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Chromatin and nucleosome structure

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ABSTRACT

Chromatin nucleosomes (mononucleosomes through pentanucleosomes) have been isolated by staphylococcal nuclease digestion of calf thymus nuclei. The peak value ellipticity is the same for all oligomers, 1900 deg cm^2, mol^{-1} at 280 nm, 23°C. The dh_{280}/dT vs T show a progressive increase in T_m of the main thermal band (73.5°C, monomer; 79°C, pentamer). Very small amounts of free DNA can be observed in the melting profiles, and shoulders at 60°C and 93°C appear and increase in magnitude as the particle size increases. The magnitude of the change, $\Delta[\theta]_{280}$, increases with oligomer size. This pattern could result from an initial unfolding of an asymmetric assembly of nucleosomes (polynucleosome superhelix) in addition to the denaturation of the internal nucleosome structure, and a subsequent or simultaneous denaturation of the double strand DNA. The extent of this unfolding appears to depend upon the size of the oligomer and therefore implies interactions between asymmetrically assembled neighboring nucleosomes.

INTRODUCTION

There is now good evidence that a repeating structural subunit, isolated from nuclei or from chromatin by the action of endonucleases, is fundamental to the structure and packaging of DNA in the eukaryotic chromosome.[4-11]

It has been suggested that this subunit represents approximately 90% of the total DNA in the eukaryotic genome.[7] These particles, known as ν bodies[6] or nucleosomes,[10] consist of 140-200 base pairs of DNA folded into an approximately spherical structure of 80Å diameter,[11] and have a DNA packing ratio of approximately 6.8:1, which is achieved as a result of interactions between the histones and DNA. However, the exact architectural arrangement of the histones, their mode of interaction with DNA and the factors which contribute to the structure and stability of the nucleosome are poorly understood.

In order to better understand the overall structure of the nucleo-

some and the interactions between subunits which contribute to the structure and stability of chromatin, monomeric nucleosomes (mononucleosomes) through oligomers of up to five (pentanucleosomes) have been isolated from calf thymus. These particles have been characterized by their circular dichroism (CD) spectra as well as their thermal denaturation profiles measured simultaneously by both hyperchromicity and circular dichroism changes at 280 nm.

Measurement of the ellipticity in the region of 280 nm, $[\theta]_{280}$, of DNA and of chromatin previously revealed that the structure of DNA in chromatin is different from that of native DNA at low salt concentration.[12-15] The CD of PS-particles,[5, 11] the products of moderate staphylococcal nuclease digestion of calf thymus chromatin, show a $[\theta]_{280}$ value between 700 and 1500, smaller than that of chromatin itself. The CD in the region of 280 nm for mononucleosomes and higher oligomers (up to pentanucleosomes) is reported herein. These studies attempt to determine how the individual nucleosomes contribute to the CD of chromatin, how interactions between mononucleosomes affect the overall structure and stability of the complex, and whether there are significant regions of free DNA connecting adjacent nucleosomes (as have been seen in some electron micrographs of chromatin).[6, 10, 16, 17] There is some evidence that shearing of chromatin, to render it soluble,[18] causes damage or rearrangement of the histones along the DNA chain. For this reason the particles were prepared directly from nuclei, without shearing, so that optical measurements could be carried out on undamaged particles.

Thermal denaturation has been widely used in the past to study the structure and stability of DNA-polypeptide complexes[19] of whole chromatin,[20] and of chromatin partially and selectively depleted of histones and some nonhistone proteins.[21, 22] This method was employed to elucidate the interactions between the proteins and DNA, and to probe the structure of the nucleoprotein complex. Furthermore, thermal denaturation has proved useful for estimating the relative amounts of regions of differing thermal stability caused by localized interactions between the proteins and DNA and regions of unbound DNA within the complex.[20]

Additional useful information can be obtained by comparing hyper-

chromicity changes with ellipticity changes as a function of temperature.[23] Since the hyperchromicity changes are sensitive only to the secondary structural changes of DNA (i.e., the unstacking and separation of base pairs), whereas the ellipticity is sensitive to both the secondary and tertiary folding of the DNA (such as any supercoiling, e.g., Ψ-DNA[24]) it is possible to obtain detailed information about the structural changes which occur prior to denaturation (sometimes known as premelting), changes which occur on unfolding of the tertiary structure of DNA, and changes due to denaturation of the double stranded DNA.

Such experiments, which have been carried out on oligomers of nucleosomes subunits from mononucleosomes to pentanucleosomes, show that there are interactions between subunits which lead to enhanced thermal stability of the DNA and which change the pattern of CD melting. In addition, measurement of ellipticity in the premelting range indicates that no premelt phenomena[22] occur in nucleosomes.

EXPERIMENTAL

Native calf thymus chromatin was prepared by the method of Maurer and Chalkley[26] and the chromatin was sheared in a Waring blender for 90 sec. at 50 volts.

Nucleosomes were prepared from calf thymus nuclei[27] by a modification of several other procedures.[7, 8, 27] Fresh calf thymus, frozen on dry ice and stored in the freezer, was put twice through a meat grinder, then was Waring blender homogenized with 0.3M sucrose, 3 mM $CaCl_2$, 5 mM tris-cacodylate pH 7.3, and twice filtered through 4 layers of cheese cloth. The homogenate was centrifuged 30 min. at 250 x g, and the pelleted nuclei were washed twice with homogenization buffer and once with digestion buffer (which differs only by having 1 mM instead of 3 mM $CaCl_2$). Partial digestion of nuclei, at DNA concentrations equivalent to OD = 50, was carried out at 37°C with staphylococcal nuclease (Worthington) at concentrations of approximately 25 units/ml of sample, and digestion times of 1 minute to 30 minutes. Acid solubility of the digests was assayed in cold 1% perchloric acid, 0.4 M NaCl, followed by centrifugation at 6000 rpm for 30 min. and measuring OD_{260}. The following approximate digestion criteria were used:[28] 10% acid soluble material yields primarily

mononucleosomes and 3% acid soluble material yields a distribution of all oligomers (mono to pentasomes). It was found that approximately 80% of the DNA contained in the nuclei was solubilized (chromatin soluble after centrifugation as above) at 10% acid solubility, and approximately 40% was soluble chromatin at 3% acid solubility. Digestion was terminated by addition of EDTA to a final concentration of 5 mM. Particles were then separated according to size on 5-20% linear sucrose gradients spun in a SW 41 rotor at 35, 000 rpm for 15 hours in a Beckman Model L centrifuge. The resultant distribution was continuously monitored by measuring OD_{260} of the liquid pumped from each tube through a flow cell (Gilford); approximately 25 fractions per tube were collected. Fractions were pooled and resedimented after concentrating by vacuum dialysis in collodion bags. Fractions were similarly collected from the second sucrose gradients, and samples were dialyzed vs. 2.5×10^{-4}M EDTA pH 7.0.

Absorption measurements were performed on a Cary 14 spectrophotometer, and CD measurements on a Cary 60 with 6001 circular dichroism accessory as previously described.[29] Concentrations of nucleosomes for optical measurements were $1.5\text{-}2 \times 10^{-4}$M nucleotide residues. Thermal denaturation measurements were carried out as previously described.[23]

DNA was isolated from the nucleosomes, for purposes of determining its molecular weight, by addition of NaCl to 2M and SDS to 1%, followed by chloroform-isoamyl alcohol (24:1) extraction of protein, dialysis in water and lyophilization to dryness.[7] DNA was run on 4% polyacrylamide slab gels, visualized with ethidium bromide (.2 μg/ml), and photographed under UV light using a red filter.[30] The absolute size of the DNA fragments was determined by comparison with PM2 phage DNA fragments from cleavage with endonuclease R Hae3 restriction enzyme.[27]

The DNA concentration of the nucleosomes was determined by dilution of the nucleosomes with 5M NaCl 1:1 and measurement of the optical density at 258 nm (ϵ_{258} = 6700).

RESULTS

Size and Homogeneity of the Particles: The DNA isolated from mononucleosomes was run on polyacrylamide gels to determine the size and homogen-

eity of the DNA in the particles. A plot of log of the number of bases versus distance migrated[31] for the PM-2 phage DNA fragments used as standards was linear between 1000 and 50 base pairs, and was used to size the DNA in the particles. An average size of 162 ± 13 base pairs for monomers, 300 ± 30 base pairs for dimers and 520 ± 55 base pairs for timers was found. Thus the higher oligomers are multiples of the monomer. Monomers through trimers were homogeneous as judged by photographs of the DNA made fluorescent by ethidium bromide intercalation. Tetranucleosomes and pentanucleosomes had small amounts of heavier and lighter oligomers in approximately equal amounts.

Circular dichroism: The circular dichroism (CD) spectra of mononucleosomes up to pentanucleosomes was measured in the region of 260 to 320 nm to determine the peak value near 280 nm. The peak value of mononucleosomes is attained at 282 nm and has a value of 1850 ± 300 deg. cm^2/mol^{-1}. This is in approximate agreement with the ellipticity of PS-particles,[5] obtained by digestion of the chromatin to approximately 50% acid solubility, where a $[\theta]_{282}$ maximum between 700 and 1500 deg. $cm^2 mol^{-1}$ was reported. The digests herein may yield a larger ellipticity due to the larger particle size resulting from digestion to only between 3% and 10% acid solubility. This value should be compared with a maximum $[\theta]_{282}$ for chromatin of approximately 4000 deg. $cm^2 mol^{-1}$ [9] (Table I).

TABLE I
CIRCULAR DICHROISM

Preparation	$[\theta]_{280}$[a]			
	at 20°C	$[\theta]^{max}$	100°C	$\Delta[\theta]_{280}$[b]
DNA	8000	8200 (47°C)	≃ 5000	200
mononucleosomes	1850	4000 (76°C)	≃ 3000	2150
chromatin	4000	6900 (73°C)	≃ 3000	2900

a. Ellipticity in deg. $cm^2 mol^{-1}$ ± 400. b. $\Delta[\theta]_{280} = [\theta]_{280}^{max} - [\theta]_{280}^{20°}$ Difference between $[\theta]_{280}$ at 20°C and at that temperature at which $[\theta]_{280}$ is a maximum, ± 300.

The circular dichroism of di- through pentanucleosomes was carefully measured in the region of 260 to 320 nm and the results indicate that the $[\theta]_{282}$ value is the same throughout this series $[\theta]_{282}$ = 1900 ± 300

(see Table II). It is interesting that there was no detectable difference in the ellipticity of the different oligomers because it has been estimated

TABLE II
CHARACTERISTICS OF OLIGONUCLEOSOMES

Oligomer	$[\theta]_{280}$ [a]	T_m [b]	$\Delta[\theta]_{280}$ [c]
1	1850	73.5	2150
2	2080	76.0	1700
3	1940	76.5	2100
4	1900	78.0	2400
5	2000	79.0	2800

a. Ellipticity in deg. cm.^{2} mol.$^{-1}$ ± 400 at 20°C. b. Temperature at which dh_{280}/dT is an absolute maximum, ± 1.5°C. c. $\Delta[\theta]_{280} = [\theta]_{280}^{max} - [\theta]_{280}^{20°}$. Difference between $[\theta]_{280}$ at 20°C and at that temperature at which $[\theta]_{280}$ is a maximum, ± 300.

that up to 90% of the chromatin is composed of nucleosomes[7] and, therefore, when infinite numbers of monomers are associated together, the CD of the particles must approach that of chromatin ($[\theta]_{282}$ = 4000). The constant value of the ellipticity also implies that in small oligomers, if there are regions of free DNA connecting the beads, they are very small and do not contribute measurably to the CD spectrum as free DNA ($[\theta]_{282}$ = 8000).

Thermal Denaturation of Nucleosomes: The thermal denaturation of mononucleosomes, simultaneously measured by means of absorption and ellipticity at 280 nm, is shown in Figures 1A and 1B. For comparison chromatin and native DNA are also shown. The derivative thermal hyperchromicity curves, dh_{280}/dT, indicate a T_m = 73°C for mononucleosomes compared with a T_m = 78°C for whole chromatin (Fig. 1A) and a T_m = 47°C for DNA. In both mononucleosomes and whole chromatin, there are two other transitions at approximately 60°C and 66-68°C. The solutions of all the oligomers are clear and show no light scattering. The transitions noted in chromatin are approximately 2-3°C lower than those reported previously.[20, 32] The shift of the T_m for mononucleosomes relative to intact chromatin is in the opposite direction from the small difference reported in the thermal denaturation of PS-particles.[11] Differences in the preparations may account

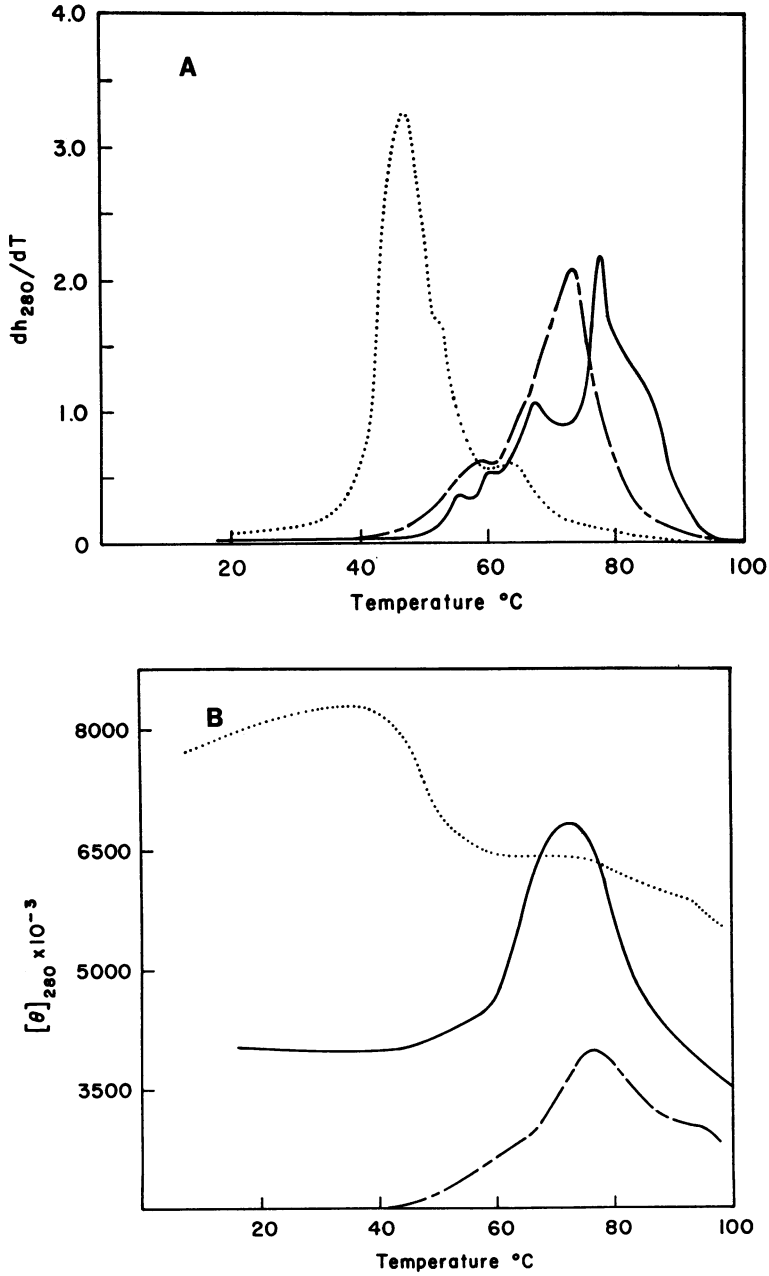

Figure 1. A. Derivative of hyperchromicity at 280 nm with respect to temperature versus temperature, dh_{280}/dT vs T, for chromatin (——); mononucleosomes (-· -); and DNA (····); in 2.5 x 10^{-4}M EDTA pH = 7.0. B. $[\theta]_{280}$, versus temperature for chromatin (——); mononucleosomes (-· -); and DNA (····) in 2.5 x 10^{-4}M EDTA pH = 7.0.

for this discrepancy.

The melting of mononucleosomes as monitored by $[\theta]_{280}$ (Fig. 1B) shows the following features: first, neither mononucleosomes nor whole chromatin shows any premelting. That is $d[\theta]_{280}/dT)_{av}$ between 20°C to 40°C is close to zero as compared with $(d[\theta]_{280}/dT)_{av} \approx 25$ for DNA. Both chromatin and mononucleosomes show a large increase in the ellipticity in the range of 60-77°C, that is in the early stages of melting. This is followed by a decrease in $[\theta]_{280}$ continuing up to 100°C. The final ellipticity for mononucleosomes and chromatin is approximately the same value, $\sim$ 3000. Denatured DNA (alone) on the other hand has a final ellipticity of $\sim$ 5000, (Table I), indicating that the DNA in chromatin and mononucleosomes is melting to a different final state than is free DNA. Finally, it should be noted that the ellipticity change between 40°C and 77°C, where the ellipticity reaches its maximum, is greater for whole chromatin than for mononucleosomes, and the maximum ellipticity for chromatin is higher than that for mononucleosomes. These experimental findings are summarized in Table I.

In order to further test these findings thermal denaturation measurements were carried out on mononucleosomes to pentanucleosomes. These results are summarized in Figures 2A-C and Table II. The hyperchromicity curves, dh_{280}/dT vs T, (Fig. 2A) clearly show three bands, characterized by the following temperatures: $T_m^1 \simeq 59°C$, $T_m^2 \simeq 68°C$, and the main band T_m^3, gradually increasing with the size of the particle, being equal to 73°C in the monomer and approximately 80°C in the pentanucleosome. In addition, in the temperature range of 40-50°C, a small amount of unbound DNA melts which tends to shift to higher temperatures as the particle size increases. Finally, another melting band, T_m^4, appears $\sim$ 90°C and becomes more pronounced and shifted to a higher temperature from mononucleosome to pentanucleosome.

The ellipticity monitored denaturation, $[\theta]_{280}$ vs T, (Fig. 2B), and its derivative curve $d[\theta]_{280}/dT$ vs T (Fig. 2C), show a pattern consistent with the findings on the mononucleosomes and whole chromatin. As the size of the particle increases, the magnitude of the ellipticity change becomes larger (from 2000 to 2800) and the maximum ellipticity attained

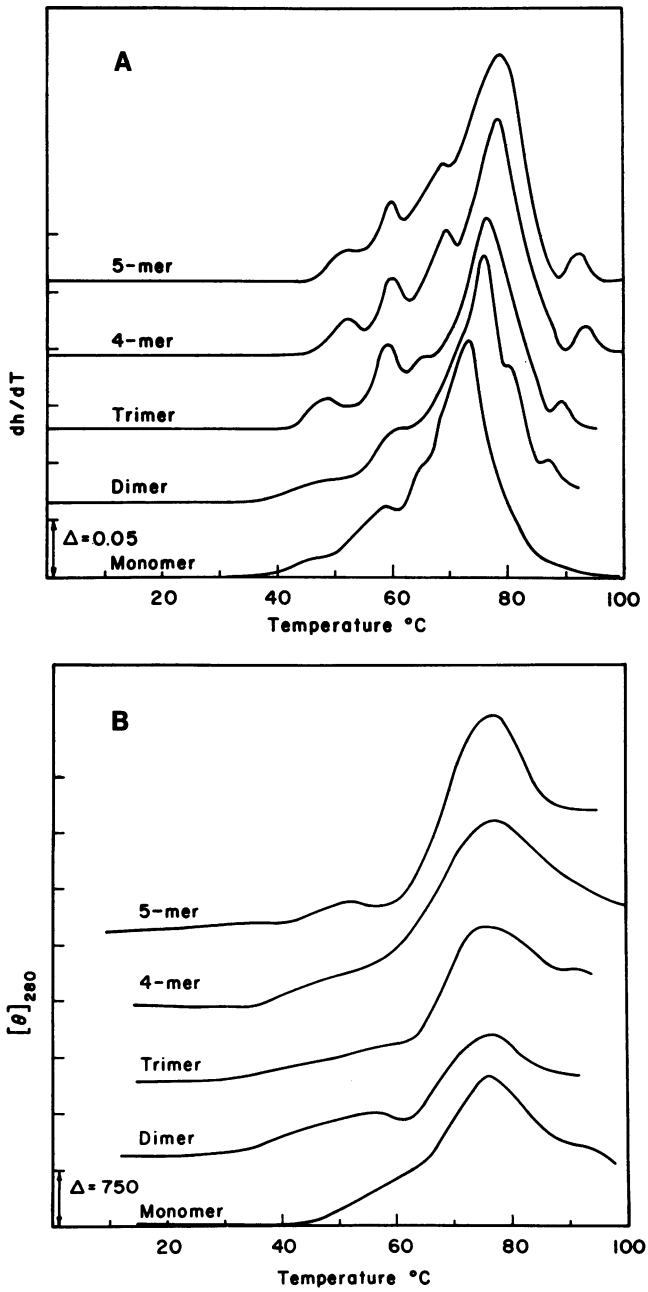

Figure 2A and B. Thermal Denaturation, at 280 nm, of mononucleosomes to pentanucleosomes as labelled. A. Derivative of hyperchromicity with respect to temperature, dh_{280}/dT versus temperature. B. $[\theta]_{280}$ versus temperature. The curves have been artifically displaced for clearer display.

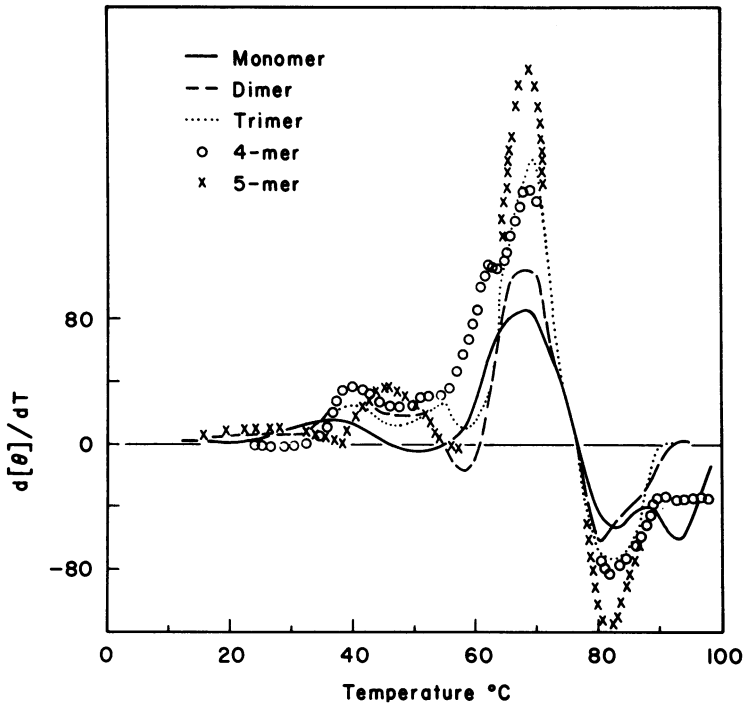

Figure 2C. Thermal Denaturation, at 280 nm, of mononucleosomes to pentanucleosomes as labelled. Derivative of ellipticity with respect to temperature, $d[\theta]_{280}/dT$, versus temperature. The curves have been artifically displaced for clearer display.

is higher (Table II). There is no clear cut pattern to the temperature of the CD maximum, but it appears to increase slightly with oligomer size. In all cases there is no premelt between 15°C and 40°C, and in each oligomer there is a small increase in the ellipticity between 40° and 60°C. It is not clear what this increase reflects.

DISCUSSION

There are several questions concerning the structure of chromatin that this study can shed light on. They concern the presence of free DNA as a spacer connecting adjacent nucleosomes, the interactions between histone complexes in different nucleosomes and the packing of nucleosomes in the chromatin chain. The thermal denaturation hyperchromicity for mononucleosomes (Fig. 2A) shows that there is only a small fraction of the DNA, approximately 5%, which melts at $\simeq$ 45-50°C. This DNA band gets progressively shifted to higher temperatures as the oligomer size increases, be-

coming 52-54°C in the pentanucleosome. This result indicates that there is only a very small amount of DNA which is extremely loosely bound to the central particle, but which becomes somewhat more protected as additional neighbors are added. The lack of premelting in the $[\theta]_{280}$ thermal denaturation measurement, and the near constant and very low value of $[\theta]_{280}$ as a function of oligomer number indicate that the amount of free DNA is quite small and in fact in chromatin, consisting of polynucleosomes, probably does not exist as such. Free DNA is apparently seen in electron micrographs[6, 10] but this may be a result of experimental manipulation and not a structure found in situ.[33] There is no concrete evidence herein for a tail of approximately 50 base pairs of free DNA which has been invoked to explain the difference between particles of 200 base pairs[7] and 130 base pairs.[34] It has been suggested[28] that there is a basic monomer of 185 base pairs which can be further digested to 140 base pairs. The preparative scheme used herein would not separate these two particles, and indeed a particle size intermediate between these two sizes was found. If there is such a readily digestible region of 45 base pairs, however, the thermal denaturation and 280 nm ellipticity indicate that it is probably an integral part of the particle and not a tail of free DNA.

The progressive increase in T_m^3 (main hyperchromicity band) of the particles as a function of oligomer size (Fig. 2A) indicates that there is an interaction between protein-protein and/or nucleic acid-protein on adjacent nucleosomes. This interaction can occur either through interactions between the protein core[41, 42] and adjacent sequences of DNA or due to the presence of H1 histone which had been earlier suggested to be involved in the maintenance of chromosome conformation.[35] This enhanced stability is also manifested by the growth of minor peaks at 68°C and 92°C in the hyperchromicity melts (Fig. 2A). These peaks may be due to the presence of H1 in the higher oligomers as it has been noted that the relative fraction of H1 increases with particle size.[9, 36]

When comparisons of melting profiles are made between nucleosomes and chromatin, it must be remembered that chromatin also contains non-histone proteins which are absent from nucleosomes.[10] Removal of non-histone proteins from chromatin has been shown to slightly alter both hyper-

chromicity and CD melting profiles.[22]

The following hypothesis is put forth to account for the observations reported herein: The large decrease in CD ellipticity (-6150), room temperature, between DNA (8000) and mononucleosomes (1850) may be due to the manner in which the DNA is wound around the histone core, plus the manner in which the small amount of loosely bound DNA is folded. Upon assembly of the mononucleosomes to form chromatin, the ellipticity increases until the value of chromatin is achieved, 4000. This change in ellipticity could be accounted for if the mononucleosomes were assembled in an asymmetric fashion in chromatin, e.g., a super helical array (tertiary structure). (The CD difference between chromatin and mononucleosomes could also be accounted for if one-third of the DNA in chromatin was present as free DNA instead of in a nucleosome-like state; however, the data indicate only a small amount of loosely bound DNA in chromatin.) However, the formation of a polysome superhelix would require a chain of more than five nucleosomes for nucleation, since $[\theta]_{280}$ is the same for the pentamer as for the monomer. A mixture of oligomers,[27] including pentanucleosome through chain lengths larger than 20, yielded a CD curve intermediate between mononucleosomes and chromatin, and therefore may include the region of superhelix initiation. This superhelix hypothesis implies that the asymmetric fold of DNA in the individual mononucleosome gives rise to a CD contribution of opposite sense than that found for the assembly of mononucleosomes.

The additional stabilization of the DNA secondary structure in oligomers and chromatin, relative to the mononucleosomes, can be seen from the hyperchromicity T_m's: chromatin, $T_m = 79^\circ C$; pentanucleosome, $T_m = 79.0^\circ C$; mononucleosome, $T_m = 73.5^\circ C$ (Table II, Fig. 1A). Thus the interaction between neighboring nucleosomes in the superhelix imparts an additional stability to the DNA double helix. This internucleosome interaction does not appear to greatly stabilize the DNA packing (tertiary structure) in the individual nucleosome, as the CD T_m's (Fig. 2B, C) are nearly identical for all oligomers.

However, this added stabilization of the DNA secondary structure can be seen to effect the CD melting properties of the nucleosomes if a compari-

son is made of the $\Delta[\theta]_{280}$ values observed during thermal denaturation. Chromatin displays a larger $\Delta[\theta]_{280}$ meltout than do mononucleosomes (3900 vs. 2150, Table I). This difference is likely to be the result of two competing processes: unfolding of the tertiary structure of DNA in the particles with the secondary structure intact (leading to an increase in $[\theta]_{280}$), and subsequent denaturation of the double helix (with a decrease in $[\theta]_{280}$). The unfolding process appears to occur at the same temperature (CD-T_m) in all oligomers, whereas denaturation takes place at progressively higher temperatures (dh-T_m) as the oligomer size increases, since the secondary structure is stabilized in the longer polynucleosomes. Therefore, the DNA in chromatin is stable to a higher temperature, allowing the attainment of a more unfolded tertiary structure and a larger value of $[\theta]_{280}$, before denaturation sets in, than is the DNA in mononucleosomes. Thus, from mono to pentanucleosome in the CD melt-out, the $\Delta[\theta]_{280}$ in this series becomes larger with increasing size of oligomer (Table II). As the $\Delta[\theta]_{280}$ for the pentamer is 2800 and that for chromatin is 3900, it is apparent that many more associated nucleosomes are necessary to reach the stability of chromatin. These data strongly indicate that nucleosomes are not assembled in a linear manner but are assembled in a condensed structure with multiple particle contacts, perhaps as a superhelix, imparting a new asymmetry to the structure. Such a possible structure is indicated in Fig. 3. Structures

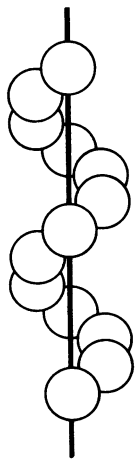

Figure 3. Schematic diagram of association of mononucleosomes (super coil).

with similar geometry can be implied from electron micrographs of strings of nucleosomes.[10,43] Similar hypotheses based on neutron scattering experiments[41,42] and X-ray diffraction studies[38] have also been put forth. The sharp increase in $[\theta]_{280}$ with respect to temperature in chromatin implies an unfolding of the particles and a breakup of the superhelical structure. Note that the CD T_m of chromatin lies a few degrees below that of the mononucleosomes (73°C compared to 76°C). The lower T_m temperature observed for chromatin could be mainly due to the unfolding of the superhelix, but the broad peak also includes the melting of the monomers at 76°C. The melting of chromatin has previously been shown to be accompanied by an increase in viscosity,[39] which might be due to the unfolding of the superhelix and then the monomers.

A recent study[40] utilizing hyperchromicity thermal denaturation of nucleosomes depleted of H1 and H5 has been reported. The melting profile of such mononucleosomes was reported to be similar to that found herein, however, the melting profile for dimers and trimers differed significantly. The predominate T_m peak (77°C) (dh_{280}/dT vs T), decreased in size and the derivative curve for a free DNA contribution increased considerably as a function of chain length. A similar appearance of loosely bound DNA in melting curves was found when chromatin from erythrocytes was stripped of H1 and H5 histones.[21] Electron micrographs[40] also showed definitive interconnective strands of DNA between nucleosome particles free of lysine-rich histones.

CONCLUSIONS

This study applies thermal hyperchromicity and circular dichroism analysis to nucleosomes to further elucidate their structure and stability. Mononucleosomes have been found to be less stable toward thermal denaturation than higher oligomers and chromatin; contain only a small fraction of their DNA as unbound sequences; show no evidence of premelting; have a $[\theta]_{280} \simeq 2000$ at 20°C; and show a smaller $\Delta[\theta]_{280}$ meltout than pentanucleosomes and chromatin. These observations may be accounted for by the following hypothesis. The hyperchromicity results imply an interaction between adjacent nucleosomes which contributes to the overall stability of

the DNA. This interaction is probably between the histones in one nucleosome with the DNA in an adjacent nucleosome or due to internucleosome histone interaction. These results also imply that there is not a significant amount of DNA spacer region or tail connecting adjacent nucleosomes, and that in chromatin the nucleosomes are probably in close contact.

The nucleosomes may possibly be arranged in an asymmetric array, i.e., superhelix, which yields its own significant CD contribution.

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