**AN ABSTRACT OF THE THESIS OF**

Feng Dong for the degree of Doctor of Philosophy in Biochemistry and Biophysics presented on July 13, 1990.

Title: Towards the Understanding of the Function of the Histone "Tails" with Respect to the Structure, Stability, and Function of Chromatin

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Abstract approved: _____
Dr. Kensal E. van Holde

By using immobilized trypsin, I have been able to prepare well-defined, stable trypsinized nucleosomes. The difficulties of lacking of control in the extent of trypsinization, which were encountered in previous studies with the use of free trypsin, have been eliminated. The nucleosomes and oligonucleosomes prepared by immobilized trypsin are suitable for biochemical and biophysical studies to analyze the function of the histone N-terminal regions ("tails"), which are removed by trypsin treatment, on chromatin structure and stability.

Studies were first conducted using the trypsinized nucleosome core particles to examine the role of the histone tails in the stabilization of the nucleosome core particle. While it was found that these tails have little effect on either the nucleosome dissociation or the conformational transition in salt, they play a very important role in determining thermal stability of the

nucleosome. The differential effects of selective removal of these tails also provided more insight about histone-DNA interactions in the nucleosome core particle.

Experiments have also been carried out to investigate the change in structure and hydration of nucleosome core particles which may be associated with the salt-dependent conformational transition. Changes in the tertiary structures are suggested to be responsible for the salt-dependent transition.

Roles of the histone tails in determination of nucleosome positions along specific DNA sequences were examined by analysis of nucleosome positioning on a specific eukaryotic gene sequence (*Lytechinus Variegatus* 5S rRNA gene) after *in vitro* nucleosome reconstitution with native and trypsinized histone octamers. Data obtained indicate that the histone tails are not required for nucleosome positioning. Results also seem to restrict the portions of histones which are responsible for determining nucleosome positions to the globular regions of $(H3/H4)_2$ tetramer, and possibly H2B. Studies with different DNA templates strongly suggest that the most important determinants of nucleosome positioning are the mechanical properties (such as bending and flexibility) of the DNA molecule.

Taking together, it seems that the N-terminal tails of the histones may play roles in stabilizing both nucleosome structure and the higher-order structure of chromatin.

# TOWARDS THE UNDERSTANDING OF THE FUNCTION OF THE HISTONE "TAILS" WITH RESPECT TO THE STRUCTURE, STABILITY, AND FUNCTION OF CHROMATIN

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## DEDICATION

This thesis is dedicated to my parents, to my parents-in-law, especially to my wife, Xiaokui Jin, and my son, James, for their love, understanding, encouragement, and unfailing support both material and spiritual.

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## Table of Contents

## List of Figures

## List of Tables

## List of Abbreviations

$A_{260\,nm}$: absorbance at 260 nm wavelength

bp: base pair

Ci: curie

CD: circular dichroism

CM-Sephadex: carboxylmethyl Sephadex

DTT: dithiothreitol

EDTA: ethylenediaminetetraacetate

EtBr: ethidium bromide

hr: hour

kcal: kilocalorie

min: minute

mn'ase: micrococcal nuclease

NMR: nuclear magnetic resonance

$OD_{260nm}$: optical density at 260 nm wavelength

PCA: perchloric acid

PMSF: phenylmethylsulphonyl fluoride

rpm: revolution per minute

rRNA: ribosomal RNA

S: Svedberg (unit of sedimentation coefficient)

SDS: sodium dodecyl sulfate

$s^{\circ}_{20,w}$: sedimentation coefficient in water at 20 °C

Tris: tris(hydroxymethyl)aminomethane

# TOWARDS THE UNDERSTANDING OF THE FUNCTION OF THE HISTONE "TAILS" WITH RESPECT TO THE STRUCTURE, STABILITY, AND FUNCTION OF CHROMATIN

## Chapter I. Introduction

### A. Chromatin Structure

Eukaryotic chromosomes are organized into arrays of repeating subunits (nucleosomes) consisting of about 200 bp of DNA and two each of the four core histones. These repeating subunits are composed of nucleosome core particles and variable lengths of spacer (linker) DNA located between them. These arrays subsequently fold into higher order structures in the presence of lysine-rich histones. Under the electron microscope, extended chromatin appears as a beaded fiber. While the lengths of linker DNA in the chromatin are different depending upon the source of organisms, the nucleosome core particles from different species are uniform structures. When chromatin is briefly digested with a non-specific endonuclease such as micrococcal nuclease or staphylococcal nuclease, particles containing multiples of 160-240 bp DNA are yielded. Such particles consisting of several nucleosome core particles and linkers are known as oligonucleosomes. If digestion is extended, oligonucleosomes are cleaved into

mononucleosomes and then eventually into nucleosome core particles containing 146 bp DNA which correspond to the beads observed under the electron microscope. The nucleosome core particle, in which a histone octamer consisting of two each of the four histones H2A, H2B, H3 and H4 is wrapped around with 1.75 turns of DNA, is the fundamental structure of eukaryotic chromatin [Shaw *et al.*, 1976; Felsenfeld, 1978; McGhee and Felsenfeld, 1980a]. The particle is cylinder-shaped with dimensions of about 5.5 nm high by 11 nm in diameter, and has a pseudo-dyad axis through the center of the histone octamer and the middle of the 146 bp DNA.

Although the formation of nucleosomes shortens the length of DNA several-fold, the packaging of large amount of genetic material in eukaryotes requires compaction to much higher levels. The next stage of compaction is achieved by wrapping the bead and thread structure into a 30-nm fiber with about six nucleosomes per turn. This 30-nm solenoide structure then supercoils further into 200-nm fibers which are observed in both metaphase chromosomes and in the nuclei of non-dividing cells. The compaction achieved by the several levels of chromatin structure makes it possible to store a vast amounts of genetic information in a nucleus. In this way, $6 \times 10^9$ nucleotides can be packaged in a human cell nucleus.

## B. Nucleosome as a Dynamic Structure in Solution — Stability, Dissociation and Reassociation

The different levels of chromatin structure in eukaryotes not only provide the way in which large amounts of genes can be compacted into a nucleus, but may play a major role in the control of gene expression as well. Since the nucleosome is the fundamental structure in chromatin, the study of nucleosome structure and function is crucial for understanding the processes in which eukaryotic gene transcription and replication occur.

Changes in environmental conditions, such as pH, ionic strength, and temperature, can produce several kinds of effects on nucleosomes. When the ionic strength is increased over a moderate range (0 - 0.7 M NaCl), nucleosome core particles undergo an apparent loss of stability as evidenced by partial dissociation [Yager and van Holde, 1984]. At the same time, a conformational transition of the remaining undissociated core particles occurs accompanying the partial dissociation. This conformational change has been observed using different biophysical means including hydrodynamic [Ausió *et al.*, 1984; Yager and van Holde, 1984], NMR [Walker, 1984], fluorescence [Daban and Cantor, 1982], and circular dichroism [Yager; 1984; Ausió and van Holde, 1986] techniques. The dissociation process of nucleosome core

particles has been studied and characterized as a reversible equilibrium of dissociation and reassociation [Ausió *et al.*, 1984; Yager and van Holde 1984; Yager, McMurray and van Holde, 1989].

Partial dissociation and conformational changes are two effects brought about by the increase in salt concentration (ionic strength) alone. Changes in environmental conditions other than ionic strength also produce significant effects. The presence of denaturing chemicals such as urea and SDS, or changes in pH of the environment cause nucleosome core particles to dissociate in different ways (see below). Since nucleosome core particles can be reassembled from the subnucleosomal structures, as well as from DNA and histones which are totally dissociated, these different ways of nucleosome disassembly and reassembly provide very useful tools in studying chromatin structure and function. The most commonly used way for reconstituting nucleosomes is to start with a DNA template and the histone octamer, as described in detail by Tatchell and van Holde (1977). Under high concentration of salt (2 M NaCl), histones and DNA can be separated completely; the totally dissociated histone octamers and DNA can then be reconstituted by a gradient dialysis process with decreasing salt concentrations until salt is completely removed. The reconstituted particles obtained in such a way have been

shown to maintain identical physical properties (histone content, circular dichroism, sedimentation coefficient, and thermal denaturation profiles) as the native particles. In addition, biochemical analysis suggested a structure indistinguishable from native particles, as evidenced by trypsin, DNase I, and micrococcal nuclease digestion patterns. Another very useful reconstitution method is to reassemble nucleosomes by adding H2A-H2B dimers to the subnucleosomal structure consisting of H3-H4 tetramers which remain bound to DNA [Sibbet and Carpenter, 1983]. In the presence of 0.35 M NaCl and 4 M urea, nucleosomes dissociate in a way which leaves this subnucleosomal structure and frees H2A-H2B dimers. By removing urea and salt gradually, the nucleosomes can be reassembled. The advantage of this method is that, by purifying the two components and replacing one of them with a modified or naturally occurring counterpart, nucleosomes containing selectively modified histone domains can be obtained. Such nucleosome structures may be very helpful for understanding the functions of specific histone domains. Nucleosomes can be reconstituted by other procedures as well, such as the method of Stein *et al.* (1979), and the DNA exchange method [FitzGerald and Simpson, 1985; Moyer *et al.*, 1989].

Nucleosomes also undergo a denaturation process upon the increase of environmental temperature. The mechanism of thermal denaturation of nucleosome core particles has

been studied by several research groups [Weischet *et al.*, 1978; Seligy and Poon, 1978; Simpson, 1979; Bryan *et al.*, 1979; Cowman and Fasman, 1980; McGhee and Felsenfeld, 1980b]. An example of nucleosome thermal denaturation curves is shown in Figure I-1 (dotted curve). A fairly good understanding of the thermal denaturation has been achieved by these studies: at low salt concentrations, the melting process can be resolved into two transitions — The first transition in the melting involves the release of about 20 to 30 base pairs at each end of the DNA [Simpson, 1979], while the second transition which melts at a higher temperature (about 10 °C higher than the first transition) reflects the melting of the rest of DNA.

## C. Salt-dependent Nucleosome Conformational Transition at Moderate Ionic Strengths

Upon increasing the ionic strength within a moderate range (up to 0.7 M NaCl), two effects on nucleosomal core particles are produced: a fast conformational change and a slower partial dissociation to free DNA and histones (see section I.B. above, and also Yager and van Holde, 1984). Although the mechanism for the partial dissociation has been fairly well investigated and understood, the detailed molecular mechanisms for nucleosome conformational transition still remain unclear. Many studies have been

Figure I-1. Comparison of thermal denaturation curves of normal and hyperacetylated nucleosome core particles. The nucleosome thermal denaturation process is visualized by plotting the excess in heat capacity (the derivative of hyperchromicity, dh/dT) versus temperature. (from Ausió and van Holde, 1986)

(·····), non-acetylated nucleosome core particles from HeLa cells;

(———), hyperacetylated core particles from HeLa cells;

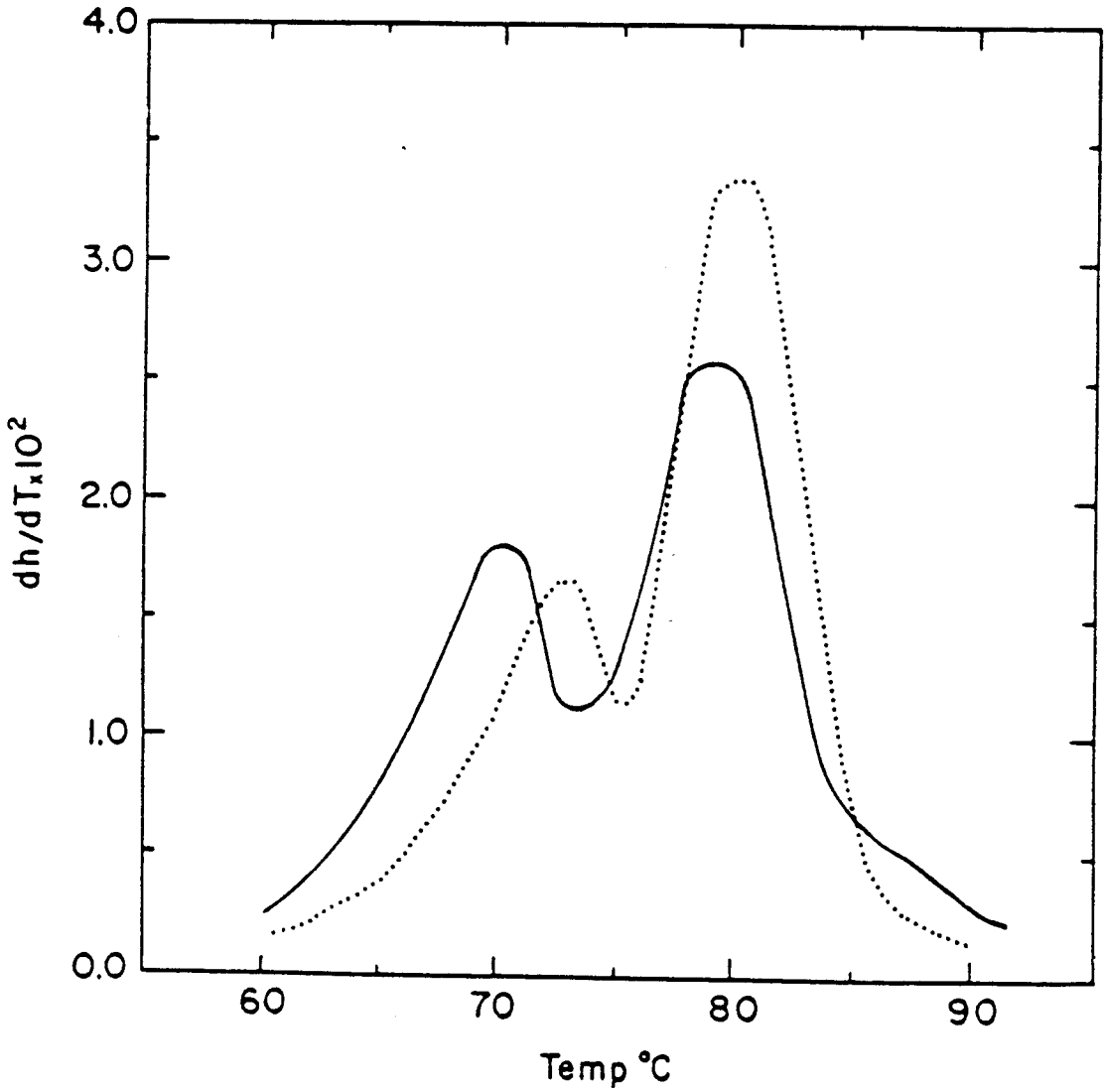


Figure I-1

carried out and several models have been proposed for this process; however, they have shown that unambiguous evidence for any of these models is extremely difficult to obtain.

Initial experimental attempts to understand this process, which were carried out by using fluorescent labels attached to the SH groups of histone H3, postulated a model for conformational transition as an extensive unfolding of the nucleosome [Dieterich *et al.*, 1979]. However, it was shown later that the cross-linking of the same reactive groups by copper phenanthroline does not stop the conformational transition. Such an observation suggests that the conformational change must be more subtle than an extensive unfolding mechanism [Ausió *et al.*, 1984]. Another model postulated to account for this conformational change proposed that the release of DNA ends from the core particle was responsible for the transition. Yet further studies using small angle X-ray scattering [Greulich, Ausió and Eisenberg, 1985] or neutron scattering [Ramakrishnan, Yager and van Holde, unpublished data] suggested that the radius of gyration of the DNA around the nucleosome core remains unchanged during the conformational transition. These results strongly argue against the mechanism of releasing of DNA ends. As an alternative, it has been assumed that the release of the highly positively-charged amino terminal regions of the histones (the histone "tails") could be responsible for the salt-dependent

conformational transition. Indeed, several lines of information suggested that these histone N-terminal regions might undergo structural changes. It has been shown by using proton NMR spectroscopy that the mobility of these histone "tails" increases with the ionic strength [Cary, Moss and Bradbury, 1978] and that they become released from tight interactions with the DNA when the salt concentration is increased from 0.2 to 0.6 M NaCl [Walker, 1984]. Nevertheless, whether the release of the unstructured regions of the histones are responsible for the salt-dependent changes in the hydrodynamic parameters of the nucleosome core particle still remains to be proven.

## D. Roles of the Histone "Tails"

### 1. Biological Roles of Histone Modifications

There is much evidence suggesting that histone modifications may have very important effects on the structure and function of chromatin. Several kinds of histone modifications have been found, including acetylation [Allfrey, 1977, 1980; Doenecke and Gallwitz, 1982], methylation [Allfrey, 1979; Paik and Kim, 1980], phosphorylation [Smith, 1982; Langan, 1978; Johnson and Allfrey, 1978; Hohmann, 1983], ADP-ribosylation [Hayashi and Ueda, 1977; Smulson and Sugimura, 1984], glycosylation [Levy-Wilson, 1983], as well as covalent linkage with the

peptide ubiquitin [Goldknopf and Busch, 1978; Busch and Goldknopf, 1981]. For a detailed review of the salient features of histone modifications, see van Holde, 1988, pp. 111-148. While each of these modifications is a complicated issue, involving different processes of modification and regulation, the general characteristics are common. All the modification reactions take place mainly in the nucleus, and are usually associated with reactions that reverse the modification processes. As a result, the overall degree of modification is controlled by the relative rates of modification and removal of the modifying groups, and therefore, determined by the steady state of the two reactions in opposing directions. It is possible that histone modifications assert their effects on chromatin function by changing chromatin conformation and stability. The strength of such effects would be therefore controlled by the level of modifications.

Each type of modification occurs by covalent addition of modifying groups onto specific chemical groups of certain amino acid residues. The modifying group and the target groups of each type of histone modification are summarized in Table I-1. An interesting finding is that almost all of the modifications in the core histones (H2A, H2B, H3, and H4) occur on the N-terminal "tails", which are the highly lysine-rich N-terminal regions of these core histones. As can be seen in Table I-1, histone

**Table I-1. Types of Histone Modifications**

| Modification | Modifying group | Target group |
|---|---|---|
| Acetylation | $CH_3-\overset{\overset{O}{\|\|}}{C}-$ | N-terminal serine, lysine |
| ADP-ribosylation | ADP-ribose | glutamate, carboxyl terminal; also maybe arginine |
| Glycosylation | fucose, mannose | unknown |
| Methylation | $CH_3-$ | Mainly lysine; Histidine, arginine, glutamate and aspartate are also possible |
| Phosphorylation | $-PO_4^{2-}$ | serine, threonine, histidine; possibly lysine and arginine |
| Ubiquitination | polypeptide chain, added by C-terminal residue | $\epsilon$-amino group of lysine the amino group becomes covalently linked with the carboxyl terminal residue of ubiquitin |

modifications always occur on one of a few amino acid residues including Lys, Ser, Arg. Among them, lysine residues are the most frequently modified and can be modified in several different ways. Because the amino acid compositions of the histone N-terminal regions of all the four kinds of core histones are highly lysine-rich, these histone "tails" provide abundant sites for histone modifications, especially for acetylation (see van Holde, 1988, pp. 111-119). It should also be noted that the large amount of lysine residues is not the only reason for the high degree of histone modification — the fact that the "globular" regions of the core histones also contain lysine residues but are not modified indicates that both the availability and the topological positions (whether on the surface or inside the histone core) of these lysine residues are important for modification. It is well expected that the large number of positive charges on the non-structural, mobile peptide chains of these histone "tails" may give them unique properties in stabilizing chromatin structure by interactions with the DNA backbone, as well as in destabilizing chromatin structure when the charges become neutralized upon histone modifications.

Among the several kinds of histone modifications, the one which has received extensive research interest during the past few years is histone acetylation [Allfrey, 1977, 1980; Doenecke and Gallwitz, 1982; Ausió and van Holde,

1986]. Because of the correlation between histone hyperacetylation and active gene processes (such as gene transcription, histone replacement, and DNA replication), it has been believed that histone acetylation may play an important role in the regulation of gene activity through its effects on chromatin structure and stability.

Acetylation is the most frequently occurring modification of the core histones. As was noted from the amino acid sequence of the histone N-terminal regions, nearly 70% of the modifiable sites on the N-terminal "tails" are lysine residues. These lysine residues, as well as the serine residues on the N-termini of H2A and H4, provide numerous sites for acetylation. During the past few years, histone acetylation has been studied by several research groups, and it has been shown that maximal acetylation of these sites could markedly reduce the net positive charges on the N-terminal domains, therefore weakening their ability to interact with DNA [van Holde, 1988, pp. 112-117]. It has also been shown that such modification can have comparable effects on the nucleosome core particles to that of proteolytic cleavage which removes the entire "tails" [Simpson, 1978; Bode *et al.*, 1980, 1983; Bertrand *et al.*, 1984].

## 2. Structural Roles of the Histone "Tails" in the Stabilization of Chromatin

There has long been interest in the question of what role the N-terminal "tails" of the core histones may have in stabilizing the nucleosome core particle structure. Since these regions contain high concentrations of positively charged residues and are the major targets of histone acetylation and phosphorylation, as discussed above, a significant role in structural stabilization might well be expected. Studies conducted by NMR techniques [Cary *et al.*, 1978] suggested that partial release of these "tails" from a rigid conformation occurs in the region of salt concentration (0.3 - 0.6 M) in which the salt-dependent nucleosome conformational transition (see section I.C. above) is observed by hydrodynamic [Ausió *et al.*, 1984; Yager and van Holde, 1984], NMR [Walker, 1984], circular dichroism [Yager, 1984; Ausió and van Holde, 1986], and fluorescence [Daban and Cantor, 1982] techniques.

The effects of acetylation at the histone "tails" on nucleosome structure and stability have also been investigated by many attempts; however, results from different laboratories were not consistent. This is probably because unequal levels of acetylation have been achieved in various studies (see van Holde, 1988). A later

study utilizing fractionated HeLa core particles which had extremely high levels of acetylation [Ausió and van Holde, 1986] provided valuable information about the characteristics of acetylated nucleosome core particles. These hyperacetylated nucleosome core particles exhibit a lower sedimentation coefficient ($s_{w,20}$ ~ 9.5 S in 0.6 M NaCl as compared to ~11.0 S in 10 mM NaCl) and a higher molar ellipticity in circular dichroism compared to the core particles with low levels of acetylation. While acetylation on the histone "tails" does not have obvious effects on either the salt-dependent nucleosome conformational changes or nucleosome dissociation, it does have an interesting effect on the thermal denaturation of the core particles. As indicated by Figure I-1, the thermal denaturation curve of the hyperacetylated core particles shows a substantial increase in the amount of DNA melting at the lower transition, as well as decreases in the melting points ($T_m$) of both of the two transitions. Although very interesting, the data are not adequate to suggest a mechanism underlying the results obtained.

There have also been several attempts to assess the importance of the N-terminal regions of the histones by the use of trypsin to selectively remove these portions of the protein from core particles or chromatin. When free histones are digested by trypsin, the digestion is more or less random and rapid because the large number of lysine

and arginine residues provide numerous sites for cleavage. However, when the nucleosome core particles are digested by trypsin under carefully controlled conditions, well-defined products can be obtained in which specific portions of the N-termini of each of the core histones (and a bit of the C-terminal regions of the histones H3 and H2A) are removed (see Böhm and Crane-Robinson, 1984, for an extensive review). Allan *et al.* (1982) have shown clearly that removal of these tails from histones in chromatin inhibits the folding of the chromatin strands into the higher-order solenoidal structure. However reports on the effects of trypsinization on structure or stability at the nucleosome core particle level have been conflicting (see Results section of Chapter II for more details). Some workers reported only minor changes in properties and behavior of trypsinized nucleosomes, while others claimed large effects. It seems that such discrepancies reflect a lack of control of the digestion processes, possibly an effect of residual protease (see Results section of Chapter II). Despite the conflicting results reported before, to study the roles of histone "tails" on chromatin structure and stability by trypsinization remains an interesting topic. If a controlled trypsinization can be achieved to prepare precisely defined trypsinized nucleosomes, such trypsinized particles should provide a suitable model system for understanding not only the roles of the histone "tails" in

stabilizing chromatin structure, but also the mechanism underlying the correlation between modification of these tails and gene activity.

## E. Nucleosome Positioning and Its Possible Physiological Significance in Gene Regulation

### 1. Arrangement of Nucleosomes along DNA Sequences

When the DNA sequence is considered in chromatin structure, one of the questions asked immediately is how the nucleosomes are arranged along the DNA strand with respect to its sequence. There are at least five different ways in which nucleosomes might be arranged along a specific DNA sequence [van Holde, 1988]. These different ways of nucleosome arrangement on DNA sequence are demonstrated as simplified models in Figure I-2 [Figure taken from van Holde, 1988, p. 290; definitions are the same as used by van Holde, 1988]. Briefly, they can be summarized as:

(a). Random arrangement - nucleosome arrangement has no specific relationship either to DNA sequence or to the positions other nucleosomes occupy.

(b). Uniformly spaced - neighboring nucleosomes are separated by a uniform DNA linker length which might result from the presence of specific linker histone or non-histone

Figure I-2

Examples of possible kinds of linear arrangements of nucleosomes on specific DNA sequences. (from van Holde, 1988)

In each case, two copies of the same region of the DNA are shown to indicate that in some arrangements nucleosomes occupy the same sequences whereas in others they do not.

Arrangements are: (a), random; (b), uniformly spaced; (c), quantized spaced, blocks correspond to H1 or other linker-associated proteins; (d), positioned, marks indicate specific DNA sites; (e) phased, dotted lines indicate junctions of DNA repeats.

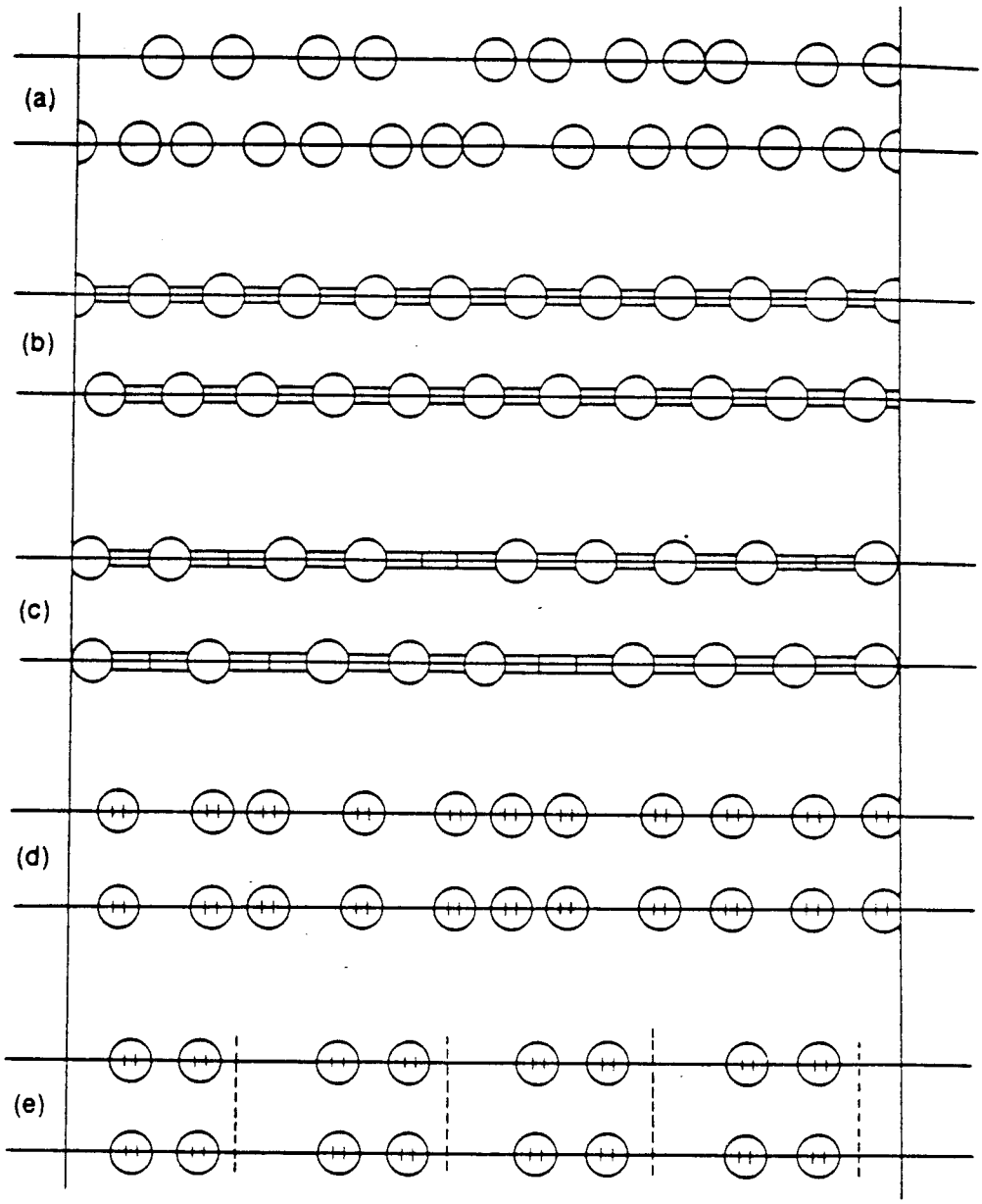


Figure I-2

proteins. Unless one of the nucleosomes in the array is positioned on a specific sequence on DNA (this might happen due to either the existence of specific DNA sequence strongly interacting with histones or the direction of some specific proteins, see below), the arrangement of the nucleosome array may not be fixed on a specific DNA sequence.

(c). Quantized spaced - nucleosomes are separated with a number of defined linker lengths. This could be achieved by a limited set of linker-associated proteins.

(d). Positioned - nucleosomes occupy specific sequences on the DNA molecules. A number of factors may direct the nucleosomes to specific sequences on DNA, such as histone-DNA interactions and interaction of nucleosome structure with sequence-specific non-histone proteins (see below for more details). This term also refers to the situation called alternative or multiple positioning, where a small number of positions are available to each nucleosome.

(e). Phased - Nucleosomes occupy the same specific positions on each repeat of a repeating DNA sequence. When nucleosomes are phased, both DNA sequence and nucleosome structures, as well as the positions the nucleosomes occupy, will repeat at the same interval (phase).

Although many different images of nucleosome arrangements can be proposed theoretically, research in the

past clearly indicates that nucleosome arrangements are well organized in all the eukaryotic genomes. Based on the results from the extensive studies in the last few years, it is well established that nucleosomes can be positioned over specific DNA sequences [Simpson, 1986; for a review]. Since the formation of nucleosomes on a specific gene sequence, as well as the interactions between such positioned nucleosomes and regulatory proteins, may influence the function of the gene, to study nucleosome positioning and the factors which affect nucleosome positioning is of special interest for understanding eukaryotic chromosome structure and the relationship of that structure to the regulation of gene active processes such as gene transcription and replication [Travers, 1987].

## 2. Studies of Nucleosome Positioning on Specific Gene Sequences

Nucleosome positioning has been examined both *in vivo* and *in vitro*. Several examples have been reported which demonstrate that positioned nucleosomes can be obtained by *in vitro* reconstitutions on short DNA sequences such as the sea urchin 5S rRNA gene sequence [Simpson and Stafford, 1983; FitzGerald and Simpson, 1985; Drew and Calladine, 1987; Moyer *et al.*, 1989], mouse satellite DNA [Böck *et al.*, 1984; Linxweiler *et al.*, 1985], *E. coli* DNA sequences

[Ramsay *et al.*, 1984], and plasmid DNA fragments [Drew and McCall, 1987]. Several methods for the identification of nucleosome positioning *in vitro* have been used, including DNase I footprinting [Simpson and Stafford, 1983], exonuclease III protection assay [Neubaver and Hörz, 1989], and restriction endonuclease mapping of nucleosome-bound DNA [Hansen *et al.*, 1989]. *In vivo* nucleosome positioning on some gene sequences has been reported too, by the method known as indirect end-labeling [Nedospasov and Georgiev 1980; Wu, 1980] or by the technique of molecular cloning of mononucleosomal DNA sequences followed by DNA sequencing of the cloned 146-bp DNA fragments [Böck *et al.*, 1984; Satchwell *et al.*, 1986].

A number of different mechanisms of nucleosome positioning have been proposed. One of the mechanisms proposed early in the attempts to understand the problem was that positioned nucleosomes may be formed by aligning nucleosomes with respect to a replication origin at regular intervals immediately after replication. The existence of irregular spacing and long linker DNAs of the nucleosomes in yeast minichromosomes, however, argue against such a mechanism [Thoma and Simpson, 1985; Thoma, 1986; Thoma and Zatchej, 1988]. It has also been proposed that some DNA sequence-specific proteins may affect nucleosome positioning *in vivo*, through interactions with specific DNA sequences and subsequently direct nucleosome formation onto

the same or a nearby DNA sequence. However, *in vitro* reconstitutions of mononucleosomes and oligonucleosomes suggest that positioned nucleosomes can be formed by DNA and histone octamers in the absence of other proteins. Another proposed mechanism in which nucleosome positioning might be influenced by the flanking boundaries established by neighboring nucleosomes or other site-specific proteins [Kornberg, 1981; Thoma and Zatchej, 1988]. This is supported by the investigation of Lohr *et al.* [Lohr, 1984; Lohr and Torchia, 1987] which suggested the possible existence of such specific proteins *in vivo*, since the nucleosomes are found to occupy different positions *in vitro* or *in vivo*. However, reconstitution of nucleosomes onto short DNA templates *in vitro* indicates that even if boundaries can affect nucleosome positioning *in vivo*, nucleosome positioning can also occur when such boundaries do not exist [Simpson and Stafford, 1983; McGhee and Felsenfeld, 1983]. In many studies, the *in vitro* reconstitution results strongly suggest that the fundamental determinants of nucleosome positioning reside in histone-DNA interactions and/or DNA sequence-dependent mechanical properties such as bending and flexibility [Trifonov, 1980; Drew and Travers, 1985; Satchwell *et al.*, 1986; Travers, 1987; Shrader and Crothers, 1989].

An example of sequence-dependent nucleosome positioning that has generated considerable interest is the

nucleosome formation on the sea urchin 5S rRNA gene sequences. Simpson and Stafford (1983) showed, by using the DNase I footprinting technique, that nucleosomes could be positioned by *in vitro* reconstitution onto a short DNA fragment containing the sea urchin 5S rRNA gene. Similar results have been reported from investigations of the effects of sequence alteration on nucleosome positioning [FitzGerald and Simpson, 1985], and carcinogen-nucleosome interaction *in vitro* [Moyer *et al.*, 1989]. However, using restriction enzyme mapping of nucleosome-bound DNA sequences, Hansen *et al.* (1989) observed multiple nucleosome positions on tandemly repeated sequences of the same 5S rRNA gene fragment, with the most preferred position occupying a sequence different from that indicated by DNase I digestion of the monomer fragment. It seems, therefore, that the positioning of nucleosomes on this sequence is more complicated than previously thought. It also remains a question whether the multiple nucleosome positioning is an inherent property of the sea urchin 5S rRNA gene sequence or a specific phenomenon because of the tandemly repeated sequences.

## F. Aims of this Research

The major focus of the work described in this thesis has been on the roles which the histone "tails" may play in

the stabilization of chromatin structure. Figure I-3 shows the strategy of the approaches which was used for the investigation reported here. The roles of the histone "tails" have been studied carefully at the following categories:

(1). On the nucleosome core particle structure and behavior in solution. Previous studies suggested possible roles of the histone "tails" in inducing the salt-dependent nucleosome conformational transition. However, the model is based on only indirect evidence (see above) and therefore needs to be tested by more direct experimental data. In order to examine the validity of this model, as well as to investigate any other roles histone "tails" may play at the nucleosome core particle level, we carried out a thorough study on the trypsinized nucleosome core particles. We also prepared and studied the "hybrid" nucleosome core particles in which either the H2A/H2B or the H3/H4 are selectively trypsinized, to address the differential effects of the H2A/H2B or H3/H4 "tails". This study is described in Chapter II.

(2). Since the research in Chapter II has shown that the release of histone "tails" cannot account for the nucleosome conformational transition in moderate salt, research has also been conducted to investigate the changes in the nucleosome core particles which are responsible for this conformational change (Chapter III). The study is

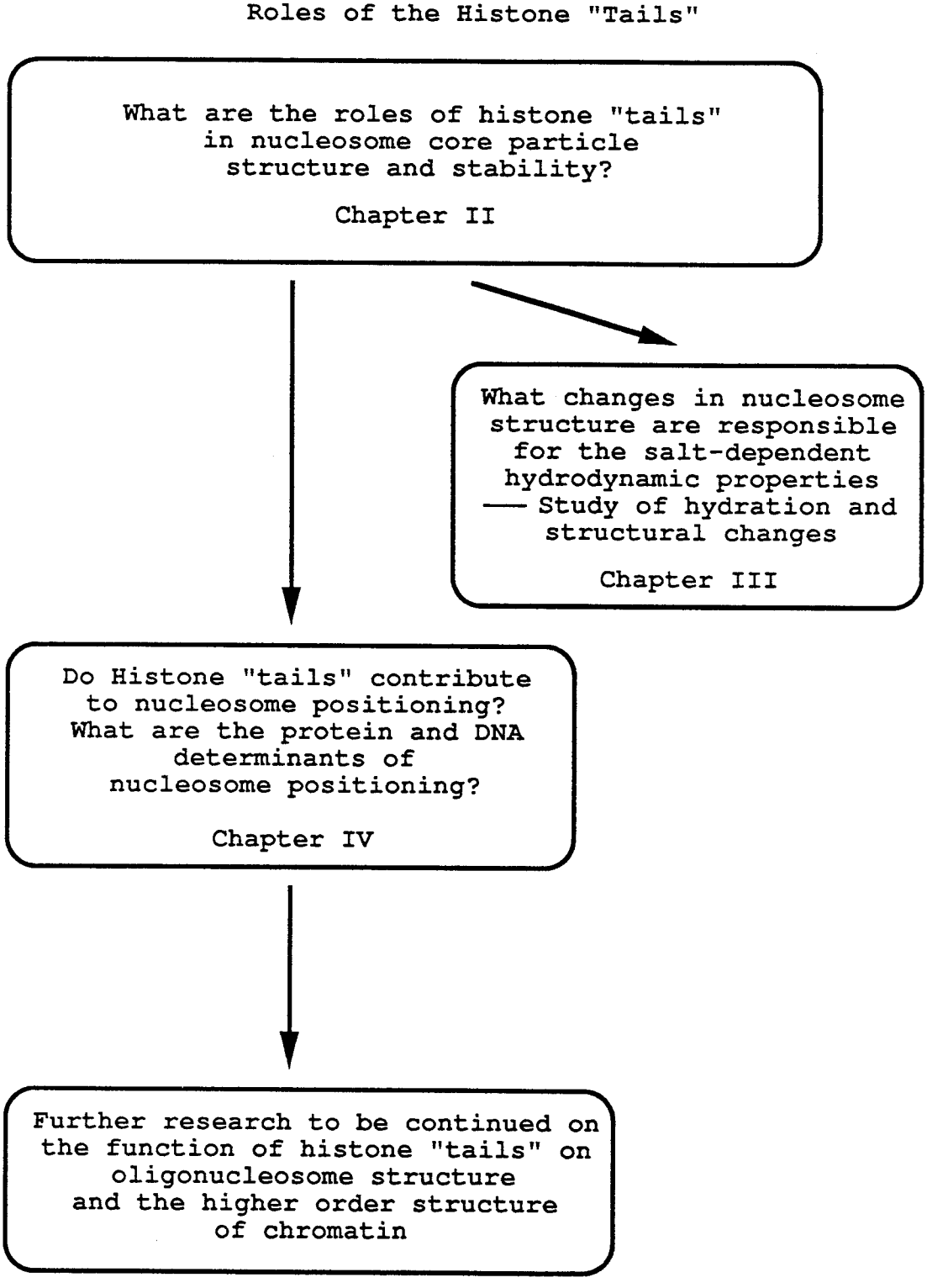


Figure I-3. Strategy of the Studies of the Function of Histone "Tails"

centered on the changes in secondary structure and hydration of the nucleosomes which associate with the conformational transition.

(3). On the nucleosome arrangement along DNA sequences. Whether histone "tails" affect the positions of nucleosomes on specific gene sequences has been an interesting question, since the answer to this question might suggest a mechanism which explains the correlation between histone hyperacetylation and active gene processes. I have investigated the effects of the histone tails on nucleosome positioning after reconstitution of native and trypsinized "tail"-free histone octamers onto a number of DNA templates containing a specific eukaryotic gene sequence, the sea urchin (*Lytechinus variegatus*) 5S rRNA gene. This research is reported in Chapter IV. DNA determinants of nucleosome positioning on this gene sequence were also examined.

# Chapter II

## Study of the Role of the Histone "Tails" in the Stabilization of the Nucleosome

## Introduction

As discussed in the Introduction (Chapter I), there has long been an interest in understanding nucleosome structure, the salt-dependent conformational transition, and dissociation in solution. The roles which the highly positively-charged histone "tails" may play on the stability and structural changes of nucleosomes have also been a focus of interest. Although there have been several lines of evidence suggesting that the release of these highly charged histone terminal regions from a "rigid" nucleosome structure into a "looser" conformation could be responsible for the nucleosome conformational transition [Cary *et al.*, 1978; Walker, 1984; see Chapter I.C. for details], the validity of this model remains to be proven.

As discussed in Chapter I, previous studies on the functions of the histone "tails" have been inconsistent. As the behavior of histone "tails" was suggested to affect nucleosome conformation and stability, it is necessary to clarify the previous unclear results on the roles of the histone "tails". It seems, at this point, a detailed and well-controlled study of the effects of histone "tails" on nucleosome core particle structure and stability becomes an important issue for the research field.

We embarked, therefore, on a very careful study of

trypsin-modified core particles, with the following criteria: (1) Proteolysis should be controlled with great care, and the products studied should be entirely free of residual active protease, and (2) We would produce "hybrid" core particles in which only one set, or the other, of H2A/H2B or H3/H4 domains was trypsinized — this is to distinguish effects involving tails of H2A/H2B or H3/H4. We have found that the proteolysis can be best controlled by the use of trypsin immobilized on glass beads. "Hybrid" nucleosomal core particles, containing specifically trypsinized H2A/H2B or H3/H4 have been prepared by a modification of the method of Sibbet and Carpenter (1983). The general plan of the preparation is shown in Figure II-1.

Using immobilized trypsin and an appropriate fractionation procedure shown in Figure II-1, we have been able to prepare, for the first time, nucleosome core particles containing selectively trypsinized histone domains. The particles thus obtained: $[(H3_T\text{-}H4_T)_2\text{-}2(H2A_T\text{-}H2B_T)]\cdot DNA$; $[(H3\text{-}H4)_2\text{-}2(H2A_T\text{-}H2B_T)]\cdot DNA$; $[(H3_T\text{-}H4_T)_2\text{-}2(H2A\text{-}H2B)]\cdot DNA$ (where T stands for trypsinized), together with the non-trypsinized controls have been characterized using the following techniques: analytical ultracentrifugation, circular dichroism, thermal denaturation and DNase I digestion. The major aims of this study were to examine the effects of the histone "tails" on the nucleosome core

Figure II-1

Schematic plan for hybrid nucleosome preparation. Histones shown by the open boxes represent native molecules whereas those shown by shaded boxes represent trypsinized histone molecules. All of the reconstitution combinations indicated by arrows have been carried out in this study.

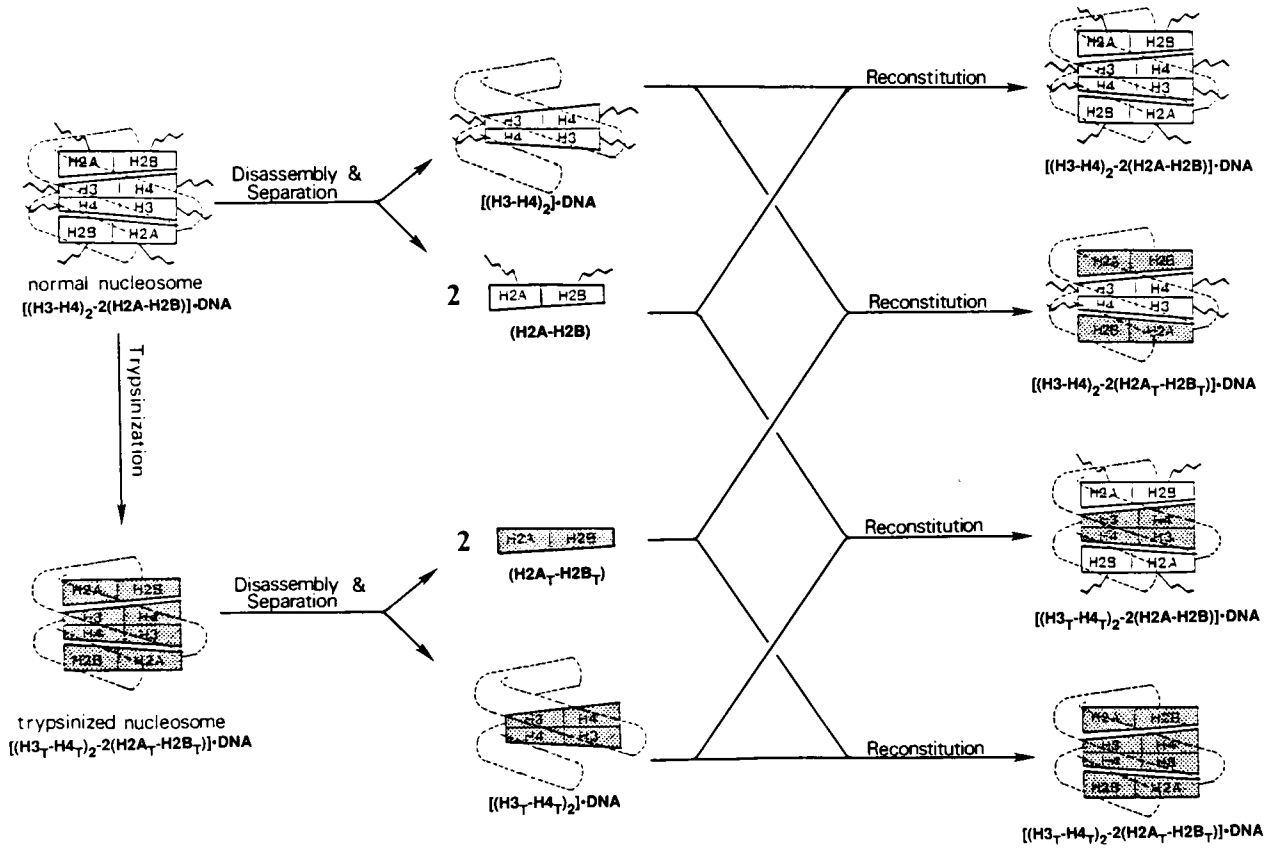
normal nucleosome
[(H3-H4)2-2(H2A-H2B)]·DNA
Disassembly & Separation
[(H3-H4)2]·DNA
2
(H2A-H2B)
Trypsinization
trypsinized nucleosome
[(H3T-H4T)2-2(H2AT-H2BT)]·DNA
Disassembly & Separation
2
(H2AT-H2BT)
[(H3T-H4T)2]·DNA
Reconstitution
Reconstitution
Reconstitution
Reconstitution
[(H3-H4)2-2(H2A-H2B)]·DNA
[(H3-H4)2-2(H2AT-H2BT)]·DNA
[(H3T-H4T)2-2(H2A-H2B)]·DNA
[(H3T-H4T)2-2(H2AT-H2BT)]·DNA

**Figure II-1**

particle in solution with respects to salt-dependent nucleosome conformational transition and nucleosome dissociation in the presence of salt. The data obtained from this analysis clearly show that nucleosome dissociation (below a salt concentration of 0.7 M NaCl) is not affected by the presence or the absence of any of the N-terminal regions of the histones. Furthermore, these histone regions make very little contribution, if any, to the conformational transition that nucleosomes undergo in this range of salt concentrations. They play, however, a very important role in determining the thermal stability of the particle, as reflected by the dramatic alterations exhibited by the melting profiles upon selective removal of these "tails" by trypsinization. The melting data can be explained by a simple model that assigns interactions of H2A/H2B and H3/H4 tails to particular regions of the nucleosomal DNA.

## Material and Methods

### 1. Preparation of Nucleosome Core Particles:

An experimental flowchart for the preparation is shown in Figure II-2. Unless otherwise mentioned, all the steps in the preparation were carried out at 0 °C on ice. All the buffers were made 0.1 mM PMSF immediately before use.

Blood from chickens (usually roosters) was collected in a beaker which contains, approximately 20% of the estimated volume for the blood (~ 100 ml blood for each chicken), an ice cold buffer of 0.15 M NaCl, 15 mM sodium citrate, 10 mM sodium phosphate, pH 7.2 (buffer I) and 50 units/ml heparin. The blood was then filtered through a funnel with a filter made of four folds of cheesecloth and immediately centrifuged for 10 min in a Sorvall GSA rotor at 3,500 rpm, 4 °C. The blood cells were gently resuspended in buffer I with the same volume as before, and were centrifuged again under the same conditions. This step was repeated once more. The pellet was then resuspended and incubated for 10 min on ice in 0.1 M KCl, 1 mM $MgCl_2$, 50 mM Tris-HCl (pH 7.5), 0.5% Triton X-100 (buffer II) in order to lyse the cells. The suspension was then transferred into 50-ml centrifuge tubes and centrifuged at 5,500 rpm in a Sorvall SS-34 rotor for 10 min at 4 °C. This step was repeated, without incubation,

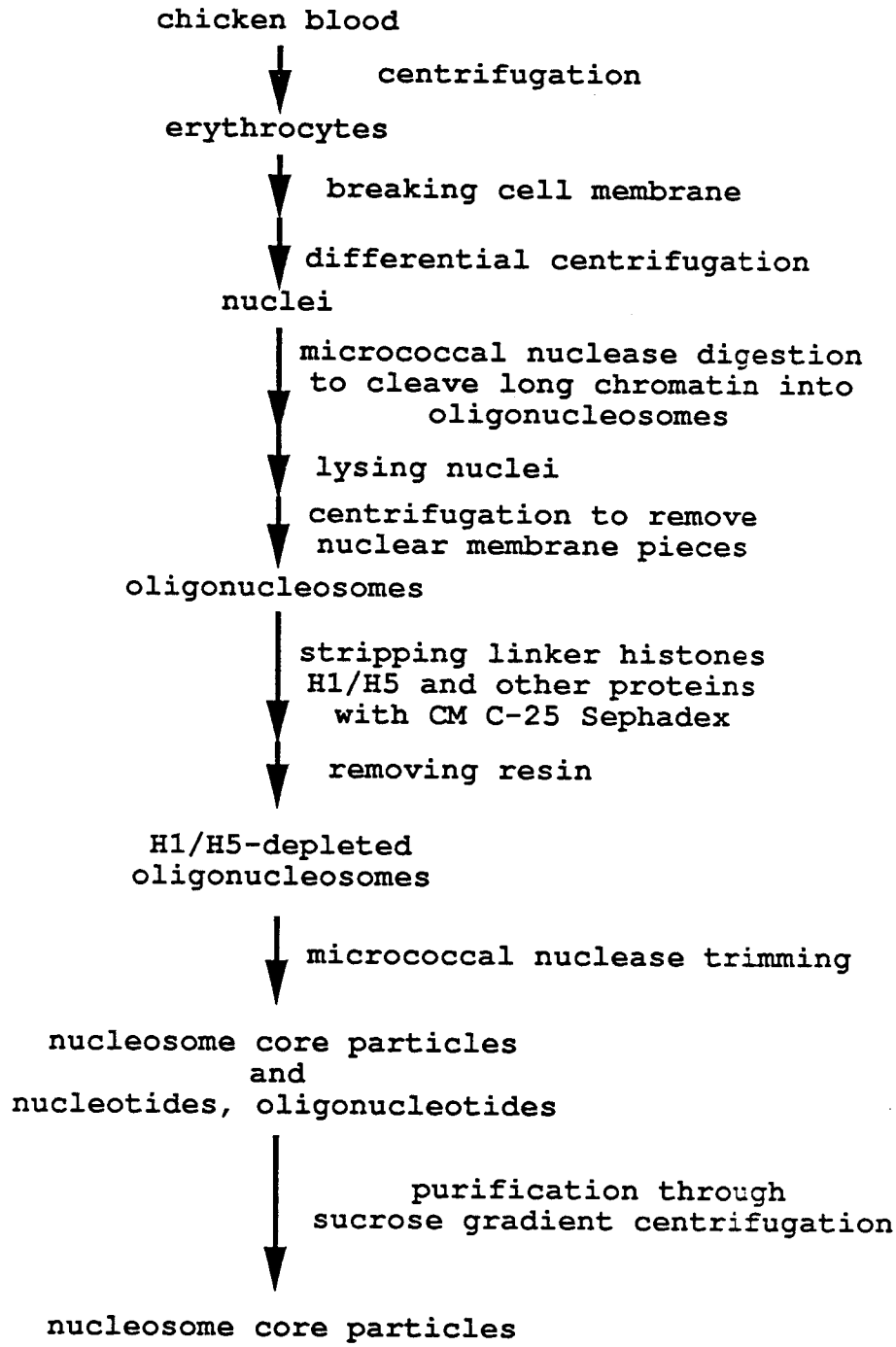


Figure II-2. Flowchart of Nucleosome Core Particle Prep.

until the pellet (chicken erythrocyte nuclei) is completely white (the step usually needs to be repeated two to four times). The pellet was then resuspended in buffer II without Triton X-100 and was centrifuged at 5,500 rpm in an SS-34 rotor for 5 min at 4 °C. After the step was repeated twice, the pellet was gently resuspended in 0.1 M KCl, 1 mM $CaCl_2$ 50 mM Tris-HCl, pH 7.5 (buffer III) and centrifuged at 5,000 rpm in an SS-34 rotor for 5 min at 4 °C. The pellet obtained was then resuspended again in buffer III and the volume was adjusted, after the $OD_{260\,nm}$ was measured by addition of 10% SDS (to 0.25%) to an aliquot of this suspension which had been lysed in the presence of 200 x volume of distilled water, so as to have an $OD_{260\,nm}$ ~ 120. This suspension of nuclei was incubated at 37 °C for 5 to 10 min and then digested with 9 units/ml micrococcal nuclease (Worthington) at 37 °C. Under such digestion conditions, chromatin is digested into oligo-nucleosomes which do not coagulate when released from nuclei. The digestion was stopped by addition of 0.5 M EDTA to a final concentration of 10 mM and the suspension was then immediately centrifuged at 10,000 rpm in an SS-34 rotor for 5 min at 4 °C. The nuclei pellet was resuspended in 0.25 mM EDTA, pH 7.5, using half of the volume used for the digestion and the suspension was gently stirred with a magnetic stirrer for 1 hr at 4 °C. The nuclear lysate was then centrifuged at 8,000 rpm in an SS-34 rotor for 20 min

(4 °C) and the supernatant was made 0.35 M NaCl by dropwise addition of a 4 M NaCl solution under sufficient stirring to minimize the sudden change of salt concentration in any portion of the chromatin solution. Sixty mg of CM-Sephadex C-25 was then added per milliliter chromatin solution, and the suspension obtained was stirred slowly for 2 hrs to strip the histones H1 and H5 as well as other non-histone proteins. The suspension was then centrifuged at 10,000 rpm in an SS-34 rotor at 4 °C for 10 min to remove the resin. The centrifugation was usually repeated in order to remove the resin completely. The clear solution thus obtained was then dialyzed overnight against 25 mM NaCl, 1 mM $CaCl_2$, 10 mM Tris-HCl, pH 7.5 (buffer IV). The chromatin solution was then adjusted to have a concentration of 3 mg/ml (DNA concentration), and digested with 90 units/ml micrococcal nuclease at 37 °C for appropriate time into nucleosome core particles. The proper time of digestion varied with different preparations; therefore, the appropriate time was determined by a pilot digestion each time in an individual preparation. Each pilot digestion was carried out by using an aliquot of the sample to be digested, and the time-course of the pilot digestion was monitored by 4% polyacrylamide gel electrophoresis of both the DNA and the native core particle. The optimum time of digestion was determined by following the narrowing of the size

distribution of the DNA during the course of digestion.

The preparative micrococcal nuclease digestion was stopped by addition of 0.5 M EDTA to 10 mM final concentration and put on ice. The nucleosome solution was then concentrated in an Amicon concentrator with an XM-50 membrane to 10 mg/ml (DNA concentration). Final purification of the nucleosome core particles was done by sucrose gradients. Sucrose gradients (5-20%) were made in 25 mM NaCl, 10 mM Tris-HCl, 1 mM EDTA, pH 7.5 (buffer V). After the solution of nucleosome core particles was loaded on top, the gradients were centrifuged in a Beckman SW-28 rotor at 25,000 rpm for 24 hrs at 4 °C. The gradients were then fractionated and the peak fractions of nucleosome core particles were pooled and dialyzed overnight against buffer V. The nucleosome solution was concentrated with XM-50 membrane in an Amicon concentrator to 10 mg/ml (DNA concentration) and stored at 0 °C on ice.

2. Trypsin Digestion of Nucleosomes

Digestions with free trypsin. Nucleosome core particles at ~ 10 mg/ml of DNA concentration (~ 200 $OD_{260\,nm}$) in buffer V without EDTA were digested with free trypsin at an enzyme/substrate (E/S) ratio of 1:1200 (W/W) at room temperature. The appropriate length of the digestion (usually 30-32 min) was established through a time-course

experiment, which was monitored with an SDS polyacrylamide gel prepared according to Laemmli (1970) (see below). An example is shown in Figure II-3.A. The reaction was stopped by addition of soybean trypsin inhibitor in a ten-fold molar excess to the enzyme.

Digestions with immobilized trypsin. Trypsin bound to DITC glass from Sigma (Sigma Chemical Company 1987 Catalog No. T-8899; DITC: diisothiocyanato) was used. Nucleosomes under the same solution conditions as above were digested at a ratio of 5 mg of glass beads per mg of nucleosome core particles. The immobilized trypsin was resuspended in buffer V without EDTA and equilibrated at 4 °C for overnight before use. Then it was thoroughly washed with buffer V without EDTA and drained. The nucleosome solution to be digested was then mixed with the glass beads and the resulting suspension was incubated under mild rotary shaking (15 ~ 25 rpm, in a vertical plane) at room temperature. As in the case of digestion with free trypsin, the optimal time of digestion (usually 60-70 min) was established with the aid of a time-course experiment (see Figure II-3.B) prior to the preparative digestion. The reaction was stopped by removal of the glass beads through filtration of the suspension through a sintered glass filter. As an additional precaution, 15 μg of soybean trypsin inhibitor was added per milliliter of the

Figure II-3

Time course of digestion of nucleosome core particles with free or immobilized trypsin. The resulting materials were analyzed by SDS-polyacrylamide gel electrophoresis.

A. - Digested with free trypsin.

B. - Digested with trypsin immobilized on DITC glass beads.

The numbers on top of each lane indicate the time of digestion in minutes. Underlined numbers represent the times of digestion selected for the final preparation of the trypsinized particles. The asterisk indicates the location of the doublet band of trypsin and trypsin inhibitor. The peptides P1, P1′, P2, P3, P4 and P5 correspond to those reported by Böhm and Crane-Robinson (1984).

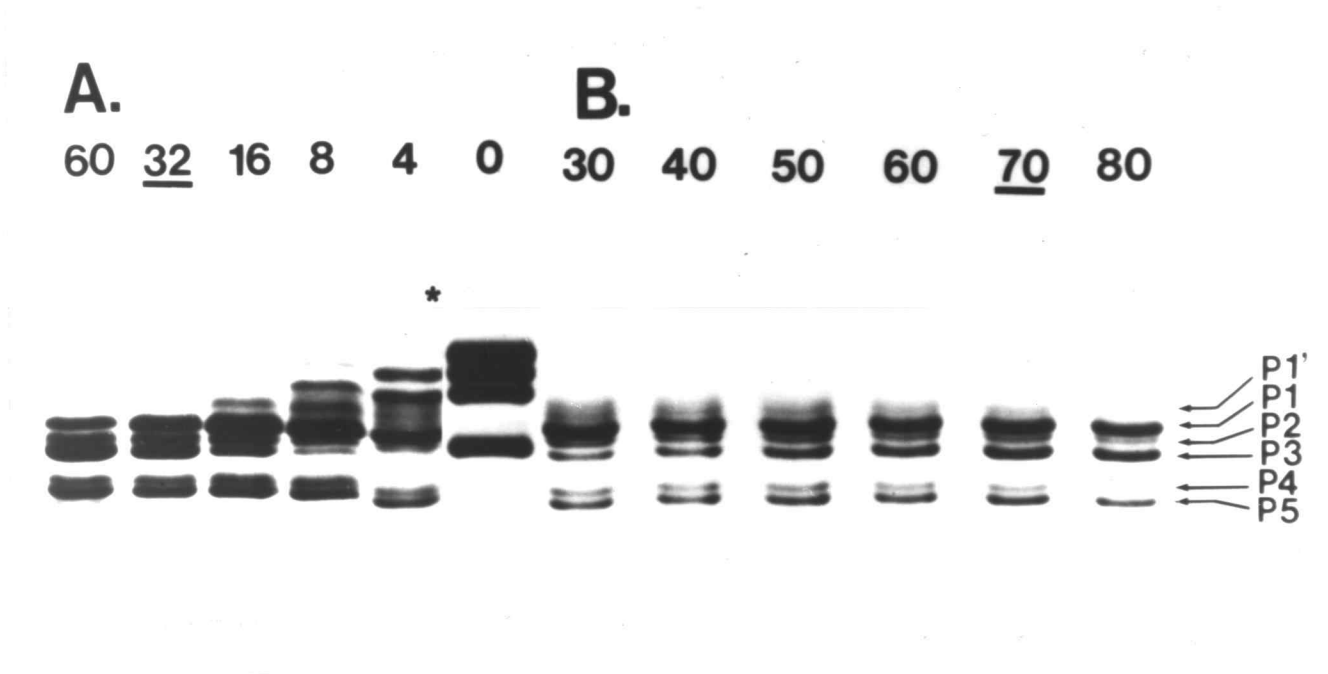


Figure II-3

clear filtrate.

Trypsinized nucleosomes obtained (both with free and immobilized trypsin) were then further purified through sucrose gradient centrifugation under the same conditions as described above for purification of the native nucleosome core particles.

3. Fractionation of Nucleosome Core Particles

Normal and/or trypsinized nucleosomes were fractionated into their subnucleosomal components [$(H3\text{-}H4)_2$]·DNA and H2A-H2B dimers] in the presence of 4M urea and 0.34 M NaCl as described by Sibbet and Carpenter (1983). To that purpose, the starting solution of nucleosome particles was mixed with an equal volume of 2x sample buffer containing 8 M urea, 0.68 M NaCl, 40 mM Tris-HCl, 2 mM EDTA, and 0.2 mM PMSF, pH 7.5. The resulting solution was applied on top of a linear 5-20% sucrose gradient in 4 M urea, 0.34 M NaCl, 20 mM Tris-HCl, 1 mM EDTA, and 0.1 mM PMSF, pH 7.5 and sedimented in a TH-641 Sorvall rotor at 40,000 rpm for 24 hr at 20 °C. The gradients were fractionated, after which the fractions were analyzed for both DNA and protein (histone) contents. Distribution of the DNA was measured by the optical density of the fractions at 260 nm, whereas that of protein was

determined by the Bio-Rad protein assay based on the $OD_{595\,nm}$ after staining protein solution with G-250 Coommassie blue. The distribution of histone components H3/H4 and H2A/H2B was visualized by a 15% SDS polyacrylamide gel [Laemmli, 1970]. Figures II-4.A and II-4.B show an example of such fractionation.

Fractions from the urea-sucrose gradient containing the $[(H3\text{-}H4)_2]\cdot DNA$ subnucleosomal structure or H2A-H2B dimers were pooled and kept at 0 °C on ice before used for nucleosome reconstitution.

## 4. Nucleosome Core Particle Reassembly

Stoichiometric amounts of the subnucleosomal particles and histone dimers obtained with the above fractionation procedure were combined in all the possible combinations, as shown in Figure II-1, so as to give the following complexes: $[(H3\text{-}H4)_2\text{-}2(H2A\text{-}H2B)]\cdot DNA$ (abbreviation = NXN), $[(H3_T\text{-}H4_T)_2\text{-}2(H2A_T\text{-}H2B_T)]\cdot DNA$ (abbreviation = TXT), $[(H3\text{-}H4)_2\text{-}2(H2A_T\text{-}H2B_T)]\cdot DNA$, and $[(H3_T\text{-}H4_T)_2\text{-}2(H2A\text{-}H2B)]\cdot DNA$, where the subindex "T" stands for trypsinized. Reconstitution was carried out through a salt gradient dialysis procedure similar to that described by Sibbet and Carpenter (1983). Immediately after mixing, the samples were dialyzed for 6 hr at 4 °C against 2 M urea, 0.34 M NaCl, 2 mM EDTA in 20 mM Tris-HCl, pH 7.5 buffer containing

Figure II-4

Sucrose gradient fractionation of partially dissociated nucleosomes.

Panel A: native nucleosome core particle.

Panel B: trypsinized nucleosome core particle.

The gradient was linear from 5 to 20% sucrose and was run at 20 oC for 24 hrs in a TH-641 (Sorvall) rotor at 40,000 rpm. The gradient contained 4 M urea, 0.34 M NaCl, 1 mM EDTA, and 20 mM Tris-HCl, pH 7.5. The shaded regions represent the DNA distribution as measured by $A_{260\,nm}$. The dashed line shows the protein distribution as determined by using the G-250 Coommassie blue Bio-Rad protein assay. The gel electrophoresis patterns show the distribution of the histone components throughout the gradient.

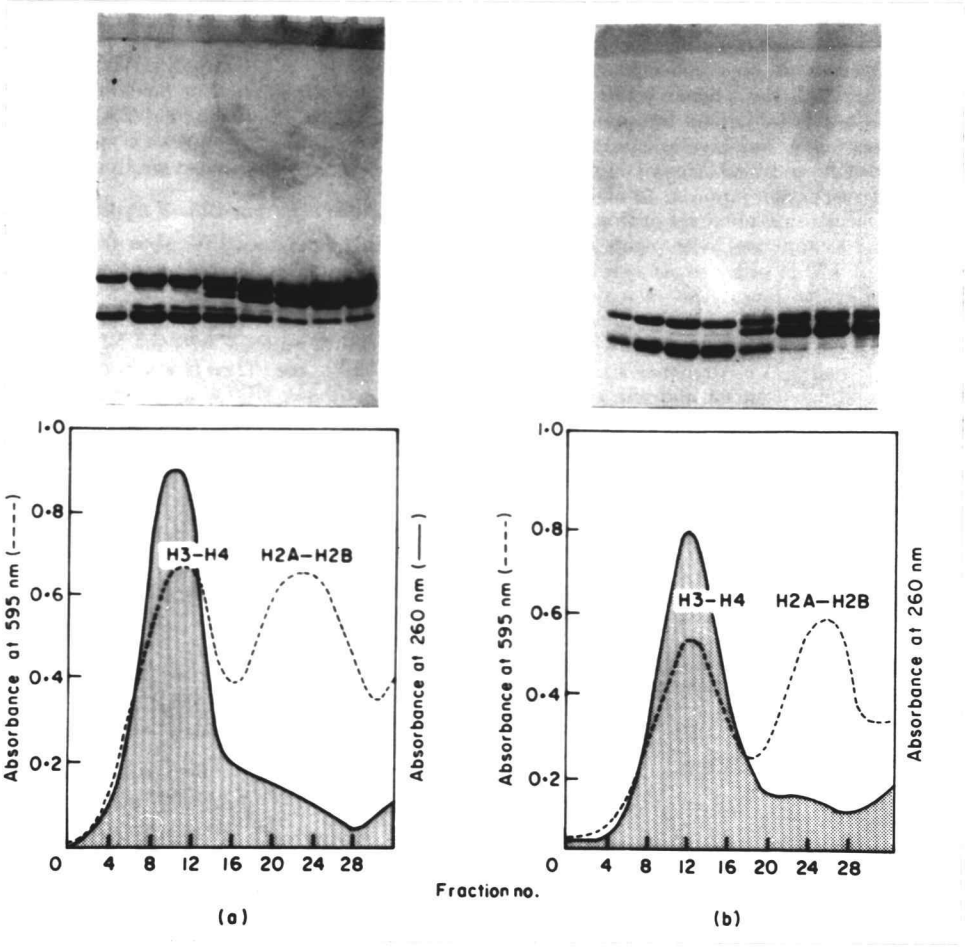
1·0
0·8
0·6
0·4
0·2
0
4
8
12
16
20
24
28
Absorbance at 595 nm (----)
Absorbance at 260 nm (——)
H3–H4
H2A–H2B
Fraction no.
(a)
(b)

Figure II-4

0.1 mM PMSF. Then they were dialyzed against the same solution but without urea for another 6 hr at 4 °C. The samples were then dialyzed overnight against buffer V containing 0.1 mM PMSF. All the dialyses were performed in Spectrapor-3 dialysis tubing (Spectrum Medical Industries, Inc., Los Angeles, CA). Finally, the samples were refractionated in 5-20% sucrose gradients in buffer V at 33,000 rpm for 24 hr at 4 °C in a TH-641 Sorvall rotor. The fractions from the middle of the nucleosome peaks were pooled, dialyzed against buffer V, and stored at 0 °C on ice for further characterization.

Besides the complexes just described, the following additional controls were also used or prepared: (1) - Normal nucleosomes which had been mixed with an equal volume of buffer VI and then immediately reassembled without any sucrose fractionation. These particles will be referred to as N-N to distinguish them from NXN, which had been additionally fractionated in sucrose gradients containing urea before being finally reassembled. (2) - Normal nucleosome core particles which had never been exposed to urea; these will be usually referred to as $N_o$. (3) - Similarly, the corresponding classes T-T, TXT, and $T_o$ of trypsinized nucleosome core particles were also prepared. All these controls are very important in order to assess the integrity of the "hybrid" nucleosomes, which have been reassembled from partially dissociated

components.

5. [$^{32}$P] End-labeling and DNase I Digestion of Nucleosomes

Nucleosome core particles at 0.225 mg/ml (~2200 nmol of 5′ ends) in 50 mM Tris-HCl (pH 7.6), 2 mM $MgCl_2$, 5 mM dithiothreitol, 0.1 mM spermidine, 0.1 mM EDTA were labeled at their 5′ termini by incubation at 37 °C for 1/2 hr in the presence of 0.5 units/$\mu$l of T4 polynucleotide kinase (BRL) and 1 mM [$\gamma$-$^{32}$P]ATP (7 Ci/mmol) (ICN). After that incubation time, the non-incorporated free label was completely removed by exhaustive dialysis against 25 mM NaCl, 10 mM Tris-HCl, pH 7.5 using a Centricon-30 microconcentrator (Amicon Corp., Danvers, MA). DNase I digestion was carried out in 100 mM NaCl, 4 mM Tris-HCl, pH 7.5, 1 mM $MgCl_2$ buffer on ice. [$^{32}$P] 5′-labeled core particles were mixed with a seven-fold excess of cold nucleosome core particles so as to give a final concentration of 85 $\mu$g/ml. The digestion was then performed at 70 DNase I (Worthington) units/ml at 0 °C on ice. At different times (usually 10, 20, 30, and 40 min), aliquots were taken and brought to 25 mM EDTA in order to stop the reaction. The sample was incubated at 100 °C for 1 minute, then cooled down on ice, after which pronase (Boehringer) was added at E/S ratio = 1:10 (weight ratio) and the mixture was further incubated at 37 °C for another

hour. Finally, the samples were mixed with an equal volume of a sample buffer containing 98% deionized formamide, 10 mM EDTA, pH 8.0, 0.4% bromophenol blue, and 0.4% xylene cyanole, incubated for 5 min at 100 °C, and then loaded on the single-stranded DNA denaturing gel channels. The low concentration of magnesium and low temperature used here for the DNase I digestions were chosen in order to minimize, as much as possible, the dissociation of the nucleosome core particles during the reaction. The analysis of the cutting patterns and the analysis of the relative digestion rates were performed as described by Lutter (1978).

6. Gel Electrophoresis

Native 4% polyacrylamide gels were prepared as described by Ausió *et al.* (1987). Denaturing gels to analyze the DNase I digestion products were prepared in 1/2 x TBE buffer (45 mM Tris-base, 45 mM boric acid, 1.25 mM $Na_2$-EDTA, pH 8.3) containing 8% acrylamide (acrylamide:bisacrylamide ratio 10:1), 7 M urea, and 0.05% TEMED. Polymerization was achieved by addition of ammonium persulfate to a final concentration of 0.1%. The running buffer was 1/2 x TBE. The dimensions of the gel were 260 mm (wide) x 460 mm (high) x 1 mm (thick). The gels were prerun until the temperature on the surface of the gel

plates was over 40 °C. At that point the samples were loaded and the run was then continued at 1500 V so as to maintain the temperature at around 50 °C. The run was stopped when the bromophenol blue was 1 cm from the lower edge of the gel. Thereafter the gels were soaked in 7% acetic acid, dried, and autoradiographed.

Gel electrophoresis of histones was performed in 15% polyacrylamide-SDS slab gels in the discontinuous buffer system described by Laemmli (1970).

## 7. UV Spectroscopy and Thermal Denaturation

UV absorption spectra and hyperchromicity measurements were carried out on a Cary 2200 (Varian) spectrophotometer equipped with a thermostated bath. The concentration of the nucleosomes was measured using $A_{260}$ = 9.5 $cm^2/mg$ [Ausió *et al.*, 1984]. Melting profiles were determined as described by Ausió *et al.* (1986).

## 8. Circular Dichroism

Circular dichroism spectra were recorded at 22 °C on a Jasco J-41 spectropolarimeter as described by Ausió *et al.* (1986). In using CD measurements to characterize the conformational changes occurring as the salt concentration is increased, the results were expressed in terms of a

quantity, $f_R$, which equals the fractional change in $CD_{maximum}$ (at 282.5 nm) observed in the core particles, as compared to the change which would be observed if the particles had completely dissociated to free DNA and histones at that concentration.

$$f_R = \frac{[\theta]_s - [\theta]_{min}}{[\theta]_d - [\theta]_{min}} \qquad (1)$$

Where $[\theta]_s$ and $[\theta]_{min}$ are the ellipticities of the nucleosome solution at a given salt concentration(s) and at 0.0-0.1 M salt (where $[\theta]$ is minimal), and $[\theta]_d$ is the ellipticity which a solution of free DNA would have, at the same salt concentration and at the same DNA concentration as in the nucleosome sample. In order to obtain values of $[\theta]_d$, we have measured the circular dichroism of the 146-bp deproteinized nucleosomal DNA between 0.05 and 0.7 M NaCl. Our results are identical with those of Ivanov *et al.* (1973). It should be noted that the quantity $f_R$ does not distinguish between DNA that has been completely dissociated from core particles and DNA that has been in some other way freed from any constraints which normally produce the depressed CD of nucleosomal DNA.

## 9. Sedimentation Velocity

Sedimentation velocity analysis was carried out on a Beckman Model E analytical ultracentrifuge equipped with photoelectric scanner and multiplexer. Sedimentation velocity experiments were routinely conducted at 40,000 rpm with an AN F rotor at 20 +/- 1 °C. The temperature was kept constant during each experiment to within 0.1 °C using the RTIC (Rotor Temperature Indicator and Controller) unit. Three cells and a counterbalance were included in each run. In every run, one internal standard consisting of native nucleosome core particles ($N_o$) in 0.1 M NaCl, 10 mM Tris-HCl, 0.1 mM EDTA, pH 7.5 buffer was included. The sedimentation coefficient ($s_{20,w}$) of $N_o$ in this buffer (11.1 S) was taken from the average of nine independent experimental determinations with a standard deviation of ±0.12 S. Thus in every experiment the apparent "s" values were corrected to $s_{20,w}$ and finally related to the internal standard being used. Such a procedure allows for a very accurate measurement of sedimentation coefficients, especially for comparative purposes, and when very subtle changes are to be measured.

The integral distribution of the sedimentation coefficients was evaluated using the method described by van Holde and Weischet (1978).

Density measurements were carried out with a

pycnometer in a room which was kept at a constant temperature (21 °C). Alternatively, in some cases the values provided in the CRC Handbook of Chemistry and Physics (Weast, 1985) were used. A very good agreement was always observed between the experimentally measured and the reported values. Partial specific volumes and related magnitudes were calculated as will be described in the next section.

## Results

### 1. Preparation of the Hybrid Nucleosome Core Particles

Nucleosome core particles containing selectively trypsinized structural domains (i.e. containing either trypsinized H2A-H2B dimer or trypsinized H3-H4 tetramer) were prepared from chicken erythrocyte core particles as outlined in Figure II-1, and described in detail in Materials and Methods. In order to prepare the "hybrid" core particles, the first step is to remove the histone tails from native nucleosome core particles by trypsinization. The initial reconstitution attempts were carried out using free trypsin in the digestions. However, the use of free trypsin brought a problem in recovering the trypsinized H2A-H2B dimers by sucrose gradient fractionation in the presence of urea. Under the conditions which were used to fractionate the H2A-H2B dimers and the subnucleosomal structure consisting of H3-H4 tetramer and DNA, the histone H2A-H2B dimer sediments on top of the gradient (see Figure II-4). Such centrifugation conditions also let residual trypsin and soybean trypsin inhibitor as well as their complex remain on top of the gradient. Therefore, after H2A-H2B dimer was fractionated from the subnucleosomal structure, it was not purified from trypsin which, in the presence of urea, seemed to recover

at least partially its enzymatic activity from the inhibition of soybean trypsin inhibitor. Under the experimental conditions of fractionation, 40 - 50 hrs at 20 °C, most of the H2A-H2B dimers were degraded by the residual trypsin activity by the end of the centrifugation. In an attempt to prevent the degradation, gel filtration on Bio-Gel A-5m (BioRad) resin was used as a substitute for the sucrose gradients. Unfortunately, similar results as with the urea-sucrose gradient were obtained. Attempts to separate the trypsin from the nucleosomes by sucrose gradient centrifugation prior to the urea-sucrose gradient fractionation also failed, probably because trypsin, which is positively charged, binds to nucleosomes. When the whole digestion mixture was centrifuged through sucrose gradients at 4 °C, immediately after stopping the trypsin digestion by addition of 10 times excess of soybean trypsin inhibitor, the nucleosome fractions obtained still retained some residual trypsin activity. Some residual trypsin activity even retained after sucrose gradient in the presence of 0.6 M NaCl. These difficulties made the initial characterization of the wholly trypsinized nucleosome core particles unreliable and usually irreproducible. The characteristics of the trypsinized particles changed from preparation to preparation, and the particles were found to be degraded with the time of storage.

The difficulties encountered with the use of free trypsin prompted us to seek for a way which can eliminate the problems in purification of trypsinized nucleosome core particles from the trypsin used for the digestion. A superior way of using immobilized trypsin bound on glass substrate was suggested by Dr. Juan Ausió (see preceding section for experimental details). This allowed complete separation of nucleosomes at the end of the reaction. Nevertheless, as an additional precaution, trypsin inhibitor was added. Wholly trypsinized nucleosome core particles prepared by the immobilized trypsin method are stable against further degradation with time, and retain their chemical composition and physical parameters unchanged almost indefinitely under storage at 0 °C on ice. Furthermore, the extent of digestion of the histones can be much better controlled when using the immobilized trypsin, since excessive incubation of nucleosome core particles with immobilized trypsin does not evidently overdigest, as is clearly shown in Figure II-3.B. As a result, the use of immobilized trypsin produce well-defined trypsinized nucleosome core particles, and the irreproducibility in the quality and characteristics of the trypsinized core particles obtained when using free trypsin was eliminated. The peptides resulted from trypsinization (peptides P1, P1′, P2, P3, P4 and P5 shown in Figure II-3) are in agreement with results of Böhm and Crane-Robinson (1984).

The amino acid sequences of the trypsinized histones are deduced based on the data of Böhm and Crane-Robinson and summarized in Table II-1.

## 2. Properties and Compositions of the Trypsinized and Hybrid Core Particles

By combining the preparation of trypsinized nucleosomes using immobilized trypsin, the sucrose gradient fractionation of subnucleosomal structures in the presence of urea as described by Sibbet and Carpenter (1983), together with appropriate reconstitution procedure in the ways illustrated in Figure II-1, we have been able to obtain the nucleosome core particles whose chemical characteristics and composition are shown in Figure II-5. The "hybrid" nucleosome core particles shown in lanes 3 and 4 of Figure II-5.A have been obtained for the first time. As can be seen in both Figures II-5.A and II-5.B, the particles thus obtained are homogeneous and retain their particulate nature. Their chemical composition in terms of the histone contents is also well defined, and in all cases the histone forms are present in stoichiometric amounts (Figure II-5.C). The remaining fragments of trypsinized histones correspond exactly to those reported by Böhm and Crane-Robinson (1984; see Figure II-3.B). One peculiar and noticeable feature of the trypsinized core particles is

**Table II-1. Trypsinized Histone Residues**

| Peptide on SDS-PAGE | Histone | Remaining A.A. Residues |
|---|---|---|
| P1′ | $H3_T$ | 21 - 135 |
| P1 | $H2A_T$ | 12 - 118* |
| P2 | $H3_T$ | 27 - 129* |
| P3 | $H2B_T$ | 24 - 125 |
| P4 | $H4_T$ | 18 - 102 |
| P5 | $H4_T$ | 20 - 102 |

*: peptide resulted from cleavage on both N- and C-termini.

Figure II-5

Characteristics of particles used in this study. Combining the different structural domains obtained as in Figure 3, we have been able to reconstitute the particles whose physical characteristics and composition are shown here.

A): Native 4% polyacrylamide gel electrophoresis to show the homogeneity and particulate nature of the reconstituted nucleohistone complexes: 1) - normal nontrypsinized nucleosome core particles ($N_o$); 2) - normal nucleosomes fractionated as in Figure 3 and reconstituted back through a salt dialysis gradient (NXN); 3) - [(H3-H4)$_2$-2(H2A$_T$-H2B$_T$)]·DNA; 4) - [(H3$_T$-H4$_T$)$_2$-2(H2A-H2B)]·DNA; 5) - completely trypsinized nucleosome core particles, fractionated as in Figure 3 and reconstituted with a salt dialysis gradient (TXT); 6) - completely trypsinized nucleosome core particles ($T_o$). D = constitutive DNA for all these particles. M = marker (*Cfo* I cut pBR322).

B. Sedimentation velocity scans at 265 nm taken after approximately 50 min at 40,000 rpm. The numbers stand for

the same samples as described in A. (The scan designated 1, 2 is actually for 1, that designating 5, 6 is actually for 6.)

C. SDS gel electrophoresis analysis of the histones in the various particles. Numbers as in A.

D. Schematic representation of a normal nucleosome core particle and the histone composition of the histone octamers in samples 1-6.

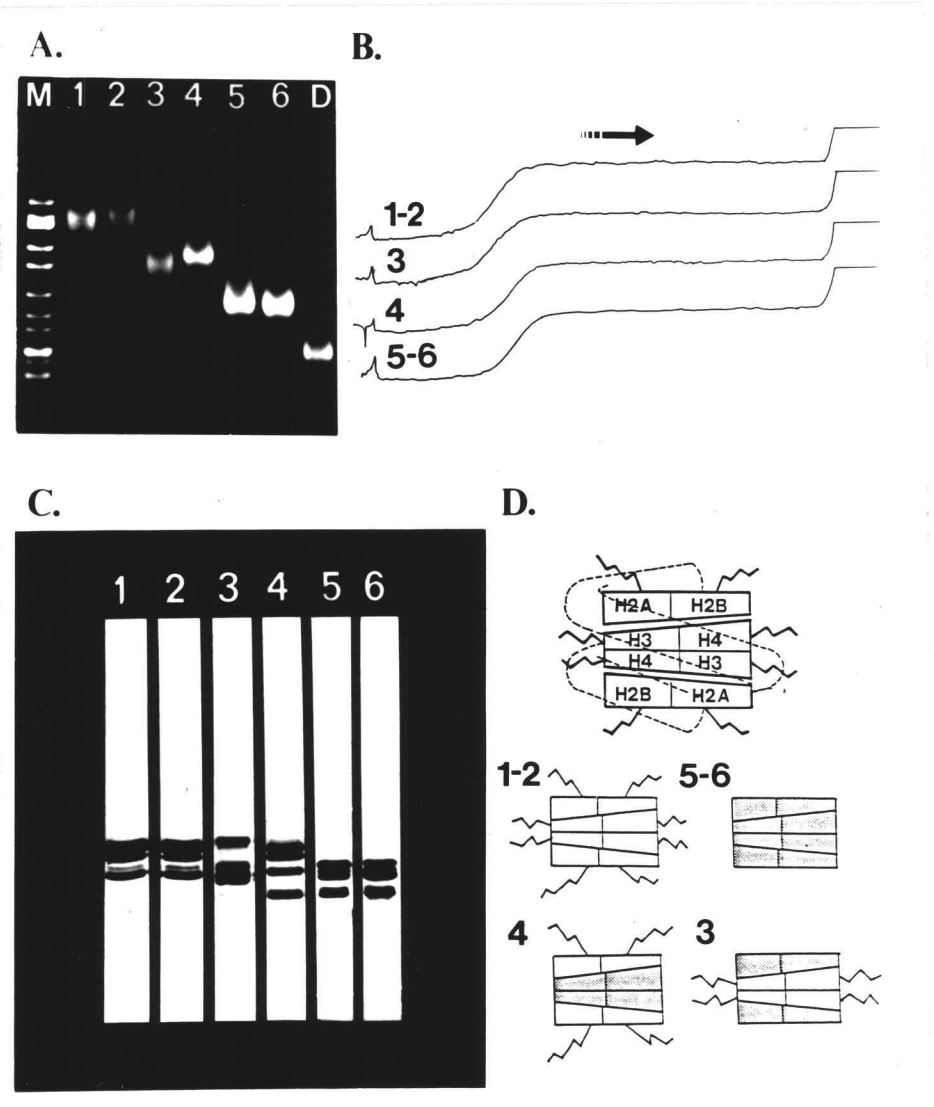
A.
M 1 2 3 4 5 6 D
B.
1-2
3
4
5-6
C.
1 2 3 4 5 6
D.
H2A
H2B
H3
H4
H4
H3
H2B
H2A
1-2
5-6
4
3

Figure II-5

their enhanced electrophoretic mobility in 4% polyacrylamide native gels (Figure II-5.A). As will be shown in the next section, the symmetry of the nucleosome particle at low ionic strength seems to be very little affected by the trypsin digestion of the N-terminal regions of the histones, and removal of the histone tails has decreased the particle molecular weight by only ~ 9%, even in the wholly trypsinized sample TXT. Such an increase in mobility can only be accounted for by an increase in the total net negative charge of the particle as a consequence of the removal of the highly positively charged histone "tails", which contain 32% (~ 15% from H2A-H2B and ~ 17% from H3-H4) of the total positive charge of the histone complex. Taking this into consideration, and in the absence of any significant structural changes for the whole particle, it is not surprising to find a marked increase in the electrophoretic mobility of the nucleosome core particles upon increase of the degree of trypsinization.

### 3. Hydrodynamic Characterization

Sedimentation coefficients of all of the kinds of particles depicted in Figure II-5 were determined at a number of different salt concentrations (Figure II-6). The differences are small, but are within the limits of experimental error because of the differential technique

Figure II-6

Sedimentation coefficients ($s_{20,w}$) as function of salt concentration for different nucleosome core particles: ( ◇ ) and ( ———— ), normal control nucleosome core particles ($N_o$); ( ◒ ), ( ◓ ), and ( —·—·— ), wholly trypsinized nucleosome core particles dissociated in 4 M urea, 0.35 M NaCl, and reassembled either within the same dialysis bag ( ◒ ) (T-T) or dissociated and fractionated in the presence of 4 M urea, 0.35 M NaCl, and further reconstituted through a salt dialysis gradient (TXT) ( ◓ ); ( ○ ), wholly trypsinized nucleosome core particles without any further treatment ($T_o$). ( Δ ) and ( —··—··— ), [(H3-H4)$_2$-2(H2A$_T$-H2B$_T$)]·DNA. ( ∇ ) and ( ····· ), [(H3$_T$-H4$_T$)$_2$-2(H2A-H2B)]·DNA; ( ■ ), the data for normal nucleosome core particles from HeLa cells (Ausió and van Holde, 1986).

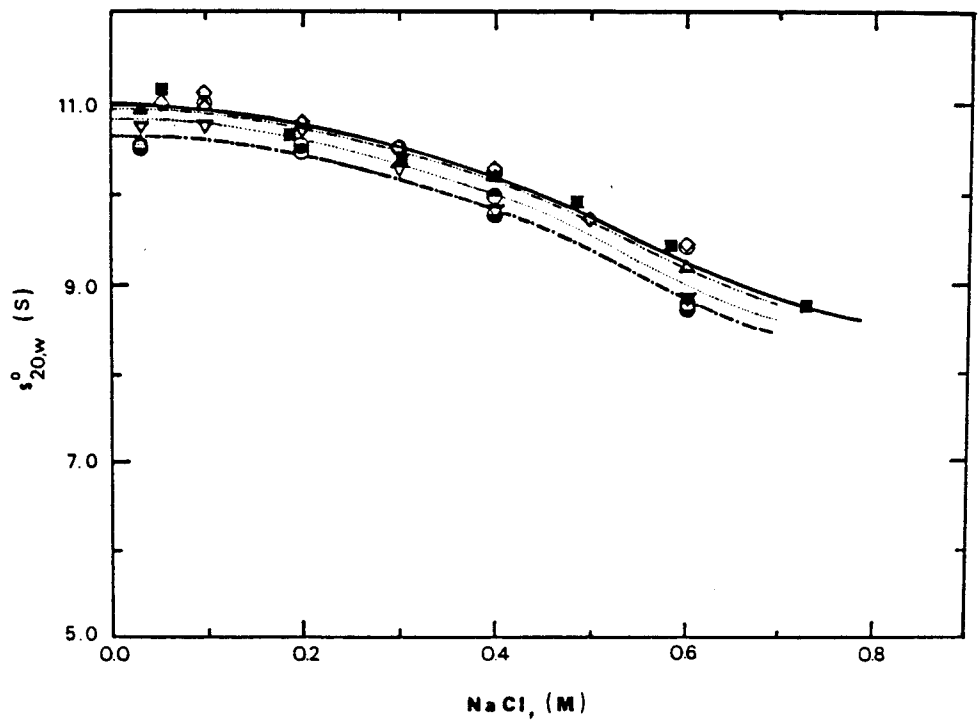


**Figure II-6**

employed (running a "standard" preparation along with each unknown -- see Materials and Methods). Since the mass of the particles has been changed by proteolysis, the frictional ratio ($f/f_o$) is a more appropriate indicator of conformational change than the sedimentation coefficient itself. The frictional coefficient (f) can be calculated from:

$$f = \frac{M_2}{s_{20,w}\, N} (\partial\rho/\partial c_2)_\mu \qquad (2)$$

where $M_2$ = the particle molecular weight, N = Avogadro's number, $c_2$ = particle concentration, and $\rho$ = solution density. The quantity $(\partial\rho/\partial c_2)_\mu$ is given by

$$(\partial\rho/\partial c_2)_\mu = (1 + \xi_1) - \rho(\bar{v}_2 + \xi_1\bar{v}_1) \equiv (1 - \phi'_2 \rho) \qquad (3)$$

Here $\phi'_2$ is an apparent partial specific volume, $\bar{v}_2$ and $\bar{v}_1$ are partial specific volumes of solute and water, and $\xi_1$ is a preferential hydration parameter (see Eisenberg, 1976).

$M_2$ was calculated from the known values for the molecular weights of the individual histones (Delange, 1976) and from the calculated molecular weight for the 146 bp DNA. In the case of the nucleosome core particles with different levels of trypsinization, the molecular weight of

their individual trypsinized histones were calculated from the data reported by Böhm and Crane-Robinson (1984). The values thus obtained were: 2.05 x $10^5$ g/mol for the normal nucleosome core particles; 1,85 x $10^5$ g/mol for the wholly trypsinized nucleosomes; 1.94 x $10^5$ g/mol for the [$(H3_T$-$H4_T)_2$-2(H2A-H2B)]·DNA; and 1.96 x $10^5$ g/mol for the [$(H3$-$H4)_2$-2($H2A_T$-$H2B_T$)]·DNA.

The buoyancy parameter $\phi'_2$ was calculated by assuming additivity of the $\phi'_2$ for the DNA (0.535 $cm^3/g$ as an average in the range 0.1 - 0.6 M NaCl) (from Eisenberg, 1976) and $\phi'_2$ for the histone octamer = 0.753 $cm^3/g$ (Eickbush and Moudrianakis, 1978). Calculations using the data of Cohn and Edsall (1943) show that the compositional differences resulting from cleavage of the histone tails have a negligible effect on $\phi'_2$ values. It has been shown by Ausió *et al.* (1984) that the partial specific values of intact core particles are independent of salt concentration over the range 0.1 - 0.6 M NaCl. The same is assumed to be true for the modified particles studied here. The values thus obtained were $\phi'_2$ = 0.650 $cm^3/g$ for the normal nucleosome [this value is in very good agreement with the one experimentally reported previously (Ausió *et al.*, 1984)]; 0.640 $cm^3/g$ for the wholly trypsinized particles; 0.645 $cm^3/g$ for [$(H3_T$-$H4_T)_2$-2(H2A-H2B)]·DNA and 0.646 $cm^3/g$ for [$(H3$-$H4)_2$-2($H2A_T$-$H2B_T$)]·DNA.

The quantity $(\partial\rho/\partial c_2)\mu \equiv (1 - \phi'_2\rho)$ was calculated from

the above values and measured on tabulated buffer densities.

The value of $f_o$ was taken to be $f_o = 6\pi\eta R_o$ where

$$R_o = \left\{ \frac{3M_2\phi \; [\eta]}{4\pi N} \right\}^{1/3} \qquad (4)$$

and thus is for a hypothetical unhydrated spherical particle. The values for $f/f_o$ are shown in Figure II-7 as a function of salt concentration. It is evident from these data, as well as those previously reported by others (Olins et *al.*, 1976) that the core particle is, even in low salt, significantly asymmetric and/or hydrated. If we correct for the hydration, assuming the value of $\xi_1 = 0.318$ (Greulich et *al.*, 1985), the values of $f/f_o$ found at the lower salt concentration used here decrease from about 1.5 to about 1.3.

Examination of the data shown in Figures II-7.A and II-7.B reveals that removal of histone tails has surprisingly little effect on the frictional properties of the core particle at low ionic strength. The differences observed at NaCl concentrations $\leq$ 0.2 M are, however, peculiar. Note that in Figure II-7.A, the $f/f_o$ for particles which have simply been trypsin-treated, but not dissociated and reconstituted ($T_o$), is significantly lower than for native core particles. This is not surprising,

Figure II-7

Change in the frictional parameter ($f/f_o$) for the different nucleosome core particles as a function of the ionic strength. The quantity $f_o$ represents the frictional coefficient of the unhydrated sphere of the same molecular weight as the particle.

A. ( ● ) and ( —— ): normal nucleosome core particles ($N_o$); ( ◑ ) and ( ---- ): wholly trypsinized nucleosome core particles without any further treatment ($T_o$); ( ···· )wholly trypsinized nucleosome core particles dissociated in 4 M urea, 0.35 M NaCl and reassembled within the same dialysis bag (T-T): ( ◒ ), or dissociated and fractionated in the presence of 4 M urea, 0.35 M NaCl and further reconstituted through a salt dialysis gradient (TXT); ( ○ ).

B. Data on hybrid particles: □ = $[(H3_T\text{-}H4_T)_2\text{-}2(H2A\text{-}H2B)]\cdot DNA$; ▲ = $[(H3\text{-}H4)_2\text{-}2(H2A_T\text{-}H2B_T)]\cdot DNA$. The lines ( —— ), ( ---- ), and ( ···· ) are taken from Panel A.

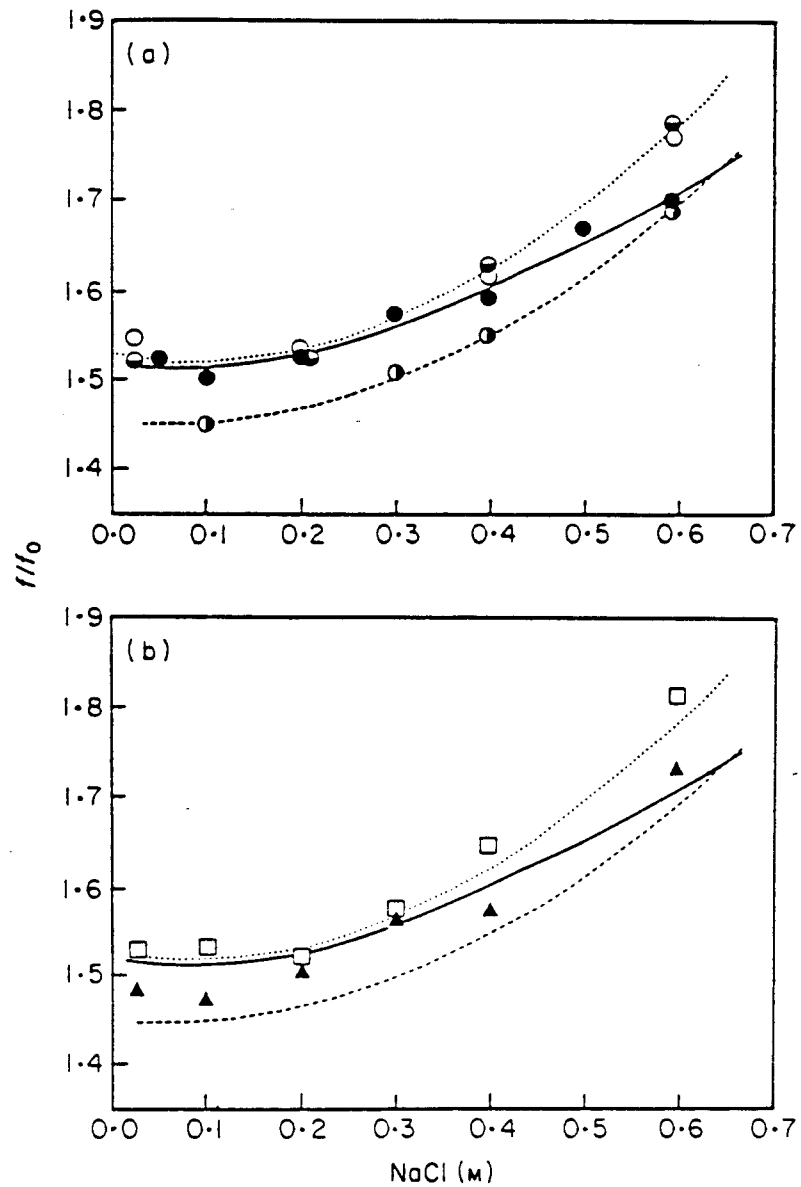


**Figure II-7**

since the tails, if partially extended at low ionic strength (Cary *et al.*, 1978) might be expected to add to the friction. But particles which have been trypsinized and then reconstituted, in either of two ways (T-T and TXT), show a higher $f/f_o$, comparable to that of the native particles (Figure II-7.A). In Figure II-7.B, it is seen that particles reconstituted with trypsinized H2A, H2B, but intact H3, H4 behave rather like trypsinized, but undissociated particles ($T_o$), whereas those in which H3 and H4 had been trypsinized behave like the reconstituted, wholly trypsinized particles (T-T and TXT). The data seem consistent with the following hypothesis: If one attempts to reconstitute after removal of H3, H4 tails, a "looser" particle, with higher frictional coefficient results. This suggests in turn that H3, H4 tails may play a role in guiding H2A and H2B into proper contacts within the nucleosome.

Regardless of these considerations, a very clear conclusion can be drawn from the effects of higher salt concentration on these particles. As both Figures II-6 and II-7 clearly show, the increase in frictional coefficient which is observed when the salt concentration is increased from 0.2 M to 0.6 M is independent of the presence or absence of the histone tails. Although there are small differences in behavior [$f/f_o$ increases somewhat more (by 17% as compared to 11%) in trypsin-treated particles], the

general conclusion is that release of the tails cannot account for the conformational transition between 0.2 and 0.6 M. Neither do the histone tails contribute to the stabilization of the core particle against dissociation into histones and free DNA in this range of salt concentration. When a detailed analysis of the sedimentation boundaries is carried out as described by van Holde and Weischet (1978), it is possible to determine the amount of the slowly sedimenting (5.2 S) free (dissociated) DNA as a function of the ionic strength. Such analyses yield indistinguishable results for all the particles analyzed here (data not shown). In other words, the amount of DNA being dissociated from the nucleosome core particles at the different ionic strengths was not dependent on the presence or absence of the N-terminal regions of the histones. Indeed, the salt- dependent dissociation behavior observed in all the cases was identical to that previously reported (Ausió *et al.*, 1984). This is in accordance with observations on trypsinized histones when studied by interaction with DNA-cellulose (Palter and Alberts, 1979).

## 4. Circular Dichroism

Figures II-8.A and II-8.B show the circular dichroic spectra of the different nucleosome core particles analyzed

Figure II-8. Circular dichroism spectra of the different nucleosome core particles. Spectra were recorded in (5 mM Tris-HCl, pH 7.5) buffer containing:

A. - 0.1 M NaCl;

B. - 0.6 M NaCl.

Lines are as follows in both panels: ( ——— ), normal nucleosome core particles ($N_o$); ( —·— ), [$(H3\text{-}H4)_2$-$2(H2A_T\text{-}H2B_T)$]·DNA; ( ···· ), [$(H3_T\text{-}H4_T)_2$-2(H2A-H2B)]·DNA; ( ---- ), wholly trypsinized nucleosome core particles without further dissociation and reconstitution ($T_o$); ( ——·—— ), wholly trypsinized nucleosomes (TXT) which had been subjected to the same fractionation and reconstitution procedures as those employed to prepare [$(H3\text{-}H4)_2$-$2(H2A_T\text{-}H2B_T)$]·DNA or [$(H3_T\text{-}H4_T)_2$-2(H2A-H2B)]·DNA.

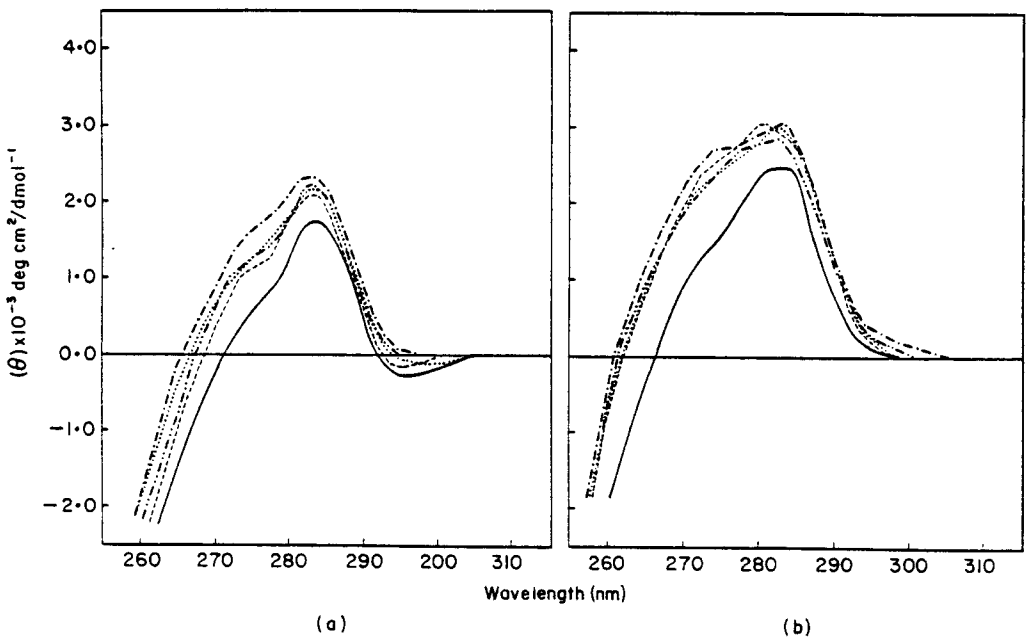
4·0
3·0
2·0
1·0
0·0
−1·0
−2·0
(θ)x10⁻³ deg cm²/dmol⁻¹
260
270
280
290
200
310
260
270
280
290
300
310
Wavelength (nm)
(a)
(b)

**Figure II-8**

in the preceding section. As can be seen in these graphs, all the trypsinized particles exhibit spectra with higher ellipticity values in the vicinity of 280 nm, both at low salt (0.1 M NaCl) and at moderately high salt (0.6 M NaCl) as compared to the spectra for normal non-trypsinized nucleosome core particles. It is difficult to analyze the dependence of this small increase on the different extents of trypsinization since the spectra almost overlap within the experimental error of measurement. It should be noted here that the overall changes in the DNA spectra observed in the past by other researchers working with wholly trypsinized nucleosome core particles have exhibited highly scattered values. We think that such scatter may reflect the problems associated with the use of free trypsin, as discussed above. As a matter of fact, the small change in spectrum observed by us agrees quite well with that reported by Lilley and Tatchell (1977) for trypsinized particles with equivalent sedimentation coefficient, or with that observed by Grigoryev and Krasheninnikov (1982). Our data, however, do not agree with the dramatic change in the CD spectrum reported by Whitlock and Simpson (1977).

The change in circular dichroism at 282.5 nm upon increasing salt concentration does not seem to be influenced at all by the removal of histone tails. This is better seen in Figure II-9, which represents the relative fraction of DNA becoming freed from the nucleosome

Figure II-9

Determination of the fraction of DNA ($f_R$) becoming freed of the nucleosomal constraints induced by its specific interaction with histones, as measured through the salt dependent increase of the maximum (at 282.5 nm) of the CD spectra. ( ○ ), wholly trypsinized nucleosome core particles ($T_o$); ( ● ), wholly trypsinized nucleosomes which have been dissociated in the presence of 4 M urea and 0.35 M NaCl and reassembled back within the same dialysis tubing (T-T); ( ◒ ), [(H3-H4)$_2$-2(H2A$_T$-H2B$_T$)]·DNA; ( ◑ ), [(H3$_T$-H4$_T$)$_2$-2(H2A-H2B)]·DNA. The solid line represents the behavior exhibited by native nucleosomes.

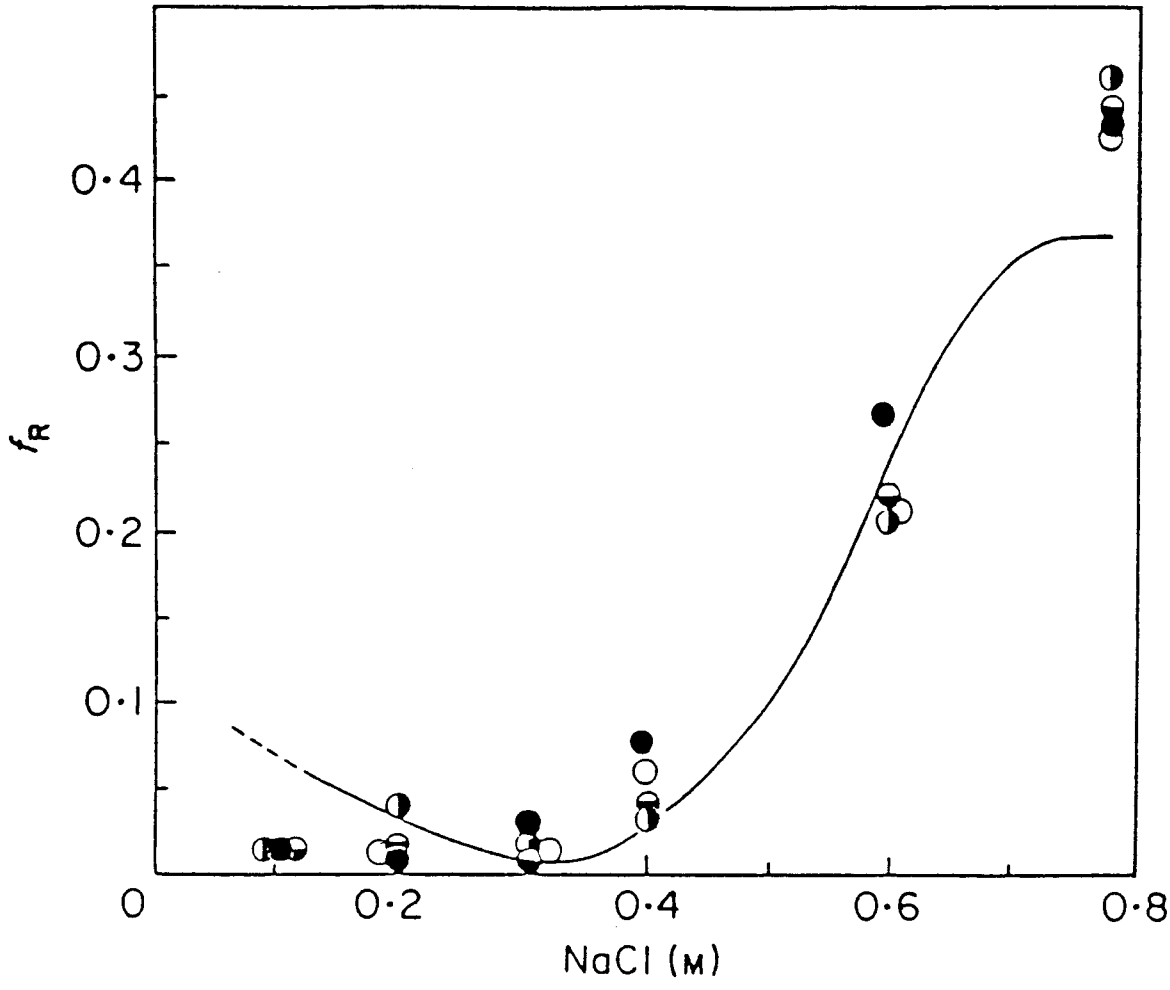


**Figure II-9**

constraints (Ausió and van Holde, 1986) as measured by the increase at the wavelength of maximum ellipticity (282.5 nm), as a function of the ionic strength of the solution. The behavior of the normal vs. the trypsinized particles is again almost identical.

5. DNase I Digestion

We have carried out DNase I digestion studies with each of the kinds of particles described above. Representative gel patterns are shown in Figure II-10. Three features are immediately obvious:

1. The positions of maximum DNase I accessibility are unchanged. Thus, the loss of any or all of the histone tails does not significantly modify the DNA winding on the nucleosome.

2. The strong distinctions between favored and unfavored cutting positions observed for the native nucleosomes are considerably blurred in the trypsinized particles. This is especially evident in lane 4, corresponding to particles from which H2A/H2B tails have been removed.

3. The overall cutting rate is higher in trypsinized samples. We have quantitated the rates of cleavage at each of the sites of maximum cutting, in the manner used by Lutter (1978). The results indicate no significant

Figure II-10. DNase I footprinting of the different nucleosome core particles.

A). Autoradiogram from gel electrophoresis of DNase I digests, on an 8% single-stranded DNA denaturing gel (10:1 crosslinking ratio), for the times indicated at top. At each time, the numbers above lane correspond to: (1) normal nucleosome core particles, ($N_o$); (2) normal nucleosome core particles dissociated in the presence of 4M urea, 0.34 M NaCl, and reassembled within the same dialysis tubing, (N-N); (3) $[(H3_T\text{-}H4_T)_2\text{-}2(H2A\text{-}H2B)]\cdot DNA$; (4) $[(H3\text{-}H4)_2\text{-}2(H2A_T\text{-}H2B_T)]\cdot DNA$; (5) wholly trypsinized core particles treated as in 2 (T-T); (6) wholly trypsinized core particles without any further fractionation or reconstitution ($T_o$).

B) and C). DNase I digestion rates (analyzed by the method of Lutter, 1978) for various nucleosome core particles.

In Panel B), 1=$N_o$; 2=N-N; 3=$T_o$; and 4=T-T.

In Panel C), $\bar{N}$ = average of the 1 and 2 in Panel B);

$\bar{T}$ = average of the 3 and 4 in Panel B);

$(3\cdot4)^T(A\cdot B)^N = [(H3_T\text{-}H4_T)_2\text{-}2(H2A\text{-}H2B)]\cdot DNA$;

$(3\cdot4)^N(A\cdot B)^T = [(H3\text{-}H4)_2\text{-}2(H2A_T\text{-}H2B_T)]\cdot DNA$.

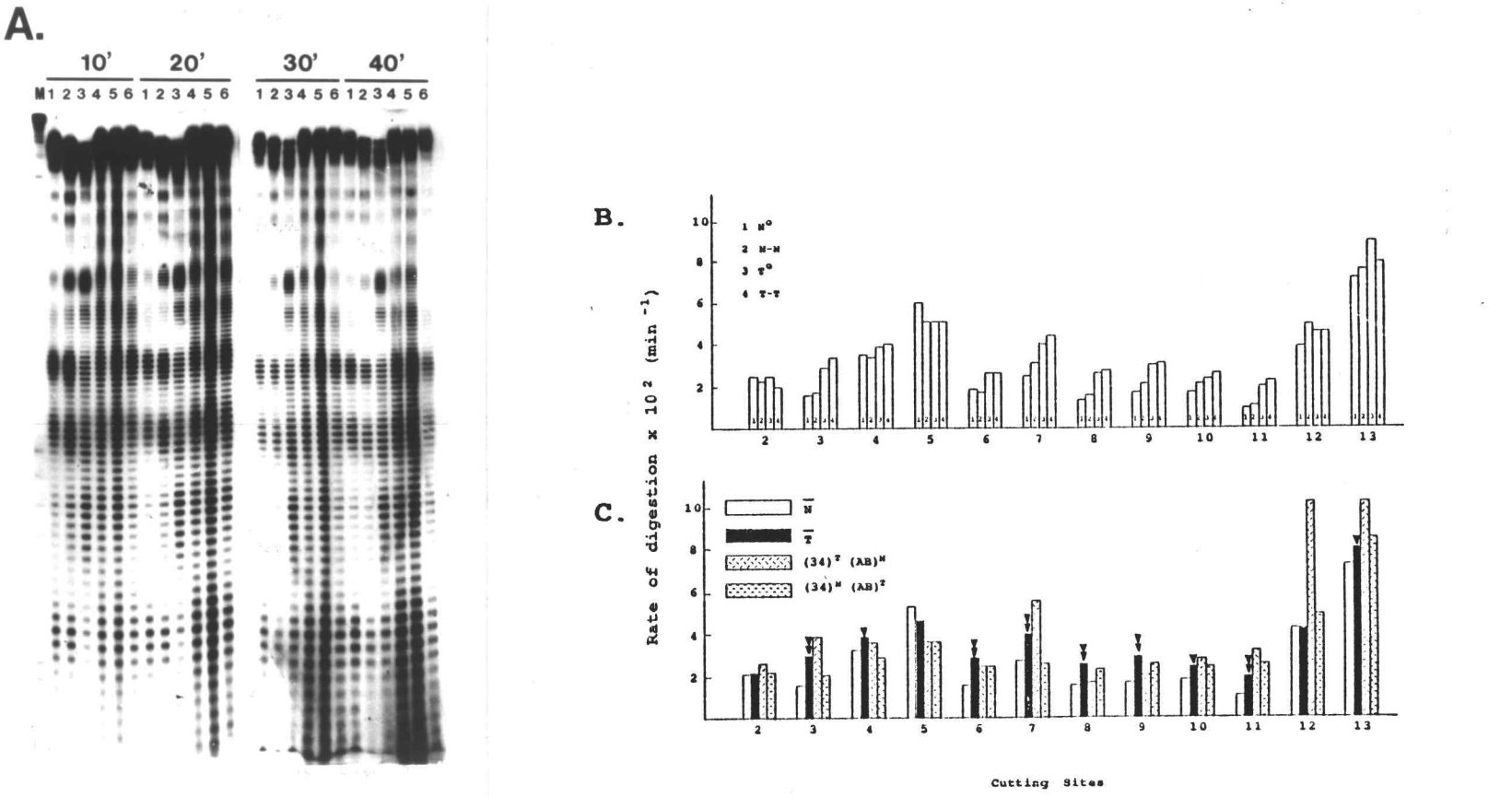
A.
10'
20'
30'
40'
M 1 2 3 4 5 6 1 2 3 4 5 6 1 2 3 4 5 6 1 2 3 4 5 6
B.
C.
Rate of digestion x 10² (min⁻¹)
Cutting Sites
(34)ᵀ (AB)ᴹ
(34)ᴹ (AB)ᵀ

Figure II-10

difference in the pattern of DNase I digestion, with the exception that sites 3, 6, 8, and 11, which are weak sites in normal nucleosomes, are not so weak (relative to other sites) in wholly trypsinized particles (Figure II-10.B). Similarity between the DNase I digestion patterns of normal and partially trypsinized nucleosome core particles was already pointed out by Lutter (1978). The same observation was made when DNase II in the presence of 1 mM $MgCl_2$ was used instead of DNase I (Grigoryev and Krasheninnikov, 1982).

Based on these data, it appears as if the relative accessibility of the cutting sites to the DNase I in the nucleosome is mainly determined by the peculiar pathway followed by the DNA around the histone core, rather than by the possible topological hindrance introduced by the presence of the amino terminal regions of the histones. In fact, it has been shown that most of those lysine residues which probably define the path of DNA on the nucleosome, are located in the globular region of the histone octamer rather than in their carboxy or N-terminal "tails" (Lambert and Thomas, 1986). Moreover, it has also been shown that these regions do not contribute to the supercoiling of the DNA in the core particle (Morse and Cantor, 1986).

## 6. Thermal Denaturation Studies

Finally, we have compared the thermal stabilities of the several classes of modified particles described above (Figure II-11). As Figure II-11.A shows, removal of all of the histone "tails" abolishes almost completely the second melting transition at 81 °C. Most of the DNA in the completely trypsinized nucleosomes melts at 76 °C, a temperature close to the first transition of the non-trypsinized particles (74.5 °C). The behavior is essentially the same for particles which have simply been trypsinized, and those which have been trypsinized and reconstituted.

Figure II-11.B reveals remarkable differences between different particles in which only one pair of histones have been trypsinized. Removal of only the H3/H4 tails has virtually no effect of the thermal denaturation profile. This must mean that the H2A/H2B tails alone can stabilize the nucleosome so as to yield a "normal" thermal denaturation pattern. If those H2A/H2B tails are removed while H3/H4 tails remain intact, a large fraction of the DNA is shifted into to first transition, at about 74 °C. In this event, the H3/H4 tails appear to retain some stabilizing influence, for about half of the DNA (70-80 bp) still melts in the 81 °C transition. Loss of both kinds of tails nearly abolishes this higher transition.

Figure II-11

Thermal denaturation data.

Panel A: ( ------ ), wholly trypsinized core particles ($T_o$); ( ——— ), wholly trypsinized nucleosome core particles treated as (T-T) in Figure 5; and ( ····· ), normal nucleosome core particles ($N_o$).

Panel B: ( ——— ), [(H3-H4)$_2$-2(H2A$_T$-H2B$_T$)]·DNA; ( —··— ), [(H3$_T$-H4$_T$)$_2$-2(H2A-H2B)]·DNA; ( ------ ) and ( ····· ) are the same as in Panel A.

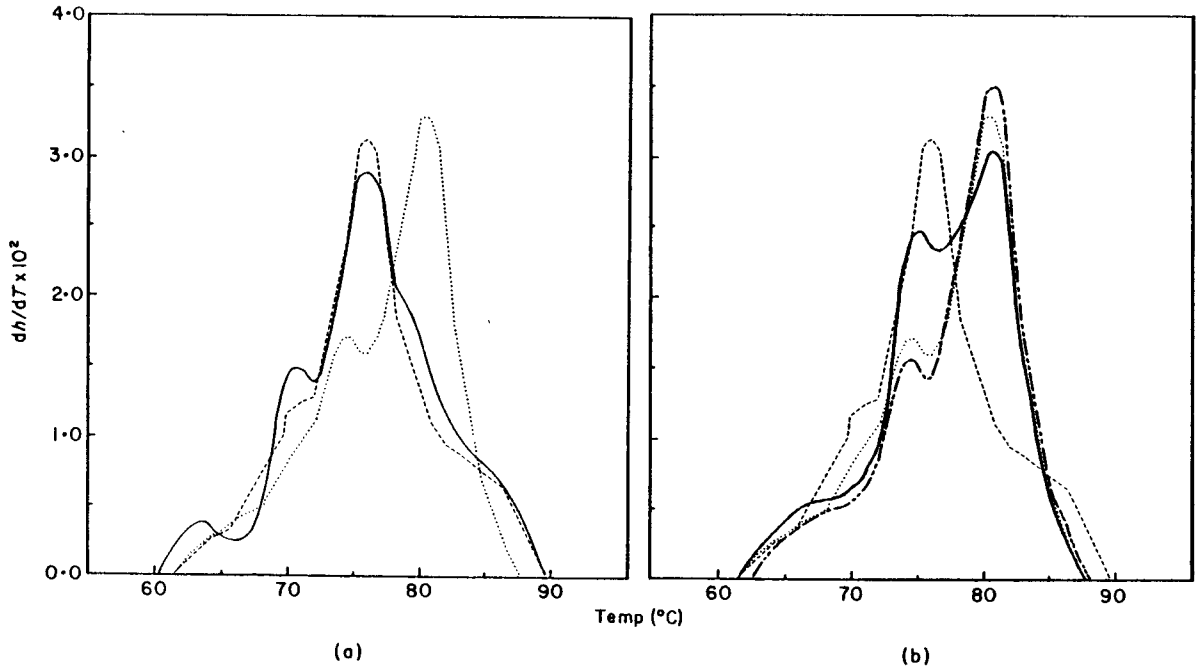


**Figure II-11**

## Discussion

These experiments have shown, by using immobilized trypsin, that it is possible to prepare core particles with precisely proteolyzed histones, which are suitable for physical analyses. We believe that many of the apparent discrepancies in such studies reported before may have arisen from improperly controlled or even continuing proteolysis. Using the method of Sibbett and Carpenter, it is possible to prepare highly homogeneous hybrid particles, in which either H2A/H2B or H3/H4 remain intact.

Hydrodynamic studies reveal that removal of histone tails, either selectively or altogether, has very little effect upon the frictional coefficient ratio of the particle. Thus, the tails cannot be contributing significantly to the frictional coefficient of the intact core particle at low ionic strength. Nor do they, from the results of CD and DNase I digestion studies, have any major effect on DNA conformation in the particle.

The conformational transition that occurs in nucleosome between 0.2 M and 0.6 M salt is not simply a reflection of the histone tail release that has been reported in this same concentration range (Cary *et al.*, 1978; Walker, 1984). Indeed, the transition, as measured either by frictional ratio or CD, occurs equally well whether histone tails are present or not. The same is also

true for the salt- dependent stability of the nucleosome particle within this salt range.

While the above experiments suggest little importance to the N-terminal histone tails, insofar as nucleosome stability is concerned, the thermal denaturation studies tell a dramatically different story. Why should **this** transition be so selectively sensitive to removal of the tails? An hypothesis can be presented which explains the peculiar effects of histone tail removal on the thermal denaturation curves (Figure II-12).

1. We assume that DNA melting in nucleosomes begins at the ends, as has been used to explain the first transition (Simpson, 1979). Approximately 20 base pairs at either end of the DNA is involved in this transition. This DNA is assumed **not** to be stabilized by any interactions with histone tails. However, its higher melting temperature (premelting transition) when compared to free DNA, shows that it must interact with some other parts of the histone octamer, most likely the globular portions of H3 and H2A (Mirzabekov *et al.*, 1978).

2. We assume that H2A/H2B tails (either or both) interact with and stabilize secondary regions about 10 bp in length, further into the core DNA (see Figure II-12). The central 70-80 bp of DNA in the coil is assumed to interact with H3/H4 tails either in whole, or at least at the two borders of this central region.

Figure II-12

A model to explain thermal denaturation behavior of modified nucleosomes. Regions on DNA are only approximately defined:

Open: no protection by tails; always melts in first transition.

Lightly stippled: interacts with H2A/H2B tails.

Heavily stippled: central region; all or at least borders interact with H3/H4 tails.

A. Presentation of the nucleosomal DNA only

B. Presentation of a nucleosome core particle with the DNA wrapping around histone octamer, showing DNA pathway with respect to the locations of individual histone domains. Relative locations between the histone subunits, and between DNA and histones, are based on the nucleosome structure deduced from X-ray diffraction studies [Richmond *et al.*, 1984] and the data of histone-DNA contact regions [Shick *et al.*, 1980]. The vertical line represents the psuedo-dyad axis of the nucleosome.

A.


B.

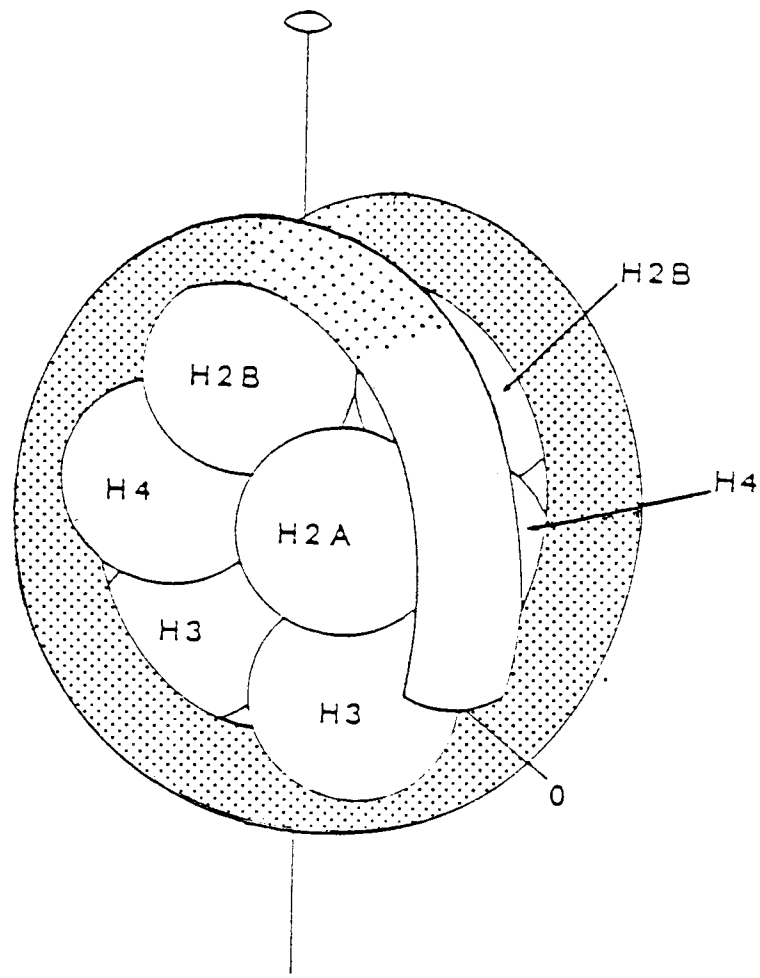


Figure II-12

Predicted consequences are as follows:

1. If H3/H4 tails are removed, the melting profile will be unchanged. Melting will proceed through the ends (first transition), but be blocked by the H2A/H2B interactions with the "intermediate zones" until the appropriate $T_m$ (~80 °C under conditions used here) is reached. Once these intermediate blocks have melted, the rest will melt.

2. If H2A/H2B tails are removed, but H3/H4 tails remain intact, the first melting transition will proceed through the entire 30 base pairs from each end. The remainder (protected by H3/H4 tails) will not melt until ~80 °C. The effect will be that the amount melting in the first transition will be increased by about 50%, as observed.

3. If **all** tails are removed, almost all of the nucleosomal DNA will melt in the first transition.

A corollary of this hypothesis is the following: If the conformational change occurring between 0.2 M and 0.6 M salt involves primarily the DNA (and its interactions) near the ends of the nucleosome, it should be unaffected, as observed, by removal of histone tails.

The model proposed agrees very well with the previous results of the proximity of histone subunits to the different regions on the DNA molecules (see Figure II-12.B), deduced from histone-DNA contact regions [Shick *et*

*al.*, 1980] and nucleosome structure suggested by X-ray diffraction data [Richmond *et al.*, 1984].

Finally, the hypothesis is consistent with observations of the effects of hyperacetylation of histone tails, which should be expected to weaken, but not abolish tail-DNA interactions. What is found is that hyperacetylated nucleosomes melt with more DNA in the first transition, and with an accompanying decrease in $T_m$ for the melting transitions (see Figure I-1). Furthermore, such modification has no effect on the salt-induced conformational change.

Our studies, together with those of Allan *et al.* (1982), demonstrate that the N-terminal tails of histone molecules may play roles in stabilizing **both** the higher order structure of chromatin, and the nucleosome itself.

## Acknowledgements

I would like to express my sincere thanks to Dr. Juan Ausió, for his cooperation and discussion in this research, as well as his help in the use of many useful biophysical techniques, in data analysis, and the understanding and presentation of research results. Acknowledgements should also be given to Drs. Jeffrey Hansen, Cynthia McMurray, Karen Miller, and Thomas Yager for their discussion, criticism and advice.

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# Chapter III

## Nucleosome Core Particles in Solution: the Changes in the Structure and Hydration at Moderate Ionic Strengths

## Introduction

Nucleosomes are highly dynamic structures, undergoing different conformational transitions and dissociations both *in vitro* and *in vivo*. An example of such conformational versatility is provided by the salt-dependent conformational transition of the nucleosome core particle at moderate ionic strength as indicated by the changes in its frictional parameters [McGhee *et al.*, 1980; Wilhelm and Wilhelm, 1980; Eisenberg and Felsenfeld, 1981; Ausió *et al.*, 1984, Yager and van Holde, 1984]. Such conformational change of the nucleosome at ionic strengths in the vicinity of physiological values (0-0.6 M NaCl) may provide a useful insight into the mechanisms involved in structural transitions of this particle associated with different functional stages of chromatin. Therefore, during the last few years, research efforts have concentrated on trying to ascertain the details of this salt-dependent behavior. In the X-ray scattering studies by Greulich *et al.* (1985), it has been shown that the radius of gyration of the particle does not change detectably within the range of ionic strength produced by 0.1-0.6 M NaCl. A similar result has been found by Ramakrishnan *et al.* [Ramakrishnan, V., Yager, T. and van Holde, K. E., unpublished data] using neutron scattering techniques. It has also been shown that release of the histone "tails" in the same salt range [Walker,

1984] cannot account for the change in the frictional parameters, since the same percent change in S is observed even if the "tails" are removed (see Chapter II). These findings, while ruling out any significant changes either at the DNA or at the histone level, emphasize the subtlety of the mechanisms involved in the salt-dependent conformational transition.

In the research described in this chapter, several experiments have been carried out in order to investigate possible subtle changes in the secondary structure of both DNA and histones under the salt conditions in which the nucleosome conformational transition occurs. This chapter also presents a detailed analysis of the changes in the virial coefficient and hydration over the same salt concentration range.

## Materials and Methods

### 1. Nucleosome Core Particles

Nucleosome core particles were prepared as described in Chapter II.

### 2. Circular Dichroism

Circular dichroism spectra were obtained and analyzed as described in Chapter II.

### 3. DNase I Digestion of Nucleosomes

Nucleosome core particles and core particle-sized DNA were digested both in 0.1 and in 0.6 M NaCl in the presence of 5 mM Tris-HCl (pH 7.5) and 1mM $MgCl_2$. For the digestion of nucleosomes, [$^{32}$P] 5′-end-labeled core particles at 10 μg/ml, in the buffer containing either 0.1 or 0.6 M salt, were brought to a final concentration of 100 μg/ml by addition of cold nucleosomes under the same buffer conditions. Digestions at 0.1 M NaCl were carried out at 0.8 units of DNase I per microgram of DNA, whereas in those carried out at 0.6 M NaCl the enzyme/substrate ratio was increased up to 10-fold (8 units DNase I/μg of DNA). At these DNase I concentrations, the same amount of total

PCA-soluble oligonucleotides could be obtained for the two conditions after 20 min digestion in 0.1 M NaCl and after 5 min digestion in 0.6 M NaCl. These are the times of digestion for the scans of DNase I autoradiograms shown in Figure III-5. In other experiments, nucleosomes were digested for different times at different temperatures as specified in the text.

The different conditions of digestion used for both ionic strengths were experimentally established through a preliminary set of pilot digestions. Such differences in enzyme/substrate requirement mainly arise from the fact that DNase I works much more slowly (both on naked DNA and on nucleosomes) at 0.6 M NaCl than at 0.1 M NaCl. Such salt-dependence in activity seems to be similar, although less strong, to that exhibited by micrococcal nuclease [Weischet *et al.*, 1979].

The analysis of the DNase I digestion patterns at high salt becomes additionally complicated from the fact that nucleosome core particles show a salt-dependent dissociation behavior [Ausió *et al.*, 1984; Yager *et al.*, 1989]. Thus, the amount of free DNA present in the sample at the nucleosome concentrations and temperature (0 °C) used for these experiments increases from approximately 5% free DNA at 0.1 M NaCl to ~10% free DNA at 0.6 M NaCl. In order to correct for the presence of the free DNA, we designed the following controls: [$^{32}$P] 5′-end-labeled 146-bp

nucleosomal DNA (deproteinized), in either 0.1 M NaCl or 0.6 M NaCl, was mixed with cold nucleosome core particles containing the same amount of salt (as in the original digestions) in a ratio of 1:10. The final total DNA concentration was 100 μg/ml. DNase I was then added to both samples and the digestion was performed under the same experimental conditions described above. After stopping the reaction, these samples were loaded on to the denaturing gel in quantities corresponding to either 5% or 10% of the total initial loading for the corresponding nucleosome core particles in 0.1 or 0.6 M NaCl. An additional control was prepared by digesting 0.5 μg/ml (5% of the amount used in the above control experiment) of [$^{32}$P] end-labeled naked nucleosomal DNA (for 0.1 M NaCl) or 1 μg/ml (10% of the same material) (for 0.6 M NaCl) in the presence of a total final DNA concentration of 100 μg/ml obtained using cold nucleosomes as above. The samples thus obtained were digested under identical conditions as those described earlier. After stopping the digestion, a volume of sample equal to that used for the original nucleosome samples was loaded onto each denaturing gel. The results from these controls (Figure III-4.B) clearly indicated that the contribution to the DNAse I pattern by the fraction of free DNA, both in 0.1 and in 0.6 M NaCl, is negligible in both cases. This would be expected from the fact that naked DNA was digested much faster than DNA in the

nucleosome under both salt conditions.

In all these digestions described above, the reaction was stopped by addition of EDTA at pH 8.0 to the sample to a final concentration of 20 mM on ice. The sample in 0.1 M NaCl was then adjusted to 0.6 M NaCl by addition of 4 M NaCl. Then both samples were incubated at 100 °C for 1 min and cooled on ice before the addition of pronase. Further treatment and preparation of samples was carried out as described in Chapter II.

4. Gel Electrophoresis Under Denaturing Conditions

Eight percent polyacrylamide denaturing gels, containing 7 M urea, were prepared in 44.5 mM Tris-borate, 44.5 mM boric acid, 1 mM EDTA (pH 8.4) buffer and polymerized in the presence of 0.1% ammonium persulfate and 0.05% TEMED at room temperature. Gels were prepared with either 10:1 or 20:1 acrylamide:bisacrylamide ratio. The lower ratio of crosslinking (10:1) was used to analyze the cutting pattern of DNase I above 60 bp, whereas the 20:1 ratio was routinely used for the analysis of the higher-mobility bands. The size of the gels was usually 26 cm (wide) x 48 cm (high) x 0.1 cm (thick). Prior to loading the samples, the gels were warmed by prerunning them until an outer surface temperature of over 40 °C was reached. The samples were then loaded onto the gel and

electrophoresis was continued at ~ 1500 V so as to maintain the temperature of the gel at around 50 °C. The gels were run until the bromophenol blue was 1 cm from the lower edge of the gel and xylene cyanole was at the middle ( for a 20:1 crosslinking ratio) or until the xylene cyanole dye was 9 cm from the same edge (for 10:1 crosslinking ratio).

## 5. Analytical Sedimentation

Sedimentation equilibrium experiments were carried out on a Beckman Model E analytical ultracentrifuge. Runs were routinely performed at 20 +/- 1 °C at 6800 or 9000 rpm. Six-channel 12-mm cells [Yphantis, 1964] were employed in all the experiments using short solution columns (80 $\mu$l sample, 90 $\mu$l buffer). Sedimentation equilibrium runs were performed at 6800 rpm at 20 +/- 1 °C. The schlieren optical system was used in all the experiments and the schlieren patterns were analyzed to measure $M_z$, according to the method of Lamm (1929) in order to obtain the apparent z-average molecular weight:

$$M_z^{app} = \frac{2RT}{\omega^2 (\partial \rho / \partial c_2)_\mu} \cdot \frac{d\ln[(1/r)(dc_2/dr)]}{dr^2} \qquad (1)$$

Density contrast variation analysis was performed as described by Eisenberg and Felsenfeld (1981). In the measurements at the different salt and sucrose

concentrations, the concentrations of the nucleosome samples were kept the same at ca. 2.2 mg/ml (concentration of total weight: DNA+histones; $A_{260nm}$ = 21). Sedimentation equilibrium runs for the density contrast variation experiments were performed at 9,000 rpm at 20 +/- 1 °C for ≥ 96 hr until there is no detectible changes in the distribution of nucleosomes along the gradient.

# Results

## 1. The Salt Dependence of the Circular Dichroism of the Nucleosome Core Particle

Upon its interaction with histones, DNA in the nucleosome exhibits an altered circular dichroism spectrum. Particularly in the spectral region of 250-300 nm, which is dominated by the DNA helix conformation, the spectrum seems to be strongly "suppressed" as compared to that exhibited by free DNA [Sahasrabuddhe and van Holde, 1974; Weischet *et al.*, 1978; Cowman and Fasman, 1980].

Two major models have been put forward to account for this phenomenon. One of them, proposed by Fasman *et al.* [Cowman and Fasman, 1978; Fasman, 1978; Cowman and Fasman, 1980], invokes the closely coiled tertiary structure of the DNA in the nucleosome particle. Alternatively, the suppression of the spectrum has been assigned to the coexistence of different secondary structures of the DNA in the nucleosome as a consequence of changes in the winding angle of the DNA in its path around the histone octamer. A detailed analysis using subnucleosomal particles containing very short DNA fragments has ruled out the first model [Mencke and Rill, 1982]. Therefore, it seems clear that the secondary structure of DNA must change upon its interaction with the histones in the nucleosome. This idea

is also supported by the DNase I digestion pattern of these particles [Lutter, 1979] when compared to B-form DNA lying on a flat surface [Rhodes and Klug, 1980].

There have been several reports indicating changes in the CD spectrum of nucleosomes at moderate salt concentrations [Wilhelm and Wilhelm, 1980; Yager, 1984]. A summary of results from our laboratory is shown in Figure III-1, which depicts how the ellipticity at the maximum in the nucleosome CD spectrum (at 282.5 nm) increases with increasing ionic strength. It is important to point out here the coincidence of this dependence with that observed for highly hyperacetylated nucleosomes [Ausió and van Holde, 1986] or for trypsinized nucleosome core particles (see Chapter II). Although these changes might, at first glance, be thought to be symptomatic of a conformational change, one must be very careful in drawing such conclusions. It must be kept in mind that, over this same salt concentration range there occurs a partial dissociation of nucleosomes, yielding increasing amounts of free DNA as the ionic strength increases [Ausió *et al.*, 1984; Yager and van Holde, 1984; Yager *et al.*, 1989]. When this phenomenon is taken into consideration and the contribution to the CD spectra by the free dissociated DNA is accounted for, the value for the maximum ellipticity at 282.5 nm remains nearly constant within the salt range analyzed here, as shown in Figure III-2. Although the CD

Figure III-1

Salt dependence of the fraction of DNA ($f_R$) becoming freed from the nucleosomal constraints at different nucleosome concentrationbs. The fraction $f_R$ were measured through the increase of the maximum of the CD spectra (at 282.5 nm). ( ● ) Nucleosome core particles at ~ 40 μg/ml of DNA (i.e. ≃ 0.8 $OD_{260}$) and at different salt concentrations were incubated at 22 °C for 14 hr before performing the measurements. ( ○ ) Nucleosome core particles at 10 mg/ml of DNA (i.e. ≃ 200 $OD_{260}$) at different ionic strengths. The dotted line was obtained from previous chicken erythrocyte nucleosome data [Yager, 1984] and from data obtained from nucleosomes prepared from HeLa cells [Ausió and van Holde, 1986]. This line which is used here as a standard was obtained under sample concentration conditions close to the one used in ( ● ), but without any long incubation time. Measurements were performed either in a 1 cm path-length cell (samples of 0.8 $OD_{260}$) or in a 50 micron path-length cell (samples of 200 $OD_{260}$).

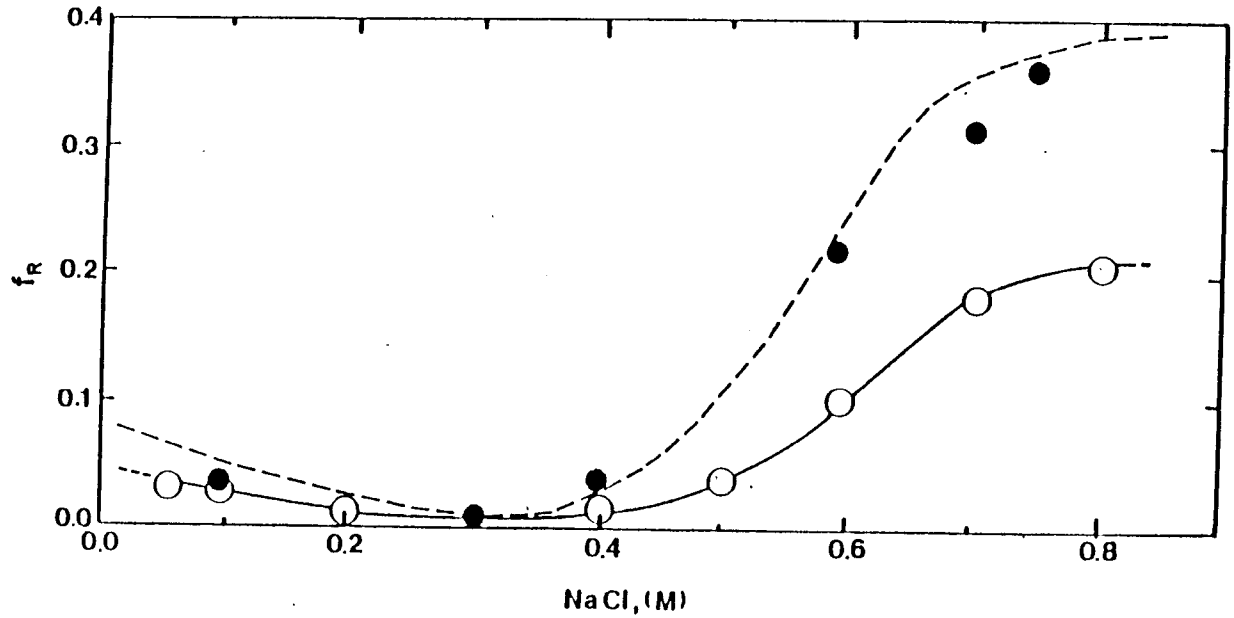


**Figure III-1**

Figure III-2. Analysis of the circular dichroism spectra of nucleosome core particle at different salt concentrations.

Circular dichroism spectra of nucleosome core particles in 0.1 M NaCl, 10 mM Tris-HCl, 0.1 mM EDTA, pH 7.5 buffer: ( ····· ) or in 0.6 M NaCl in the same buffer: ( ------ ). The line: ( ——— ) corresponds to the CD spectrum in 0.1 M NaCl after subtraction of 8% of the CD spectrum of naked nucleosome-size DNA in the same salt. The line ( -··-·· ) corresponds to the spectrum presented in ( ------ ) after correction for 18% free DNA at this ionic strength. The values for the amounts of free dissociated DNA under the different salt conditions were obtained from Ausió *et al.* [1984]. In both cases, the spectra for the naked DNA used for the corrections were obtained under identical experimental conditions as those used for the original nucleosome samples. The concentration of the samples was always very close to 0.8 $OD_{260\,nm}$.

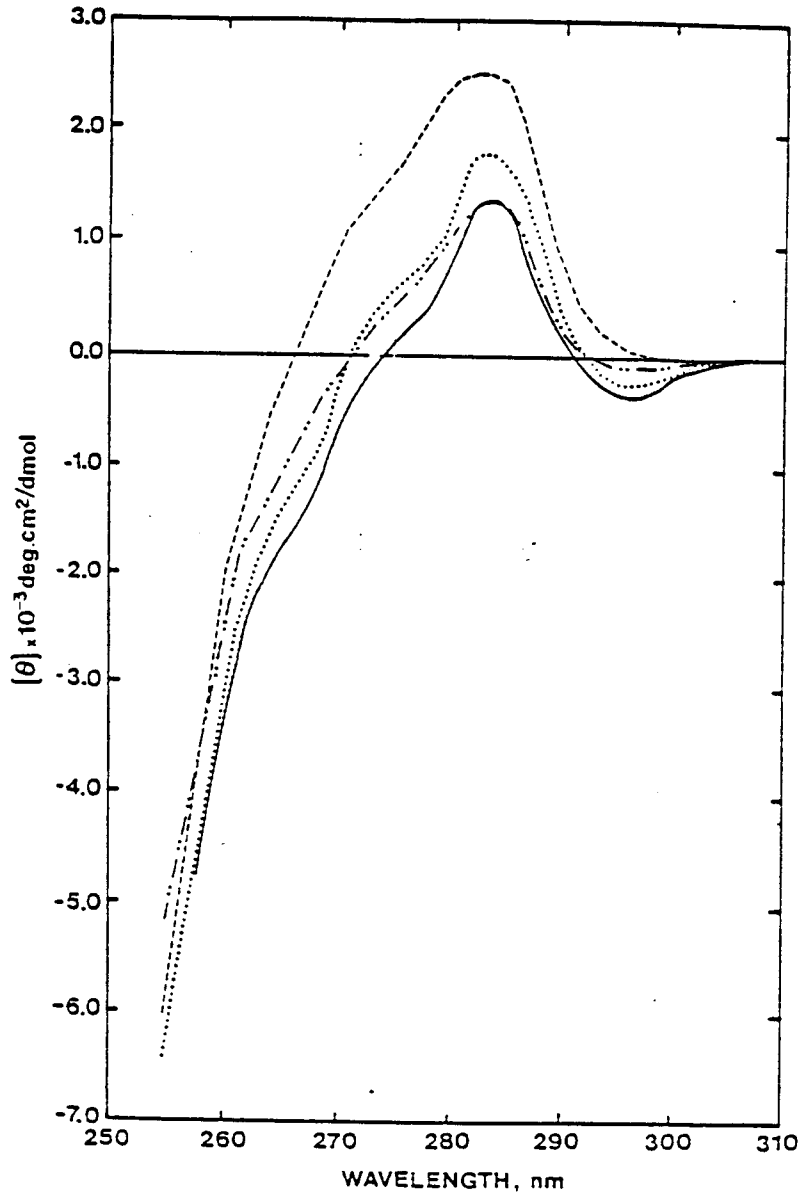
3.0
2.0
1.0
0.0
-1.0
-2.0
-3.0
-4.0
-5.0
-6.0
-7.0
250
260
270
280
290
300
310
WAVELENGTH, nm
[θ] x 10⁻³deg.cm²/dmol

**Figure III-2**

spectra are quite similar after such correction, some differences remain. For example, the negative peak at 296 nm, which is present at low salt, has significantly decreased at 0.6 M NaCl even after correction for the free DNA. Although the source of this negative ellipticity has never been explained, it may be related to some conformational constraints induced on the DNA by the N-terminal regions of the histones, since this band is also abolished by complete trypsinization of these histone regions (see Chapter II) or when they are extensively acetylated [Ausió and van Holde, 1986]. There are also some slight differences in the spectra in the region between 260-280 nm. Baase and Johnson (1979) have interpreted the change in ellipticity at 275 nm in terms of changes in the winding angle of the DNA. The differences observed here, between 0.1 M and 0.6 M NaCl (see Figure III-2) may indicate a small change in this parameter. The change in ellipticity we observe at 275 nm, after correction, would only correspond to a 4-5% of the total change in the winding angle per base pair inferred by Baase and Johnson (1979). The contribution of such a small difference is very difficult to detect at 282.5 nm and, under the low sample concentrations used here, should fall within the experimental error.

That most of the ellipticity change shown in Figure III-1 is caused by dissociation is supported by the salt

dependence of the ellipticity at this wavelength when highly concentrated nucleosome samples are used (see Figure III-1, open circles). In this case, the overall change in ellipticity as function of salt concentration is smaller, as would be expected from the concentration effects on the salt dissociation behavior of the nucleosome core particles [see Figure 5 in Ausió *et al.*, 1984]. However, the increase in ellipticity at 282.5 nm expected merely on this basis (the presence of ~5-6% free DNA) is still somewhat smaller than the experimental value (11-12%) (see Figure III-1). This is in agreement with a ~5% secondary structure change as predicted from the changes in the ellipticity at 275 nm mentioned above.

Below 260 nm, the spectra again change very little with increased salt. We find that the ellipticity at 222 nm of nucleosomal core particles becomes more negative by about 3% as the salt is increased from 0.1 M to 0.6 M (Figure III-3). Although interpretation of such a small change in terms of a change in $\alpha$-helical content is hazardous for a particle containing DNA as well as proteins, it would be noted that this change is in the same direction, and of the same order of magnitude, as that reported by Prevelige and Fasman (1987) for free histones. In any event, the data rule out any significant change in protein secondary structure.

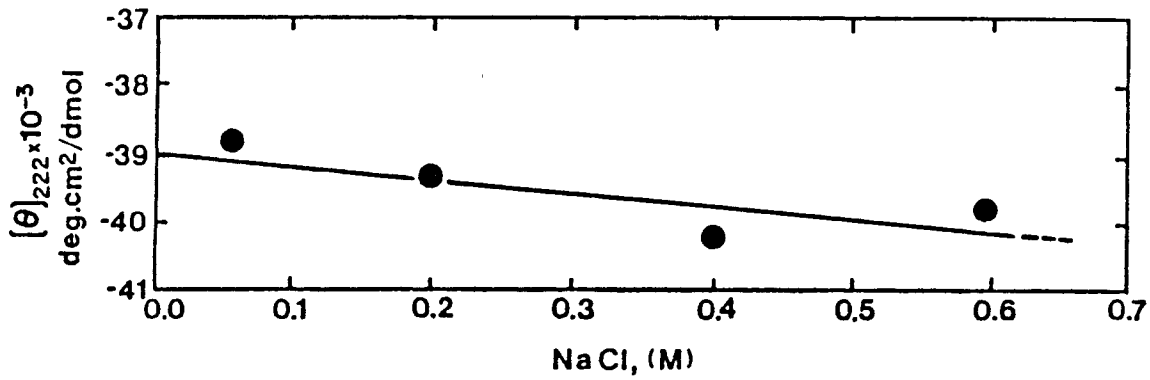


Figure III-3

Ellipticity at 222 nm of the nucleosome core particles as a function of the ionic strength. Experimental conditions are the same as in Figure III-2.

## 2. The DNase I Pattern of Digestion of Nucleosome Core Particles in 0.1 and in 0.6 M NaCl

The DNA in the nucleosome core particles, when digested with DNase I at low salt exhibits a very characteristic pattern of digestion [Noll, 1974a; Noll, 1974b]. This pattern arises from the local interactions between the histones and the DNA on its path around the histone core [Lutter, 1979]. Thus, the DNase I pattern of digestion of the DNA in the nucleosome is very different from that exhibited by DNA when in solution, or when interacting with a flat surface [Rhodes and Klug, 1980]. It should then be possible to monitor any changes in the tertiary structure of the DNA in the nucleosomes by the use of this enzyme. With this aim we decided to digest nucleosomes in 0.1 M NaCl and in 0.6 M NaCl with DNase I. However, one of the first problems encountered was the low rate of digestion at 0.6 M NaCl when compared to 0.1 M or lower ionic strength. Nevertheless, by increasing the enzyme-to-substrate ratio, it is possible to partially overcome this problem (see Materials and Methods). Figure III-4.A shows the DNase I digestion patterns of nucleosome core particles in 0.1 and 0.6 M NaCl at different temperatures. It is obvious from this figure that the patterns of digestion exhibited by the DNA in the nucleosome under the two ionic strengths are different.

Figure III-4. DNase I digestion patterns of nucleosome core particles in 0.1 and 0.6 M NaCl at different temperatures.

Panel A.

DNase I digestion patterns of nucleosomes in 0.1 M (lanes 1, 3, and 5) and in 0.6 M (lanes 2, 4, and 6) NaCl at different temperatures. Temperatures used for the digestions were 4 °C (lanes 1 and 2), 20 °C (lanes 3 and 4), and 37 °C (lanes 5 and 6). See Materials and Methods for details of digestion conditions. Gel electrophoresis was carried out under denaturing conditions at a 20:1 acrylamide to bis-acrylamide ratio. Numbers on side of gel represent the sites of the 10-bp repeating digestion pattern.

Panel B.

Control experiments to show the contribution of the free DNA dissociated from nucleosome core particles to the overall DNase I pattern of the nucleosomes. Gel electrophoresis was carried out under the same conditions as in Panel A. DNase I digestions of labeled nucleosome and deproteinized nucleosomal DNA were carried out at 0 °C on ice as described below. Lanes are:

M = size marker prepared by DMS-treated 195-bp DNA fragment from the *L. variegatus* 5S rRNA gene sequence [Simpson and Stafford, 1983].

#1 and #2 = DNase I digestion of nucleosome core particles in 0.1 M and 0.6 M NaCl at 0 °C.

#3 and #4 = labeled nucleosomal DNA of 5% or 10% amount of the labeled nucleosome (DNA weight) used in lanes #1 and #2, in the presence of excess of cold nucleosomes, were digested with DNase I under the same conditions as used for the nucleosome core particles (see Materials and Methods).

#5 and #6 = labeled nucleosomal DNA of 100% amount of the labeled nucleosome used in the nucleosome digestions were digested in the presence of excess cold nucleosomes in 0.1 and 0.6 M NaCl, respectively. Then, samples were loaded at amount corresponding to 5% (for 0.1 M) or 10% (for 0.6 M) of the amount of samples in Lane #1 or #2 (see Materials and Methods).

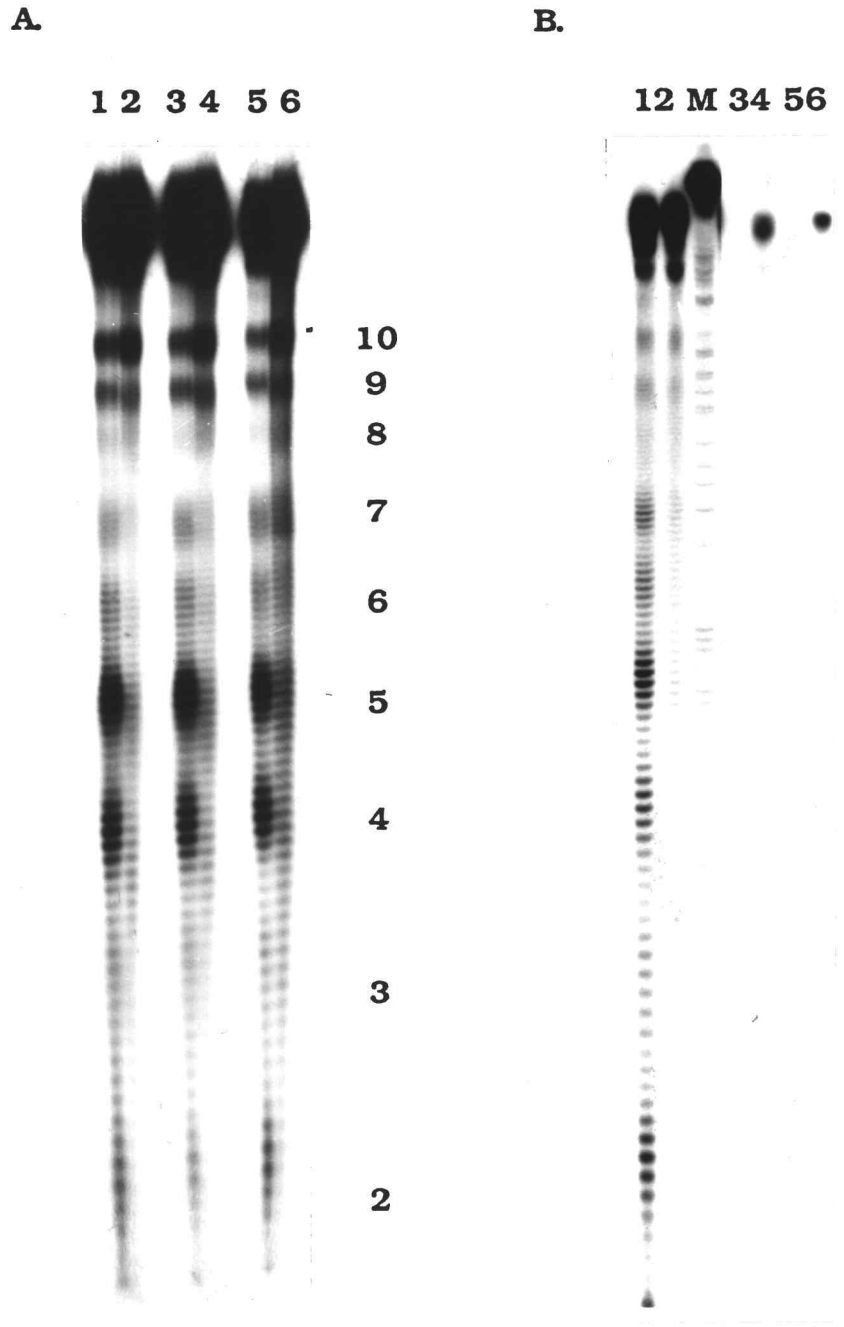


Figure III-4

From inspection of Figure III-4.A, two major differences become immediately apparent. The first is a significant weakening of the sharpness of the 10-bp repeat in all the regions along the DNA molecule, especially in the regions within 60 bp from the ends of the DNA. It seems as if the 10-bp repeat has blurred so as to resemble the digestion pattern exhibited by free DNA in solution. Secondly, the maximal cutting positions of some sites of the 10-bp repeating patterns are visibly shifted. Both differences are more obviously shown by the laser densitometer scans of the autoradiograms of the DNase I digestion patterns (see Figure III-5.A). At this point it is important to mention that all necessary experimental precautions have been taken in order to avoid any possible artifacts in gel electrophoretic analysis arising from the difference in salt concentration between the samples which were digested in 0.1 or 0.6 M NaCl, respectively. In each experiment, the nucleosome samples (both radioactively labeled and cold) for digestions in 0.1 M and 0.6 M NaCl were taken from the same batch, and after digestion the sample in 0.1 M salt was adjusted to 0.6 M by addition of concentrated NaCl. After that, the two samples were treated identically and loaded side by side on the gel. We performed more than ten independent experiments and all of them reproduced the observations mentioned above. We also included several controls in order to avoid artifacts arising from the

Figure III-5. Analysis of the fragment length distribution of DNase I footprinting of nucleosome core particles at different salt concentrations.

A. Scans of a 10:1 acrylamide:bis-acrylamide denaturing gel to show the DNase I digestion patterns of nucleosome core particles at 0 °C. Scans (from top to bottom) are corresponding to lanes #1, #2, and M of Figure III-4.B. The numbers on top indicate the hypersensitive sites in the 10-bp repeating patterns. Numbers marking the individual peaks in the scan of the marker lane (M) represent the lengths (in nucleotides) of the single stranded DNA molecules.

B. Graph of the fragment length distribution for the DNase I digestion patterns in 0.1 M ( ○ ) and in 0.6 M NaCl ( ● ). Gels of both 10:1 and 20:1 cross-linking ratios were used in order to analyzed DNA fragments (single-stranded) ranging from 20 to 146 nucleotide in length. The excess in length over 10.0 x n bases (where n is an integer) has been plotted as a function of band number n [Lutter, L. C., 1979].

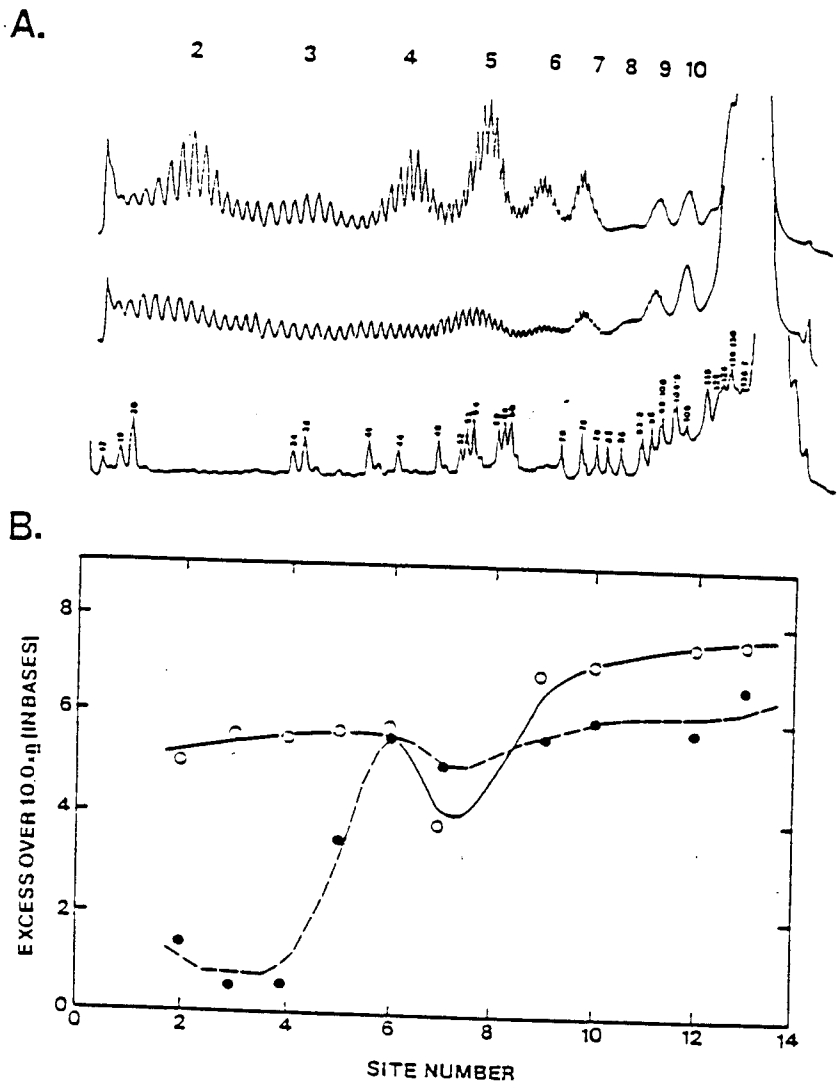
A.
2
3
4
5
6
7
8
9
10
B.
EXCESS OVER 10.0 [IN BASES]
SITE NUMBER
0
2
4
6
8
10
12
14

**Figure III-5**

different degree of DNA dissociation at the different salts and temperatures used [Ausió *et al.*, 1984]. Figure III-4.B shows an example of such control experiments, which evaluates the contribution of free DNA present in 0.1 M or 0.6 M NaCl at 4 °C to the overall DNase I digestions of nucleosomes under such condition. From these controls, the contribution of free DNA to the DNase I patterns was found to be negligible both in 0.1 and 0.6 M NaCl. As a matter of fact, when free 5′ end-labeled nucleosomal DNA (in an amount corresponding to 5% labeled DNA in the digestion of nucleosome samples) was digested in the presence of 100 μg/ml cold nucleosomes (which is the total nucleosome concentration in the digestion of nucleosome samples) at 0.1 M NaCl under the same digestion conditions used for end-labeled nucleosomes in this salt and then run side by side in the same gel, the lane corresponding to this amount of free DNA did not show any bands by the time that the nucleosome counterpart was fully autoradiographed (see lane 4 in Figure III-4.B). The same was also true when an amount corresponding to 10% 5′ end-labeled free nucleosomal DNA was digested in the presence of 0.6 M NaCl (lane 5). (The values for the estimated amount of dissociated DNA at 0.1 and 0.6 M NaCl were taken from the data of Ausió *et al.*, 1984.) The same results were obtained in an additional control experiment, when labeled free nucleosomal DNA in an amount equal to the total amount of the labeled DNA present

in the labeled nucleosome sample was digested in the presence of excess cold nucleosomes (labeled free DNA/cold nucleosomes = 1:10, total DNA concentration = 100 μg/ml), and then loaded onto the gel in quantities corresponding to 5% or 10% of the starting sample.

Figure III-5.A shows the scans of one of the gels of 5′-end labeled nucleosomes digested at 0 °C in the presence of 0.1 and 0.6 M NaCl. These data have been used in order to obtain the data presented in Figure III-5.B, plotted in the manner described by Lutter (1979). The interpretation of this latter figure is not so simple as in Lutter (1979). It is important to point out here that our digestion conditions are different from those used by Lutter. In our case, we have decreased both the amount of divalent ions and the temperature so as to minimize the dissociation effects. Clearly, as is evident in both Figure III-4 and III-5, the spacing of positions of maximum cleavage depends on salt concentration and these differences change markedly in different regions of the core particle. In particular, the data indicate significant changes in the pitch of the DNA in 0.6 M NaCl at around 40 to 60 bp from the 5′ end. Indeed, it has been shown that the site at ~20 bp from the center of the core DNA exhibits an enhanced sensitivity toward micrococcal nuclease when the temperature of the digestion is decreased to 4 °C [Huang and Garrard, 1986; Libertini *et al.*, 1988]. The DNA molecule in the nucleosome seems to

adopt a strongly kinked configuration around this position as evidenced by the intercalating behavior of methylene blue [Hogan *et al.*, 1987]. Furthermore, the regions with altered DNase I patterns observed by us agree fairly well with the sites of DNA bending observed by Richmond *et al.* (1984).

## 3. The Virial Coefficient and the Hydration of the Nucleosome Core Particle at Moderate Ionic Strengths

When X-ray scattering is used to analyze the nucleosome salt-dependent conformational transition [Greulich *et al.*, 1985], no change in the radius of gyration of the particle is observed within the salt range 0.1-0.6 M NaCl. Yet, a significant decrease in I(0) (intensity at 0 angle) is observed when the salt concentration was increased from 0.1 M to 0.6 M. Since I(0) is proportional to the apparent molecular weight ($M^{app}$) multiplied by a "contrast factor":

$$I(0) \cong (\partial\rho_{el}/\partial c_2)^2_{\mu} \; M \; {}^{a}_{2} \; {}^{pp}c_2 \qquad (2)$$

$\rho_{el}$ = electron density

$(\partial\rho_{el}/\partial c_2)^2_{\mu}$ = electron density increment

then a decrease in I(0) must therefore be accompanied by a corresponding decrease in either $M_2^{app}$ (apparent molecular weight) of the particle or in the contrast factor. A similar drop in I(0) has been observed by neutron scattering [Ramakrishnan, V., Yager, T. and van Holde, K. E., unpublished data]. At the high particle concentrations required by these techniques (~ 10 mg/ml), the salt-dependent dissociation of DNA from the nucleosome core particles cannot account for the decrease in $M_2^{app}$, since under such concentrations the amount of dissociated DNA even at 0.6 M NaCl and room temperature is estimated to be quite small (≤ 4%) [Ausió *et al.*, 1984]. It is also important to mention here that below 0.8 M NaCl there is no loss of mass due to selective histone dissociation [Yager and van Holde, 1984].

The fact that the frictional parameters of the particles increase without any change in the radius of gyration, at the same time as the zero angle scattering decreases, prompted us to undertake a careful hydrodynamic analysis of the virial coefficients of the nucleosome particles at 0.1 and at 0.6 M NaCl. The results of such analyses are shown in Figure III-6. From this graph it is possible to evaluate both the molecular weight of the particles and their virial coefficients according to the

Figure III-6

Dependence of the reciprocal of the apparent molecular weight (z-average) on concentration, for the nucleosome core particles in 0.1 M NaCl ( ○ ) or in 0.6 M NaCl ( ● ). The concentration of the nucleosome core particles in the sample was calculated from the absorbance of the sample at 260 nm using an extinction coefficient for the nucleosome core particle = 9.5 $cm^2 \cdot mg^{-1}$ [Ausió and van Holde, 1986].

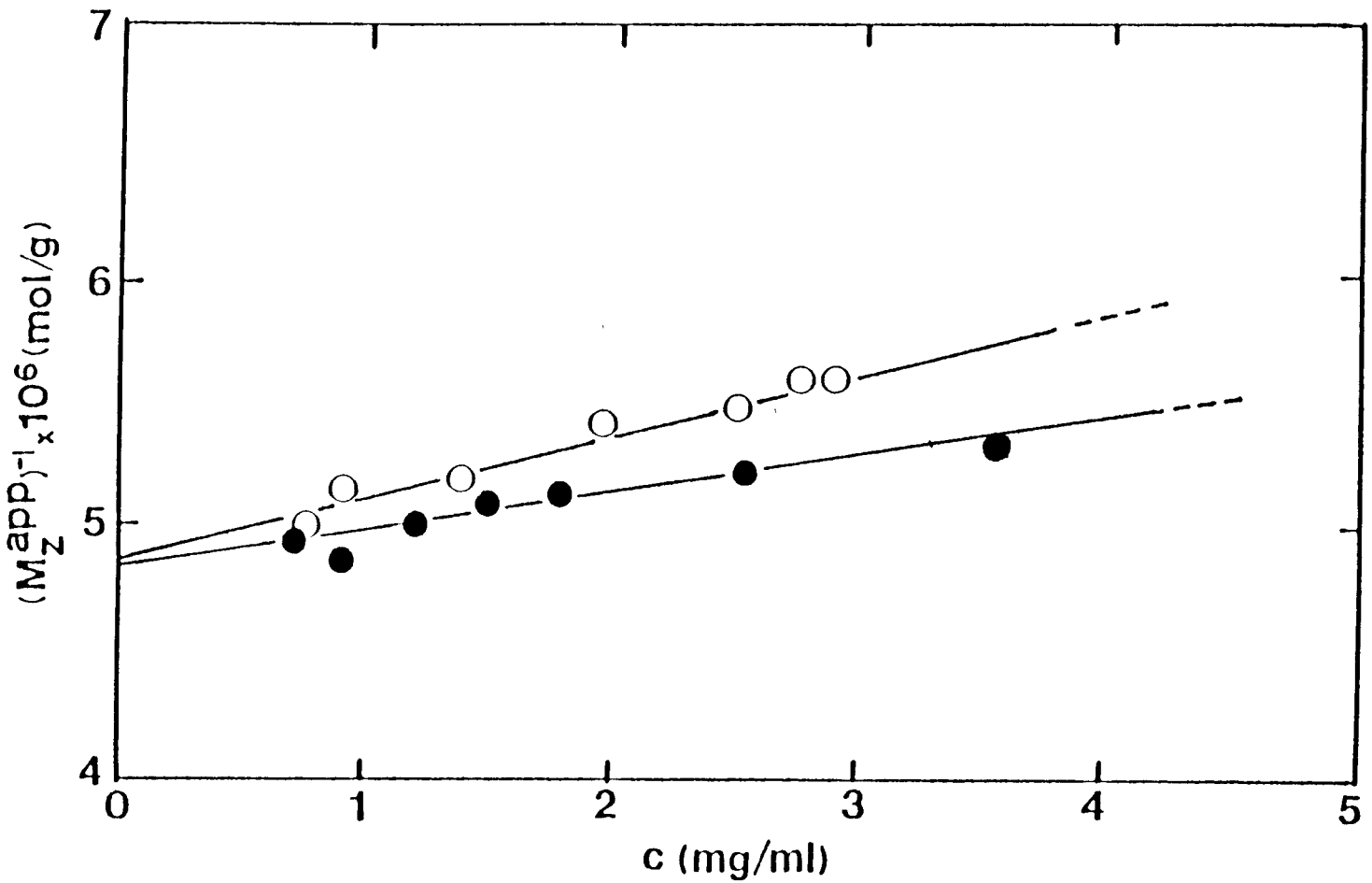


Figure III-6

equation:

$$(M_z^{app})^{-1} = M_z^{-1} + 4Bc_2 \qquad (3)$$

where, $M_z = M_2$ for homogeneous samples.

B = colligative second virial coefficient defined as in Roark and Yphantis (1969)

The molecular weight (z-average) estimated from both sets of data was 205,800 g/mol which is in excellent agreement with the value calculated from the composition of the particle: 205,400 g/mol for a nucleosome with a 146-bp DNA. The virial coefficients were found to be: $6.1 \times 10^{-5}$ ml mol/g$^2$ for nucleosomes in 0.1 M NaCl, and $3.8 \times 10^{-5}$ ml mol/g$^2$ for nucleosomes in 0.6 M NaCl in 10 mM Tris-HCl, pH 7.5 buffer. These experimental values for B compare very well with the theoretical values calculated from either:

$$B = B_{ex} + B_D \quad \text{or} \quad B' = B'_{c_x} + B_D \qquad (4)$$

where $B_{ex}$ = contribution to the virial coefficient due to the excluded volume, which for a sphere of radius R is:

$$B_{ex} = \frac{16N\pi MR^3}{3M_2^2} \qquad (5)$$

Alternatively, we may express the excluded volume in

terms of the partial specific volume $\bar{v}_2$:

$$B'_{c_x} = \frac{4\bar{v}_2}{M_2} \qquad (6)$$

In equation (3), $B_D$ is the Donnan contribution to the virial coefficient, and is given by:

$$B_D = \frac{(Z \cdot \delta)^2}{4M_2C_3} \qquad (7)$$

where Z is the total net charge of the particle, $C_3$ is the molar concentration of the salt in the buffer, and $\delta$ is the effective fraction of charge remaining after counterion condensation, which for DNA is equal to 0.24 [Record *et al.*, 1978; Manning, 1978]. In these calculations we have used R for the nucleosome core particle = 5.5 nm, $\bar{v}_2$ = 0.670 $cm^3/g$ (see below) and $Z^-$ = 78 (292 negative charges due to the phosphates in the DNA - 214 positive charges due to the arginine and lysine residues of the histones). The values estimated from these parameters were: B = 6.3 x $10^{-5}$ ml mol/$g^2$ and B′ = 3.4 x $10^{-5}$ ml mol/$g^2$ in 0.1 M NaCl and B = 4.6 x $10^{-5}$ ml mol/$g^2$ and B′ = 1.7 x $10^{-5}$ ml mol/$g^2$ in 0.6 M NaCl. Such values, although they should only be taken as approximate estimates, agree very well with the experimental ones. The difference observed for the virial

coefficients between 0.1 and 0.6 M NaCl, however, cannot explain the drop in I(0) seen by the X-ray scattering, since the change is in the wrong direction. Nucleosomes are less non-ideal in high salt, and therefore the apparent molecular weight [and I(0)] should increase with salt at these high concentrations.

Yet another explanation for the change in I(0) could arise from a dramatic change in the hydration of the particle upon increase of the ionic strength of the buffer from 0.1 to 0.6 M NaCl, which would affect the "contrast" factor. To check for this alternative possibility, we conducted a series of density contrast variation experiments in the ultracentrifuge [Eisenberg and Felsenfeld, 1981; Greulich *et al.*, 1985]. The results of such equilibrium sedimentation analysis are summarized in Figures III-7 and III-8. Figure III-7 shows data for the experimental quantity $M_z(\partial\rho/\partial c_2)_\mu$ versus the density of a series of sucrose solutions ($\rho$), in 0.1 M, 0.3 M, and 0.6 M NaCl. The slope of a curve like the plots in Figure III-7 allows us to evaluate the preferential hydration parameter of the nucleosome particle ($\xi_1$), which represents water "preferentially bound" to the particles (grams of water bound per gram of particles), according to the equation

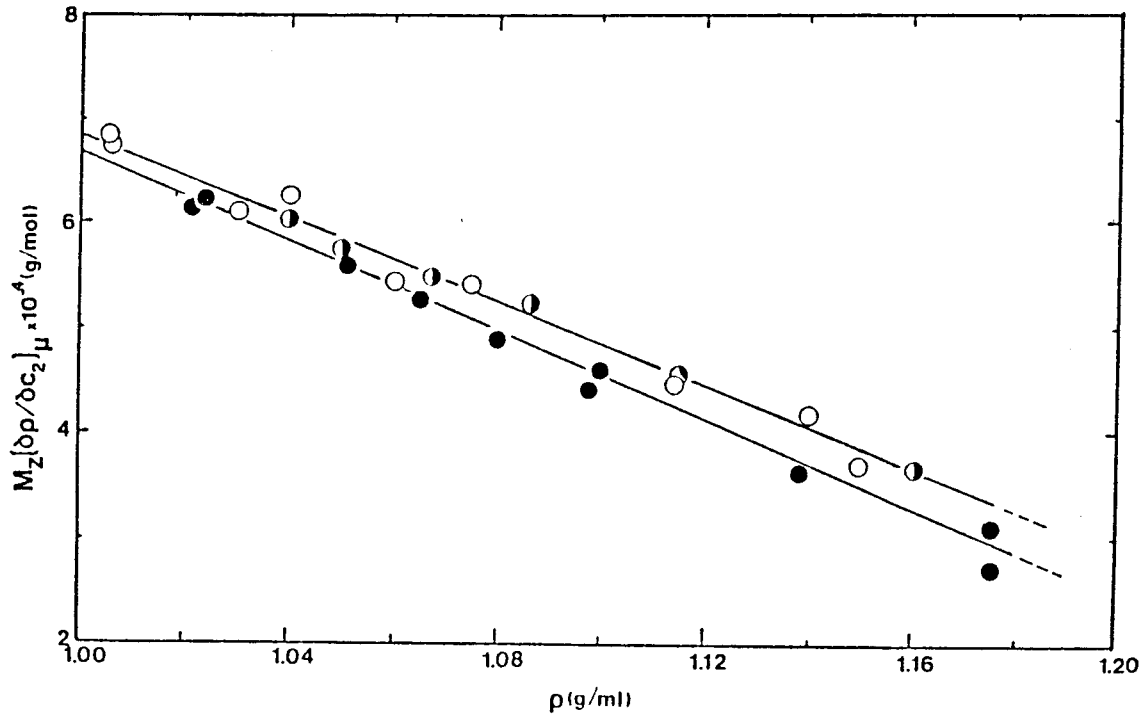


Figure III-7

Plots of $M_z(\partial\rho/\partial c_2)_\mu$ from sedimentation equilibrium versus solution density ($\rho$). Experiments were carried out in sucrose at ( ○ ): 0.1 M NaCl, ( ◑ ): 0.3 M NaCl, or ( ● ): 0.6 M NaCl in 10 mM Tris-HCl, pH 7.5 buffer.

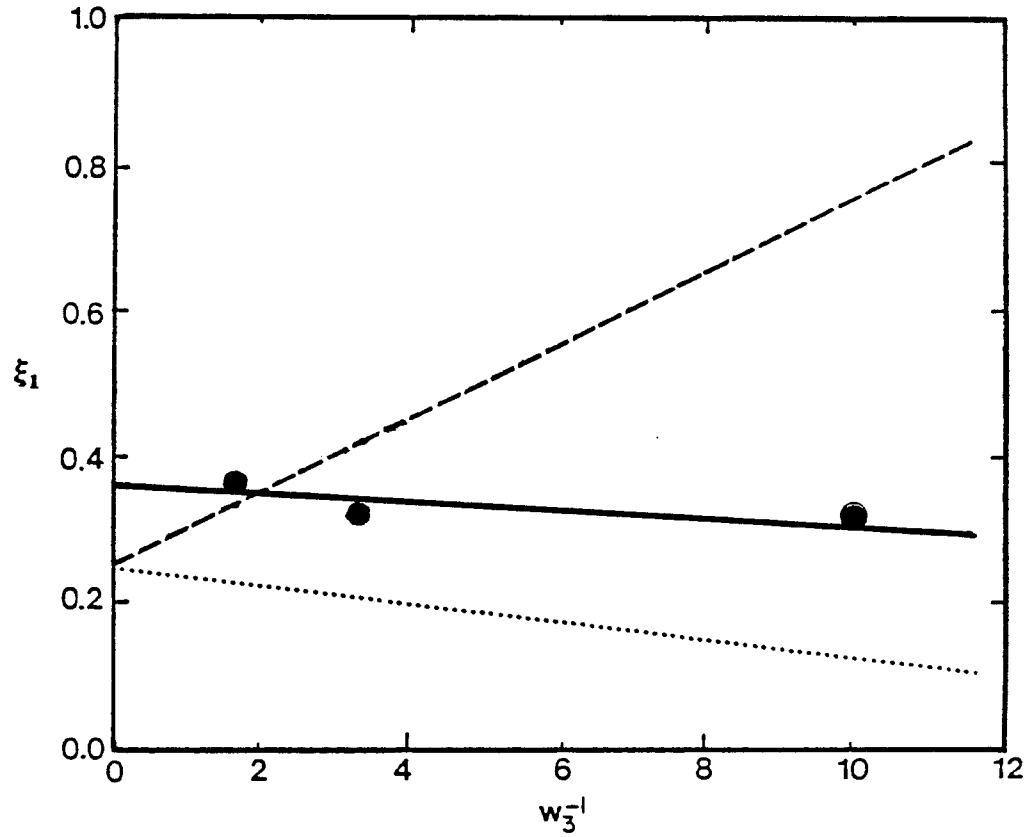


Figure III-8

Change of the preferential interaction parameter $\xi_1$ as a function of the reciprocal NaCl weight molality $W_3^{-1}$ for the nucleosome core particle ( ●, ○ ); ( ● ): present data; ( ○ ), from Greulich et al. (1985). Curve ( ------ ): DNA in NaCl; curve ( ····· ): bovine serum albumin in NaCl [data taken from Pundak and Eisenberg, 1981].

[Eisenberg, 1976; Eisenberg and Felsenfeld, 1981]:

$$(\partial\rho/\partial c_2)_\mu = (1 + \xi_1) - \rho(\bar{v}_2 + \xi_1 \bar{v}_1) \qquad (8)$$

Here $\rho$ = density of the buffer; $c_2$ = concentration of the nucleosomes, and $\bar{v}_2$ and $\bar{v}_1$ are the partial specific volumes of the particle and water, respectively. Given that $M_z$ = 205800 and $\bar{v}_1 = 1$, the $\bar{v}_2$ and $\xi_1$ values obtained from the intercepts and slopes of the lines shown in Figure III-7 were: $\bar{v}_2 = 0.667$ ml/mg and $\xi_1 = 0.301$ g $H_2O$/g nucleosome for nucleosomes in 0.1 and 0.3 M NaCl. Likewise, $\bar{v}_2$ and $\xi_1$ were found to be 0.675 ml/mg and 0.361 g $H_2O$/g nucleosome for nucleosomes in 0.6 M NaCl.

The preferential hydration parameter can also be expressed in terms of decomposition as [Eisenberg, 1976; Reisler *et al.*, 1977]:

$$\xi_1 = B_1 - \frac{B_3 - E_3}{W_3} \qquad (9)$$

where $B_1$ = water binding (hydration), $B_3$= salt binding, $E_3$ = salt exclusion by Donnan effects, and $W_3$ = molality of the salt in the buffer. The absolute hydration of the particle can thus be evaluated from the intercept of a plot of $\xi_1$ versus $W_3^{-1}$ (see Figure III-8).

The hydration value thus obtained for the nucleosome (~0.37 g $H_2O$/g nucleosome) is slightly higher than that

observed for typical globular proteins such as BSA or even for naked DNA (see Figure III-8). Yet, from the salt dependence on the preferential hydration parameter, $\xi_1$, it seems unlikely that the changes in hydration observed here could, only by themselves, account for the changes in I(0) observed with the small angle scattering.

## Discussion

The two simplest mechanisms to account for the salt-dependent conformational change of the nucleosome core particle are schematically represented in Figure III-9. Both models satisfy the experimental observation made by different groups [McGhee *et al.*, 1980; Wilhelm and Wilhelm, 1980; Eisenberg and Felsenfeld, 1981; Ausió *et al.*, 1984; Yager and van Holde, 1984] of an increase in the frictional parameters of the particle as the salt increases in the range of ionic strength below the point at which selective histone dissociation from the DNA begins (around 0.8 M NaCl) [Burton *et al.*, 1978]. The first of these models, proposed by Ausió *et al.* in 1984, was based on the earlier observations made by Cary *et al.* (1978), and later by Walker (1984), that the N-terminal regions of the histones become mobile and eventually dissociate from the tight interactions with DNA in this salt range. However, it has recently been shown in this work that complete removal of these histone "tails" does not abolish the salt-dependent change in frictional coefficient (see Chapter II).

The second model (release of DNA ends), which had been proposed by Harrington (1982), seems at first sight more suitable inasmuch as it would represent an intermediate stage in the DNA dissociation process observed in this range of ionic strength [Ausió *et al.*, 1984; Yager and van

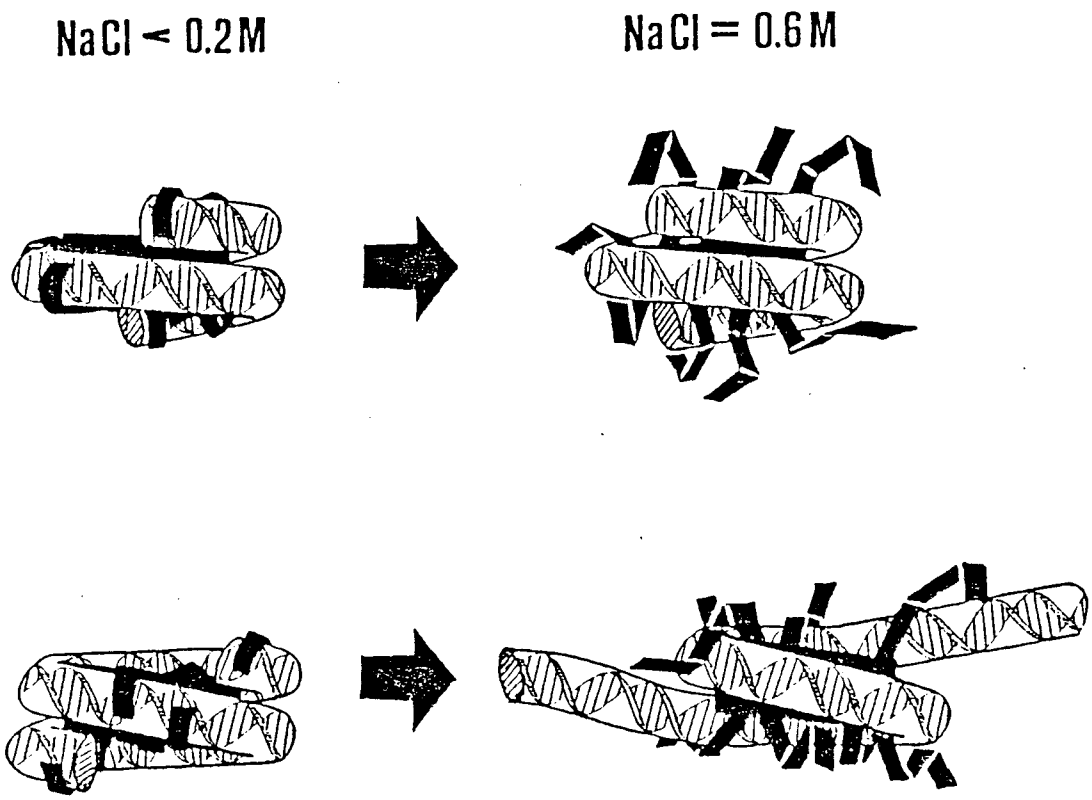


**Figure III-9**

**Schematic representation of two possible models in order to account for the salt dependent changes of the frictional parameters of the nucleosome core particles.**

**A. Release of the N-terminal region of the histones.**

**B. Partial release of the flanking DNA ends.**

Holde, 1984]. However, this model is in serious conflict with the constancy of the radius of gyration [Greulich *et al.*, 1985; Ramakrishnan, Yager and van Holde, unpublished data]. Obviously, other more complicated models might be constructed. Nevertheless, all of them would have to fulfill the seemingly existing paradox of an important change in the frictional parameters of the particle while maintaining constant its radius of gyration. As was mentioned in the Introduction, subtle conformational changes may be involved in this structural transition. In this context, we have carefully checked for possible changes in the secondary structure of both histones and DNA. From the circular dichroism experiment in the preceding experimental section, we find that only ~ 5% change is observed in both cases. In the case of the spectrum region above 250 nm, we have shown that the changes in the spectra as a function of salt concentration are mainly due to the partial DNA dissociation from the particle at different salt concentrations. This is true for the low concentrations of the sample normally used in this kind of experiments, and therefore rules out the correlation between the changes in the CD spectrum above 250 nm and those observed for the frictional parameters of the particle, as had been proposed before [Wilhelm and Wilhelm, 1980]. Our interpretation is in very good agreement with the observation made by Walker (1984), using long H-1 stripped polynucleosome chains. In

that case (where the DNA dissociation should be almost completely abolished), it was found that the CD spectrum did not change upon increasing the salt from low salt to 0.7 M NaCl. The interpretation given by Walker to such findings, based on the proton NMR studies, was a decrease in the supercoiling of the DNA as a consequence of the histone "tail" release[1] [Walker, 1984]. This conclusion, although supported by the changes in the sedimentation coefficients of the same kind of polynucleosomes, does not necessarily need to be invoked in order to properly interpret the CD in view of the data presented here. Nevertheless, our DNase I digestion patterns at 0.6 M NaCl seem to corroborate also the relaxation of the supercoiling of the DNA, especially by loosening its histone interaction constraints at the flanking ends of the nucleosome particle, as observed by Walker (1984). As a matter of fact, from the CD results obtained at high concentrations of the sample and also from the changes in $[\theta]_{275\ nm}$, a slight but noticeable change in the secondary structure of the DNA (~5%), as mentioned above, seems to take place within this salt range. This change in the DNA seems also to be accompanied by an almost quantitatively identical change in the secondary structure

---

[1]: It has been shown that the histone "tails" have no secondary structure or CD [Diaz and Walker, 1983]; therefore, there is no change in the secondary structure of the histones when the "tails" dissociate from tight contacts with DNA [Walker, 1984].

of the histone octamer detected by the changes in the CD spectrum at 222 nm.

The findings that there is very little change in the histone and DNA secondary structure, together with the previous observations that the radius of gyration of DNA in the nucleosome remains unchanged within the salt concentration range in which the salt-dependent conformational transition occurs, suggest a model involving the changes in the tertiary and/or quaternary (the steric arrangement of histone subunits) of the histone octamer. Figure III-10 shows a simplified diagram of the model to demonstrate how such changes could increase the overall size of a nucleosome core particle, and therefore increase its frictional coefficient, while maintaining the DNA radius of gyration unaffected. At this point it is difficult to relate the changes in the secondary structure of the histones (observed in this study) to any major change in the tertiary structure of the histone octamer, such as that proposed by Chung and Lewis (1986) when using reconstituted nucleosomes containing fluoresceinated H4. It seems possible that some kind of tertiary or quaternary structural changes, such as the rotation of the H4 domains proposed by these authors, might account for all or part of the change in frictional parameters. However, it seems unlikely that the transition involving H3, postulated by Cantor *et al.* (1981), can explain our data, since studies with H3-H3

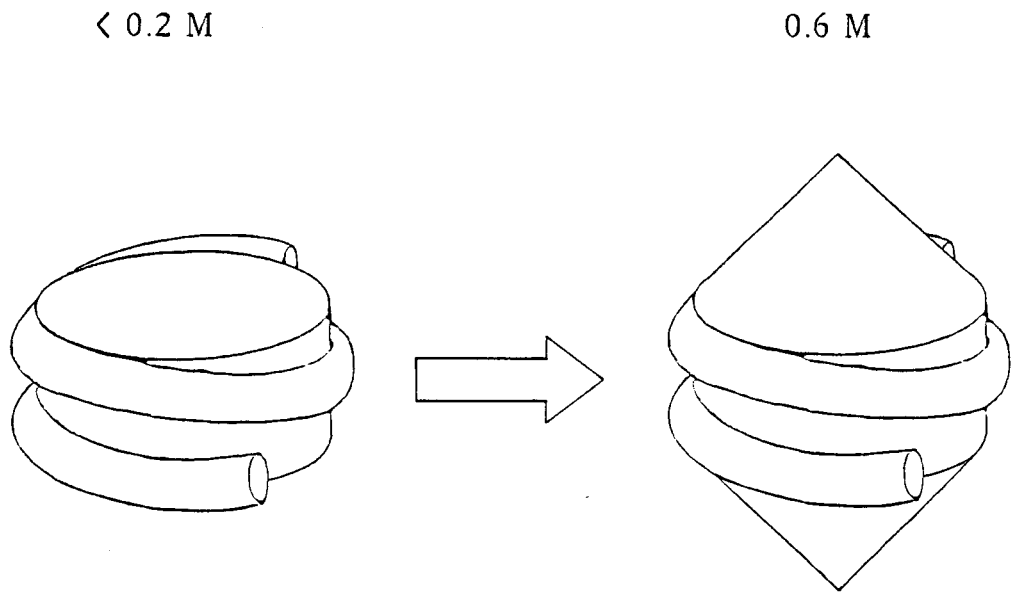


Figure III-10. Diagram of a model demonstrating possible tertiary and/or quaternary structural changes of the histone octamer during the salt-dependent nucleosome conformational transition.

crosslinked histones show no effect on the transition [Ausió *et al.*, 1984].

Any change in tertiary structure must also involve the DNA conformation, as evidenced by the changes in DNase I pattern in 0.6 M NaCl mentioned above. The loss of the 10-bp periodicity toward the ends of the pattern suggest a loosening or relaxation of the DNA in these flanking regions. On the other hand, an important shift in the sites of the DNase I cleavage is also observed, which seems to have a major effect in the pitch of DNA in the region up to 40 to 60 bp from the 5′ ends. We do not have at present any clear interpretation of this phenomenon.

We have shown that the virial coefficients of the nucleosome particle can be accounted for from the known dimensions and volume of the particle when the contribution of the charge is also taken into consideration. Our predicted values for B and the observed ones are in very good agreement. They are, however, significantly smaller than the value of B = 2.8 x $10^{-4}$ ml mol/$g^2$ previously reported by Greulich *et al.* (1985), using small angle X-ray scattering. In the present case, we do see a difference in the virial coefficient between 0.1 and 0.6 M NaCl, which is expected as a consequence of the reduced Donnan effect in high salt. Furthermore, we observe a significant change in the preferential hydration parameter in going from 0.1 to 0.6 M NaCl (see Figure III-8). Such a change can not only

affect the $\Delta I(0)/c_2$,[2] but also the frictional parameters of the particle. Indeed, the values for $f/f_o$ for the nucleosome core particle in 0.1 M NaCl ($f/f_o$ =1.50) or in 0.6 M NaCl ($f/f_o$ = 1.70) [data from Chapter II] change to $f/f_o$ = 1.30, and $f/f_o$ = 1.40 at 0.1 and 0.6 M NaCl, respectively, when the $\xi_1$ values found here are taken into account.

Thus, it seems likely at this point that the salt-dependent conformational transition of the nucleosome core particle arises from the confluence of several subtle alterations on the physical properties of the particle involving the secondary structure of its chemical components (histones and DNA) and also the global extent of hydration. However, the exact relationship of these changes to the tertiary structural changes exhibited by the whole particle, as envisaged from the circular dichroism, DNase I digestion, and sedimentation behavior pattern remains yet to be established.

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[2]: The change in preferential hydration parameter affects the $\Delta I(0)/c_2$, following the relations established by equations (2), (8), and:

$$(\partial\rho_{el}/\partial c_2)_\mu = \ell_2 - \frac{\ell_1 + w_3\ell_3}{1 + w_3}\,[1 - (\partial\rho/\partial c_2)_\mu] - \frac{\ell_1 - \ell_3}{1 + w_3}\,\xi_3$$

where $\ell_i$ are electrons per gram of component i, and $w_i=c_i/c_1$ are molalities (gram of component i per gram of principal solvent, water). See Eisenberg (1981) and Greulich *et al.* (1985) for more details.

**Acknowledgements**

I would like to gratefully acknowledge Dr. Juan Ausió for his advice, discussion, as well as his active involvement in this research project. Credit should be given to him for his cooperation in the design of the research and in the analysis of experimental data, for his discussion and help in understanding the results, and for the calculations on the virial coefficients and the preferential hydration parameters he performed for nucleosome core particles.

Special thanks should also be addressed to Colleen Nelson, who was actively involved in this study and was responsible for the performance of DNase I footprinting of nucleosomes at different temperatures (Figure III-4.A).

Acknowledgements also extend to Dr. Jeffrey Hansen and Dr. Karen Miller for their discussions and advice.

# Chapter IV

## DNA and Protein Determinants of Nucleosome Positioning on a Eukaryotic Gene — the Sea Urchin 5S rRNA Gene Sequence

## Introduction

Nucleosome positioning on specific gene sequences has drawn much interest in the research field of chromatin structure and function, as well as of gene transcription and replication. Positioning of nucleosomes has been examined on many DNA sequences both *in vivo* and *in vitro* (see Chapter I.E. for details). However, while results on nucleosome positions on specific gene sequences have been accumulated, the determinants which cause nucleosomes to occupy certain positions on a DNA sequence still remain unclear. Although several mechanisms have been proposed to address the problem of how nucleosome positions are determined, yet there are no working mechanisms established.

During the last few years, much effort has been concentrated on understanding the potential roles of the special DNA sequence or sequence-related characteristics on the determination of nucleosome positions. Much evidence suggests that the histone-DNA interactions or DNA sequence-dependent mechanical properties, such as bending and flexibility, may be responsible for positioning nucleosomes on DNA sequences [Trifonov, 1980; Drew and Travers, 1985; Satchwell *et al.*, 1986; Travers, 1987; Shrader and Crothers, 1989].

While there has been much interest in the DNA sequences that determine the positioning of nucleosomes, very little

attention has been paid to the histone determinants. The main problem involved has been the difficulties to prepare well defined modified histones. The use of immobilized trypsin to prepare precisely defined "tail"-free histone octamers, combined with the sea urchin 5S rDNA sequences used for the previous studies of nucleosome positioning [Simpson and Stafford, 1983; FitzGerald and Simpson, 1985; Hansen *et al.*, 1989], provide a very useful system for analyzing the histone and DNA determinants as well as giving a solution to the confusing results of the nucleosome positioning on the 5S rRNA gene sequences (see Chapter I.E.2, Introduction).

In order to determine whether the existence of multiple positions is an exclusive property of the tandemly repeated templates, as well as to understand better the mechanisms responsible for the determination of nucleosome positions, we have examined the distribution of native and trypsinized nucleosomes present, after salt dialysis reconstitution, on both tandemly repeated DNA templates and several monomeric templates derived therefrom by restriction endonuclease digestion, containing different permutations of the sea urchin 5S rRNA gene sequence. In all cases, a major nucleosome position, as well as a number of minor positions have been observed, which indicates that the generation of multiple positions is an inherent property of the 5S rRNA gene sequence. Interestingly, all positions observed differ

by multiples of 10 bp. Data obtained under different reconstitution conditions demonstrate that the observed distributions of nucleosomes on these DNA templates is an equilibrium distribution. This study has also examined the positioning of histone octamers from which histone "tails" had been removed by tryptic digestion. Results indicate that the histone "tails" are not determinants of nucleosome positioning. While the results suggest that the mechanical properties of the 5S rDNA are the fundamental factors determining nucleosome positioning, they are insufficient to direct all nucleosomes into a single position.

## Materials and Methods

1. Preparation of DNA templates.

Both monomer fragments and tandemly repeated DNA templates containing the sea urchin 5S rRNA gene sequence were derived from plasmids p5S207-12 or p5S172-12 [Simpson *et al.*, 1985; Hansen *et al.*, 1989; also see Figure IV-1.A.]. The plasmids are composed of a 3559-bp pAT153 vector with DNA sequences of either 207-12 or 172-12 (see below) inserted at the *Ava* I site (position 1424 on the pAT153). Plasmids were purified from *E. coli* HB101 by the alkaline lysis method [Micard *et al.*, 1985] followed by cesium chloride gradient banding. Briefly, the bacteria were cultivated in LB medium at 37 °C [Maniatis *et al.*, 1982], then harvested by centrifugation in a Beckman J-4 centrifuge for 30 min at 4,200 rpm. The cells obtained from every 500-ml culture were then digested with 16 mg lysozyme in 10 ml GET buffer (50 mM glucose, 25 mM EDTA, 10 mM Tris-HCl, pH 7.8) for 10 min at room temperature, after which the suspension was mixed with 2x volume of a solution containing 0.2 M NaOH, 1% SDS, and subsequently homogenized briefly until the suspension became jello-like. The suspension was incubated on ice for 10 min, then mixed with

Figure IV-1.

DNA templates used for oligonucleosome (Panel A) and mononucleosome (Panel B) reconstitutions. See Experimental Procedures for details of preparation. Shaded areas represent the major nucleosome position on the sea urchin 5S DNA sequence (position 1-146). Restriction sites are: A=*Ava* I; E=*Eco*RI; X=*Xmn* I; Aa=*Aat* II; Al=*Alu* I; M=*Msp* I; R=*Rsa* I; and H=*Hha* I.

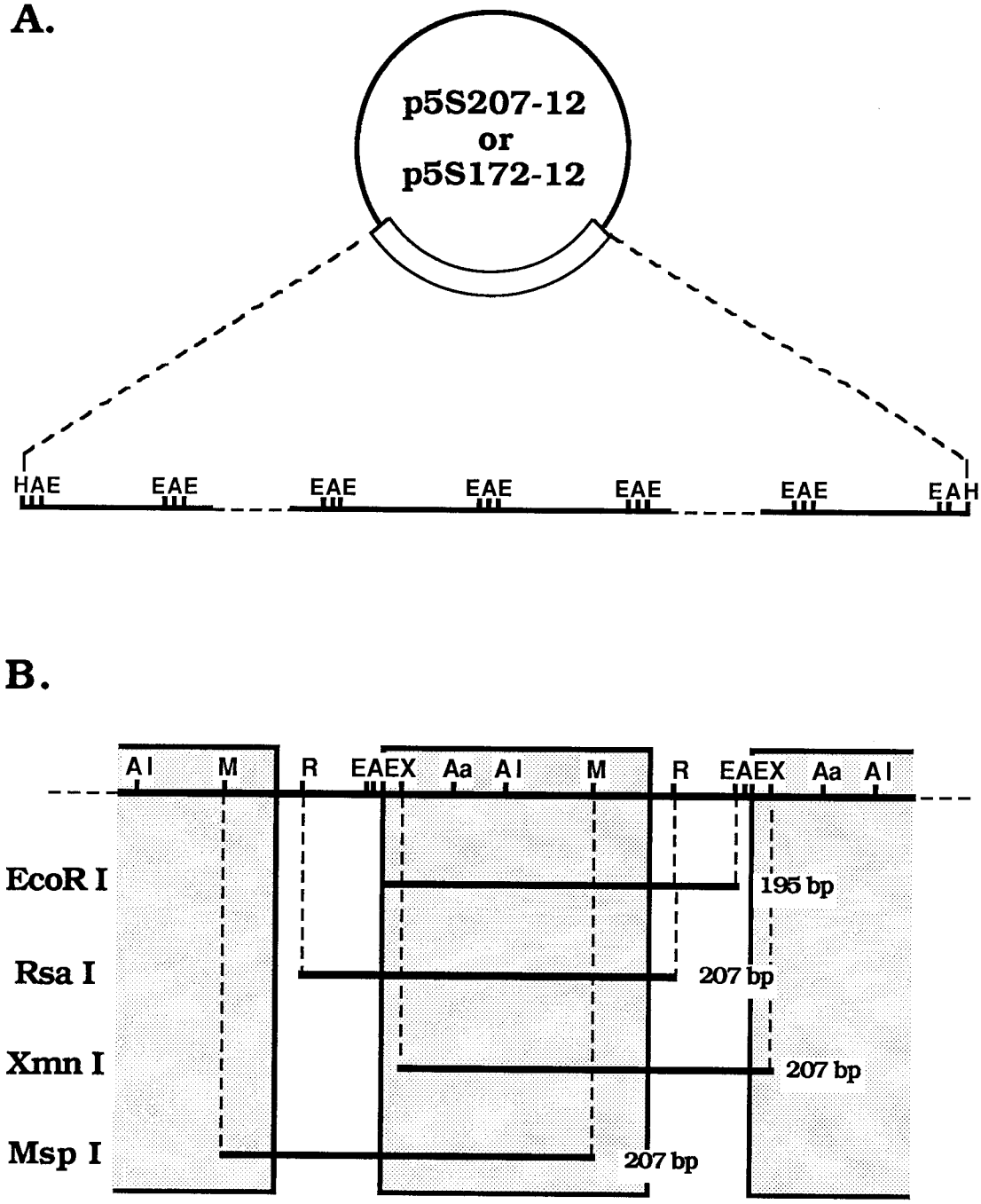
A.
p5S207-12
or
p5S172-12
HAE
EAE
EAE
EAE
EAE
EAE
EAH
B.
AI
M
R
EAEX
Aa
AI
M
R
EAEX
Aa
AI
EcoR I
195 bp
Rsa I
207 bp
Xmn I
207 bp
Msp I
207 bp

Figure IV-1

one half volume of 5 M potassium acetate[3], and the precipitate was removed by centrifugation in a Sorvall SS-34 rotor at 10,000 rpm for 15 min. The plasmids were recovered by ethanol precipitation, further purified by phenol/chloroform extraction, and recovered again after repeated ethanol precipitations. Finally, the plasmids were centrifuged in cesium chloride gradients in order to remove RNA completely [Maniatis *et al.*, 1982].

Oligonucleosome templates consisting of 12 repeats of a 207-bp (207-12 template) or a 172-bp (172-12 template) DNA sequence were prepared by *Hha* I restriction enzyme digestion of plasmids p5S207-12 or p5S172-12. After digestion with *Hha* I, the plasmid vector DNA was cut into small pieces (<400 bp in size). The oligonucleosome templates were then purified from the plasmid vector DNA fragments, by exclusion chromatography on Ultrogel A2 [Hansen and Rickett, 1989]. Under proper conditions (elution by natural hydraulic pressure generated from the column itself, ~6 ml/hr; room temperature), a 115-ml column with dimensions of 0.75 cm (diameter) x 65 cm (high) provides complete separation of the oligonucleosome templates from the plasmid vector DNA fragments (<400 bp).

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[3]: 5 M potassium acetate solution was prepared (for 50 ml) by mixing: 30 ml 5.0 M potassium acetate, 5.75 ml glacial acetic acid, and 14.25 ml glass distilled $H_2O$. The resulting solution is 3 M with respect to potassium and 5 M with respect to acetate. (see Maniatis *et al.*, 1982)

*Eco*RI and *Msp* I monomer templates were prepared by restriction endonuclease digestion of the tandemly repeated 207-12 oligonucleosome template, followed by purification through 2%-low melting agarose gels [Maniatis *et al.*, 1982]. Agarose gels were used here instead of Ultrogel A2 column chromatography, since digestion of 207-12 oligonucleosome template with *Eco*RI or *Msp* I yields both the mononucleosome template (195 bp for *Eco*RI monomer, 207 bp for *Msp* I monomer), and shorter DNA fragments (12 bp and 36 bp fragments in *Eco*RI digestion; 95 bp and 148 bp fragments in *Msp* I digestion). It is impossible to separate the mononucleosome template from these short DNA fragments by an Ultrogel A2 column, since they all come out at the end of elution.

*Rsa* I and *Xmn* I monomer templates were prepared by restriction endonuclease digestion of the intact plasmid p5S207-12, and subsequent purification through Ultrogel A2 column chromatography [Hansen and Rickett, 1989]. The digestions yield the *Rsa* I or *Xmn* I mononucleosome templates, as well as longer DNA fragments (680-bp and longer fragments in *Rsa* I digestion; 1834-bp and 1932-bp fragments in *Xmn* I digestion), which can be removed completely from the 207-bp monomers by Ultrogel A2 column chromatography (data not shown; also see Hansen and Rickett, 1989).

2. Preparation of Native and Trypsinized Histone Octamers.

Both native and trypsinized histone octamers were obtained from their respective nucleosome core particles by hydroxylapatite column chromatography [Simon and Felsenfeld, 1979]. The experimental procedures are briefly summarized as follows:

Native nucleosome core particles were purified from chicken erythrocytes using the method described in Chapter II. Trypsinized core particles were prepared from native core particles by digestion with immobilized trypsin, followed by purification through sucrose gradients (see Chapter II for details).

In order to purify native and trypsinized histone octamers, the nucleosome core particles (native or trypsinized) in 2.2 M NaCl, 0.1 M $K_{3/2}H_{3/2}PO_4$, pH 6.7 were loaded on the hydroxylapatite column which was pre-calibrated with the same buffer. The histone octamers were then eluted with the buffer whereas DNA molecules were bound onto hydroxylapatite. The histone octamers (both native and trypsinized) were concentrated with a Centricon-30 micro-concentrator if necessary, and were kept at ~2 mg/ml in order to minimize the possibility of dissociation.

3. Nucleosome Reconstitutions.

Reconstitution of both oligonucleosomes and nucleosome monomers was carried out using the method of step-wise salt dialysis [Tatchell and van Holde, 1977; Hansen *et al.*, 1989]. Template DNA in TE buffer(10 mM Tris-HCl, 0.25 mM $Na_2EDTA$, pH 7.8) was made to 2.0 M NaCl, and mixed with histone octamers. Samples were then dialyzed over a 24-hr period against progressively lower NaCl concentrations, and finally into TE buffer. The salt gradient dialysis is usually carried out as: 1.5 M NaCl in TE - 4 hr; 1.0 M NaCl in TE - 4 hr; 0.75 M NaCl in TE -3 hr; 0.5 M NaCl in TE - 3 hr; and TE buffer - overnight (> 12 hr) (protocol I). DNA concentrations used in all the reconstitutions were 40-50 μg/ml. A ratio of 0.9 moles histone octamer/mole DNA repeat was used in all the reconstitutions to minimize the possibility of association of more than one octamer with each repeat of the sequence.

Two other gradient dialysis protocols were also used for the study of nucleosome positioning versus different reconstitution kinetics. All three protocols are summarized in Table IV-1. DNA concentrations and histone/DNA ratio used for different protocols were kept the same as mentioned above for potocol I.

**Table IV-1. Salt Dialysis Reconstitution Protocols**

| Protocol | I | II | III |
|---|---|---|---|
| NaCl concentration and dialysis time | 1.5 M - 4 hr<br>1.0 M - 4 hr<br>0.75 M - 3 hr<br>0.5 M - 3 hr<br>TE* - > 12 hr | 1.0 M -6 hr<br>0.6 M - 12 hr<br>TE - 6 hr | 1.0 M - 6 hr<br>0.6 M - 12 hr<br>TE - 4 hr |

General procedure: Mix histone octamer with DNA in 2 M NaCl

Dialyze over 24 hr period into TE buffer

*: TE buffer = 10 mM Tris-HCl,

0.25 mM $Na_2EDTA$,

pH 7.8

4. Determination of Nucleosome Positioning.

Nucleosome positions were determined by the method of restriction endonuclease mapping of nucleosome-bound DNA sequences, as described previously [Hansen *et al.*, 1989]. Briefly, both nucleosome monomers and oligonucleosomes were digested into nucleosome core particles with micrococcal nuclease. In order to prevent salt-dependent sliding of nucleosomes, digestions were always carried out in 10 mM Tris-HCl, 1 mM $CaCl_2$ buffer. Unless otherwise mentioned, the concentration of nucleosomes used was about 0.6 mg/ml, and the digestions were carried out at 37 °C using enzyme/substrate ratio of ~79 units/mg nucleosomes (DNA weight). The proper time for each individual digestion was determined by a time-course experiment under the same reaction conditions. After histones were removed from the DNA by pronase digestion (at 0.5 mg enzyme/mg histones) in the presence of 0.5% SDS for 3 hr at 37 °C, the micrococcal nuclease digestion products were electrophoresed through 6% preparative polyacrylamide gels[4], the nucleosome-bound DNA

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[4]: 6% preparative polyacrylamide gels for the purification of nucleosome-bound DNA were prepared in the presence of 6% 29:1 acrylamide:BIS, 1x TBE (90 mM Tris, 90 mM borate, 2.5 mM EDTA, pH 8.3), and 0.08% TEMED. Gels were polymerized by addition of ammonium persulfate to a final concentration of 0.1%. Gels were made with dimensions of 155 mm (high) x 100 mm (wide) x 2 mm (thick). Running buffer was 1x TBE.

(146 bp) was then purified by the method of Maxam and Gilbert (1980), and subsequently digested with restriction enzymes that cut within the 5S rDNA repeat. Restriction digests were electrophoresed on 6% polyacrylamide gels, stained with 1 μg/ml ethidium bromide, and photographed under UV illumination. The sizes of the fragments in each restriction digest were obtained from densitometer tracings of the photograph negatives, and used to deduce the positions of bound nucleosomes. The relative amounts of nucleosome positions were obtained from the areas of the corresponding fragment peaks, after correction for differential EtBr staining.

5. Examination of Special Structural Features of the 5S rRNA Gene Sequence

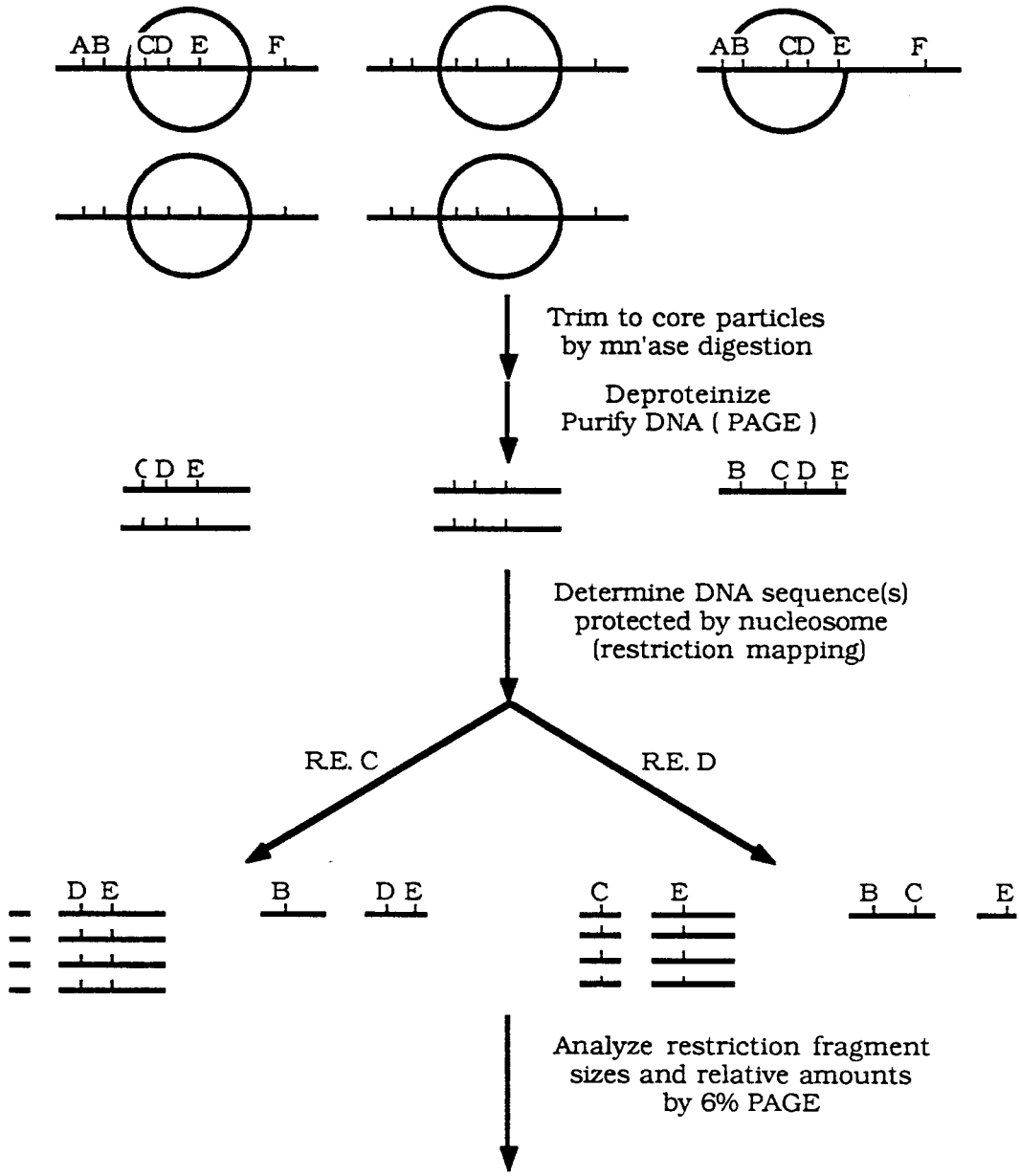
Several permutations of the 5S rDNA sequence (all are 207 bp in length) were generated by digestion of 207-12 DNA template with different restriction endonucleases. Resulting permuted DNA fragments were then analyzed by 6% polyacrylamide minigels (155 mm long, twice of the length usually used). DNA bending patterns in the DNA sequence were determined based on the differences in mobility of these sequences (which are of the same length).

## Results

The rationale for determining nucleosome positions on specific DNA sequences by restriction endonuclease mapping of nucleosome-bound DNA is as follows (see Figure IV-2). After micrococcal nuclease digestion, mononucleosomes or oligonucleosomes are trimmed into nucleosome core particles containing 146 bp nucleosomal DNA. Nucleosome positions can be deduced if the sequence of the 146 bp protected DNA is determined. The easiest way one can think of would be to sequence the monoclonal nucleosomal DNA fragments. However, since the DNA sequence of the template is known, there is an alternative way to determine the nucleosome-protected sequences, which requires much less experimental effort. This is done by restriction endonuclease mapping of the nucleosome-bound DNA: If all the nucleosomes occupied the same position on a DNA template (see Figure IV-2, only the first four nucleosomes drawn in the scheme are considered), and these nucleosomes were trimmed to 146-bp core particles by micronuclease digestion, two characteristic fragments (adding up to 146 bp) would be generated by digestion of the isolated core particle DNA with each restriction enzyme that cuts within the nucleosome-bound DNA. On the other hand, no further cuts would be made by those enzymes whose cleavage sites lie outside the nucleosome-bound DNA. By analyzing the sizes

Figure IV-2.

Diagram showing the rationale for the determination of nucleosome positioning by restriction mapping of the nucleosome-bound DNA sequences. The sites indicated by small marks represent cleavage sites by of restriction enzymes — from left to right: R.E. A, B, C, D, E, and F.




Sizes of restriction fragments reflect positioning;

Relative amounts of restriction fragments reflect distribution of nucleosomes among positions.

**Figure IV-2**

of the fragments generated with the digestions of the restriction enzymes which cut the DNA, the sequence of the nucleosome-bound DNA can be deduced with respect to its position on the DNA template. This method also works well when multiple nucleosome positions are present, in which case a number of fragments would be generated by each restriction digest. Furthermore, the relative intensities of these bands will reflect directly the quantitative distribution of nucleosomes on the DNA template. In contrast, if the nucleosomes were completely randomly distributed on the DNA templates, a smear of DNA would be seen after restriction enzyme digestion. By using this approach, we have been able to determine both the number and relative affinity of nucleosome positions present on a number of different permutations of the sea urchin 5S rRNA gene sequence.

Since a major goal of this work was to determine whether the multiple positions observed previously are present only on the tandemly repeated 207-12 template (see Chapter I.E.), we have determined nucleosome positioning on a related oligonucleosome template containing much shorter linker DNA lengths (172-12) as well as on four different monomer fragments derived from restriction nuclease digestion of the tandemly repeated 207-12 source. Oligonucleosome templates 207-12 and 172-12 are composed of twelve tandem repeats of a sequence containing the sea

urchin 5S rRNA gene (Figure IV-1.A). Each repeating unit consists of 12 bp of connecting DNA plus the 5S DNA fragment (195 bp for 207-12 template, 160 bp for 172-12 template, respectively) carrying the nucleosome positioning sequence [Simpson *et al.*, 1985]. As shown in Figure IV-1.B, the 195-bp fragment derived from *Eco*RI digestion of the 207-12 template is composed of sequences 1-195 of the sea urchin 5S DNA, while the 207-bp *Rsa* I-derived monomer template extends from position 165 of one repeat to position 165 of the adjacent repeat. They both contain sequences 1-146, which is the major nucleosome position observed for the 207-12 oligonucleosomes [Hansen *et al.*, 1989]; however, they differ in the sequences that flank each side of this important positioning region. In contrast, the 207-bp *Xmn* I template is composed of the sequences extending from position 10 of a repeating unit to position 10 of the adjacent repeat, while the 207-bp *Msp* I template starts at position 118 of a repeat and extends to 118 of the adjacent repeat. Thus, neither of these templates contains the complete 146-bp sequence of the major nucleosome position observed for the 207-12 oligonucleosome template. All four monomer fragments, however, selectively contain some of the alternate minor nucleosome positions reported for the repeated 207-12 DNA.

## Micrococcal Nuclease Digestion of Mononucleosomes

The DNA products obtained from micrococcal nuclease digestion of reconstituted *Eco*RI, *Xmn* I, and *Msp* I mononucleosomes are shown in Figure IV-3. As was observed previously for the 207-12 oligonucleosomes [Hansen *et al.*, 1989], digestion of either the *Eco*RI or *Rsa* I mononucleosomes at equivalent enzyme concentrations yields a single stable fragment with apparent length of 153 bp when measured against pBR322/*Hha* I or pBR322/*Msp* I fragments as standards. This differs from the value of 146 bp traditionally associated with the nucleosome core particle. In order to distinguish whether this anomaly is caused by a specific feature of the 5S DNA, or is due to micrococcal nuclease-dependent sequence specificity, a set of size standards composed of fragments derived from the 5S DNA itself was used to quantitate micrococcal nuclease products (see Figure IV-4). With such calibration, the nucleosomal DNA is found to migrate at exactly 146 bp. This indicates that the 146-bp core particle DNA derived from nucleosomes positioned on the sea urchin 5S rDNA sequence exhibits aberrant migration on polyacrylamide gels.

In contrast to the single stable digestion product obtained with mononucleosomes derived from reconstitution of the *Eco*RI and *Rsa* I fragments, micrococcal nuclease

Figure IV-3.

Micrococcal Nuclease digestion of the reconstituted nucleosomes on the *Eco*RI (A), *Xmn* I (B), and *Msp* I (C) monomeric templates.

Reconstituted nucleosomes were digested with micrococcal nuclease; resulting DNA fragments were electrophoresed on 6% polyacrylamide gels after proteins were depleted by 2% SDS. Digestions were carried out at 0.5 mg/ml nucleosome concentration (DNA weight), and 41 units/ml (for *Eco*RI mononucleosomes) or 4.5 units/ml (for *Xmn* I and *Msp* I mononucleosomes) enzyme concentrations.

The numbers on top of each lane indicate the time of digestion in minutes. DNA standard used in Panel A is pBR322/*Hha* I, while that in Panel B and C is pBR322/*Msp* I.

Sizes of DNA standards are shown in bp.

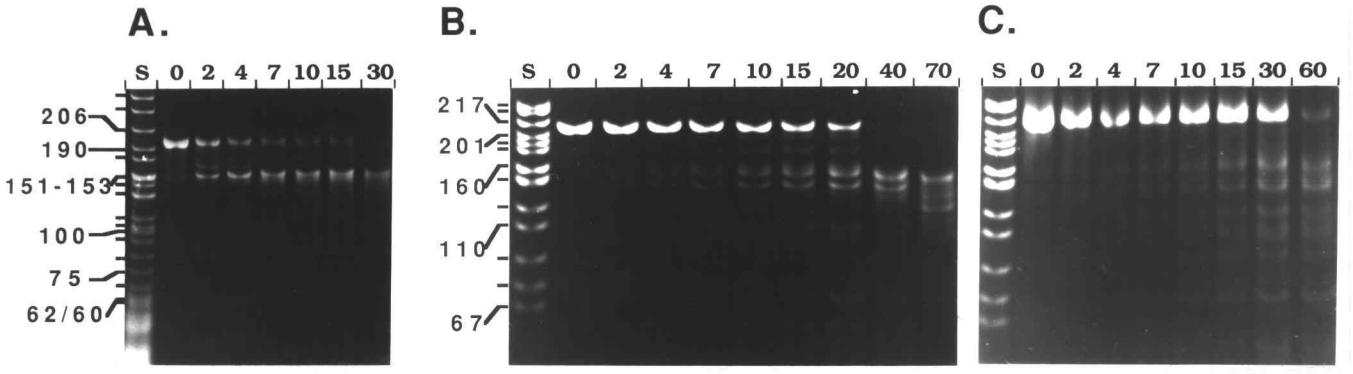

A.
S 0 2 4 7 10 15 30
206
190
151-153
100
75
62/60
B.
S 0 2 4 7 10 15 20 40 70
217
201
160
110
67
C.
S 0 2 4 7 10 15 30 60

Figure IV-3

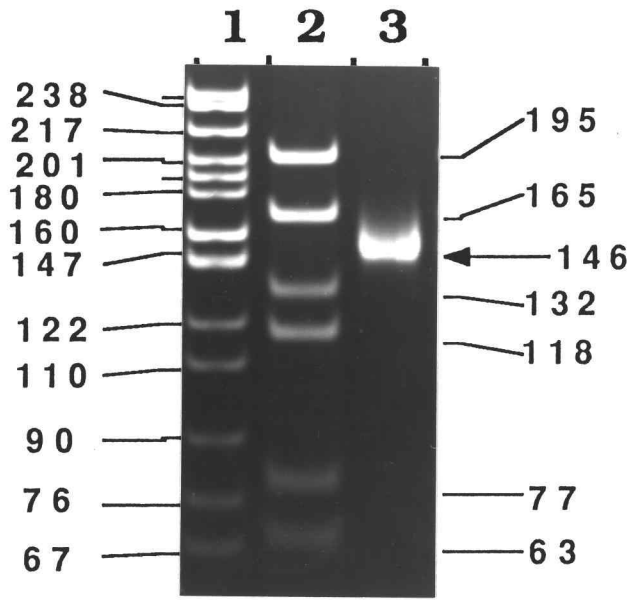
Figure IV-4.

Abnormal mobility of the sea urchin 5S rDNA fragments on polyacrylamide gel electrophoresis.

(a). Nucleosomal DNA obtained from the nucleosomes on the DNA templates containing 5S rRNA gene sequence (from *Eco*RI mononucleosomes in the example), as well as DNA standards composed of restriction fragments of the 5S rDNA sequence, were compared with plasmid DNA standards for differences in migration mobilities on a 6% polyacrylamide gel. Lanes are: #1 = DNA size standards derived from plasmid, pBR322/*Msp* I; #2 = DNA size standards derived from sea urchin 5S rRNA gene sequence; #3 = nucleosome-bound DNA purified from micrococcal nuclease digest of *Eco*RI mononucleosomes. Sizes of DNA standards are shown in bp.

(b). DNA fragment size (bp, in logarithm scale) as a function of migration distance (mm on laser-densitometer scans of the polyacrylamide gel) for the gel shown in Panel A. Heavy line and ( ○ ), pBR322/*Msp* I (lane #1 on gel); dashed line and ( □ ), 5S rDNA fragments (lane #2 on gel); dotted vertical line indicates the migration distance of nucleosome-bound DNA (lane #3 on gel). Note that all the 5S rDNA fragments migrate consistently slower than the plasmid DNA with same size.

A.




B.

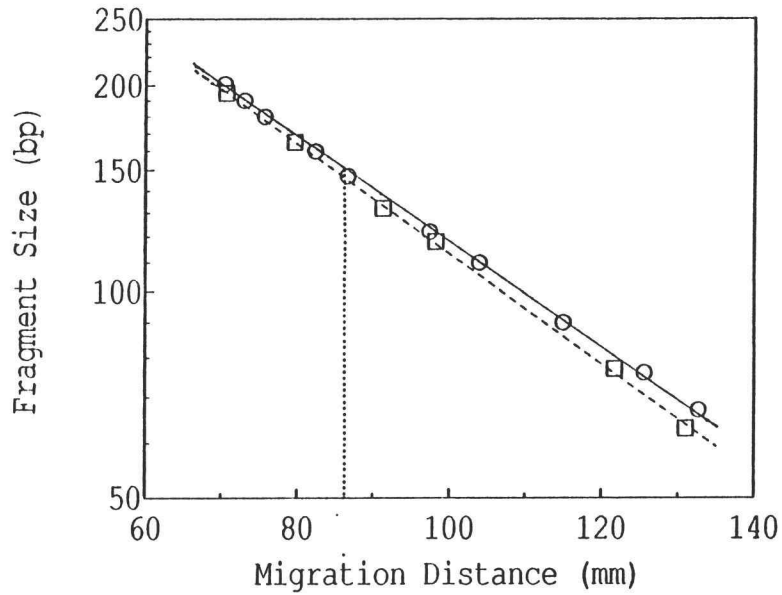



Figure IV-4

digestion of the mononucleosomes reconstituted onto *Xmn* I and *Msp* I templates yields both 146-bp DNA fragments and some fragments smaller than 146 bp (see Figures IV-3.B, 3.C), even when using 10-fold lower enzyme concentrations. The *Xmn* I monomers trim to fragments which are found, when properly calibrated with respect to the 5S DNA standards (see above), to be 146 bp and 136 bp in length, with many smaller fragments also present. In the case of *Msp* I mononucleosomes, we find no clear kinetic stop, and a much larger fraction of the total products migrating at < 146 bp.

## Determination of Histone Octamer Positioning on Mononucleosomes

The core particle DNAs obtained by micrococcal nuclease trimming of reconstituted mononucleosomes were each subjected to redigestion by several different restriction endonucleases. Examples of gel electrophoresis patterns obtained are shown in Figure IV-5. Figure IV-6 depicts a scan of lane 6 in Figure IV-5.B. Pairs of bands are observed which sum to 146 bp, the length of the uncut DNA. Note that if one member of a pair is very small, it is difficult to observe on the gel, since pair members are present in equimolar quantities, but stain in proportion to the mass of each. Furthermore, with some positions the

Figure IV-5.

6% polyacrylamide gel electrophoresis for restriction enzyme mapping of nucleosome-bound DNA obtained from *Eco*RI (A), *Rsa* I (B), *Xmn* I (C, lanes 2-5) mononucleosomes, and the 136-bp subnucleosomal DNA from *Xmn* I mononucleosome reconstitute (C, lanes 7-10).

The letters on top of each lane represent the restriction enzymes used (same as in Figure IV-1). The lane labeled by "U" in each panel is the unrestricted nucleosome-bound DNA used in each case. DNA standards are p5S207-12/*Hha* I (Panels A and B) and pBR322/*Msp* I (Panel C). Sizes of DNA standards are shown in bp.

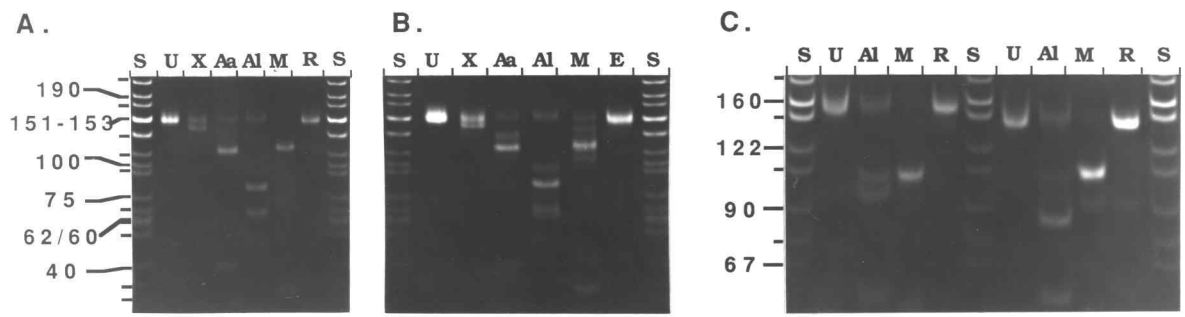

A.
S U X Aa Al M R S
190
151-153
100
75
62/60
40
B.
S U X Aa Al M E S
C.
S U Al M R S U Al M R S
160
122
90
67

Figure IV-5

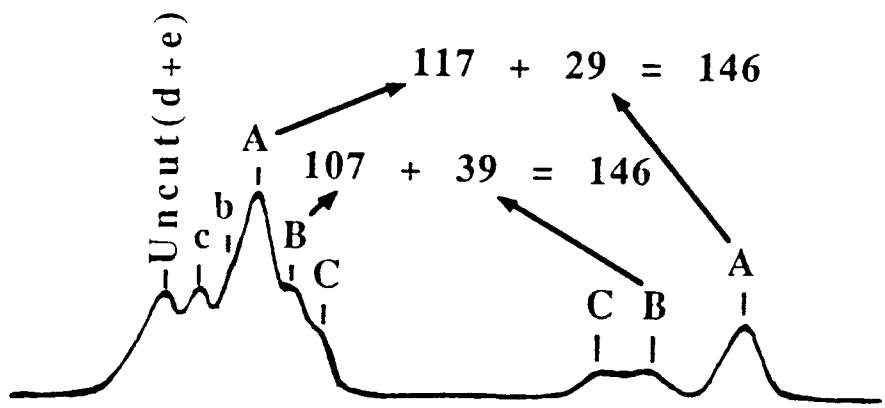



**Figure IV-6.**

**Quantitation of restriction digests of core-particle DNA. Shown is the densitometer scan of lane 6 of Figure IV-5.B.**

**Numbers indicate fragment sizes derived from calibration with the 5S rDNA standards. Arrows indicate two pairs of bands that sum to 146 bp. Letters indicate nucleosome positions that correspond to observed fragment sizes (see Table IV-2).**

*Msp* I site is absent, leading to no further cleavage of the core particle DNA.

To test the possibility that some or all of the fragments observed in Figure IV-5 resulted from the DNA sequence specificity of micrococcal nuclease, we isolated core particle size DNA from a partial digest of the **free** 207-12 DNA template by the same method used for purification of nucleosome-bound DNA (see above), and incubated it with the same restriction enzymes. Although a number of discrete bands appeared in each digest, in no case did they migrate with the same mobility as any of the fragments generated from restriction enzyme digestion of the nucleosome-bound DNA (see Figure IV-7). Thus, the observed presence of multiple bands is **not** an artifact of sequence-specific micrococcal nuclease cleavage, but instead is due to multiple nucleosome positions present on the reconstituted templates. An additional potential concern of the micrococcal nuclease approach is whether the isolated core particle DNA samples used for restriction analysis represent a majority of the nucleosome population present prior to micrococcal nuclease digestion. In the case of the tandemly repeated 207-12 template, it has been observed routinely that greater than 75-80% of the nucleosome-bound DNA can be recovered in the core particle band [Hansen *et al.*, 1989]. For the 172-12 template, and the *Eco*RI and *Rsa* I templates used here, similar recoveries

Figure IV-7

Control experiment for restriction mapping using free 207-12 DNA template.

A. Micrococcal nuclease digestion of the 207-12 free DNA.

The 207-12 *free* DNA template was digested with micrococcal nuclease for different length of time to yield many DNA fragments including the fragments with the size of nucleosomal DNA (146 bp). The numbers on top of each lane indicate the time of digestion in seconds (") or minutes (′). Lane marked "S" is DNA size standard, pBR322/*Msp* I.

B. Comparison of restriction mapping of nucleosome-bound DNA and core particle-size DNA isolated for the micrococcal nuclease digest of free 207-12 template DNA. The core particle-size DNA was purified from a partial digest of 207-12 DNA by the method of Maxam and Gilbert (1980). DNA fragments with size between 140 and 150 were all excised and purified.

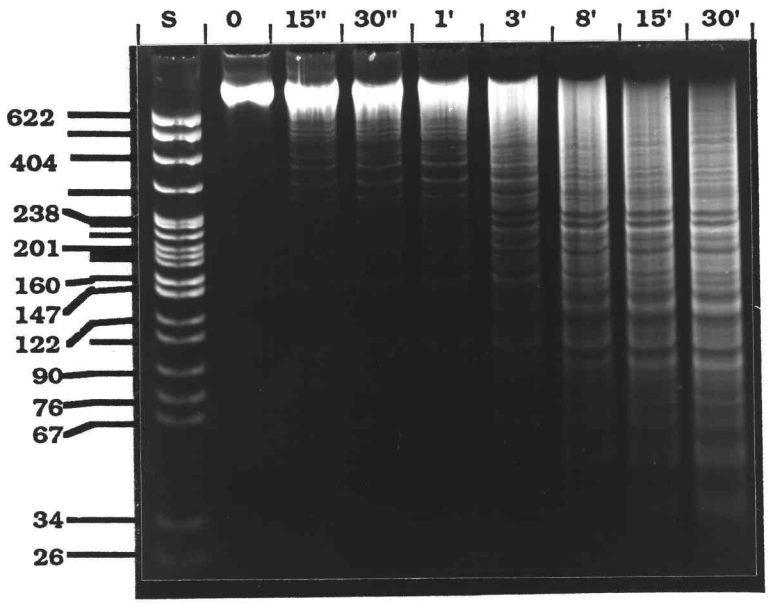
lanes labeled with "pBR322/*Msp* I" = DNA size standards;

lanes labeled with "N" = restriction mapping of 146 bp DNA from free 207-12 template;

lanes labeled with "D" = restriction mapping of nucleosome-bound DNA from 207-12 oligonucleosomes.

Restriction enzymes used for mapping are as shown on top of lanes. Sizes of DNA standards are shown in bp in both panels.

A.




B.

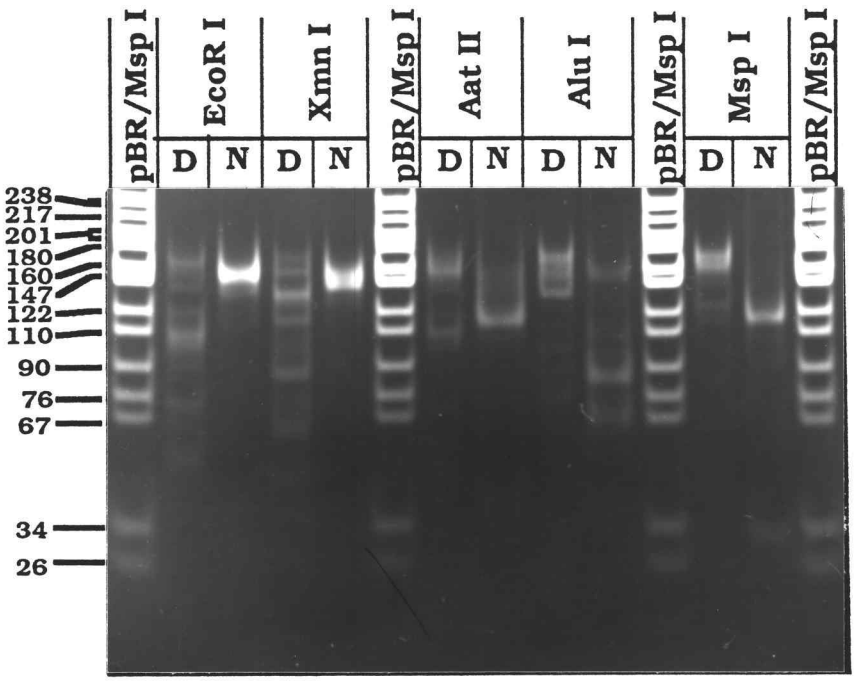



Figure IV-7

are obtained. However, in some experiments, we have restricted core particle DNA isolated from incomplete digests (where the recovered core particle band represents a much smaller percentage of the original population) and obtained identical positioning results. Thus, for the tandemly repeated templates, and the *Eco*RI and *Rsa* I monomer templates, the positions determined reflect accurately the total population of nucleosome positions present originally after reconstitution. However, the susceptibility of the *Xmn* I and *Msp* I reconstitutes to overdigestion (Figure IV-3.B, C) indicates that in these cases, it is possible that we may be observing the positions of only the most stable nucleosomes.

By correlating the lengths of the restriction nuclease-generated fragment pairs with the known cleavage sites, we can establish the positions occupied by the histone cores on the various mononucleosomes. In addition, the relative intensities of bands provides a semi-quantitative comparison of position preferences. Consider first the *Eco*RI and *Rsa* I mononucleosomes: in each of the restriction digests, the sizes of the major fragment(s) are the same for both *Eco*RI and *Rsa* I templates, while some (but not all) minor fragments are shared by both templates. The actual nucleosome positions, as well as the relative amount present in each position are shown in Table IV-2.A. For both templates, approximately

**Table IV-2. Nucleosome Positioning on Mononucleosomes and Oligonucleosomes**

| Template | Protocol[a] | Major Position[b] | Minor Positions[b] |
|---|---|---|---|
| A. Monomers | | | |
| EcoR I | I | A | C, E > B > D |
| Rsa I | I | A | B, C, c > b > d, e |
| Xmn I | I | —— | A′, B > C > D |
| Msp I | I | —— | d/e > c |
| B. Multimers | | | |
| 172-12 | II | A | B, C > b, c, d, E, e |
| | I | A | C > B, b, e > c, D, d, E, F, f, G, g, H, h |
| 207-12 | II | A | same as above |
| | III | A | same as above |

(a). The protocols for nucleosome reconstitutions are as shown in Table IV-1.

(b). Abbreviations used for nucleosome positions are: A=1 to 146; B=10 to 156; C=20 to 166; D=30 to 176; E=40 to 186; F=50 to 196; G=60 to 206; H=70 to 9′(9′ is the 9th bp on the 5′ flanking sequence — i.e., the adjacent downstream repeat); b=-10 to 136(-10 is the 10th bp form position 1 on the 3′ flanking sequence — i.e., the position 197 on the adjacent upstream repeat); c=-20 to 126; d=-30 to 116; e=-40 to 106; f=-50 to 96; g=-60 to 86; h=-70 to 76; A′=10 to 146. The error inherent in the assignment of these positions is +/-2 bp.

50% of the nucleosomes occupy the sequence 1-146. Interestingly, the less favored positions present in both templates are found to differ from the major position by multiples of 10 +/- 2 bp. Although many of the same minor positions are present in both cases, the relative amount of the position present in each template is different. For example, a significant fraction of the total number of nucleosomes occupy sequence 20-165 on the *Eco*RI monomer fragment, but are present on this sequence to a lesser extent on other templates. In addition, in the case of *Rsa* I mononucleosomes (where the DNA sequence extends on both sides of the major position - see Figure IV-1.B), the total fraction of nucleosomes found on the sequences 3′ to the major position (i.e., positions B through H) is greater than the fraction found on the sequences 5′ to the major position (positions b through h).

The *Xmn* I and *Msp* I monomeric templates do not carry the entire DNA sequence from bases 1 to 146, which we observe to be the major nucleosome position on both the *Eco*RI and *Rsa* I monomeric templates as well as the 207-12 oligonucleosome template [Hansen *et al.*, 1989]. The greater susceptibility of *Xmn* I and *Msp* I mononucleosomes to overdigestion by micrococcal nuclease suggests that the nucleosomes reconstituted onto these two templates may be less stable than the nucleosomes found on templates which contain the preferred 1-146 nucleosome position (Figure IV-

3). In the case of *Xmn* I mononucleosomes, two discrete populations of DNA fragments are found to be protected from micrococcal nuclease digestion: one of the two protected DNA fragments migrates as core particle-sized DNA, while the other is 10 bp shorter (Figure IV-3.B). These two fragments have been separated; restriction enzyme mapping (Figure 5.C) of each reveals that these particles containing core particle-size DNA occupy mainly the positions 10-155 and 20-165 (Table IV-2.A). Perhaps not surprisingly, the particles which retain only 136 bp of DNA after micrococcal nuclease digestion correspond to structures in which the nucleosome has occupied the sequence 10-146 (Table IV-2.A, position indicated by A′). Thus, histone core preference for the major position (1-146) is sufficiently strong to yield a stable 136-bp subnucleosomal particle lacking 10 bp of DNA at one end in an amount of about 30% of the reconstitutes. Nucleosomes reconstituted onto the *Msp* I monomeric template appear to be the least stable: only very weak protection of nucleosome core particle-size DNA is observed. As a result, even though positioned nucleosomes are observed on this fragment, it is virtually impossible to make even semi-quantitative conclusions of the distributions present on the *Msp* I template.

## Positioning on Tandemly Repeated Templates

Nucleosome positions observed on the 172-12 oligonucleosome template, as well as on the 207-12 template reconstituted using three different pathways in dialysis (see Table IV-1), are shown in Table IV-2.B. Both templates consist of tandemly repeated sequences containing the preferred nucleosome position sequence; however, the 172-12 template has 35 bp less linker DNA between the repeats. Results indicate that the most favored position on both oligonucleosome templates is the same as the major position (position 1-146) found for the *Eco*RI and *Rsa* I mononucleosomes, and is independent of dialysis protocol. The fact that identical nucleosome positioning can be achieved by salt dialysis reconstitution using different kinetic pathways indicates that the observed positioning patterns represent an equilibrium distribution of positioned nucleosomes. This conclusion is supported further by the findings that the distribution of positions on the 201-12 template is independent of the histone/DNA input ratio. Although multiple positions are observed in both cases, minor positions on the 172-12 template that are greater than 40 bp away from either side of the major position are not detected. On the other hand, we observe minor positions on the 207-12 template as far as 70 bp from either side of the major position. These results suggest that, in the

oligonucleosome templates, "borders" imposed by nucleosomes located at the preferred position (1-146) can influence the overall distribution of nucleosomes present on other sites. As was observed for the *Rsa* I monomer (Table IV-2.A), the total fraction of nucleosomes occupying sequences 3′ to the major position is greater than the fraction occupying the sequences 5′ to the major position.

## Positioning of Trypsinized Histone Cores

In order to understand better the regions of the histone octamer that function in sequence-dependent nucleosome positioning, histone octamers from which the "tail" segments of the histones have been removed by trypsin digestion (see Chapter II) have also been reconstituted onto each of the previously described DNA templates. The densitometer scans of restriction mapping of the nucleosomal DNA from the native (panel B) and trypsinized (panel C) reconstituted *Eco*RI mononucleosomes are shown in Figure IV-8. In this example, as well as with all other templates (data not shown), we observe absolutely identical positioning patterns for native and trypsinized histone octamers. These results indicate that, under these conditions, the histone "tails" are not determinants of nucleosome positioning.

Figure IV-8.

Comparison of nucleosome positioning in native and trypsinized reconstitutions.

A. 6% polyacrylamide gel electrophoresis showing restriction enzyme mapping patterns of the nucleosomal DNA purified from the reconstituted trypsinized nucleosomes on the *Eco*RI monomeric DNA template. Restriction enzymes used and DNA size standard are the same as in Figure IV-5.A.

B. Densitometer scans for the 6% polyacrylamide gel shown in Figure IV-5.A.

C. Densitometer scans for the 6% polyacrylamide gel shown in Panel A.

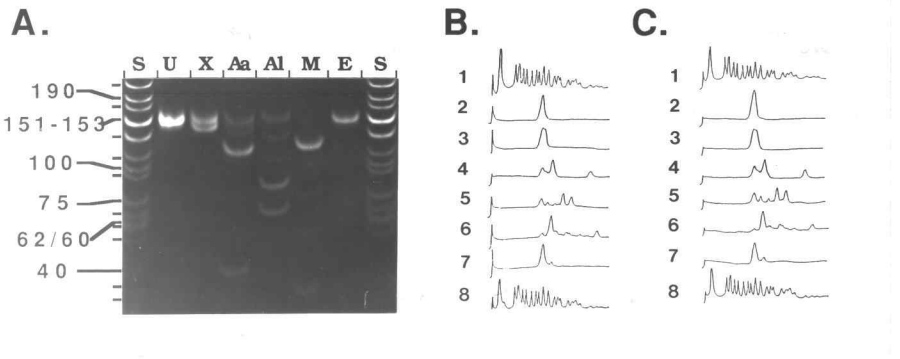



**Figure IV-8**

## Special DNA Structural Properties of the 5S rDNA Sequence

A number of 207-bp DNA sequences have been examined for abnormal mobilities on polyacrylamide gels. Results are shown in Figure IV-9. These different permutations of 5S rDNA sequence are shown in Panel A, aligned with respect to their positions in the 207-12 DNA from which they were derived. If there were no bends in the 5S rDNA sequence, all the permuted 207-bp fragments would migrate identically although they are different in nucleotide sequence. However, if there was a bend, these different permutations would carry the bend at different positions; some would have the bend near the ends of the sequences, whereas others would have it in the middle. For the 207-bp fragments derived by cleavage of the restriction enzymes which cut at or near the bend, the bend would have minimum effects on the fragments obtained. On the other hand, for the permutations derived by the restriction enzymes whose cleavage sites are away from the bend, the bend would remain somewhere in the middle of the fragments generated and reduce the mobilities of the DNA fragments on polyacrylamide gels. The fragment with the bend in the center would have the slowest mobility. Figure IV-9.B shows the results of the 6% polyacrylamide gel electrophoresis to test a number of permuted 207-bp fragments for migration mobility. The data clearly

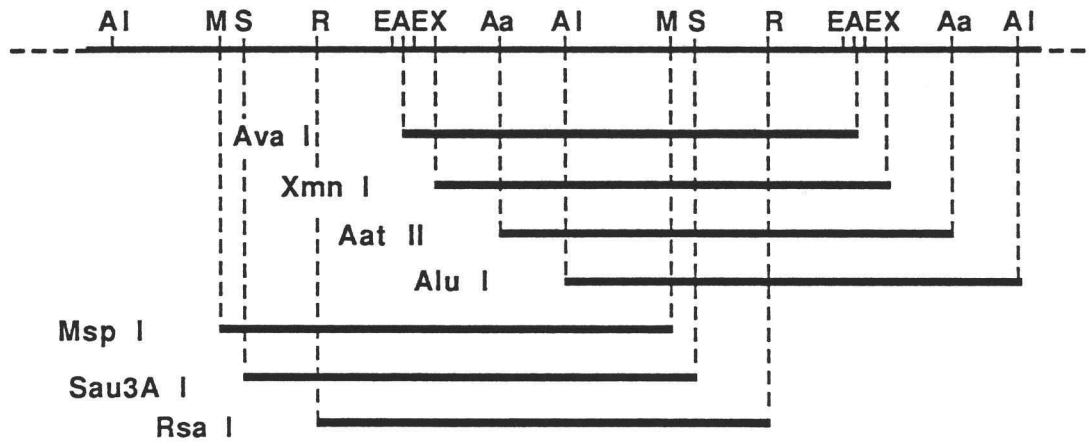
Figure IV-9.

Analysis of DNA bending by using permuted 207-bp DNA sequences.

A. Diagram showing the sequences of permuted 207-bp fragments used with respect to the repeated 207-12 DNA. Fragments are named after the restriction enzymes by which they are prepared.

B. 6% polyacrylamide gel electrophoresis to analyze the mobilities of the 207-bp permuted DNA sequences. Lanes are: S = DNA size standards, pBR322/*Msp* I; #1 = *Ava* I fragment; #2 = *Xmn* I fragment; #3 = *Aat* II fragment; #4 = *Alu* I fragment; #5 = *Msp* I fragment; #6 = *Sau*3A I fragment; and #7 = *Rsa* I fragment. Sizes of DNA standards are shown in bp.

A.




B.

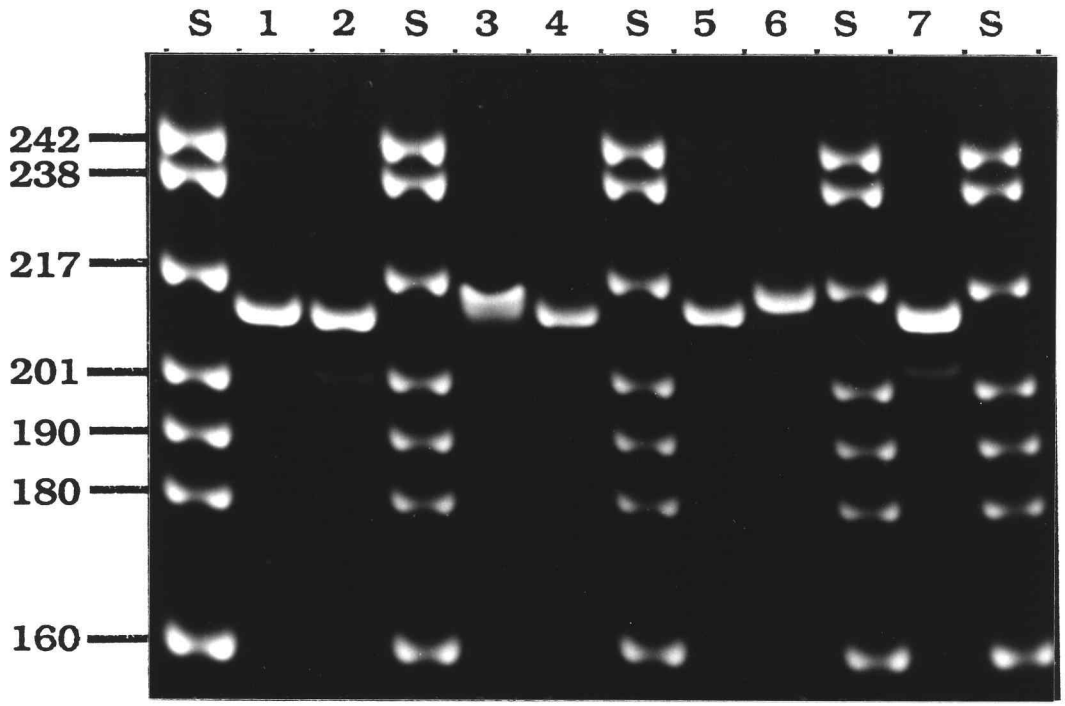



Figure IV-9

indicate that the permuted sequences exhibit different gel mobilities; however, the situation is more complicated than the simple example above. The gel patterns in Figure IV-9.B suggest that there are more than one bends in the 5S rRNA gene sequence, seemingly one between *Alu* I and *Msp* I sites, and another near *Xmn* I site.

## Discussion

A number of permutations of the 5S DNA sequence have been used to examine the DNA determinants of nucleosome positioning. For each template tested, multiple nucleosome positions are observed, although the major position is always found to be the same (sequence 1 to 146). The presence of multiple translational nucleosome positioning frames on each of several different restriction fragments containing the 5S rRNA gene indicates directly that multiple nucleosome positioning is an inherent property of this DNA sequence. Furthermore, these results indicate that multiple nucleosome positioning observed previously on tandemly repeated dodecamers of the 5S rDNA sequence [Hansen *et al.*, 1989] is not due to its repeated structure, or the salt-dependent folding that it undergoes during reconstitution. While multiple translational nucleosome positions have been observed previously on both natural [Linxweiler and Hörz, 1985; Clarke *et al.*, 1985] and synthetic [Shrader and Crothers, 1989] DNA sequences, it is not yet clear whether this is a general property of all sequences that position nucleosomes. That the phenomenon is not restricted to chromatin reconstituted *in vitro* is suggested by the early observation of Ponder and Crawford (1977) who obtained evidence for multiple positioning of nucleosomes in animal viruses, as well as by the results reported for nucleosome

positioning on mouse satellite DNA *in vivo* [Zhang and Hörz, 1984]. The observation that all minor positions are found at distances of multiples of 10 base pairs from the major position suggests strongly that the mechanical properties of DNA molecules, such as bending and flexibility, play an important role in determining nucleosome positions (i.e., a shift by multiples of 10 base pairs on a DNA will keep the same direction of DNA bending around the nucleosome core and the same side of the DNA molecule interacting with the histones). Consistent with this notion, gel electrophoresis demonstrates that the core particle DNAs from our reconstituted nucleosomes (see Figure IV-4), as well as the permuted 207 bp monomer templates (Figure IV-9) exhibit aberrant migration, consistent with the presence of one or more DNA bends in the sequence. Computer simulations (see Figure IV-10) using refined dinucleotide wedge angles [Bolshoy *et al.*, 1990] also indicate a significant (40 degree) bend centered at about sequence 65, and less pronounced bends near positions 20 and 160 of the 5S rDNA sequence. Interestingly, Shrader and Crothers (1989) have also observed multiple nucleosome positions differing by 10 bp on sequences known to be composed of a series of DNA bends.

Indeed, there exists a large body of evidence suggesting that alignment of a DNA bend(s) with specific region(s) on the histone octamer surface may account for

Figure IV-10. Computer modeling for the sea urchin 5S rRNA gene sequence. DNA bending model was generated using the data of dinucleotide wedge angles from Bolshoy *et al.* (1990). Parameters of DNA twist and pitch used were 10.2 bp/turn (corresponding to the average bp/turn for the DNA molecule in nucleosome, obtained from Figure III-5.B) and 34 Å/turn, respectively. DNA sequence used was the sequence 1-195 of the cloned sea urchin gene fragment.

A. van der Waal's model as viewed from the direction from which the strongest bending pattern was observed. The DNA molecule is oriented from 1-195 when counted from bottom to top.

B. Panels 1-5: ball and stick models of the same image, as viewed from different directions to give a three-dimensional impression. Panel 6: diagram of a cubic box to demonstrate the directions from which 1-5 were viewed. The DNA molecule was so placed that the strongest bending pattern (Fig IV-10.A) could be viewed from the top (z-axis).

1). from the center of the top surface (z-axis), same as the viewing direction for Figure IV-10.A;

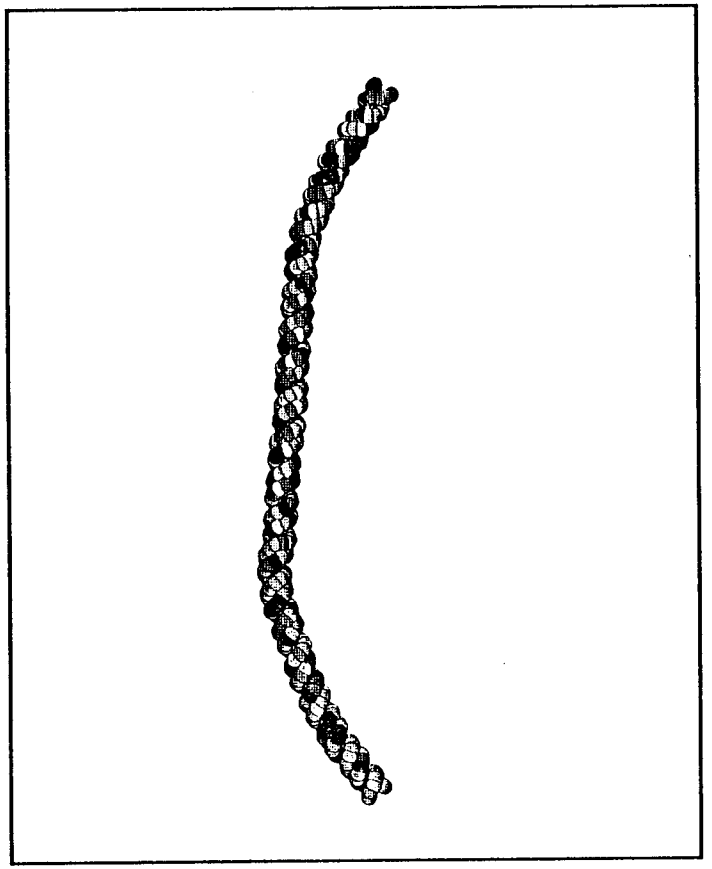
2). from the center of the right surface (y-axis);

3). from the middle of top front edge (in the x-z plane, 45° from each axis);

4). from a point on upper front surface, 30° from x-axis and 60° from z-axis;

5). from the center of the front surface (x-axis).

A.



B.

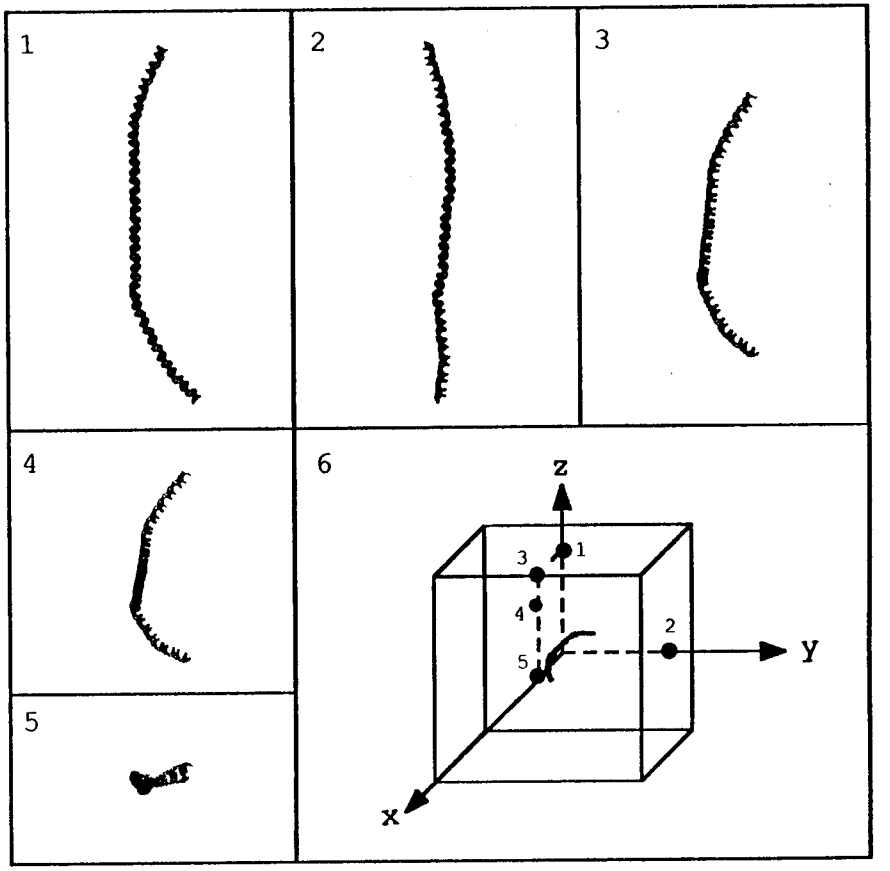



Figure IV-10

sequence-dependent nucleosome positioning [Drew and Travers, 1985; Satchwell *et al.*, 1986; Travers, 1987; Shrader and Crothers, 1989; FitzGerald and Simpson, 1985]. However, the fact that we observe as many as 14 other sequences occupied by positioned nucleosomes (in the 207-12 oligonucleosomes), indicates clearly that alignment of the bend at many regions **other** than the most preferred position is also sufficient to form a positioned nucleosome. The observation that each of these alternative positions is displaced by a multiple of about 10 bp from the most preferred position suggests that it is not the location of the strongest bending so much as the correct orientation of the bends (or the overall bending) with respect to the histone core surface that is most important in favoring positioning.

While the major nucleosome position and most of the minor positions are shared by all of the DNA templates used in this study (except the *Msp* I fragment), the relative distributions of nucleosomes between the minor positions are variable. For example, nucleosomes are found to occupy the sequence 20-165 in both the 172-12 and 207-12 oligo-nucleosome templates, as well as the *Rsa* I, *Xmn* I and *Eco*RI monomer fragments. However, it is only on the *Eco*RI monomer template that a significant percentage of the total nucleosomes are found to occupy this sequence. This observation is important because the *Eco*RI fragment has been used extensively in previous studies of nucleosome

positioning on the sea urchin 5S DNA sequence. A reexamination of DNase I patterns of *Eco*RI mononucleosomes generated in our own laboratory [Moyer *et al.*, 1989] as well by others [Simpson and Stafford, 1983; FitzGerald and Simpson, 1985], indicates a strong protection border at position 145, as well as a significant (but secondary) border at position 165. Thus, in retrospect, we feel that the previous studies of nucleosome positioning using DNase I footprinting are in fact quite consistent with the results reported here using a different experimental approach. An additional control experiment has also been conducted, in order to test the possibility that different nucleosome positioning results might be obtained because of the different salt conditions used by Simpson and Stafford (1983) or by Hansen *et al.* (1989) and our studies reported here. The experiment was carried out using *Eco*RI mononucleosomes following the procedure of restriction enzyme mapping described above, but using the two different salt conditions for micrococcal nuclease trimming (see Figure IV-11 and Table IV-3 for results). Although differences were found for the minor positions (Table IV-3), results clearly indicate that the major position remains the same (~50% on 1-146) in both cases. It is also important to note, from the positioning results of the mononucleosomes, that we find no evidence for an "end effect" as a significant determinant of nucleosome positioning *in vitro*;

Figure IV-11. Effects of micrococcal nuclease digestion conditions on results of nucleosome positioning.

6% polyacrylamide gel electrophoresis for restriction enzyme mapping of nucleosome-bound DNA obtained from micrococcal nuclease digestions of *Eco*RI mononucleosomes under different salt conditions.

Condition 1 = condition used by Hansen *et al.* (1989), as well as in this study, for micrococcal nuclease trimming of nucleosomes:

1 mM $CaCl_2$,
10 mM Tris-HCl,
pH 7.8.

Condition 2 = condition used by Simpson and Stafford (1983) for DNase I footprinting studies:

10 mM $MgCl_2$,
3 mM $CaCl_2$,
100 mM NaCl,
10 mM Tris-HCl,
pH 7.8.

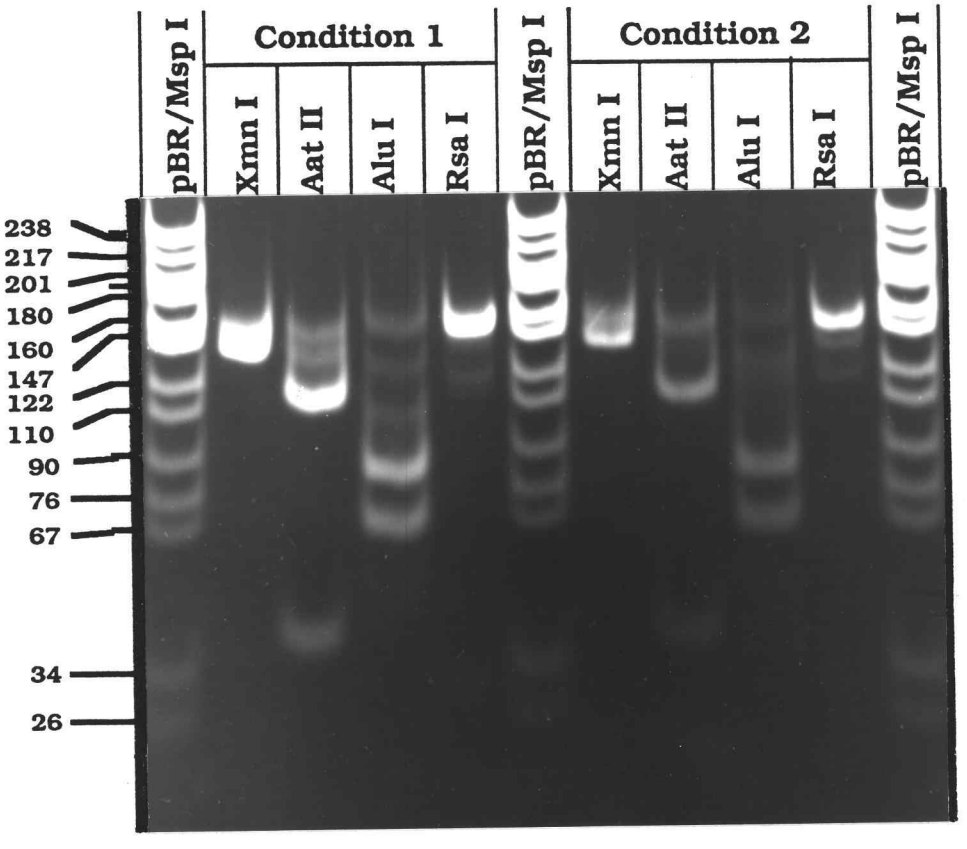

pBR/Msp I
Condition 1
Xmn I
Aat II
Alu I
Rsa I
pBR/Msp I
Condition 2
Xmn I
Aat II
Alu I
Rsa I
pBR/Msp I
238
217
201
180
160
147
122
110
90
76
67
34
26

Figure IV-11

**Table IV-3. Nucleosome Positioning on EcoR I Mononucleosome under Different Micrococcal Nuclease Digestion Conditions**

| Mn'ase Digestion Condition[a] | Major Position[b] | Minor Positions[b] |
|---|---|---|
| Hansen et. al., (1989) and this study | A | C, E > B, D |
| Simpson and Stafford (1983) | A | D, E > B, C |

(a): See figure legend of Figure IV-11 for digestion conditions.

(b): Abbreviations for nucleosome positions are same as in Table IV-2.

the same fraction of nucleosomes are found in the same major position (50%) regardless of whether this sequence is at the end of the template (*Eco*RI monomer), or in the middle (*Rsa* I monomer, oligonucleosome templates). In addition, the fraction of nucleosomes in each minor position differs with templates; however, there is no clear co-incidence for increase or decrease in the fraction of nucleosomes in a given position whether or not this position is at the end of a template.

Our analysis of nucleosome positioning on the 207-12 template as a function of different reconstitution protocols demonstrates that the positions assumed by nucleosomes on the 5S rDNA after salt dialysis reconstitution represent an equilibrium distribution, in agreement with the findings of Shrader and Crothers (1989). This provides a means to estimate the energy differences between different nucleosome positions. At equilibrium, fractions of nucleosomes distributing into different positions reflect free energy differences between those positions, as defined by the Boltzmann equation: $N_i/N_j = e^{-(Ei-Ej)/RT}$. We find in most cases that the major position (1-146 bp) is occupied by about 50% of all nucleosomes. In contrast, the number of nucleosomes distributed into any particular minor position corresponds to about 5-20%. This means that the free energy differences between major and minor positions are only of the order of 0.5-1.3 kcal/mol. This result does not mean that the total

binding energy for a nucleosome is small; it only compares energy differences between favored positions. Actually, the fact that we see no evidence for randomly positioned nucleosomes argues for rather large values of the binding energy for the positioned nucleosomes as compared to randomly chosen positions.

The results obtained with reconstitutions utilizing trypsinized histone octamers demonstrate that the histone tails play no role whatsoever in determining nucleosome positions, consistent with other evidence suggesting that the histone tails contribute very little to the overall stability of the nucleosome core particle (Chapter II). Such results suggest that the histone determinants, if there are any, must reside in the central globular region of the histone octamer. Furthermore, the generation of a nucleosomal particle on the *Xmn* I monomer fragment in which only 136 bp DNA interact with histones indicates that the regions of the histone octamer that contact the DNA entry and exit points are also not essential for formation of a positioned nucleosome. A similar finding, utilizing reconstitution on a fragment of *E. coli* DNA, was reported by Ramsay *et al.* (1984). Since DNA exit and entry contacts are presumed to involve H2A [Shick *et al.*, 1980], these results seemingly restrict the portions of the histones involved in selecting DNA positions to the globular portions of H4, H3, and possibly H2B. However, the finding of more than 12

different translational nucleosome positioning frames on the 207-12 template differing in free energies by only 0.5-1.3 kcal/mol indicates that there is no single specific histone core-DNA sequence interaction required for formation of a positioned nucleosome. Instead, the single most important positioning determinant appears to be the correct rotational orientation of the DNA with respect to the core surface. Based on the above observations for DNA and protein determinants of nucleosome positioning, we suggest the following model: the inner portion $(H3/H4)_2$ of the histone core positions itself with respect to bends and/or flexibility of the DNA. While one position is favored in any sequence, alternative positions displaced by multiples of 10 bp, which allow the same "face" of the DNA to interact with the core, may be nearly as favorable. The histone tails may be freely modified, and it is even likely that H2A and H2B may be removed without disturbing the positioning.

Finally, the observations that histone "tails" do not affect nucleosome positioning argue against changes in nucleosome positioning as a mechanism by which covalent modification of histone tails influence nuclear functions. Our results, together with the previous findings that histone "tails" have little influence on the behavior of nucleosome core particles in solution within and near physiological conditions (although the tails do stabilize core particles as demonstrated by the stabilization of

nucleosomes towards thermal denaturation) (see Chapter II), suggest that the correlation between histone modifications on the "tails" (such as hyperacetylation) and gene activation processes might be accomplished at the higher order structure of chromatin. This notion is further supported by the studies of Allan *et al.* (1982), which demonstrated possible roles of the histone tails in the stabilization of chromatin higher order structure.

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