moderate ionic strengths. Work

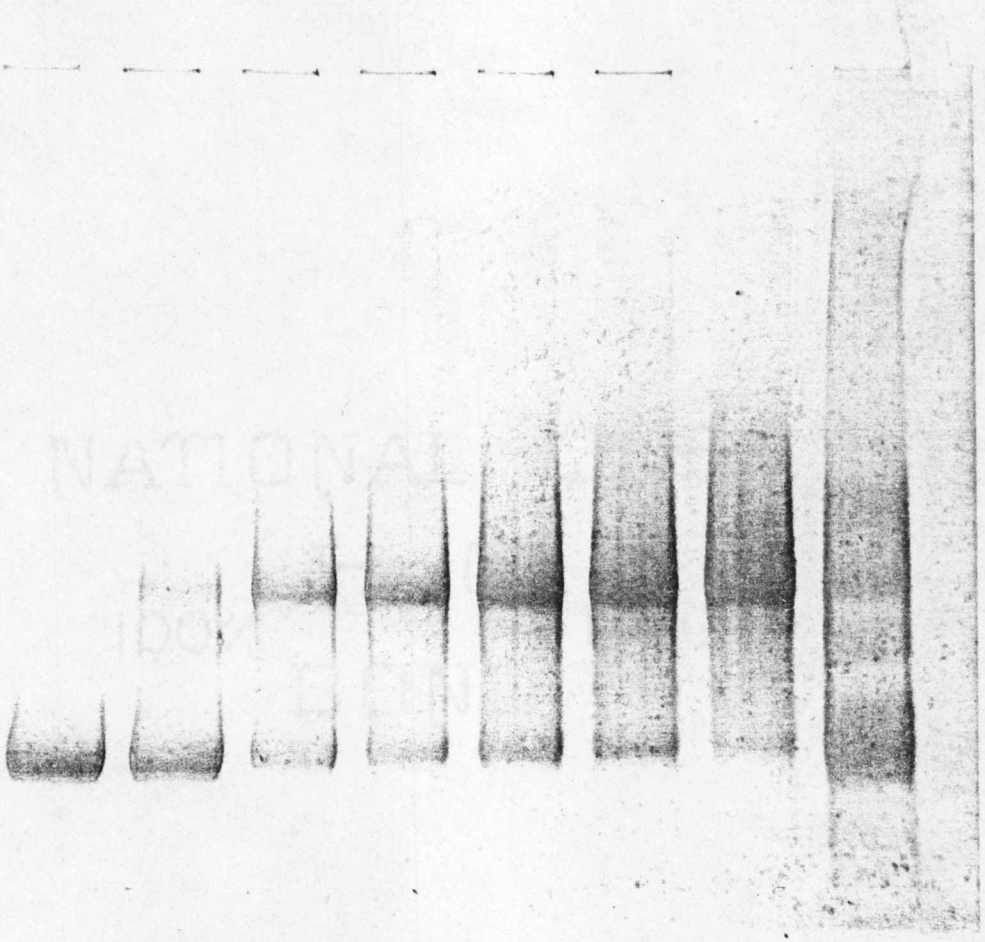

Figure 14

Electrophoresis of nucleosome-HMG 14 complexes in 0.1 × TBE plus 5 mM NaCl. Left to right 0, 1, 1.25, 1.5, 1.75, 2.0, 2.25 moles HMG 14 per mole nucleosomes.

from several labs (112-114) suggests that HMG 14 and 17 are associated with active chromatin, and work from Weintraub's laboratory suggests that HMG 14 and 17 preferentially associate with active regions upon reconstitution (114). In addition, the reported stoichiometry of HMG to nuclear protein (~3%) suggests that not all nucleosomes can have HMG bound. The simplest explanation of this paradox is that HMG binding sites exist on all nucleosomes as an inherent structural property of the nucleosome. The preference of HMG binding to active regions may be due to a variety of additional features attributed to active chromatin; notably, acetylation of histones, undermethylation of DNA, and a more open or accessible chromatin structure.

Of most immediate interest is the large mobility shift seen when HMG proteins bind to nucleosomes, and the high degree of cooperativity in the binding. Using molecular weights obtained from sequence data for chicken HMG 14 and 17, the binding of two HMG proteins to a chicken nucleosome core particle adds about 10% additional mass to the nucleosome. This small amount of additional mass is insufficient to account for the observed mobility shift, which corresponds to an incremental mass increase of 30–50%. This assumes, of course, that the HMG adds a spherical shell of protein to the nucleosome. Another possible cause is a change in shape. However, conformational changes would require large axial ratios to account for the observed mobility change. This is not supported by either the sedimentation results or electron microscopy on the complexes (Figure 15). The remaining possibilities include charge effects due to the binding of highly charged HMG proteins to the highly charged nucleosome core particle. However, the HMG proteins have both positively and negatively charged

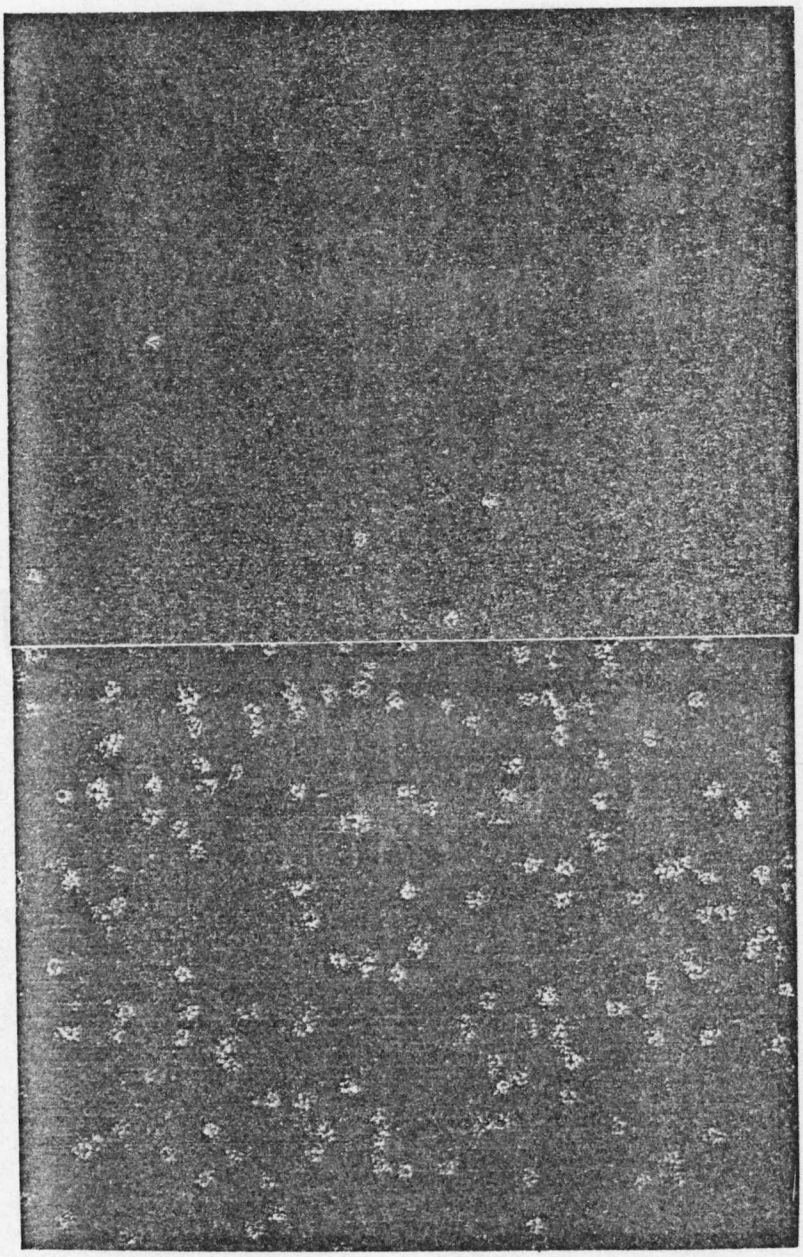

Figure 15

Dark field electron micrographs of nucleosomes in TBE buffers.
Top: 0.1 × TBE, Bottom: 0.3 × TBE.

residues, in nearly equal proportion. Neutralization of HMG positive charges by the abundance of negative DNA phosphate residues would leave additional HMG negative charges to balance those lost from DNA. In addition, the number of DNA phosphates that remain free to interact ionically is very large, compared to the number of those that might be neutralized by HMG amino groups. Thus, none of the likely changes, alone, can account for the observed mobility shift. The resulting picture is that the mobility shift of nucleosomes, induced by the binding of HMG proteins, must include contributions from added mass plus conformation changes plus charge alterations.

The cooperativity of HMG binding to nucleosomes implies a conformational change in the particle. In the results described, cooperative binding initially requires the compact form of the nucleosome. Once that conformational state is conferred on the nucleosome, cooperative binding of two HMG proteins can occur. This suggests the cooperative binding site for HMG proteins requires specific juxtaposition of the DNA and histone. Simple binding of HMG to DNA or histones would occur with equal, or even enhanced, facility in swollen or unfolded nucleosomes.

An important point here is that HMG 14 and 17 have a random coil structure in solution and may bind to the nucleosome in extended form. This is due to the enrichment for helix-destabilizing proline and glycine residues in their sequences. Thus, the cooperative binding mode may occur with HMG proteins extended up to twice the diameter of the nucleosome. Biophysical characterizations of the complex and recent neutron scattering results by Uberbacher et al. (136) are consistent with the HMG proteins folding around the nucleosome and altering the shape very little upon binding. These models and ideas will be covered

in greater detail in Chapter 3. The structure of the nucleosome provides several possible ways for the binding of a single HMG protein to alter the second HMG binding site for maximum cooperativity. Two favorable examples would be the potential surface charge neutralizations upon binding of the first HMG; or structural displacement of the DNA and histone contacts favoring the binding of a second HMG. Clearly the available evidence supports several kinds of models and further work is necessary to narrow the possibilities.

CHAPTER 3

HMG BINDING SITE MAPPING

Introduction

The HMG proteins are nuclear non-histone proteins eluted from nuclei with 0.35 M salt (86). HMG 14 and 17 are reported to be associated with active regions of chromatin, and are thought to confer DNase I sensitivity on active chromatin regions (112-115). In the previous chapter, it was shown that HMG 14 and 17 bound specifically and cooperatively to nucleosome core particles. The indications from staining of electrophoretic gels were that nucleosome cores specifically bound 2 HMG 14 or 17 proteins per nucleosome. It was decided to map the position and number of bound HMG proteins to confirm this stoichiometry.

Nucleosome core particles reconstructed from synthetic DNA and purified chicken inner histones meet all the criteria of nucleosome core particles and mimic their behavior in a variety of biochemical and biophysical studies (34, 35, 137, 138). They have the particular advantage of providing high resolution DNA analysis on electrophoretic gels. Using reconstructed nucleosome cores, binding HMG 14 or 17, and digesting with DNAse I, the binding sites on the nucleosomal DNA have been determined.

Methods

Reconstructed nucleosome core particles were prepared as described in Chapter 2. Briefly, purified chicken inner histone octamers were mixed with synthetic poly dA·dT (P.L. Biochemicals) in 2 M salt and

buffer. The ratio of inner histone to DNA was 0.68 mg histone per milligram of DNA. The mixture was step dialyzed in decreasing salt to 10 mM Tris-HCl pH 7.0. The reconstructed chromatin was digested with micrococcal nuclease to produce monomer nucleosomes, and nucleosome core particles were purified on sucrose gradients.

HMG 14 and 17 were purified as described in Chapter 2. Briefly, HMG proteins were extracted from chicken erythrocyte nuclei in 0.35 M NaCl and selectively precipitated with TCA. The crude HMG 14 and 17 was separated into purified HMG 14 or 17 by chromatography on CM-Sephadex.

End Labelling

Nucleosome core particles with or without bound HMG 14 or 17 were labeled at the 5' ends of the DNA using γ-^{32}P-ATP (New England Nuclear) and T4 Polynucleotide Kinase (Miles) (34). The labelling reaction contained 0.32 nmol nucleosome core particles, 0.2 nMol γ-^{32}P-ATP, and 20 units/ml polynucleotide kinase, in 50 mM Tris-HCl, 5 mM $MgCl_2$, 4 mM dithiothreitol, pH 7.0. The reaction ran for 1 hour at 37°C and was chilled on ice until digestion with DNase I.

DNAse I Digestion

Digestion of the samples with DNase I immediately followed labelling since DNase I requires magnesium. The sample mixture containing the kinase reaction on ice was warmed to 20°C and pancreatic DNase I (Worthington) was added to a final concentration of 1 unit/ml. A less concentrated stock of DNase I was made by carefully diluting the concentrated stock to 100 units/ml in reaction buffer. Care was taken in pipetting, and mixing, as DNase I is sensitive to surface denaturation. The DNase I digestion was incubated at 20°C for the times indicated in

the figures, then EDTA was added to a final concentration of 5 mM (pH 7.0) to stop the reaction, and the samples were cooled on ice until prepared for electrophoresis.

Electrophoresis

Samples of poly dA·dT nucleosomes were deproteinized for electrophoresis by heating to 100°C for 1 minute, followed by digestion with Proteinase K (Merck), 10 μg/ml, for 1 hour at 37°C. Electrophoresis was done on 18 cm × 43 cm × 0.75 mm polyacrylamide gels (34). Gel solution contained 20 ml of 30% acrylamide and 1% bisacrylamide, 21g of ultrapure urea (Schwarz-Mann), 5 ml of 10 × TBE, water to bring volume to 50 ml, 0.25 ml 10% ammonium persulfate in water, and 25–40 μl of TEMED. This produced a 12% acrylamide gel containing 7 M urea.

After pouring, the plates were wrapped in plastic film to prevent dehydration and the gel was allowed to cure overnight. After curing, the gels were pre-electrophoresed at 500 volts for 2 hours or more until current was reduced to less than 10 ma. Samples containing the deproteinized DNA were mixed 1:1 with sample loading solution containing 10 M urea with bromophenol blue and xylene cyanol dyes as markers. Maximum volumes of 20 μl per well were loaded on the gel. Electrophoresis was carried out for 4 hours at 1000 volts. Electrode buffer was 1 × TBE. High resolution electrophoresis of native chicken DNA was carried out on gels slightly different from those described for poly dA·dT. The gels are modifications of the method of Lutter (139), whose formulations failed to polymerize in our laboratory. The gels were identical to those used for poly dA·dT except that the ratio of acrylamide to bisacrylamide was increased from 30:1 to 6:1. In

addition, the higher amount of bis required additional ammonium persulfate, 300 µl for 50 ml of gel. The resulting gel contained 12% acrylamide 2% bis, and produced resolution on chicken DNA identical to that obtained with poly dA·dT on conventional, low bis gels.

Autoradiography

After electrophoresis the gel containing labelled DNA was allowed to remain on one of the glass plates for support. The gel was covered with a single layer of plastic film and all air bubbles were rolled out with a 25 ml pipet. The wrapped gel and glass plate were transferred to a darkroom and half sheets of Kodak XR-5 film were placed over the gel and weighted with additional glass plates. The stack of plates was placed into a light-tight acrylic box (Woodchucks Inc.) and allowed to expose at -20°C for 12 hours. Thinner gels (0.4 mm), required less time to expose, typically 1–4 hours, depending on the extent of labelling. Double sheets of film could also be placed on the gel with some loss of resolution on the top layer of film.

Results

Samples of reconstructed nucleosomes were mixed with HMG 14 at a ratio of 2.63 HMG 14 molecules per nucleosome. Reconstructed nucleosome core particles with and without bound HMG 14 were labelled at the 5' end of the DNA with γ-^{32}P-ATP and digested with DNAse I for the times indicated. Figure 16 shows the results of electrophoresis on a denaturing DNA gel and detection of the bands by direct autoradiography. The radiogram displays labelled DNA fragments corresponding to every possible length generated by the DNAse I digestion. Resolution is one

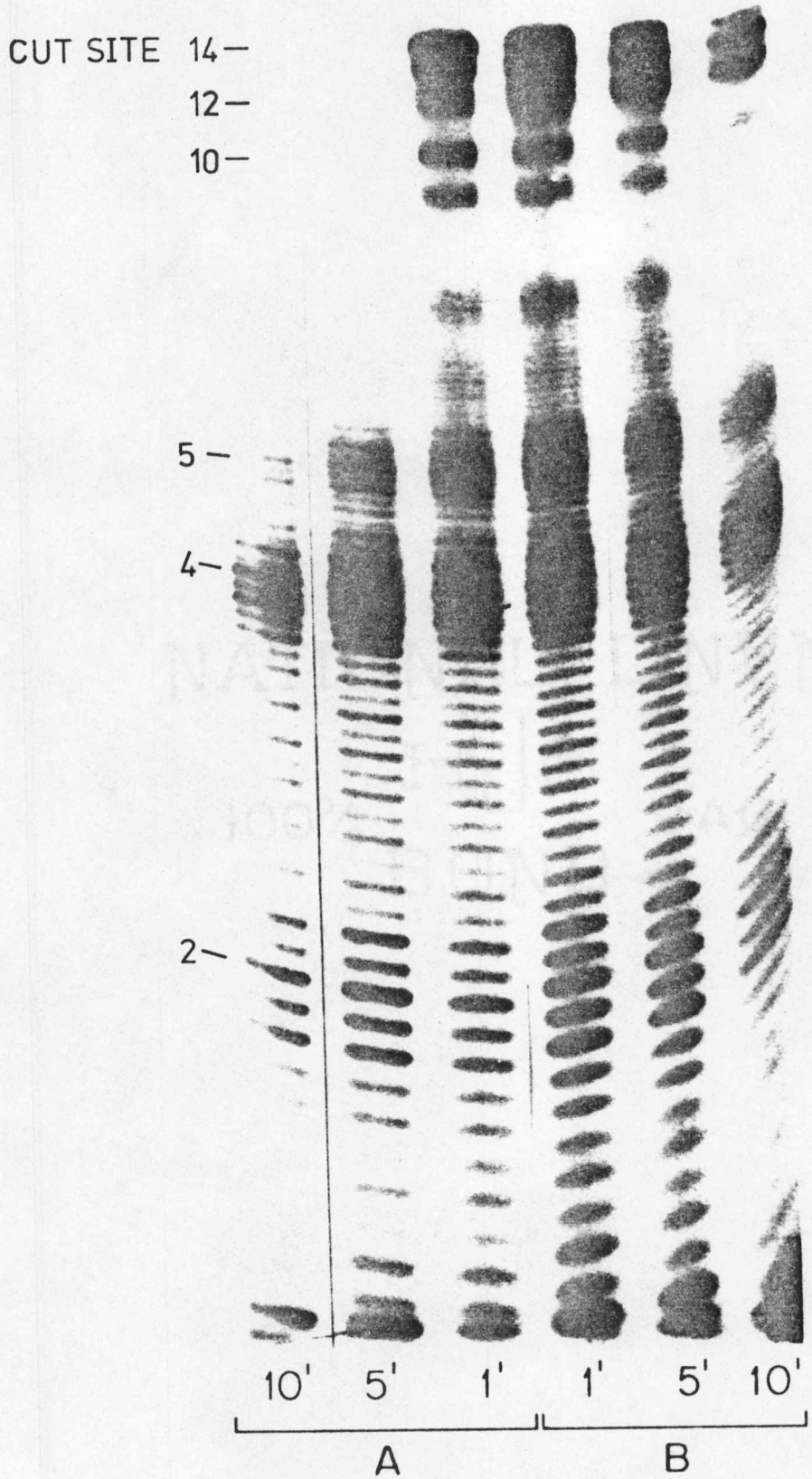

Figure 16

Autoradiogram of nucleosomal DNA from DNAse I digested nucleosomes. A: nucleosomes, B: nucleosomes + HMG 14. Digestion times are shown.

nucleotide. Inspection of the intensity of individual bands in corresponding lanes, for nucleosomes with and without HMG proteins, shows intensity differences. The band at cutting site 12 is much less intense for nucleosomes with bound HMG 14. The same is true at cutting site 2. Band intensities of cutting sites 4, 5 and 14 are more intense for nucleosomes with HMG 14 bound.

In an end labelling experiment however, the frequency of cutting is not directly proportional to band intensity because some DNA strand lengths are lost from the analysis by second DNAse I cuts, closer to the 5' end, that effectively remove the label. The true cutting frequency at each site can be determined by dividing the band density at each site by its own band density plus the densities of all bands farther from the 5' end (32). This correction assumes that cutting at any given site is independent of cutting at any other site. Figure 17a shows a scan of the gel in Figure 16, normalized to represent equal amounts of radioactivity loaded in each lane. Figure 17b shows a diagram of the corrected cutting frequencies for the results shown in Figure 16. The correction agrees qualitatively with the scan on the gel but also reveals a reduced cutting frequency at site 13. The results show that the presence of bound HMG 14 on the nucleosome inhibits the digestion of nucleosomal DNA, by DNase I, at sites 1, 2, 12 and 13. The binding of HMG 14 also appears to enhance the DNAse I cutting at sites 4, 5, 9 and 10.

Similar results were obtained with HMG 17 bound to nucleosomes. Figure 18 shows the results of a DNAse I digestion experiment comparing nucleosomes with bound HMG 14 or 17. The results show the nucleosomes with bound HMG are more resistant to digestion with DNAse I than

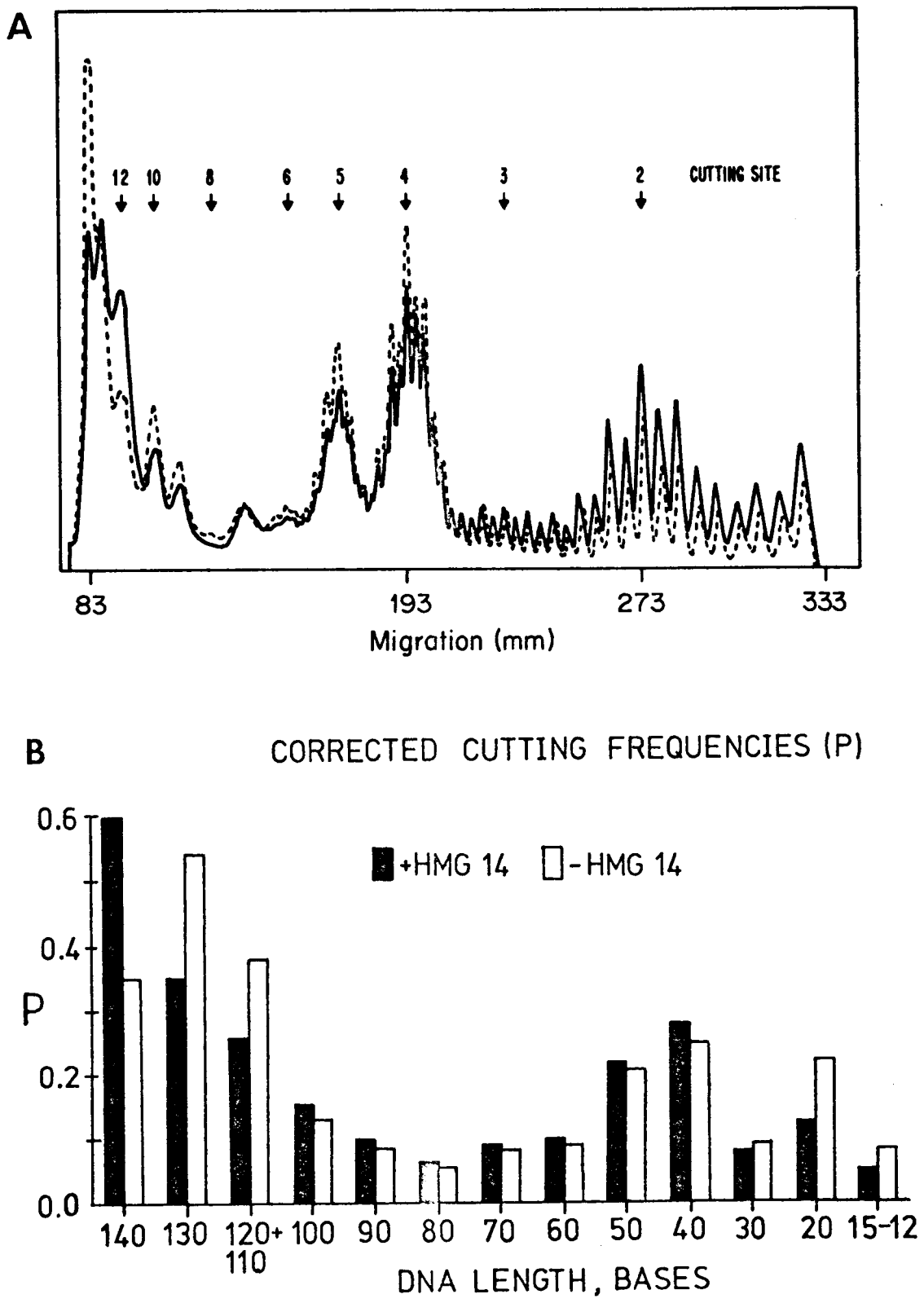

Figure 17

Diagrams of DNAse I cutting frequencies. A; scan of autoradiogram in Figure 16. ---- +HMG 14, —— -HMG 14. B; corrected cutting frequencies for selected sites.

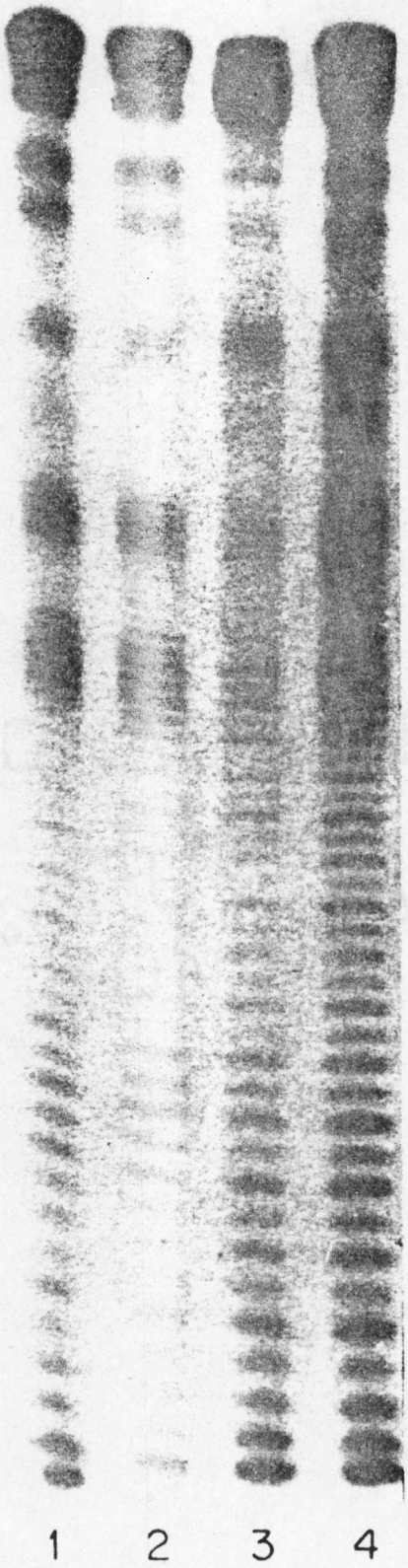

Figure 18

Autoradiogram of DNAse I digested nucleosome-HMG complexes. 1, nucleosomes with bound HMG 14; 2, nucleosomes; 3, nucleosomes + HMG 17; 4, nucleosomes + 1:1 mix of HMG14 and 17.

nucleosomes without HMG. In addition, the protection against digestion afforded by HMG 17 is very similar to, if not greater than, HMG 14.

Discussion

The results show that nucleosomes containing 2 bound HMG proteins, HMG 14 or 17, are protected against DNase I digestion between two specific points along the DNA. The simplest interpretation is that one HMG protein is bound to the DNA at each site, restricting access of the DNAse I to the DNA. In addition to the protection at two sites, bound HMG appears to enhance the cutting frequency at other sites. This could be explained by the release of DNA from protection by histone proteins upon binding of the HMG.

The protection against DNAse I, at two places along the DNA, is more easily visualized when the protection sites are mapped onto a superhelical coil of DNA, such as that modelled by Finch et al. (37). Figure 19 shows a superhelical coil of DNA with the sites of protection against, and enhancement of, DNAse I digestion mapped onto the DNA. One can see immediately that the sites of protection and enhancement lie adjacent to each other on different turns of the supercoil. The analysis of the digestion results also clearly shows the effect of bound HMG 14 or 17 is limited to specific sites along the DNA. Since the DNA was in fact an alternating A-T copolymer, the specific binding of HMG 14 or 17 must also involve other components of the nucleosome surface, such as proximity to certain histones or features of general nucleosome conformation. The binding is not DNA sequence specific in these experiments.

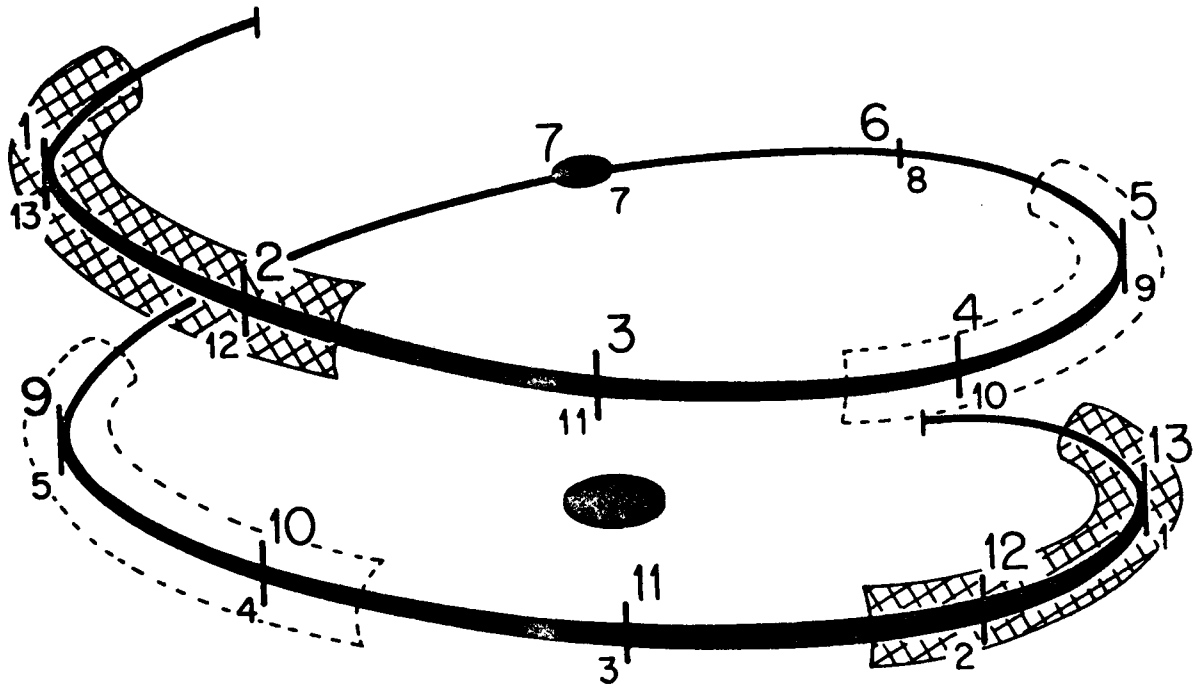

Figure 19

Mapping of altered DNAse I accessibility onto nucleosomal DNA. Cross-hatching represents protection, dashed lines represent enhancement.

However, the analysis of cutting site protection is not sensitive to very small differences in binding position, on the order of 1-5 base pairs, that may be due to DNA sequence.

Inspection of Figure 19 shows sites of protection and enhancement at two regions, on opposite sides of the nucleosome. These two regions are related symmetrically. This implies that the binding of HMG 14 or 17 may be symmetric with respect to the nucleosome, a constraint imposed as a result of nucleosome structure.

Since it is known that HMG 14 and 17 have an extended conformation in solution, it is likely that they bind to the nucleosome in their extended form rather than forming a globular structure. In the case of extended binding, each HMG could wrap around the nucleosome as much as one full turn. This suggests an interesting illustration of the binding mode using Figure 19. An extended HMG protein might bind to the nucleosome between sites 1 and 2 with its known N-terminal DNA binding region. The carboxy-terminal end of the HMG protein would extend around the nucleosome binding to sites 9 and 10. The path of the HMG protein would stay on the upper side of the supercoil as viewed in the figure. The second HMG protein to bind would begin by binding to the second site in the same way, but would wrap around the nucleosome, next to the lower side of the supercoil, as viewed in the figure. In this way, two HMG proteins could be accommodated on the particle without the necessity of two HMG strands occupying the space between supercoils, which is known to be small.

The two sites found by DNase I digestion analysis agree with the finding of two sites by analysis on particle gels as presented in

Chapter 2. This combined evidence argues persuasively for two specific HMG binding sites on the nucleosome core particle.

Evidence from other workers indicates that the ends of the DNA on the nucleosome core particle exist in an altered helical conformation relative to the DNA in the central region of the supercoil (34, 140). It is possible that the binding sites for HMG 14 and 17 depend on this structural feature. However, it has not been shown if this altered twist of the DNA helix exists on nucleosomes contained within long oligonucleosome structures.

It has been reported that DNA sequences, from active chromatin, are as much as two orders of magnitude more sensitive to DNAse I digestion than non-active regions (141). This sensitivity has been reported to be conferred by HMG 14 and 17 (112). It is clear from the analysis of results presented here, that bound HMG 14 and 17 do not confer an increased DNAse I sensitivity upon reconstructed nucleosomes. Other workers have failed to find DNAse I sensitivity in monomer nucleosomes isolated from actively transcribing chromatin (142). This point remains unresolved and requires further investigation.

The presence of 2 HMG binding sites on the nucleosome core particle is supported by results from at least two other laboratories. Sandeen et al. (119), have reported that nucleosomes will specifically bind up to 2 HMG 17 molecules. In addition, their paper shows DNAse I protection at three specific sites along the DNA in chicken nucleosomes. They also reported nucleosomes titrated with only 1 HMG per nucleosome produce a particle gel band corresponding in mobility to 2 HMG per nucleosome, that is enriched in active DNA sequences (119). Albright et al. (143), have reported the protein makeup of different chromatin fragments.

Their reconstitutions of HMG 17 onto a mixture of mononucleosomes and oligonucleosome support the model of two specific HMG binding sites on nucleosome cores. Uberbacher et al. (136) have done neutron scattering experiments on nucleosome core particles titrated with 2 HMG 14 molecules per nucleosome. Their results show only very small changes in the radius of gyration for the DNA and virtually no change in the protein radius of gyration. This agrees well with the model proposed previously in which the binding of HMG was thought to displace the DNA in selected regions, resulting in the enhanced digestion by DNAse I. The neutron scattering results further constrain the model for HMG binding and suggest that HMG proteins bind very close to the surface of the histone octamer or within the surface of the histone octamer rather than to the outside of the DNA supercoil.

The specific binding of 2 HMG 14 or 17 molecules, to specific sites on the nucleosome core particle, strongly argues for a legitimate role for HMG proteins in the structure of chromatin. Their association with actively transcribing chromatin argues for a role in the function of chromatin as well.

CHAPTER 4

BIOPHYSICAL CHARACTERIZATION OF THE NUCLEOSOME-HMG COMPLEX

Introduction

Several studies have established the existence of discrete HMG-nucleosome complexes (118, 119, 143). The results presented in Chapters 2 and 3 explored the binding of HMG 14 and 17 as a function of ionic strength. In addition, the two binding sites for HMG 14 and 17 were mapped onto the nucleosomal DNA. The next direction taken was to study the nucleosome, before and after HMG proteins were bound, to determine what effects the binding had on the structure of the nucleosome. It was clear from the particle gel results that a conformational change and surface charge effects were both possible. More sensitive techniques were needed to look at the components of the nucleosome separately and to give information on more subtle binding effects not readily discerned by electrophoretic techniques. For these experiments, thermal denaturation and circular dichroism were chosen. Thermal denaturation is a sensitive measure of the stability of the DNA helix. Circular dichroism is a sensitive measure of the conformation of both the DNA, and protein, in a sample. Sedimentation velocity was also chosen as a check on the results of the particle gels. Sedimentation is sensitive to shape and with proper solvent conditions can provide information on charge and mass.

Throughout the study, the working hypothesis was that HMG 14 and 17, as components of actively transcribing chromatin, would contribute to the structural alterations of chromatin necessary for RNA polymerase

to transcribe the DNA. The hypothesis included the idea that DNA-histone contacts would be weakened before passage of the polymerase, to allow uncoiling of the DNA superhelix and dissociation of the histone octamer. This hypothesis was based on several existing lines of evidence: First, electron micrographs of transcribing chromatin spread by the "Miller technique" failed to show typical beaded structure of the chromatin being transcribed at high rates (117, 144-146). They also revealed low packing ratios for the DNA, suggesting an extended conformation (117, 144-146). Second, DNAse I sensitivity of active regions (141, 142) suggested a more accessible structure for the chromatin. Third, in vitro transcription experiments showed chromatin was transcribed more slowly and the transcription products were much smaller than for DNA alone (147-149). These lines of evidence suggested a destabilization mechanism was required that would provide less of a barrier to passage of the polymerase. HMG 14 and 17 were likely candidates for destabilizing proteins because of their association with active chromatin, and their highly charged amino acid composition.

The initial thermal denaturation studies on HMG-nucleosome complexes revealed a stabilization of the nucleosome when HMG 14 or 17 was bound. This result was unexpected, but subsequent studies in a wide variety of solvent compositions gave the same results.

Circular dichroism studies also showed stabilization of the nucleosomal DNA when HMG 14 or 17 was bound. The stabilization seen was in addition to that seen when DNA is associated with histones to form the nucleosome. The observed change in signal, suggests that the stabilization of DNA by HMG occurs by a mechanism similar to that found in histone-DNA interactions.

Sandeen et al. (119) reported a very small difference in sedimentation coefficient between nucleosomes with and without bound HMG 17. They attributed this difference to a slight increase in frictional coefficient balancing the small increase in mass when HMG 17 was bound.

My sedimentation experiments also show a very small difference between the sedimentation of nucleosomes with or without bound HMG.

Methods

Nucleosomes and HMG proteins were prepared as described in Chapter 2. Nucleosomal DNA was prepared from isolated nucleosomes by digestion with 10 μg/ml proteinase K (Merck) for each milligram of nucleosomes. The digestion was done at 37°C for one hour. The proteinase K was removed by 3 extractions using 10 mM Tris-HCl buffer saturated phenol mixed 1:1 with chloroform. The sample was extracted once with ether to remove residual phenol and chloroform. The ether was evaporated with a stream of dry nitrogen and the DNA was precipitated with 1/10 volume of 2M Na acetate and 2 volumes of cold absolute ethanol. The sample was frozen in liquid nitrogen to enhance precipitate formation, thawed, and the DNA pelleted at 15,000 x g for 30-45 minutes. The pellet was resuspended in 10 mM Tris pH 8.0 and used as a stock solution. DNA integrity and size distribution were monitored by denaturing gel electrophoresis as described in Chapter 2.

Thermal Denaturation

Thermal denaturation was done in a Gilford model 2000 equipped with thermal programmer, automatic baseline correction and manual offsets.

Data for each of 4 samples and temperature were collected and analyzed by computer. Analysis included calculation and plotting of hyperchromicity and the first derivative of the denaturation profile as a function of temperature. Samples were melted in 1 cm path, 2 mm window cuvettes. Sample volume was typically 300 µl. Samples were degassed under vacuum for 3-5 minutes. Sample heating was accomplished with the Gilford chamber heating blocks and a water/glycol bath controlled by the thermal programmer. Heating rate was approximately 1°C per minute. Absorbance was monitored at 260 nm.

Circular Dichroism

Circular dichroism was monitored with a JASCO J-40A spectropolarimeter. Standard round cells of 1 cm path containing a volume of 500 µl were used for some samples. Other samples of smaller volume were placed in a standard rectangular cuvette clamped to the sample cell holder. Throughout a given experiment, the cuvettes were left clamped to the removable cell holder to ensure proper realignment after sample changes. Data for each experiment were collected and analyzed by computer. Data collection resolution was 10 points per nanometer. All experiments were carried out at 20°C. Sample absorbance was checked immediately prior to taking CD spectra. Buffer blank spectra were collected for each sample, and subtracted during analysis.

Sedimentation

Sedimentation velocity runs were carried out in the Beckman Model E analytical ultracentrifuge. Samples were run in double sector cells in the AN-G rotor. Absorbance at 264 nm was monitored with the photo-

electric scanner system. Speeds and temperatures will be given in the individual figure legends in the Results section.

Results

Thermal Denaturation

Thermal denaturation of nucleosomes provides an increase in absorbance at 260 nm that is easily monitored spectroscopically. The absorbance change is sharp and occurs at a temperature much higher than for naked DNA of the same length and composition. Under some conditions, thermal denaturation of nucleosomes shows a second, earlier melting region. The early melting region can be produced by melting nucleosomes that have a DNA length greater than 146 base pairs, or by melting nucleosome core particles in buffers containing EDTA. Figure 20 shows the melting profile of nucleosomes with and without bound HMG 14 or 17 in EDTA. The first derivative plot is shown. This type of plot allows the calculation of melting temperature, and the resolution of small changes in slope, to be made more easily. Both HMG 14 and 17 cause two changes in the melting profile. First, the bound HMG suppresses the early melting peak. One HMG bound per nucleosome gives half the suppression of two HMG's bound. Second, the melting temperature of the main peak is increased by bound HMG.

To determine if the suppression of the early melting transition was a general phenomenon, nucleosome-HMG complexes were melted in other buffer systems. Figure 21 shows the melting profile of nucleosome HMG complexes in 0.1 × TBE. Figure 22 shows the melting profile of complexes in 0.3 × TBE. The suppression of the early melting region and

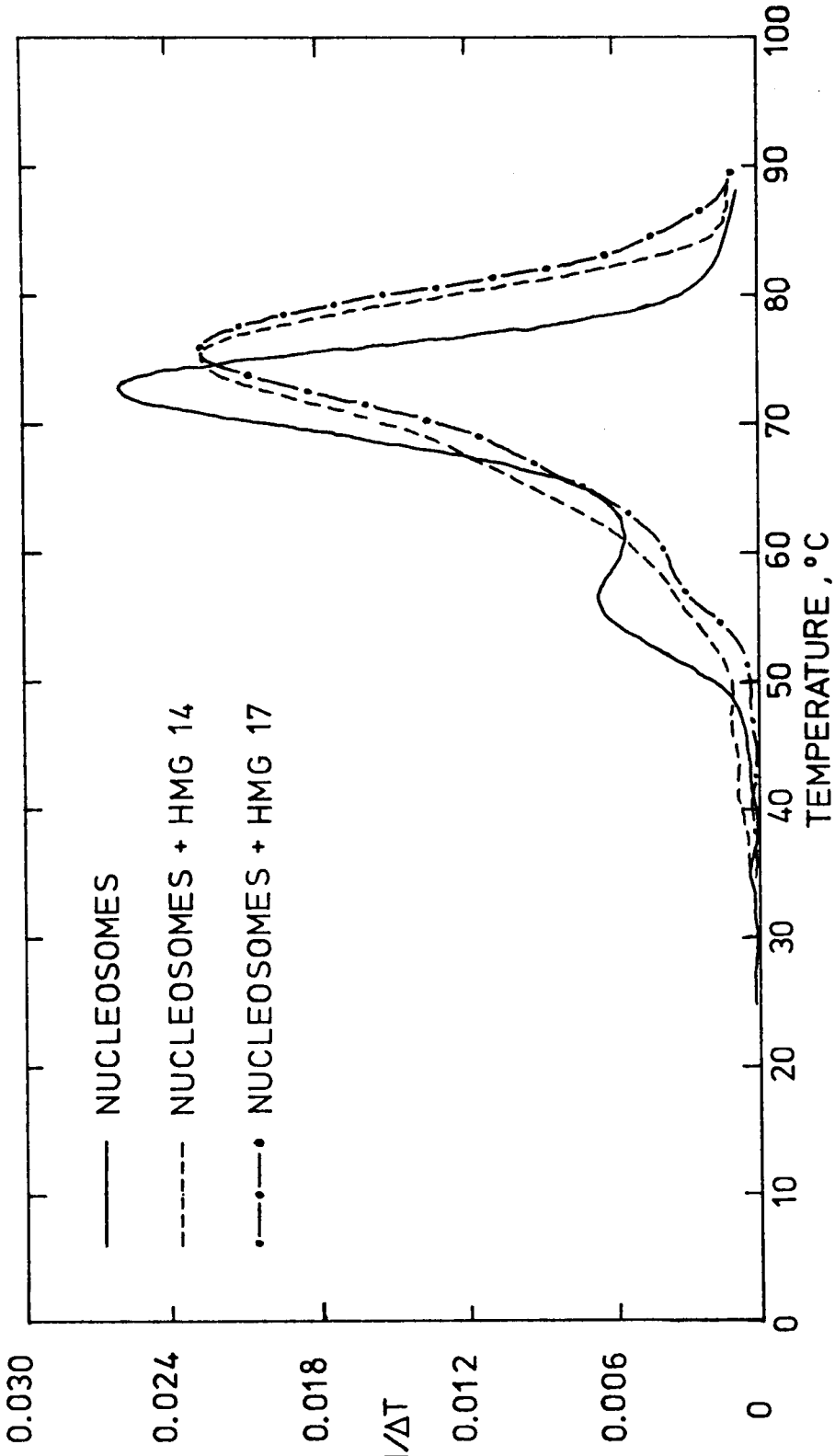

Figure 20

Thermal denaturation profiles of nucleosome-HMG complexes in 0.2 mM EDTA. Stoichiometry is 2 moles HMG protein per mole nucleosomes.

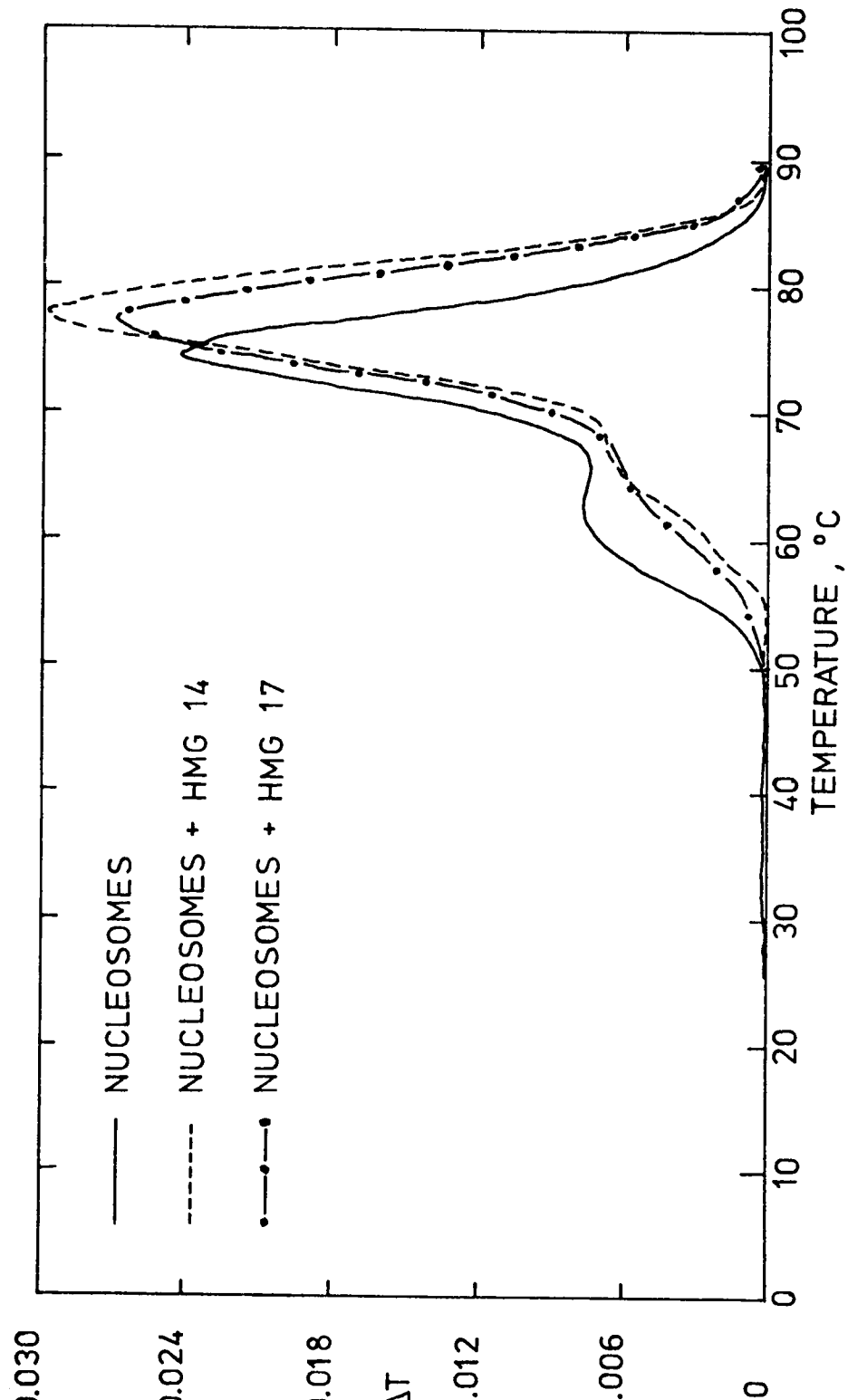

Figure 21

Thermal denaturation profiles of nucleosome-HMG complexes in 0.1 × TBE buffer. Stoichiometry is 2 moles HMG protein per mole nucleosomes.

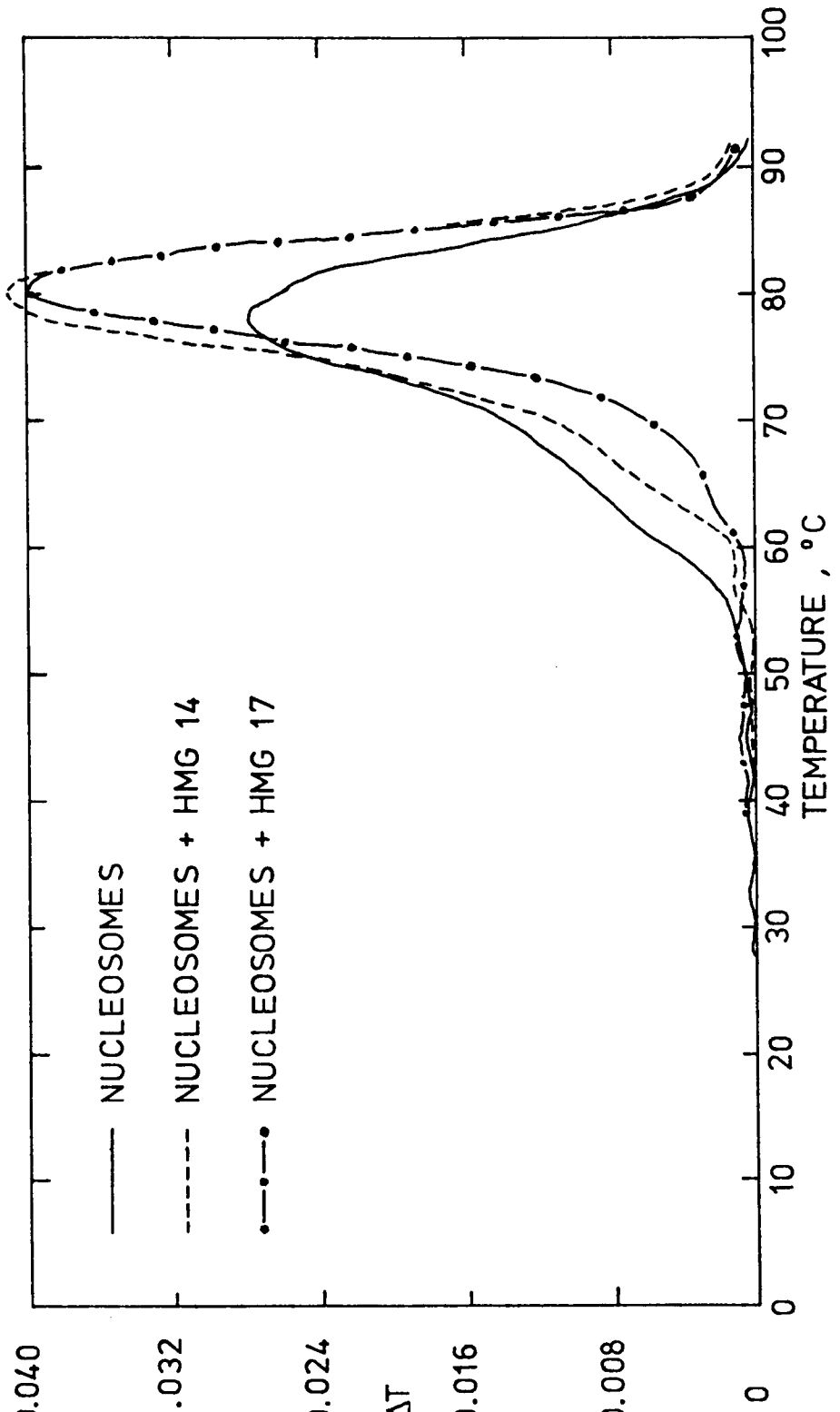

Figure 22

Thermal denaturation profiles of nucleosome-HMG complexes in 0.3 × TBE buffer. Stoichiometry is 2 moles HMG protein per mole nucleosomes.

a small increase in melting temperature of the main melting peak can be seen in both concentrations of buffer at pH 7. The higher ionic strength of 0.3 × TBE also stabilizes the nucleosome and partially masks the stabilization by bound HMG protein. However, the additional stabilization by HMG protein is readily observed. At this point, it was discovered that the early melting peak was only observed when nucleosomes were melted in buffers containing EDTA. Melting of nucleosomes in low concentrations of phosphate, cacodylate and acetate buffers showed only a single sharp peak corresponding to the main melting transition. As a result, TBE or EDTA buffers were used for the remaining experiments. This was done to exploit the suppression of the early melting region as a measure of the stabilizing effect of bound HMG protein.

Figure 23 shows the melting profiles of nucleosomes in low concentrations of phosphate or cacodylate buffer at pH 7. The concentrations used were identical to literature values of other investigators who observed early melting transitions for nucleosomes in these solvents (150-152). There are at least two possible reasons for the difference between my results and those of other authors. First is DNA length. The DNA in the nucleosome core particles used in my experiments was checked on long denaturing DNA gels against known 146 bp DNA markers. Although the other investigators checked their DNA lengths, there is some question about the accuracy of the measurements. DNA lengths greater than 146 base pairs could give early melting peaks due to the presence of these more easily denatured DNA tails.

A second possibility was the presence of contaminating metal ions in the solvent. The presence of an early melting peak, only in EDTA, suggested the chelation of metal ions that otherwise would stabilize the

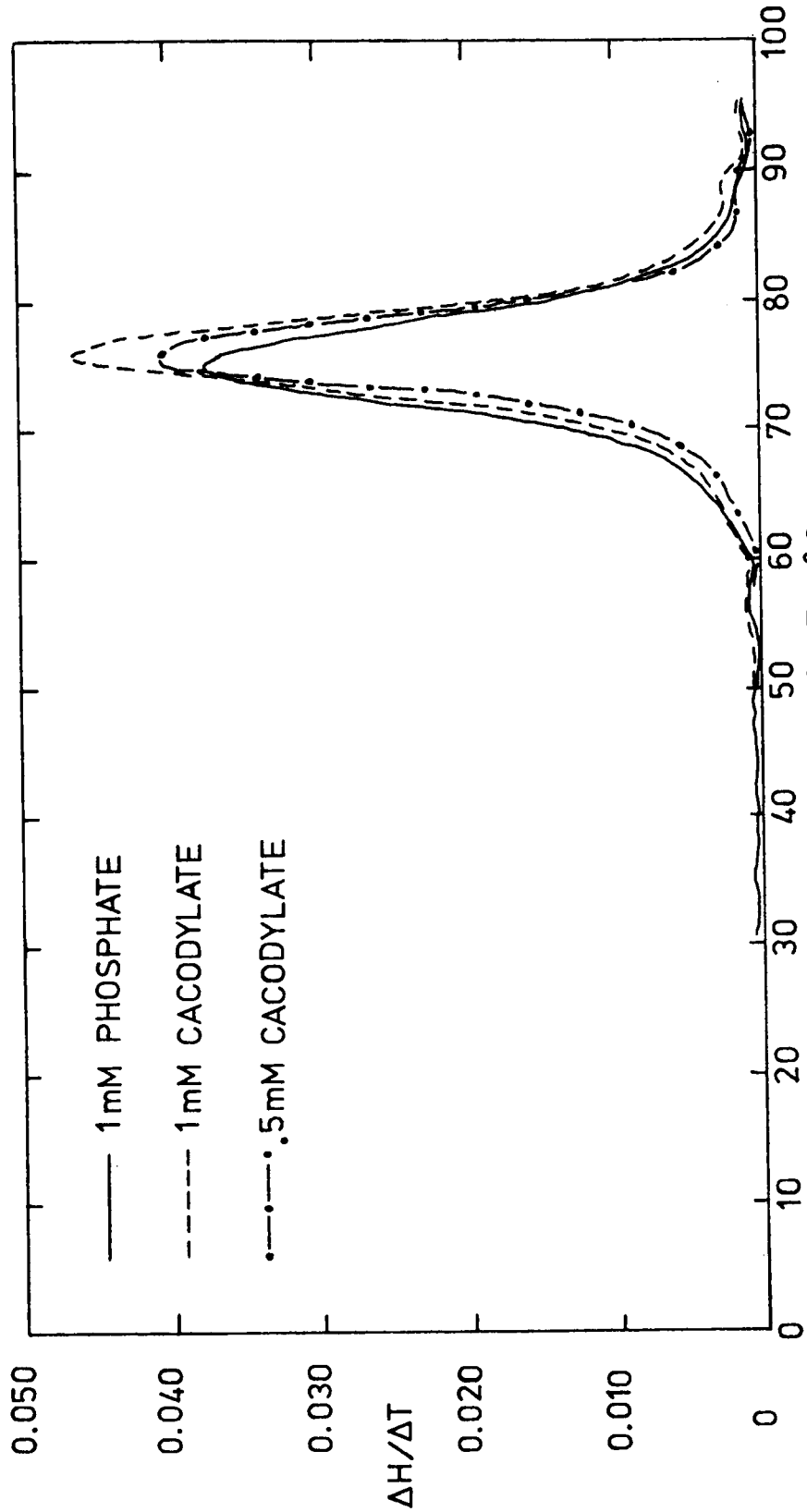

Figure 23

Thermal denaturation profiles of nucleosome in phosphate and cacodylate buffers.

nucleosomal DNA. All water used was from a glass distilled stock. The still feed line was deionized water. Conductivity of the glass distilled water was typically 0.8 μ MHO. Obviously, no gross contamination was involved. As a precaution, samples were obtained of each buffer; including the EDTA, glass distilled water, and glass distilled water rinses of tubes and containers used in the preparation of samples. These solutions were collected into acid washed vials and subjected to atomic absorption analysis for Mg^{++}, Ca^{++}, Fe^{++}, Cu^{++}. No contaminating ions, above the limits of detection, could be found in any of the samples. It was particularly disturbing to note that the molar concentrations of nucleosomes used in these melts was very low. A concentration of 100 μg/ml contains 4.85×10^{-10} moles of nucleosomes. This molar value is at or near the limits of detection for contaminating metal ions, by atomic absorption. It became clear that if the nucleosome could be stabilized by one or two atoms of a contaminating metal, it would be impossible to test for the presence of that contamination. Also, effects due to trace contaminants could not be ruled out. However, it is unlikely that the nucleosome could be stabilized by so few atoms of contaminating metal. Residual negative charges on the DNA phosphates are two orders of magnitude higher in concentration and would be expected to dilute binding of any contaminating metal at the ends of the DNA. A satisfactory explanation for the presence of early melting peaks, only in EDTA, remains to be found.

The results of a number of experiments in different buffers are collected in Table V. The general features include early melting peaks at temperatures 10° to 15°C below the main melting peak. In the presence of HMG protein, the early melting peak is suppressed. It melts

Table VI

THERMAL DENATURATION OF NUCLEOSOMES AND DNA

Sample	Buffer[1]	Melting Temperature	
		Early Peak	Main Peak
Nucleosomes	0.5 mM cacodylate pH 7.0	-	76°C
	1.0 mM cacodylate pH 7.0	-	76°C
	0.5 mM phosphate pH 7.0	-	77°C
	1.0 mM phosphate pH 7.0	-	75°C
	0.5 mM acetate pH 4.5	-	62°C
	0.2 mM EGTA pH 7.0	-	70°C
	0.2 mM EDTA pH 7.0	55°C	72°C
	0.1 × TBE pH 8.3	62°C	74°C
	0.3 × TBE pH 8.3	shoulder, 65°C	77°C
Nucleosomes + 1 HMG-14	0.2 mM EDTA pH 7.0	shoulder, 67°C	78°C
Nucleosomes + 1 HMG-17	0.2 mM EDTA pH 7.0	58°C	72°C
Nucleosomes + 2 HMG-14	0.2 mM EDTA pH 7.0	shoulder, 65°C	79°C
	0.1 × TBE pH 8.3	shoulder, 65°C	77°C
	0.3 × TBE pH 8.3	shoulder, 67°C	79°C
	0.5 mM acetate pH 4.5	-	70°C
Nucleosomes + 2 HMG-17	0.2 mM EDTA pH 7.0	shoulder, 58°C	74°C
	0.2 × TBE pH 8.3	shoulder, 64°C	76°C
	0.3 × TBE pH 8.3	-	80°C
	0.5 mM acetate pH 4.5	-	70°C
146 bp DNA	0.2 mM EDTA pH 7.0	-	39°C
	0.1 × TBE pH 8.3	-	51°C
	0.3 × TBE pH 8.3	-	58°C

[1]Buffer Compositions: Cacodylate: Sodium Cacodylate/Cacodylic Acid
Phosphate: Disodium Phosphate/Monosodium Phosphate
Acetate: Sodium Acetate/Acetic Acid
0.1 × TBE: 9 mM Tris Base, 9 mM Boric Acid, 0.25 mM EDTA

at a higher temperature and may appear as a shoulder of the main peak. In the presence of HMG protein, the main melting peak is shifted up in temperature.

Circular Dichroism

CD measurements were made on nucleosomes, with and without bound HMG protein, to detect conformational alterations in the nucleosome upon binding of HMGs. The region of the spectrum between 260 nm and 300 nm contains information on the conformation of the DNA helix. The region of the spectrum between 210 nm and 240 nm contains information on the conformation of proteins, specifically, the amount of α-helix and β-sheet structure. Figure 24 shows the CD spectra of nucleosomes with and without bound HMG 14 in 0.1 × TBE buffer. Spectra in 0.3 × TBE are shown in Figure 25. In both spectra, the DNA region is suppressed in the samples containing HMG 14. This suppression is similar to that seen when free DNA is complexed with histone proteins in solution (60, 153). The protein region shows little change in α-helical structure. The small change seen could be explained by the addition of more protein, as HMG, resulting in less total α-helix in the sample. HMG 14 has no α-helical structure and would tend to "dilute" the amount of α-helix in the sample. Clearly no large conformational changes are taking place. The DNA spectrum does not show release of DNA from histones, instead it shows further suppression of the DNA signal. The protein spectrum indicates no unfolding of the individual histones. The overall picture is one of increased stability of the nucleosome upon binding of HMG 14.

Comparing the spectrum in 0.1 × TBE with that in 0.3 × TBE, it is clear that the altered electrophoresis patterns at these ionic strengths

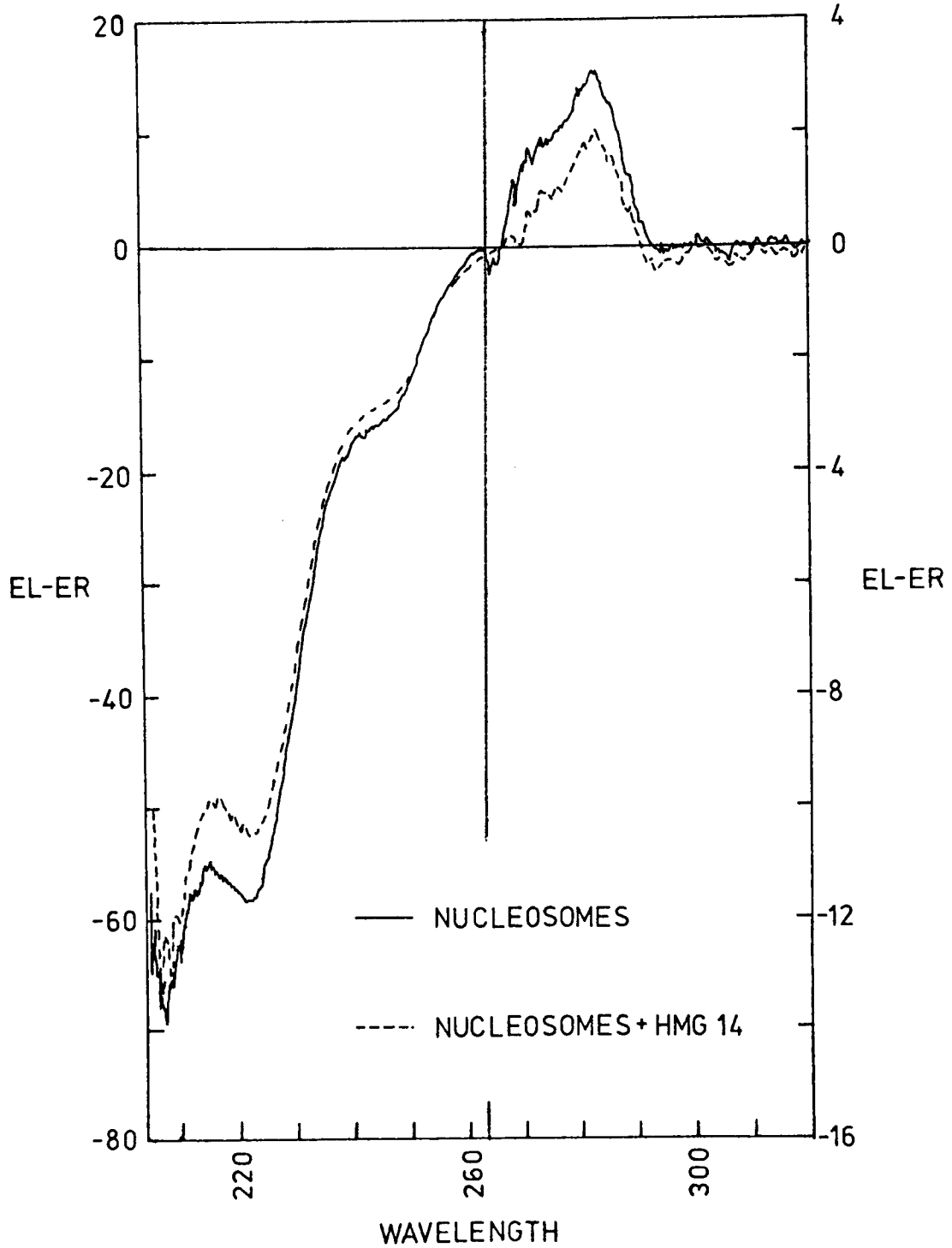

Figure 24

CD spectra of nucleosomes ± HMG 14 in 0.1 × TBE. Note more sensitive scale used for EL-ER at wavelengths above 262 nm. Stoichiometry was 2 HMG 14 per nucleosome.

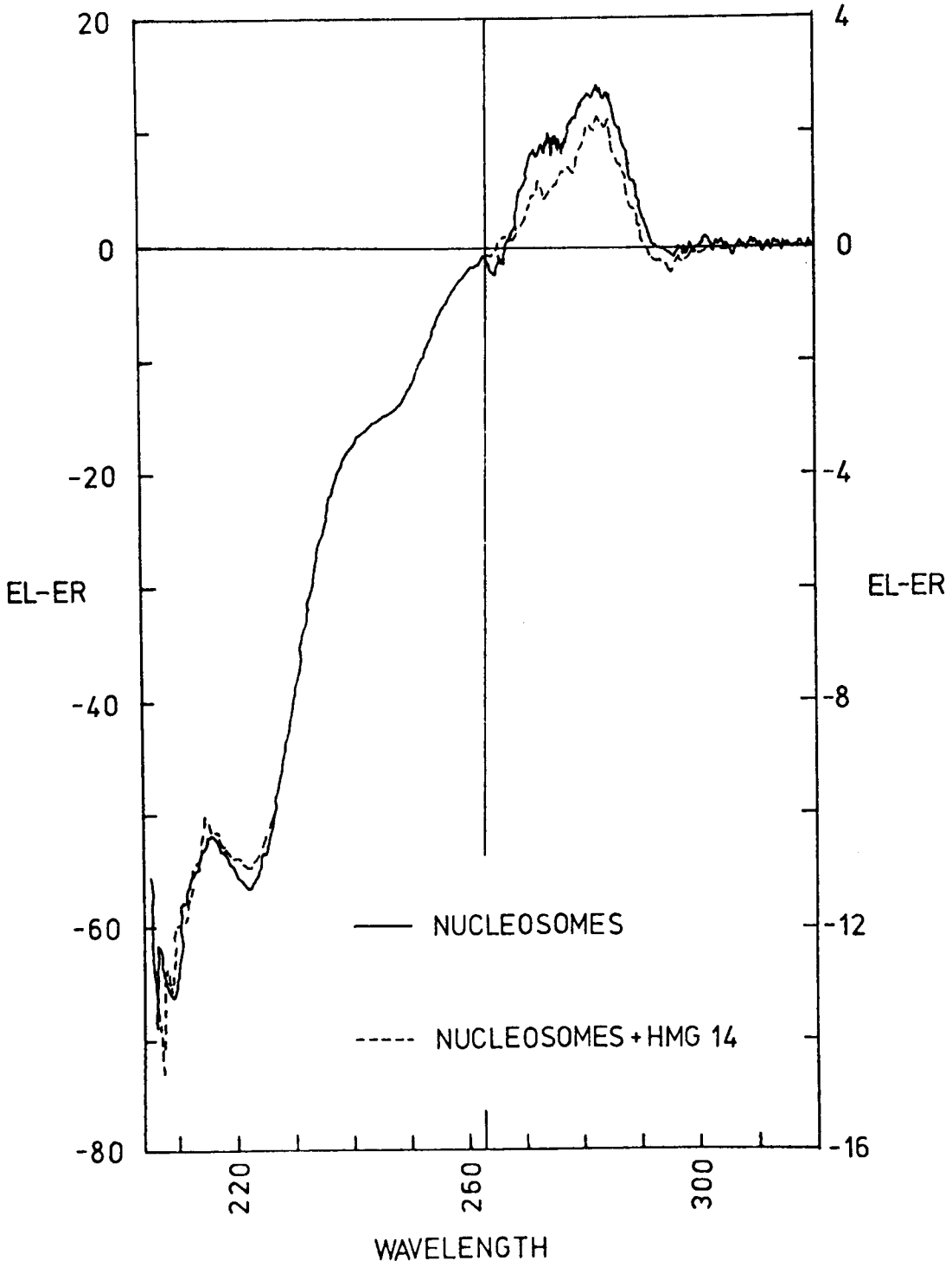

Figure 25

CD spectra of nucleosomes ± HMG 14 in 0.3 × TBE. Note the more sensitive scale used for EL-ER at wavelengths above 262 nm. Stoichiometry was 2 HMG 14 per nucleosome.

do not arise from major conformational changes in the structure of the nucleosome. The protein region of the spectrum shows no increase in α-helix or β-sheet. This might have been expected if HMG 14 or 17 assumed either of the regular secondary structures found in globular proteins. Major alterations in histone secondary structure upon binding of HMG-14 or 17 were not detected. Additional results, presented in Chapter 5, show the nucleosomal histones are still capable of major alterations in secondary structure when HMG 14 or 17 is bound.

Sedimentation

Nucleosomes in 0.1 × TBE or 0.3 × TBE and nucleosomes with bound HMG-14 were sedimented in the analytical ultracentrifuge to determine the mass and shape of the complex. From results reported by Sandeen et al. (119) it was known that HMG-nucleosome complexes showed only a small decrease in sedimentation coefficient, (S), relative to nucleosomes. With this in mind, experiments were planned to use the technique of difference sedimentation (154) to measure, accurately, any difference in S value, and to gain information on the affinity of HMG proteins for the nucleosome. Initial centrifuge runs showed variable S values for the nucleosome in TBE buffers. At low concentrations of TBE, S values were typically 1–1.5 S less than the value expected at these ionic strengths. S values in TBE also varied within an experiment by up to 1–1.5 S. Extreme care in sample preparation, temperature control, and solvent stabilization with 0.5% sucrose failed to reduce the variability observed. Boundary profiles revealed no heterogeneity in the sample. Profiles were sharp, single component curves. Boundary hypersharpening was noted at low ionic strength and was eliminated by adding 0.5%

sucrose to the sample. Changing pH from 8.3 to 7.0 did not reduce the variability between samples.

Difference sedimentation was done by loading nucleosomes in the reference sector and nucleosome-HMG 14 complexes in the sample sector of a double sector cell. The scanner electronics subtract the reference boundary from the sample boundary. The resulting chart output is the first derivative of the difference in boundary position and gives a sharp peak. The area, height and position of the peak can be used to calculate the difference in S value to a lower limit of 2%, and the binding constant for HMG. Peaks were obtained in two experiments. Both were very small, and of opposite polarity. These results indicated a very small difference in S value between nucleosomes and nucleosome-HMG complexes. The opposite polarities indicated variability between runs that precluded accurate measurement of the true S value.

Electron Microscopy

In an effort to determine the cause of variable S values, nucleosomes and nucleosome-HMG complexes were observed in the electron microscope to compare morphologies in TBE and standard buffers. Figure 26 shows electron micrographs of nucleosomes and nucleosome-HMG complexes. The appearance of the particles in each micrograph is the same as normally observed for nucleosome core particles under a variety of conditions. The particles are a two-dimensional representation of an oblate ellipsoid. The negative stain has penetrated the center of the particle in each case and the particles appear to be homogeneous in size and shape. The micrographs show no large structural alterations that would explain the variability in sedimentation. Samples of nucleosomes

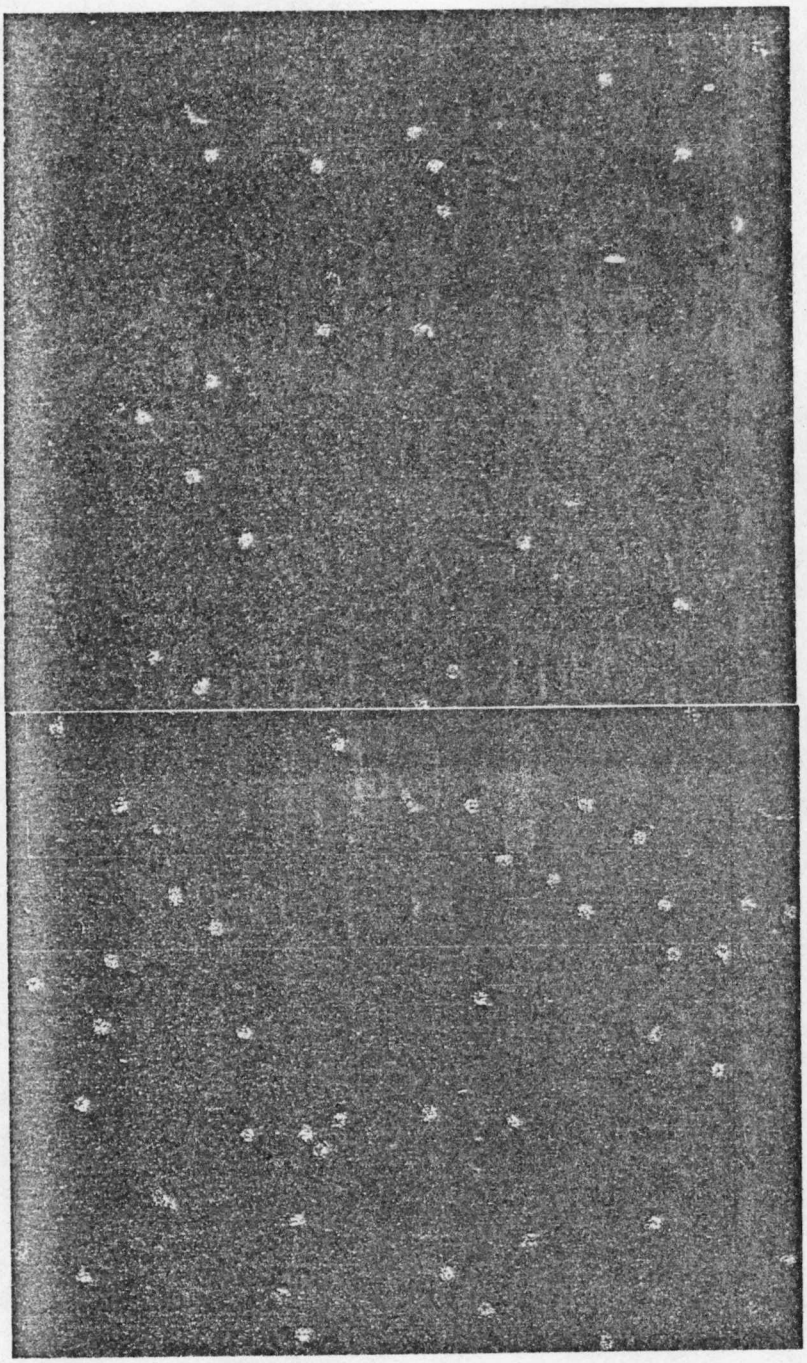

Figure 26

Dark field electron micrographs of nucleosomes and nucleosome-HMG complexes. Buffer is 0.2 mM EDTA. Top: nucleosome, Bottom: nucleosome-HMG complex in a 1:2 ratio.

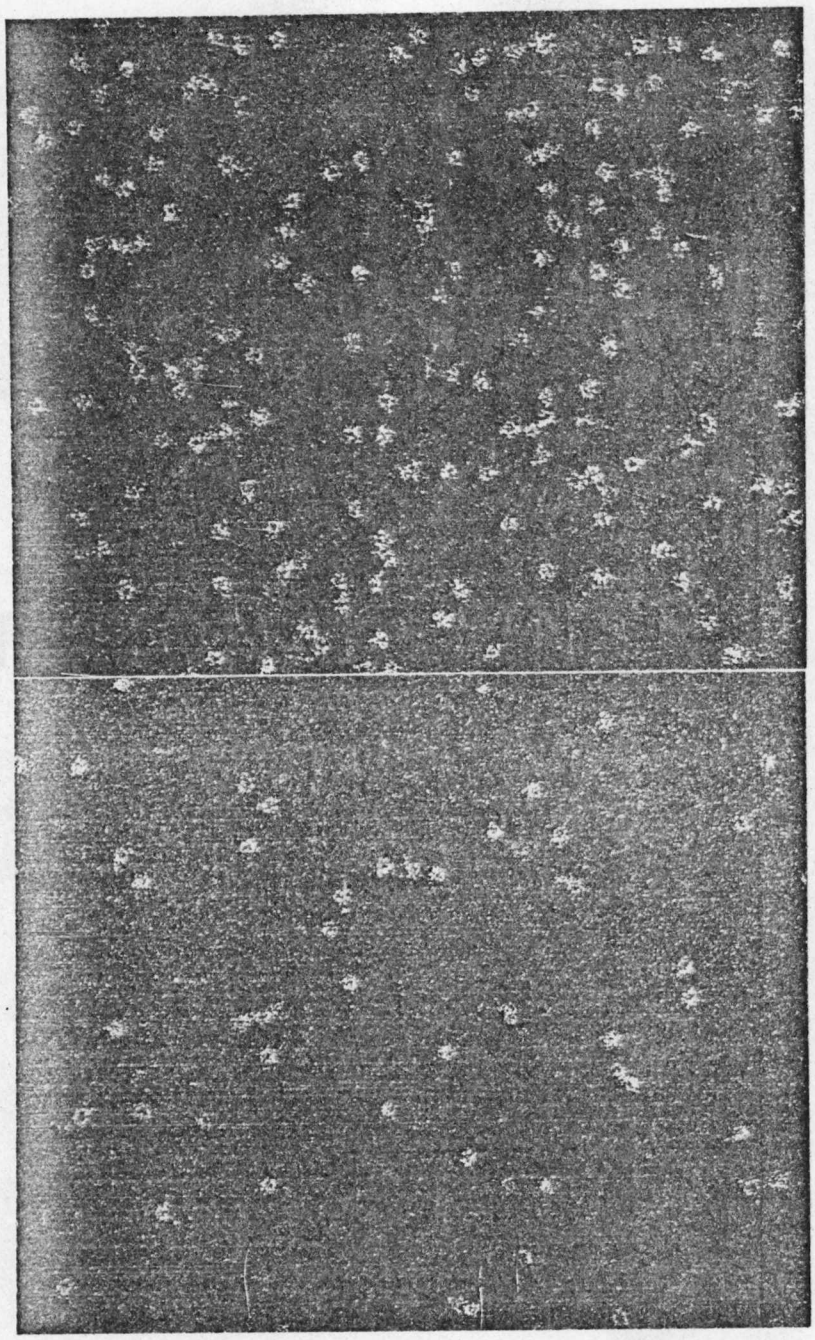

Figure 26 (continued)

Buffer is 0.3 × TBE. Top: nucleosomes, Bottom: nucleosome-HMG complexes in a 1:2 ratio.

in TBE buffer appear slightly larger in diameter than those in EDTA. This could partially account for the lower S value of nucleosomes in TBE buffer. Clearly, nucleosome-HMG complexes look very much like nucleosome core particles. The slightly rougher surface appearance of nucleosome core particles in TBE buffer may be the result of preparation for microscopy in TBE buffer, or effects of the electron beam that are not as pronounced in EDTA.

Discussion

The results of thermal denaturation studies and circular dichroism measurements clearly show that nucleosomes are more stable when HMG 14 or 17 is bound. The suppression of the early melting peak in melting studies suggests that HMG 14 and 17 strengthen the interaction of DNA ends with histones, through a shielding or neutralization of DNA phosphates in a manner analogous to raising the ionic strength of the solvent. Another possibility is that binding of HMG 14 and 17 might supply new contacts between DNA and histone that stabilize the DNA. This is consistent with results presented in Chapter 3 showing HMG binding sites near the ends of the DNA. The increased accessibility of DNAse I to certain regions of the DNA is still consistent with stabilization of the early melting region. The regions of increased accessibility are located away from the ends, near the center of the DNA sequence. Another feature of the stabilization is the higher melting temperature for the main peak when HMGs are bound to the nucleosome. This observation argues for an increased stability of the entire particle. This is consistent with the model proposed in Chapter 3 for

HMG coiling around the nucleosome. The wrapping of HMG 14 or 17 around the nucleosome has the potential for stabilizing interactions over much of the DNA length.

The circular dichroism results also show increased stabilization of the DNA. The reduced signal from the DNA region of the spectrum suggests further protein DNA interactions, like those seen between DNA and histone, that are known to stabilize DNA. In the protein region of the spectrum, the binding of HMG 14 or 17 contributes no additional α-helical or β-sheet structure to the overall signal. This is important because it is the first evidence against the formation of regular structures within the HMG-proteins upon binding to the nucleosome. This supports the idea of HMGs wrapping around the nucleosome in their extended form. HMG binding also does not reduce the existing α-helix and β-sheet within the histones. Reduction of regular secondary structure within the histones might be expected if HMGs destabilized nucleosomes. This was not seen. Effects on histone-histone contacts could not be determined.

The sedimentation results were variable; however, it was clear there were no major structural changes in the nucleosome upon binding of HMG 14. This result is in agreement with results reported by other investigators (119), and argues against major unfolding of the nucleosome that might be expected if HMG binding was a destabilizing event.

Electron microscopy of nucleosomes with bound HMG 14 confirmed that the complex was very similar to nucleosome core particles and that no major structural changes had taken place upon binding of the HMG protein.

The results presented, strongly suggest that binding of HMG 14 and 17 increases the stability of nucleosome core particles. Further, HMG-binding does not cause major structural alterations in the nucleosome. These results are surprising in view of current ideas concerning mechanisms of transcription in eukaryotic cells. HMG 14 and 17 are reported to be associated with actively transcribing chromatin (112-115). It is unexpected that structural proteins would act to stabilize the nucleosome in a region of chromatin where disruption of nucleosome structure may be essential for passage of the polymerase, and its ability to "read" the DNA sequence (117, 155). It is possible that the effect of HMG 14 and 17 is different when they bind to long oligonucleosome length stretches of chromatin. However, previous structural studies on nucleosomes have been excellent models for the behavior of long chromatin (58, 79). Recently, McGhee et al. (156) have shown that HMG 14 and 17 do not release chromatin from its condensed form. This is consistent with the idea that HMG 14 and 17 do not significantly destabilize long chromatin. It is clear that additional investigation on the interaction of HMG 14 and 17 with long stretches of chromatin is necessary. It is equally clear that the stabilization of nucleosomes by bound HMG 14 or 17 must be accomodated in models of chromatin structure and function.

CHAPTER 5

PROBES OF CONFORMATION AND BINDING

Introduction

Complexes formed between nucleosome core particles and the High Mobility Group (HMG) proteins 14 and 17 have been studied as a possible model for the interaction of HMG 14 and 17 with active chromatin (118-119). HMG 14 and 17 bind to nucleosome core particles at two specific sites (118-119). The binding is cooperative and alters the mobility of nucleosome core particles on polyacrylamide gels (118,119, 136,143). It has been shown in Chapter 4 that the binding of HMG 14 or 17 to nucleosome core particles stabilizes the particle against thermal denaturation and suppresses the DNA signal in circular dichroic spectra of nucleosomes. Electron microscopy, sedimentation (119,156), and neutron scattering (136) results indicate only very subtle structural alterations occur in the nucleosome after the binding of HMG 14 or 17.

From the available evidence, two questions emerge. First, in the absence of major structural alterations, does the binding of HMG 14 or 17 alter the response of the nucleosome to structural perturbations from outside the complex. This question addresses whether nucleosomes with bound HMG 14 or 17 are poised to undergo structural changes that may be required for transcription. Second, what portions of nucleosome structure are responsible for the cooperativity of HMG binding. This question addresses both histone substructures and overall nucleosome conformation.

Urea was used previously to induce nucleosome structural transitions that were studied as models for nucleosome transitions that might occur in transcribing chromatin (126, 157-159). The results of those studies showed that nucleosomes do unfold in a characteristic manner and that the maximum level of histone unfolding still permitted the histones to bind DNA. This was consistent with electron micrographs showing the absence of beaded structures corresponding to nucleosomes in transcribing regions (117, 144-146). It was also consistent with immunological evidence showing histones were still present on actively transcribing regions of DNA (117, 160). Based on the previous studies, it seemed appropriate to use urea to characterize the unfolding of nucleosome-HMG complexes. Structural alterations, induced by urea in nucleosome-HMG complexes, were monitored by circular dichroism, gel electrophoresis, and thermal denaturation. The results clearly show HMG 14 and 17 stabilize the nucleosome against unfolding induced by urea.

Trypsin digestion and formaldehyde crosslinking were used to study the portions of nucleosome structure involved in the cooperativity and specificity of HMG binding. Light digestion of nucleosome core particles with trypsin specifically removes the amino-terminal tails of the histones (49, 50). The resulting particle remains capable of organizing DNA into a supercoil and behaves like a slightly less stable nucleosome (50, 161). Removing the histone tails abolished the specificity of HMG binding to nucleosomes, however HMG 14 and 17 could still bind non-specifically.

Crosslinking nucleosomes with formaldehyde primarily involves the lysine residues on the histone amino-terminal tails. The crosslinking

reaction links histones to each other and links DNA to histone. This effectively freezes the nucleosome into the conformation it held at the time it was crosslinked. Crosslinking permitted the study of the role of histone tails in HMG binding and the role of nucleosome conformational changes in the cooperativity of HMG binding.

The results of crosslinking experiments indicate that HMG 14 and 17 will bind specifically to crosslinked nucleosomes; and that highly crosslinked nucleosomes still change from cooperative to non-cooperative HMG binding when ionic strength is lowered.

Methods

Nucleosome core particles from chicken erythrocyte chromatin were prepared as described in Chapter 2. HMG 14 and 17 were taken from stock solutions made by dissolving lyophilized HMG 14 or 17 in glass distilled water. Stock concentrations for HMG 14 and 17 were 4 mg/ml. Urea stock solutions were made by dissolving ultrapure urea (Schwarz-Mann or Bethesda Research Labs) to a concentration of 10 molar in glass distilled water. Next, the 10 molar urea was passed through a 5 cm × 25 cm column of mixed bed resin (Biorad AG 501X8) to deionize the urea. The resulting urea solution was filtered through 0.2 μ Duropore filters (Millipore Corp.). The conductivity of the deionized, filtered urea stock was typically less than 5 μMHO and was deionized again if conductivity exceeded 20 μMHO. Concentration was checked by refractive index measurements (126). The concentrated urea stock was monitored for conductivity and pH before each use. Shelf life of the deionized stock

was one week at room temperature or 24 hours if refrigerated and then heated to redissolve urea crystals.

Pancreatic Trypsin-TPCK and Soybean Trypsin Inhibitor were obtained from Worthington. Trypsin was stored at a concentration of 1 mg/ml in 1 mM HCl. Soybean Trypsin Inhibitor was stored at a concentration of 5 mg/ml in 10 mM Tris-HCl buffer, pH 8.0.

Formaldehyde (37% aqueous) containing less than 12.5% methanol as preservative, was used to prepare solutions for crosslinking nucleosomes. Commercial formaldehyde stocks containing greater than 12.5% methanol were less effective in crosslinking reactions.

Circular Dichroism

Circular dichroism was monitored with a JASCO J-40A spectropolarimeter. All spectra were taken at 20°C. Samples were placed in a 1 cm path, round cell 500 µl volume. Blanks were collected prior to each sample run, and subtracted during analysis. Spectra were collected and analyzed by computer. Sampling resolution was 10 points per nanometer. A 5-point smoothing routine was performed on all spectra during analysis.

Thermal Denaturation

Thermal denaturation was done in a Gilford model 2000 equipped with thermal programmer, automatic baseline correction and manual offsets. Data for each of 4 samples and temperature was collected and analyzed by computer. Samples of 300 µl volume were placed into 1 cm path, 2mm window, cuvettes. Samples were degassed under vacuum. Heating rate was 1°C per minute. Sample heating was done by the Gilford chamber heating blocks and a water/glycol bath controlled by the thermal programmer.

Absorbance was monitored at 260 nM. Temperature ranges are given in the figures.

Electrophoresis

SDS-polyacrylamide, gels (162) were used to monitor the extent of trypsin digestion and crosslinking. Assays of HMG binding in urea were done by electrophoresis on polyacrylamide gels containing urea. HMG binding to trypsinized or crosslinked nucleosomes was monitored by electrophoreses on non-denaturing polyacrylamide gels. All gels were formed in a vertical slab apparatus (Biorad or Hoefer) to make gels 12 × 14 × 1.5 mm in size.

SDS gels were composed of a separating gel of 15% acrylamide, and a stacking gel of 7.5% acrylamide. The gel was polymerized with ammonium persulfate and tetraethylmethylene diamine (TEMED). Gel buffers and electrode buffers were those of Laemmli (162).

Urea gels and non-denaturing gels were identical except for the presence of urea, added in place of water, in the gel formulation. Urea gels did not have urea in the electrode buffer. The gels were 6% acrylamide made up in Tris-borate-EDTA buffer as described in Chapter 2.

After electrophoresis, gels were stained for protein in a solution of methanol:water:acetic acid, 5:5:1, with Coomassie Blue; or for DNA, with Ethidium Bromide (1 μg/ml). Destaining was done for 2-4 hours in the stain solution minus the stain. Final destaining and storage was done in 10% acetic acid, 7.5% methanol.

Trypsin Digestion

Digestion of nucleosome core particles with trypsin was done as follows: 100 μg of nucleosome core particles was mixed with 5 μg of

trypsin in a total volume of 102 µl. The nucleosomes were in 10 mM Tris-HCl pH 8.0, at a concentration of 1 mg/ml. Trypsin was in 1 mM HCl at a concentration of 0.5 mg/ml. The reaction was incubated at 37°C and samples of 10 µl were removed at various times. The 10 µl time points were added to iced tubes containing 10 µg of Soybean Trypsin Inhibitor. At each time point 3 µl of SDS gel sample buffer was added to the sample. SDS sample buffer contains 50% glycerol, 25% 2-mercaptoethanol, 10% SDS, 0.325 M Tris-HCl pH 6.8, and 0.005% Bromophenol Blue. After SDS gel electrophoresis, the stained gel was observed and the appropriate digestion time corresponding to complete loss of the histone amino termini was chosen. This digestion time was used for a second bulk reaction to produce a stock of digested nucleosomes. Trypsin and trypsin inhibitor were separated from nucleosome core particles by chromatography on Sephadex G-100.

Crosslinking

Nucleosome core particles were crosslinked with formaldehyde using a modification of the method of Burch and Martinson (163). Nucleosomes at a concentration of 150 µg/ml were dialyzed exhaustively against 2.5 mM Na Phosphate, 20 mM NaCl, 0.1 mM EDTA pH 6.7. Typical sample volume was 2 ml. After equilibration, the dialysis buffer was changed to the same buffer containing a final concentration of 8% formaldehyde. The sample was allowed to equilibrate for 16 hours at 4°C, then the sample was exhaustively dialyzed against several changes of the dialysis buffer without formaldehyde. The extent of crosslinking was monitored on SDS-polyacrylamide gels. Satisfactory crosslinking was represented by an SDS gel showing all 4 histones and the DNA migrating as a single

200,000 molecular weight band. Lower concentrations of formaldehyde produced a 200,000 molecular weight band but the DNA migrated at the positon of free nucleosomal DNA at 100,000 molecular weight. Higher concentrations of formaldehyde produced small amounts of nucleosome oligiomers, and were avoided. Removal of residual formaldehyde by dialysis was monitored by an extremely sensitive, and rapid colorimetric test (164). The method employs the reagent pararosaniline and can be assayed on a visible light spectrophotometer.

Results

Gel Electrophoresis

The first question asked was whether unfolding of the nucleosome, as induced by urea, would alter the specificity or cooperativity of HMG binding. Nucleosome core particles were combined with HMG 14 at molar ratios of 0 to 3 HMG 14 per nucleosome in 0.1 × or 0.3 × TBE buffer containing 1 M urea. The results of electrophoresis on 5% polyacrylamide gels, containing the same buffer as the sample and 1 M urea, are shown in figures 27A and 27B. The gel shown in figure 27A shows the discrete, non-cooperative mode of HMG binding to nucleosomes in 0.1 × TBE buffer. This pattern is identical to the pattern seen on gels run in the presence of 0.1 × TBE without urea (see figure 13A). Figure 27B shows the results of electrophoresis in the presence of 0.3 × TBE and 1 M urea. This gel shows the cooperative mode of HMG binding to nucleosomes. It is identical to the same gel run in the absence of 1 M urea (see figure 13B). These results show that mild unfolding of the nucleosome does not alter the specificity or the

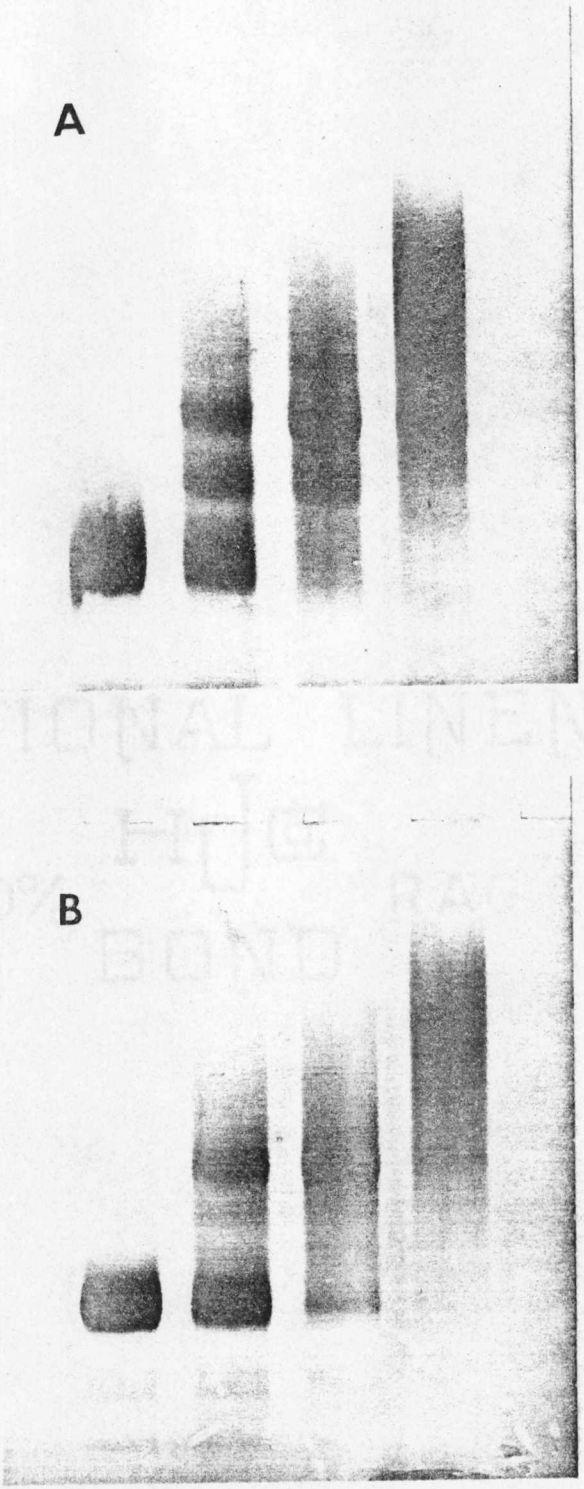

Figure 27

Electrophoresis of nucleosome-HMG 14 complexes in the presence of 1 M urea. A: 0.1 × TBE 1 M urea. B: 0.3 × TBE 1 M urea. Both gels, left to right, 0, 1, 2, 3 moles HMG 14 per mole nucleosomes.

cooperativity of HMG binding. The sites of HMG binding remain intact and accessible. Control experiments in which urea was added to the nucleosome core particles prior to HMG binding showed identical results. HMGs are known to exchange among binding sites in solution. The type of complex seen on gels is determined solely by the solvent conditions, as presented in Chapter 2.

In the next set of experiments, nucleosome-HMG 14 complexes were mixed with 0.1 or 0.3 × TBE containing 4 M urea. This concentration of urea is known to unfold the nucleosome extensively and to disrupt the the majority of histone α-helix (126).

Figure 28A shows the results of electrophoresis in the presence of 0.1 × TBE and 4 M urea. The nuclesomes without added HMG migrate as discrete bands. As HMG 14 is added, the lanes show diffuse staining in the region normally occupied by discrete bands of nucleosome-HMG complexes. This diffuse staining is interpreted as non-specific binding and closely resembles the type of staining seen when HMG 1 and 2 are nonspecifically bound to nucleosome core particles (129). Figure 28B shows the results of electrophoresis in 0.3 × TBE and 4 M urea. This gel shows that more extensive unfolding in urea prevents discrete binding even at higher ionic strength. The results of electrophoresis in 4 M urea demonstrate that extensive disruption of nucleosome structure by urea destroys the specific and cooperative binding of HMG 14 to nucleosome core particles. The results of electrophoresis in 1 M urea demonstrate that mild unfolding of the nucleosome by urea has no effect on HMG binding.

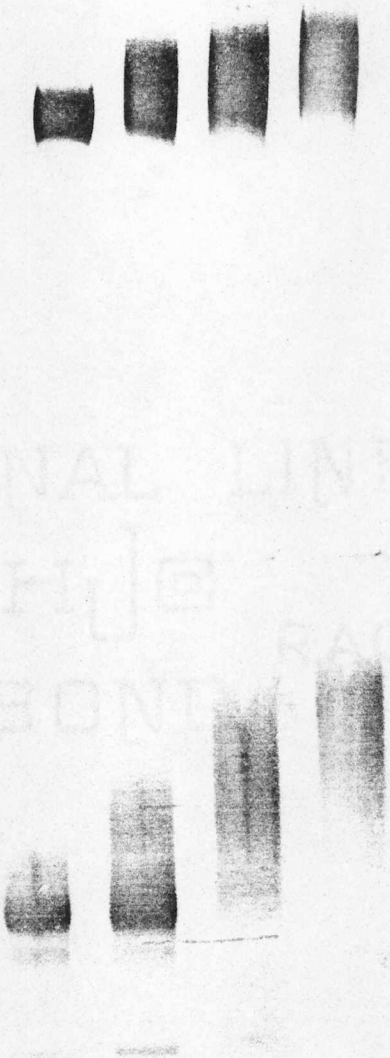

Figure 28

Electrophoresis of nucleosome HMG-14 complexes in the presence of 4 M urea. A: 0.1 × TBE 4 M urea. B. 0.3 × TBE 4 M urea.

Thermal Denaturation

The next series of experiments were done to see if HMG 14 could stabilize the nucleosome against thermal denaturation in the presence of urea. From the electrophoresis results, it was known that discrete nucleosome-HMG complexes existed in 1 M urea. The first melting studies were done to determine if complexes formed in the presence of urea were less stable even though the HMG was discretely bound. Mild unfolding of the nucleosome in 1 M urea is sufficient to alter some histone-histone contacts and reduce the main melting temperature at which the entire structure is destroyed (126,157-159).

Nucleosomes complexed with two HMG 14 molecules per nucleosome in 0.1 or 0.3 × TBE were melted in the presence or absence of 1 M urea. The results are shown in figures 29A and 29B. In figure 29A, nucleosomes in 0.1 × TBE and 1 M urea show a prominent early melting region at about 57°C and a main melting region at 72°C. The presence of HMG 14 at a molar ratio of 2 HMG 14 per nucleosome suppresses the early melting peak and shifts the main peak to 74°C. The same sample without urea shows even greater suppression of the early melting shoulder by HMG 14. The main melting temperature for the sample without urea was 77°C, indicating the urea was destabilizing the nucleosome when present in the sample.

Figure 29B shows a similar stabilizing effect of HMG 14 on nucleosomes in 0.3 × TBE in 1 M urea. Again, the early melting shoulder is suppressed by the addition of HMG 14, and is suppressed further by HMG 14 in the absence of urea. The results strongly indicate that HMG 14 is stabilizing the nucleosome against the effects of both urea and thermal denaturation.

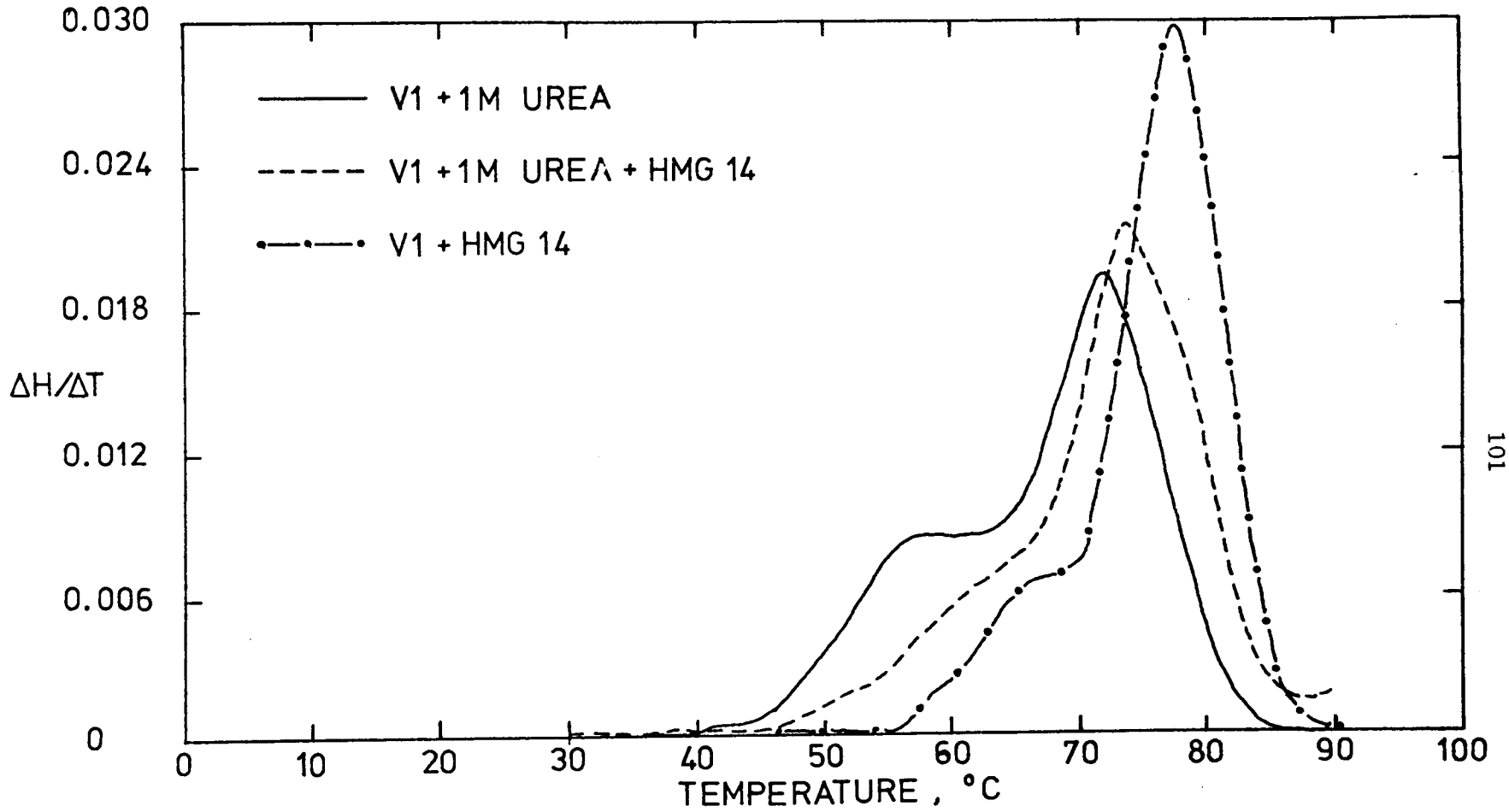

Figure 29A

Thermal denaturation profiles of nucleosomes and nucleosome-HMG 14 complexes in 1 M urea, 0.1 × TBE.

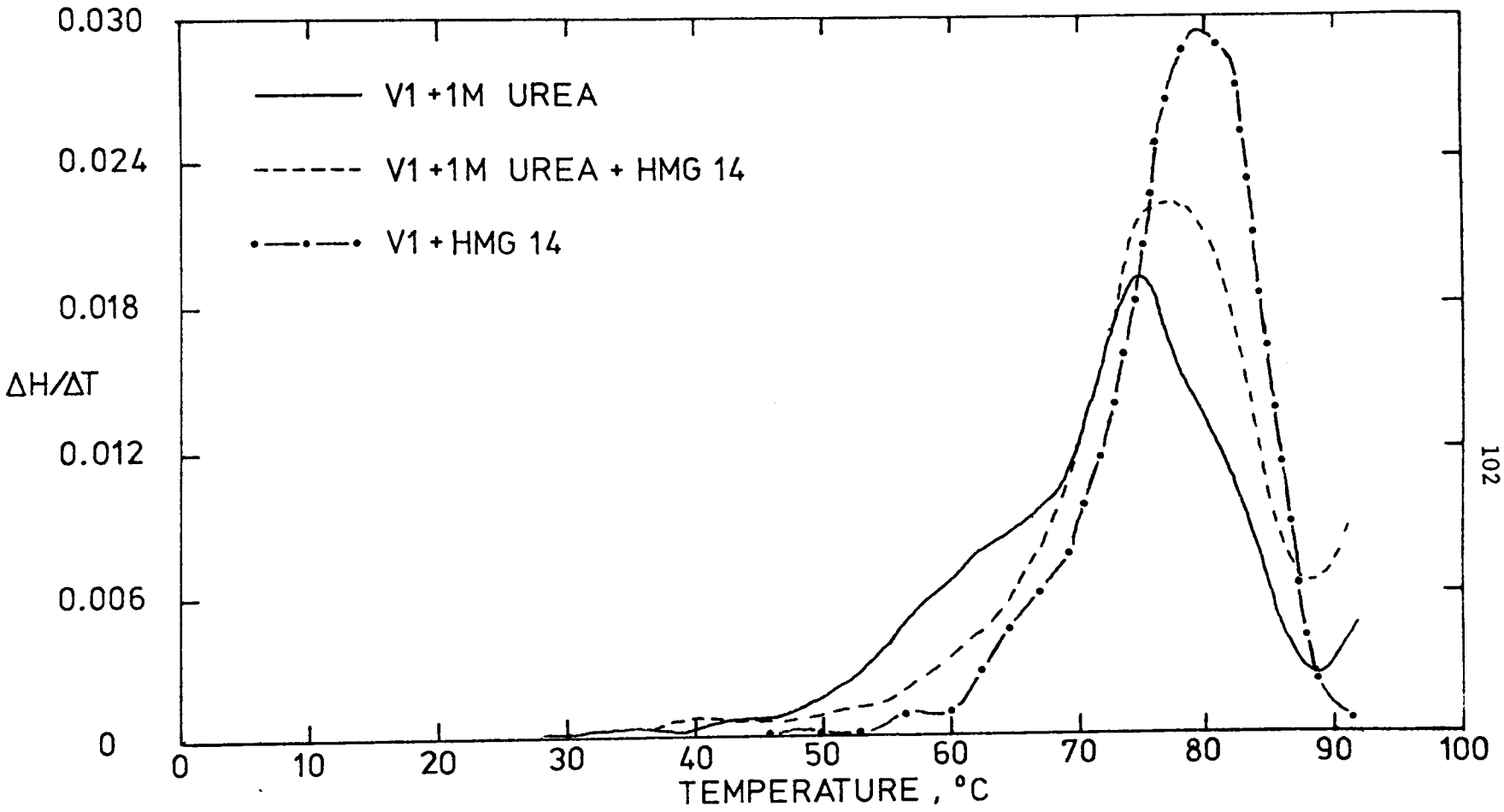

Figure 29B

Thermal denaturation profiles of nucleosomes and nucleosome-HMG 14 complexes in 1 M urea, 0.3 × TBE.

Circular Dichroism

Previous studies using circular dichroism have revealed much about the alterations in nucleosome structure in the presence of urea (126,157). Suppression of the signal from DNA in 260-300 nm region of the spectrum is characteristic of nucleosomes. It occurs when free DNA is complexed with histones to form a stable nucleosome structure. This signal suppression is reversed by high concentrations of urea. Urea is known to destroy nucleosome structure by destabilizing histone-histone contacts, and histone α-helix.

Circular dichroism was used to study the effects of bound HMG on the stability of nucleosomes in the presence of urea. Figure 30 shows the spectra of nucleosomes, with and without bound HMG 14, in the presence of 0.1 × TBE and urea. The spectra of nucleosomes and nucleosome-HMG 14 complexes in 0.3 × TBE and urea is shown in Figure 31. The DNA signal is suppressed by the presence of HMG 14 in both 1 M and 4 M urea, indicating greater stability. The stabilizing effect of HMG 14 is still apparent in both 1 M and 4 M urea at the higher ionic strength of TBE.

The results shown in figures 30 and 31 point to increased protein-DNA contacts, known to stabilize DNA, when HMG 14 is bound to nucleosomes in the presence of urea. In addition, small changes are observed in the protein α-helix region of the spectrum, between 200-240 nm, in both figures. In this case, the more negative the signal, the greater the amount of α-helix in the sample. The samples containing 4 M urea appear to show slightly more α-helix in the presence of HMG 14 than those without HMG, indicating greater stability in the presence of HMG 14. These changes are not seen in the 1 M urea samples

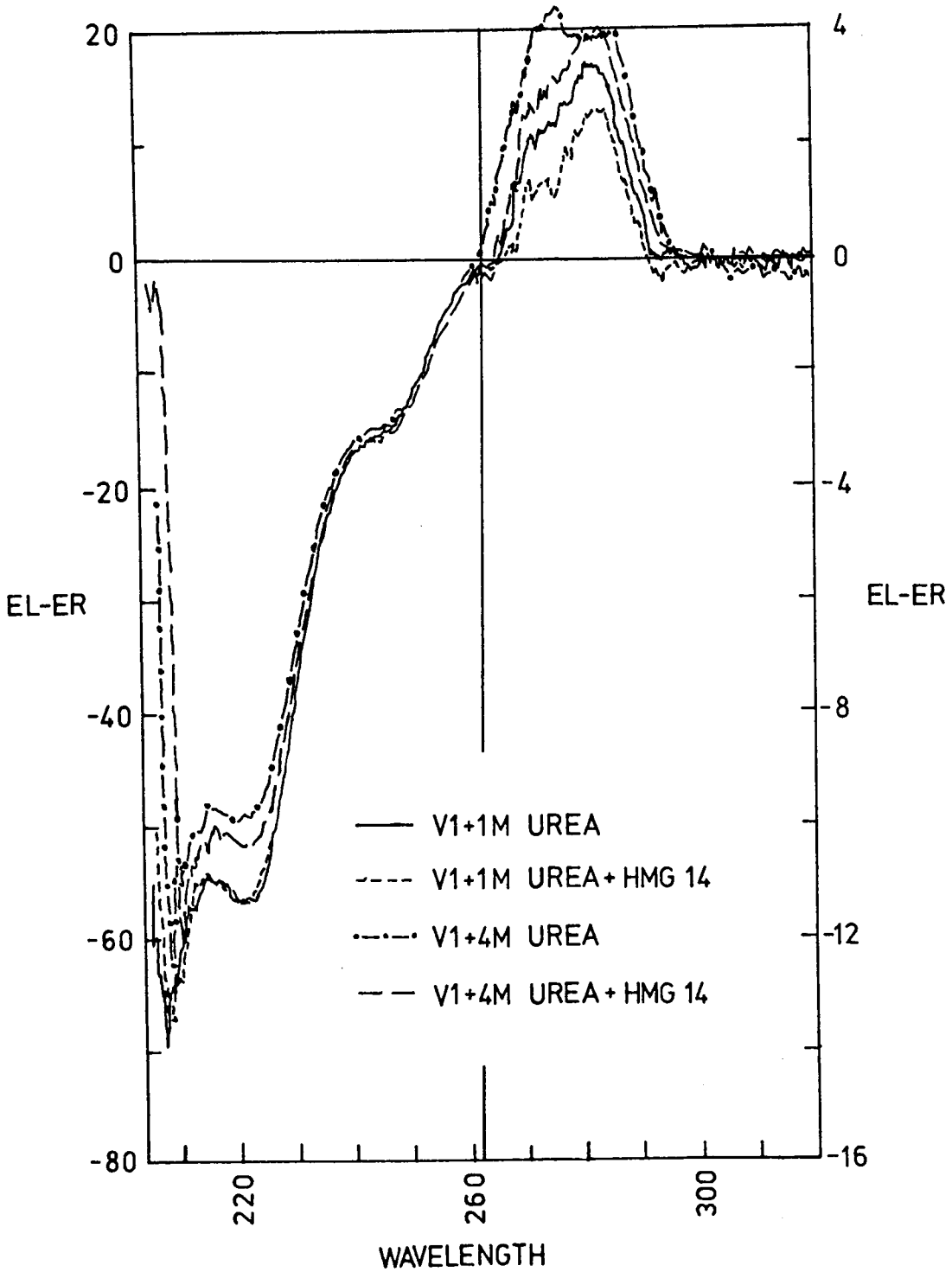

Figure 30

Circular dichroism of nucleosome-HMG 14 complexes in 0.1 × TBE and urea. Nucleosomes in 0.1 × TBE ± 2 HMG 14 per nucleosome.

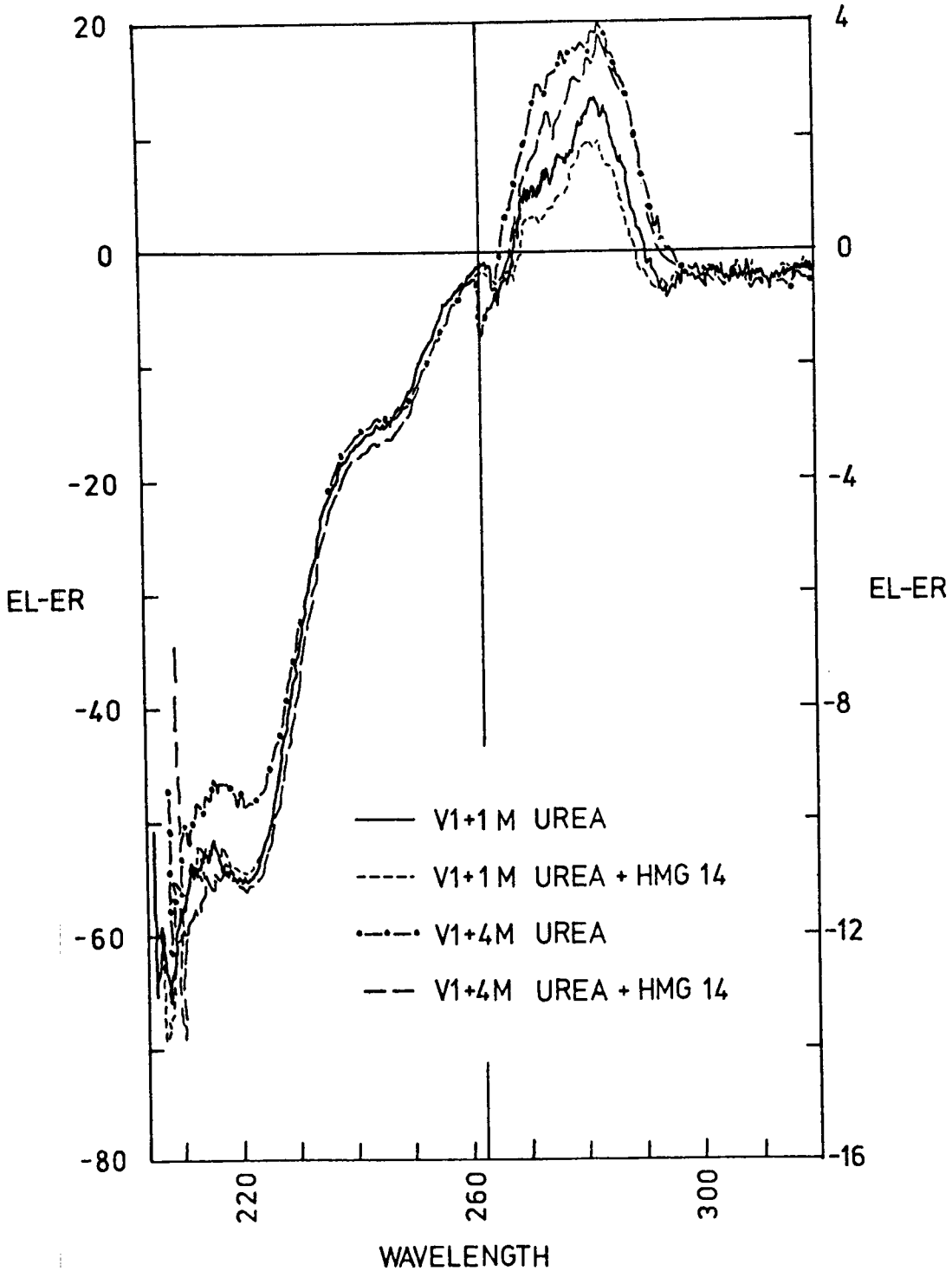

Figure 31

Circular dichroism of nucleosome-HMG 14 complexes in 0.3 × TBE and urea. Nucleosomes in 0.3 × TBE ± 2 HMG 14 per nucleosome.

due to the minimal effects of 1 M urea on histone α-helix. It is pertinent that HMG 14 has no secondary structure and contributes little to the circular dichroic spectra of the samples in this region.

The results of the circular dichroism studies provide evidence for stabilization of the nucleosome, by HMG 14, against the destabilizing effects of high urea concentrations known to unfold the nucleosome.

Trypsin Digestion

Removal of the histone amino-termini by digestion with trypsin permits assessment of their role in the binding of HMG proteins to nucleosomes. HMG 14 and 17 are equivalent in function and for these experiments HMG 17 was used.

Nucleosome core particles were digested with trypsin as described in methods, purified on Sephadex G-100, and complexed with 0, 1 or 2 HMG 17 molecules per nucleosome. The samples were run on a 6% polyacrylamide gel. The results are shown in Figure 32. The trypsin treated nucleosomes migrate more slowly than undigested control nucleosomes. This may be the result of the nucleosome unfolding slightly after removal of the histone tails, as reported by others (50). In addition, the nucleosome-HMG 17 samples show a diffuse staining in the region normally occupied by discrete bands of nucleosome-HMG complexes. This diffuse staining indicates non-specific binding of HMG to the nucleosome after removal of the amino-terminal tails of the histones. Either the loss of the amino-termini or the slight unfolding of the nucleosome after removal of the tails could explain the non-specific binding pattern observed. The histone tails may be required for the specific binding of HMG protein because of direct

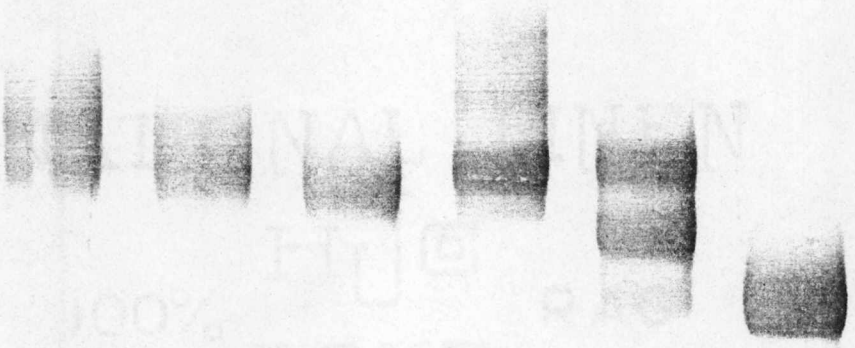

Figure 32

Electrophoresis of trypsin digested nucleosome-HMG 17 complexes. Left to right, trypsinized nucleosome complexed with 2, 1, 0 moles HMG 17 per mole nucleosomes; undigested nucleosomes complexed with 2, 1, 0 moles HMG 14 per mole nucleosomes. Gel contains 0.1 × TBE buffer.

involvement in the binding site. An alternative explanation would require the presence of the tails to maintain a compact nucleosome structure necessary for the existence of the binding site.

Crosslinking

Formaldehyde crosslinking of nucleosomes was done to determine if HMG binding required mobile histone amino-termini. Crosslinking was also used to determine if a large conformational change in the nucleosome was the reason for the non-cooperative HMG binding mode at low ionic strength.

Nucleosome core particles were crosslinked with 6% or 12% formaldehyde at high ionic strength (20 mM) as described in Methods. This was done to preserve the structure known to be present during the cooperative binding. Formaldehyde was removed and crosslinked nucleosomes were mixed with HMG 17 and diluted to low ionic strength in 0.1 × TBE. Control nucleosomes, not crosslinked, were prepared the same way and all samples were electrophoresed on a 6% polyacrylamide gel containing 4 M urea and 0.1 × TBE. The results are presented in Figure 33. The gel was run in urea to demonstrate the compact nature of the crosslinked nucleosomes when compared to the non-crosslinked, unfolded controls. The gel shows the higher mobility of crosslinked nucleosome core particles and the absence of higher oligomers of nucleosomes. This gel also demonstrates retention of the discrete, non-cooperative binding mode of HMG protein with crosslinked nucleosomes. Crosslinked nucleosomes with bound HMG 17 show the same banding pattern, in 0.1 × TBE and 4 M urea that is shown by nucleosome-HNG complexes in the absence of crosslinking and urea.

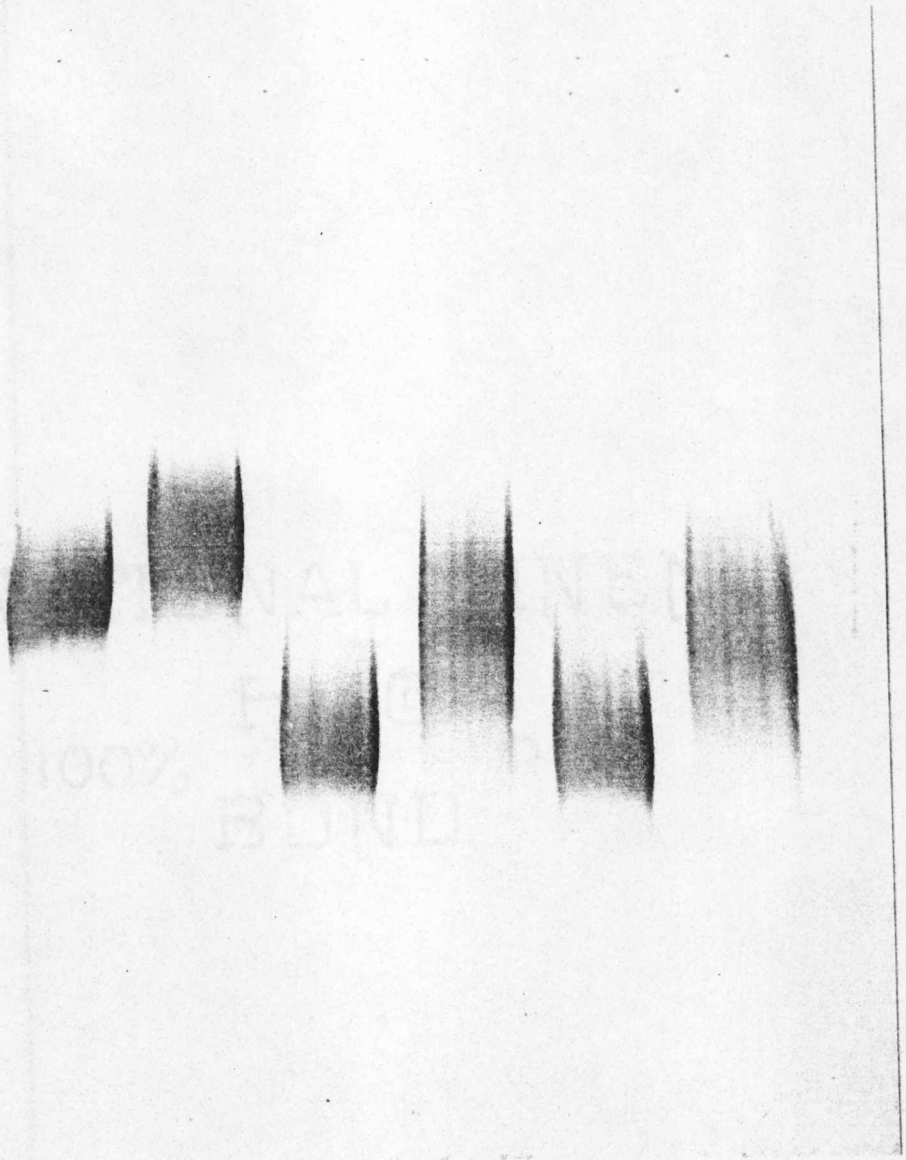

Figure 33

Electrophoresis of crosslinked nucleosomes complexed with HMG 17 in 0.1 × TBE, 4 M urea. 1 and 2, nucleosomes with 0 or 2 HMG 17 per nucleosome 3 and 4, nucleosomes crosslinked with 6% formaldehyde, with 0 or 2 HMG 17 per nucleosome; 5 and 6 nucleosome crosslinked with 12% formaldehyde with 0 or 2 HMG 17 per nucleosome.

Figure 34 shows the results of an experiment combining HMG 14 with crosslinked nucleosomes. The samples were also run on a 6% polyacrylamide gel in 0.1 × TBE and 4 M urea. Again, the crosslinked nucleosomes show discrete, non-cooperative binding of HMG protein.

These results reveal two important points. First, extensive crosslinking of the nucleosome does not prevent the binding of HMG 14 or 17. Second, extensively crosslinked nucleosomes are still able to respond to solvent conditions to produce non-cooperative HMG binding.

Discussion

The results shown in this chapter clearly indicate that nucleosome core particles are stabilized by HMG proteins against unfolding and denaturation induced by urea. The results also show that unfolding of the nucleosome by 4 M urea, or the loss of histone amino-termini, prevents specific binding of HMG proteins. The results of the crosslinking studies indicate that HMG proteins still bind to nucleosomes in an ionic strength dependent manner. The binding mode is still able to shift from cooperative to non-cooperative when nucleosomes are extensively crosslinked.

The loss of HMG binding specificity in the presence of high concentrations of urea may be the result of nucleosome unfolding. Urea is known to cause swelling of the nucleosome and to loosen interactions between histones. Histone secondary structure is also affected, causing the loss of α-helix. Any one of these effects could be sufficient to prevent specific HMG binding. It is known that HMG proteins bind to the outside of the nucleosome in two specific sites. Based on previously

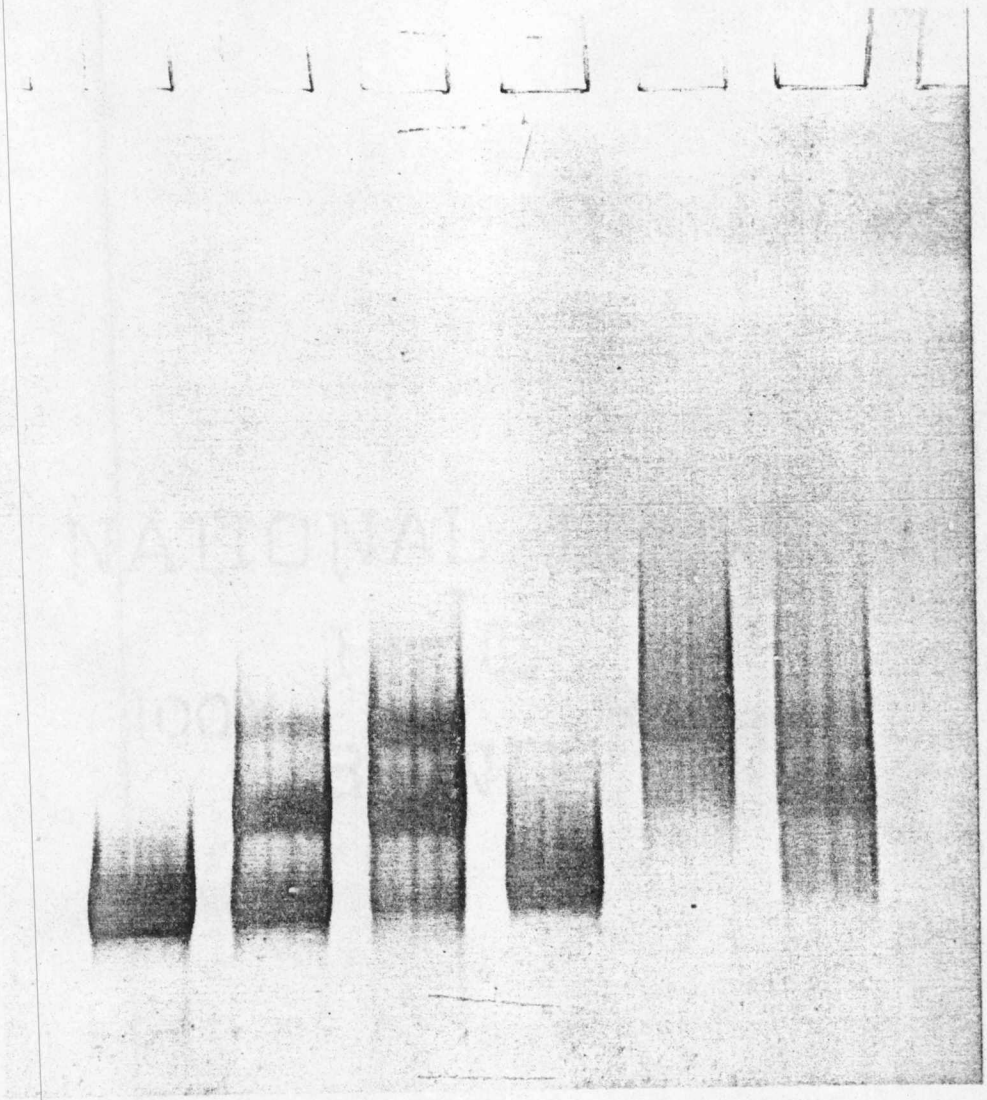

Figure 34

Electrophoresis of crosslinked nucleosomes complexed with HMG 14 in 0.1 × TBE, 4 M urea. Left to right, nucleosomes with 0, 0.75, 1.5 HMG 14 per nucleosome; crosslinked nucleosomes; crosslinked nucleosome with 2 or 1 HMG 14 per nucleosome.

cited neutron scattering studies, it is likely the HMG proteins bind in their extended form. If multiple contact sites are required for specific HMG binding, as previously proposed in Chapter 3, any movement of histones or DNA could destroy the binding site. These results support the model presented in Chapter 3 which requires binding to both histones and DNA. It is unlikely that the loss of specificity in binding is due to effects of urea on HMG proteins. HMGs have no secondary structure and are highly charged proteins known to bind chromatin ionically. In addition, control experiments in which nucleosome-HMG complexes were sedimented in the presence of 6 M urea showed no loss of HMG protein from the nucleosomes.

Removal of N-terminal regions of the histones also results in a loss of specific binding. It is possible the amino termini are required for HMG binding as part of the binding site. Another explanation could be the swelling of the nucleosome that occurs after the removal of histone amino termini. As described above, this swelling could alter the HMG binding sites preventing specific binding. The removal of histone tails also supports the idea of a binding site incorporating both DNA and histone contacts, since removal of the histone amino termini would not alter binding to DNA alone.

The partial unfolding and destabilization of nucleosomes by urea results in lower melting temperatures, or changes in circular dichroism, of both DNA and protein. These effects are reduced or prevented if HMG protein is bound to the nucleosome. In the case of thermal denaturation, two kinds of stabilization are occurring. First, the melting of the ends of the DNA, giving rise to the early melting peak, is suppressed almost entirely by bound HMG protein. The DNA ends could be

directly stabilized by ionic interactions between DNA phosphates and the basic amino acids of the HMGs. This could effectively counteract the disruption of hydrogen bonds by increased temperature, and the weakening of hydrophobic interactions by urea. Hydrophobic interactions have a significant role in stabilizing the DNA helix. The presence of direct linkages between DNA phosphate and HMG basic residues, or the presence of a high number of HMG positive charges stabilizing the DNA by charge attraction, could oppose the weakening of hydrophobic interactions. Second, the stabilization of the main melting transition results from the stabilization of both DNA and histone by HMG protein. The main melting transition results from the catastrophic loss of the remaining DNA structure and the loss of histone secondary structure. These results are consistent with the model of HMG proteins extending across a large area of the nucleosome surface rather than a more localized region.

The results of circular dichroism on nucleosome-HMG complexes in urea also support stabilization of the nucleosome by HMG protein. The increase in signal from the DNA region of the spectrum, in the presence of urea, is reduced or suppressed when HMG protein is bound to the nucleosome. This DNA signal suppression is identical to the type of DNA signal reduction seen when histone octamers are added to free DNA, organizing and stabilizing the DNA.

Addition of urea also causes reduction of the large negative peak in the α-helical region of the protein spectrum. This change indicates a small reduction in the amount of α-helix in the histones. The presence of HMG protein on the nucleosome suppresses or reverses this change, restoring all or part of the α-helix lost due to urea effects.

This stabilization supports the idea of HMG proteins binding to a larger area of the nucleosome and preventing the loss of protein secondary structure as part of the overall stabilization of the nucleosome. The effect may be entirely mechanical in nature, with the HMG wrapped around the nucleosome, adding to the forces holding the structure together. However, the high charge density of HMG 14 and 17 argues for a contribution from the basic and acidic residues on the HMGs. Further neutalization of DNA phosphates, or the presence of additional protein charges at the surface of the histones, may enhance the binding of DNA to the histone core.

Crosslinking of the nucleosome with formaldehyde, fixes the nucleosome with covalent bonds between very closely opposed sites. Crosslinking that is extensive enough to prevent unfolding of the nucleosome in high urea concentrations, or the dissociation of DNA by SDS, should prevent major structural alteration. The results of adding HMG protein to crosslinked nucleosomes shows that major structural alterations of the nucleosome are not required for HMG binding. Mobile histone tails may not be required either. It is probable that they are crosslinked extensively by formaldehyde and are not free to interact normally. Comparing these results with the trypsin digestion data suggests the histone amino termini may be necessary for specificity in HMG binding but do not interact directly with the HMG proteins. However, as stated previously, the swelling of the nucleosome after loss of the histone tails may explain the loss of HMG binding specificity.

The crosslinking results also show that the transition from cooperative to non-cooperative HMG binding does not require major structural changes in the nucleosome. Nucleosomes crosslinked at high ionic

strength, where cooperative binding is seen, give the non-cooperative binding pattern when shifted to low ionic strength. This could be explained by a minor structural change that alters the HMG binding site. It is also possible that the cooperative binding of HMG proteins is sensitive to ionic strength alone and that subtle alterations in solvent charge, or the random coil structure of HMG proteins, may be enough to determine the type of binding observed.

From the results of the studies presented here three main points emerge: First, nucleosomes are stabilized against unfolding by the presence of bound HMG 14 or 17. Second, HMG proteins cannot bind specifically to nucleosomes that are partially unfolded or swollen. Third, the transition from cooperative to non-cooperative HMG binding, influenced by ionic strength changes, does not require major alterations in the conformation of the nucleosome.

CHAPTER 6

SUMMARY

The studies described in the preceding chapters were directed toward understanding the structure of nucleosome-HMG complexes, and the effects of HMG binding on the nucleosome core particle. The discovery by Weintraub and co-workers (112) that HMG 14 and 17 conferred DNAse I sensitivity on active genes, made the study of complexes between HMG proteins and chromatin essential. Previous studies of nucleosomes as a model for chromatin made the study of nucleosome-HMG complexes attractive, both technically and as a meaningful extension of prior studies. The well-characterized nucleosome was likely to allow clear-cut evaluations of HMG binding. Biophysical methods of study were chosen in order to obtain the most information about complexes available in small amounts and without any assayable function.

Chapter 2 provided evidence for the formation of discrete complexes between nucleosome core particles and HMG 14 or 17. Assayed on electrophoretic gels, the titration of nucleosomes with known molar ratios of HMG 14 or 17 resulted in discrete complexes corresponding to 1 or 2 HMG proteins per nucleosome. The binding occurred equally well between HMG proteins and nucleosomes reconstructed from purified histones and a synthetic, alternating copolymer DNA. This aspect was important because it confirmed that histones and DNA were the only necessary components for formation of the complex with HMG proteins. Synthetic DNA would have none of the modifications or tightly bound proteins found in vivo and the histones would have no contaminating proteins because of high salt washes and sucrose gradient purification.

Another discovery was the shift from non-cooperative to cooperative HMG binding when the ionic strength was raised above 1-2 mM. This immediately suggested that HMG binding to nucleosomes could be modified by the environment of the complex. It further suggested that a conformational change might be involved in the binding of HMG 14 and 17 to nucleosomes. This was strengthened by the fact that the shift to cooperative binding occurred at the same ionic strength where the nucleosome was known to shift from an open to a compact native conformation.

Chapter 3 described the use of high resolution DNA gels, and DNAse I digestion of nucleosome-HMG complexes, to map the binding sites of HMG 14 and 17 on the nucleosome. The results showed that bound HMG 14 or 17, at a molar ratio of 2 per nucleosome, protected the nucleosomal DNA at 2 symmetrically located sites.

Combined with the titration results from Chapter 2, the mapping results confirmed the presence of two equivalent HMG binding sites on the nucleosome core particle. The results generated a model for HMG binding to the nucleosome core particle at sites near each end of the nucleosomal DNA, and possible binding sites on the histone surface at sites near the center of the DNA supercoil.

The mapping results immediately posed new questions about how the HMG molecule binds to the nucleosome. Since HMG 14 and 17 were known to have a random coil conformation in solution, it was not known if they folded or remained extended upon binding to the nucleosome. Evidence presented in later chapters argues for the absence of secondary structure in bound HMG and suggests a model for HMG binding around the surface of the nucleosome adjacent to the DNA supercoil.

One additional finding was that digestion with DNAse I proceeded more rapidly in nucleosomes without bound HMG protein. This suggested that the preferential DNAse I sensitivity of active chromatin required a feature not present in reconstructed nucleosomes.

Chapter 4 presented results showing that when HMG 14 or 17 was bound, nucleosomes were stabilized. Thermal denaturation showed that the ends of the nucleosomal DNA melted at a much higher temperature when HMG was bound. This is consistent with HMG binding sites near the ends of the DNA. The melting temperature of the remaining DNA was also higher. This indicated stabilization of the entire nucleosome by bound HMG protein. Circular dichroism showed increased stabilization of nucleosomal DNA upon binding of HMG protein. In this case, no outside perturbation was used. The addition of HMG 14 or 17 to nucleosomes caused suppression of the signal in the DNA region of the CD spectrum consistent with increased stability and organization of nucleosomal DNA.

These results were surprising. The working hypothesis was that HMG 14 and 17, as structural proteins of actively transcribing chromatin, would act to prepare the chromatin for transcription by destabilizing DNA-histone interactions. The results obtained would be more consistent with a reorganization role for HMG proteins.

Further studies using sedimentation and electron microscopy confirmed that no major change in axial ratio or nucleosome conformation occurred when HMG protein bound to nucleosomes.

The results of Chapter 4 indicate that HMG proteins might exert effects at the level of oligonucleosome lengths of chromatin. Unpublished work by Gerard J. Bunick has shown bound HMG 14 greatly increases the solubility of nucleosome core particles in the presence of magnesium.

This is consistent with chromatin fractionation procedures using magnesium that show enrichment for active nucleosomes in the magnesium soluble fraction (117). One attractive possibility is that HMG 14 and 17 stabilize the individual nucleosome, while altering the response of longer stretches of chromatin to magnesium. Further studies on longer stretches of chromatin containing bound HMG 14 and 17, are needed.

Chapter 5 describes experiments that probed conformational changes in the nucleosome HMG complex. Results from gel electrophoresis in various ionic strengths showed a shift form non-cooperative to cooperative binding. Coupled with the large mobility shift on gels after HMG binding, these results suggested a conformational change was taking place as a result of HMG binding and solvent conditions. Changes in conformation of the complex were induced by addition of urea to the sample. These experiments were done to see if the nucleosome, though stabilized by HMG protein, was still poised to undergo structural changes triggered by other factors. The results showed that 1 M urea had no effect on the titration of nucleosome core particles with HMG 14 or 17. The addition of 4 M urea destroyed specific binding of HMG proteins, giving only a smear on the gel. This was attributed to unfolding of the nucleosome to a degree that destroyed the proximity of DNA and histone necessary for the HMG binding site. This showed that a compact nucleosome structure was required for specific binding.

Thermal denaturation and circular dichroism both showed nucleosomes were stabilized against the effects of added urea when HMG 14 or 17 was bound. Melting temperatures were higher, indicating greater stability of both DNA and histone. Circular dichroic spectra showed urea caused a

loosening of the DNA on the nucleosome that was reversed by the addition of HMG protein.

Trypsin digestion of nucleosomes was done to see if histone amino termini were necessary for HMG binding to the nucleosome. The results showed the loss of histone termini destroyed the specific binding of HMG protein. The loss of specificity could be the result of losing structures required for the binding site. It could also be the result of nucleosome structural changes that are known to occur after trypsin digestion. The definitive experiment would have maintained a compact nucleosomal structure after removal of the histone tails. This was not feasible because of the immediate conformational change resulting from trypsin digestion and the inability to remove digested histone tails from previously crosslinked nucleosomes.

Crosslinked nucleosomes were used to see if the transition from cooperative to non-cooperative HMG binding required a conformational change in the nucleosome. The results showed that extensive crosslinking of the nucleosome did not prevent the ionic strength induced shift from cooperative to non-cooperative binding of HMG proteins. These results suggested that the conformational change was very subtle and not prevented by crosslinking. Another possibility was that the binding mode of HMG 14 or 17 is determined by ionic strength alone and that the folding of HMG protein over the surface of the nucleosome requires a threshold of supproting ions for complete cooperative binding.

Much work remains to be done on the interaction of HMG proteins and chromatin. Future studies should focus on longer stretches of chromatin to determine the binding stoichiometry on oligonucleosomes. Binding studies on reconstructed active genes using modern recombinant DNA

techniques are possible, as are competition studies between inactive and active chromatin, for HMG 14 or 17. Ultimately, these proteins must be functionally tested by addition to an in vitro transcription system containing chromatin. Only then, may the effects of HMG protein on polymerase recognition, initiation, and active gene structure, be assessed.

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