Effect of non-histone proteins on thermal transition of chromatin and of DNA

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ABSTRACT

The effect of chromatin non-histone protein on DNA and chromatin stability is investigated by differential thermal denaturation method. 1) Chromatin (rat liver) yields a multiphasic melting profile. The major part of the melting curve of this chromatin is situated at temperatures higher than pure DNA, with a distinct contribution due to nucleosomes melting. A minor part melts at temperatures lower than DNA which may be assigned to chromatin non-histone protein-DNA complex which destabilized DNA structure. 2) Heparin which extracts histones lowers the melting profile of chromatin and one observes also a contribution with a Tm lower that of pure DNA. In contrast, extraction on non-histone proteins by urea supresses the low Tm peak. 3) Reconstitution of chromatin non-histone protein-DNA complexes confirms the existence of a fraction of chromatin non-histone protein which lowers the melting temperature when compared to pure DNA. It is concluded that chromatin non-histone proteins contain different fractions of proteins which are causing stabilizing and de-stabilizing effects on DNA structure.

INTRODUCTION

Native eukaryotic chromatin contains DNA, RNA, histones and non-histones proteins (NHP). It was suggested that chromatin is made up of a linear array of spherical particles, the nucleosomes, containing DNA around a core of oligomers of histones (1-5). The possibility that NHP may have an active role in gene expression emerged from experiments using chromatin reconstitution and DNA-RNA hybridization (6). It was shown that NHP modify transcription in a manner characteristic of the origin of the tissue.

The present experiments have been undertaken to reach a better under-standing of how the non-histone proteins interact and influence chromatin structure and particularly DNA structure. The changes in chromatin and DNA structure may be related to biological processes of gene activation or repression. It was suggested that NHP might be bound to histones and some of them bound to DNA (7-11). By their binding to nucleosomes and to DNA a few NHP could induce modification of chromatin structure and

superstructure (12,13). From prokaryotic systems some information is accumulating about the importance of DNA specific proteins binding including RNA and DNA polymerases, repressor and unwinding proteins on binding and on the stability of DNA which is presumably related to their role in gene expression (14). Among the NHP some proteins seem to be involved in the regulation of gene expression (15,16,10).

In the present work, we have investigated thermal denaturation profile of various chromatin preparations and of NHP-DNA reconstituted complexes. Previous investigations indicated that the thermal denaturation profile of isolated chromatin was either bimodal or multiphasic reflecting the melting of DNA stabilized to different extent by histones (17-19). In reconstituted chromatin each of the individual histone displays a definite contribution to the magnitude of thermal transition (20-25). In all these studies on thermal denaturation, the formation of complexes stabilized DNA structure and the melting was observed at higher temperatures.

Polyanions including polystyrene sulfonate (26), sodium dodecyl sulfonate (27) and heparin (28) selectively extract histones from chromatin. This causes a marked destabilization of nucleohistone complexes (29,30) and an increase of the residual chromatin template activity (31-33). The complexes obtained after removal of histone by heparin and the reconstitution of DNA-NHP were investigated in the present studies to determine to which extent NHP are involved in DNA and chromatin stability. Evidence is presented indicating that the stability of DNA is affected in different ways by the presence of non-histone proteins. Some proteins contribute by increasing the stability of DNA in chromatin whereas other fractions of NHP have a destabilizing effect on DNA ordered structure. This is reflected in a relatively complex temperature denaturation profile in which thermal transitions at temperatures lower than DNA are clearly observed. This destabilization of DNA conformation was confirmed in reconstitution experiments in which an artificial complex NHP-DNA was investigated and also on a partial chromatin from which histones were removed by heparin. These studies indicate that NHP include protein species with a wide range of binding characteristics and which probably affect the conformation of DNA differently.

MATERIALS AND METHODS

Preparation of Chromatin : Chromatin was prepared from Wistar rats according to the method of Reeder (34) modified by De Pomerai et al. (35) which appeared to be the most suitable method for obtaining of product of large size, high template activity and closest to the native state. Two livers were homogeneized in 30-35 ml 0.25 M sucrose 5mM MgCl$_2$ - 10 mM Tris-HCl (pH 8.0) in a Potter homogenizer, filtered on cheesecloth and centrifuged at 1200 g and 4°C for 15 mn. The pellet was then homogeneized in 9 ml of the same sucrose medium containing 0.1 % triton X$_{100}$ and 40 ml 2.2 M sucrose, 5 mM MgCl$_2$ - 10 mM Tris-HCl (pH 8.0) again in a Potter homogenizer. The homogenate was then layered on the same volume of the same medium in 35 ml centrifuge tubes of a SW 25 rotor. Nuclei were centrifuged at 21.000 rpm at 4° for 30 minutes. The nuclei were lysed with 20 ml of 5 mM Tris-HCl buffer A (pH 8.0) containing 0.1 mM EDTA, 1 mM β-mercapto ethanol, 5 mM sodium bisulfite, 12.5 % glycerol. Chromatin was pelleted at 15,000 g for 15 minutes and suspended overnight in a small volume of the same buffer and diluted in 2 mM NaCl. In control experiments chromatin was prepared in the presence of 0.1 mM phenyl-methylsulfonylfluoride (PMSF) maintained at all steps of preparation. The final solvent contained 0.1 mM EDTA, 1 mM or 2 mM NaCl.

Extraction of Chromatin proteins by heparin : Chromatin samples containing 10 mg of proteins were mixed with various amounts of heparin (Sigma) for 18 hours at 4°. The samples were centrifuged 48 hours at 100,000 g. The pellets were solubilized in buffer A and dialyzed against 2 mM NaCl. For electrophoresis proteins were extracted from the pellets with 7 M urea, 3 M NaCl, 10 mM tris-HCl buffer, pH 8.0, 1 mM β-mercapto ethanol (36). Samples containing 100 μg of protein were used for electrophoresis performed according to the technique of Panyim and Chalkley (37), modified by Shaw and Huang (38), which allows a good separation of histones from NHP (Fig. 4.A). Electrophoresis were also performed according to Laemmli (39) which gives a good resolution of the NHP (Fig. 4.B).

Extraction of chromatin proteins by urea : Chromatin samples were solubilized for 18 hours at 4° in the following solution : 5 M urea 10 mM Tris HCl (pH 8.0) 0.1 mM EDTA, 1 mM β-mercapto ethanol. The samples were centrifuged 48 hours at 100,000 g. The pellets were solubilized in buffer A and dialyzed against 2 mM NaCl.

Association of NHP with DNA : DNA was prepared according to Marmur (40). NHP were prepared as follows : rat liver nuclei were prepared by the technique of Chauveau et al. (41). They were washed 4 times by centrifugation in 0.25 M sucrose containing 10 mM $MgCl_2$. NHP were extracted according to Kamiyama and Wang (15).

DNA and NHP were mixed at a ratio of 1:1 in 2 M NaCl. The NaCl concentration was lowered to 0.1 M NaCl in 10 steps of 8 hours each. Finally, the mixture was dialyzed against 2 mM NaCl.

Preparation of nucleosomes : Nucleosomes were prepared by digestion of purified rat liver nuclei with Staphylococcal nuclease (Worthington) as described by Shaw et al. (42). Nuclei prepared according to Chauveau et al. (41) were washed once with the washing buffer : 0.3 M sucrose, 3 mM $CaCl_2$, 5 mM Tris-cacodylic acid buffer adjusted to pH 7.3 and once with the same buffer containing only 1 mM Ca^{2+}. Digestion was performed for 90 sec. at 37°C with 10^8 nuclei and 200 U of nuclease per ml. A volume of 4 ml of the supernatant was applied on a Biogel A5 column equilibrated with a 10 mM tris-cacodylate buffer pH 7.3. containing 0.7 mM EDTA. Nucleosomes were identified by polyacrylamide gel electrophoresis, sucrose gradient centrifugation and by a electron microscopy (performed by Dr. S. Bram).

Thermal denaturation : The melting experiments were performed by measuring the increase in absorbancy at 260 nm of different chromatin and DNA samples in a cuvette in which the evaporation is minimized by the use of a teflon stopper. The reference cuvette containing the solvent and equipped with a thermocouple was heated under identical conditions at the rate of 0.5°C / minute. The changes in absorbancy as a function of temperature and its first derivative (dA/dT versus T) were measured and recorded simultaneously. The obtained derivative melting profile were normalized for the total hyperchromaticity change. The chromatin samples (containing EDTA) were adjusted with 2 mM NaCl, pH 7.

RESULTS

Thermal denaturation of chromatin : Figure 1 shows the first derivative thermal denaturation profile of soluble chromatin. This profile is multiphasic and at least 6 major peaks can be observed corresponding to the following melting temperatures : peak 1 : 56°C - peak 2 : 64°C - peak 3 : 71°C - peak 4 : 74°C - peak 5 : 78°C and peak 6 : 82°C.

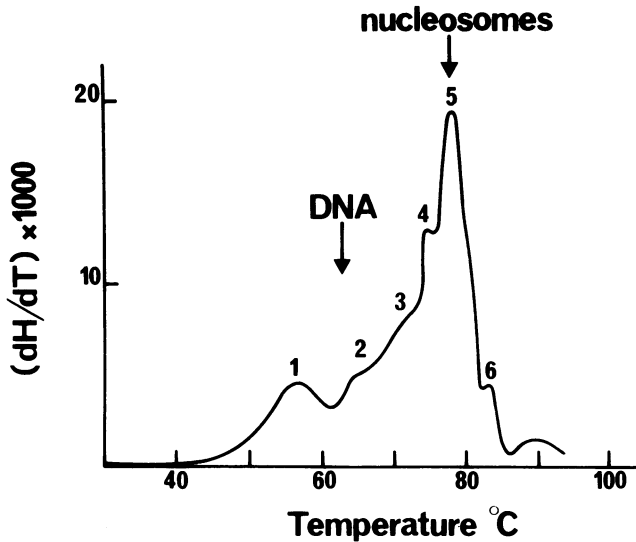To establish the origin of different thermal transitions and whether

FIGURE 1 : Thermal denaturation profile of chromatin. Rat liver chromatin was prepared as described in the "Methods", and diluted about 200 times with 2 mM NaCl, 0.1 mM EDTA. An identical melting profile was observed when PMSF was included in all steps of chromatin preparation.

NHP are involved in the structure of chromatin, we have first identified the peaks corresponding to the thermal transitions of free DNA and of nucleosomes.

The melting profile of pure rat liver DNA is shown in Fig.2. The T_M of this DNA is 63°C under our experimental conditions (Fig.2). It corresponds to the peak 2 of soluble chromatin (at 64°C). It suggests the presence of some free DNA regions in chromatin. The thermal transition occuring at about 71°C (peak 3) is probably also due to free DNA between nucleosomes. The presence of nucleosomes and also of other proteins is probably increasing the stability of this internucleosomal DNA, which melting occurs at T_M of 64°C and 71°C (see "Discussion").

The thermal transition of mononucleosomes is shown in Fig.3. The denaturation profile of mononucleosome has a maximum at 74°C and corresponds probably to the peaks 4 and 5 of whole chromatin, which occur at about 74°C and 78°C respectively. The increase in melting temperature between the mononucleosomes and the polynucleosomes is also in agreement with previous melting studies as a function of oligomer size (25). The broad shoulder with a maximum at 63°C corresponds to free DNA present in the nucleosome preparation.
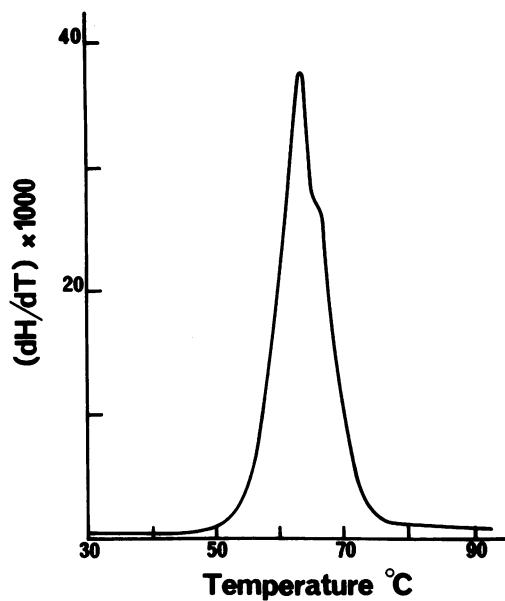
FIGURE 2 : Thermal denaturation profile of rat liver DNA. DNA was prepared according to Marmur (40). Solvent conditions as in Fig.1.
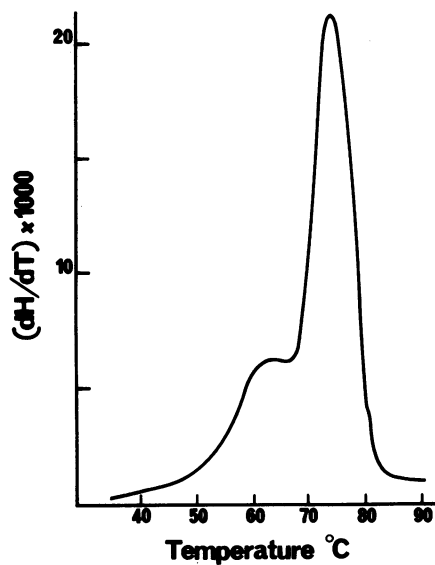

FIGURE 3 : Thermal denaturation profile of rat liver nucleosomes. Nucleosomes were prepared by the method of Shaw et al. (42). Solvent used as in Fig.1.

Effect of heparin on chromatin thermal denaturation profile : In a previous work, it was shown that histones are selectively extracted from chromatin by heparin at low concentration (28), see Fig.4. When a heparin chromatin ratio of 1:20 (w/w) was used, part of histones H1, H3 and H4 were extracted (Fig. 4.A). The thermal denaturation profile was broader but presented a maximum at 78°C corresponding to that of the T_M of polynucleosomes.(Fig.5.) This suggests that the non extracted histones are sufficient to maintain a nucleosome like structure. Histones H1 and H4 and most of H3 were extracted when 1:10 (w/w) of heparin was used. The
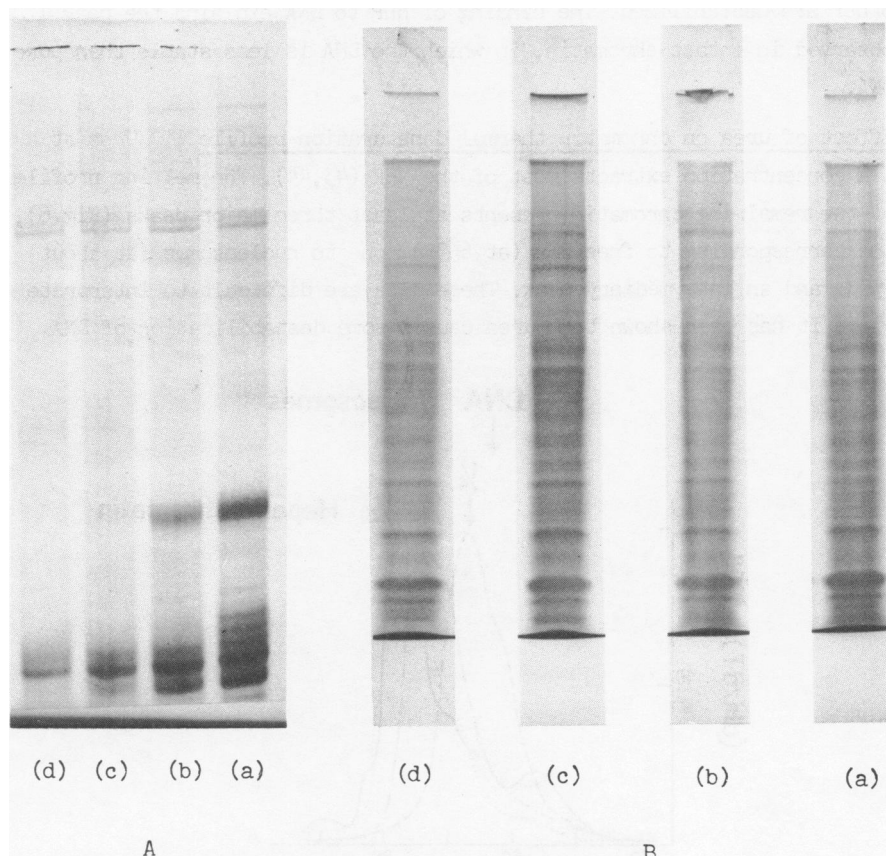

FIGURE 4 : Electrophoresis of proteins left in chromatin after the extraction by heparin.
A - Electrophoresis performed according to the technique of Panyim and Chalkley (37) modified by Shaw and Huang (38).
B - Electrophoresis performed according to Laemmli (39).
(a) Proteins from non-treated chromatin. Proteins remaining after heparin treatment at heparin-proteins ratios (w/w) of (b) 1:20 - (c) 1:10 - (d) 1:4. In electrophoresis performed according to Laemmli the histones ran out of the gels.

T_M of this chromatin decreased to 73°C and the peak was broad. This indicates that a part of DNA is not bound to protein and that another part of chromatin DNA is less stable that in nucleosomes, due to the presence of NHP which were not extracted by heparin (Fig. 4.B.). At a 1:4 (w/w) ratio heparin removed most of the histones and some NHP. The maximum of the peak is at 66°C. (Fig.5.) The peak is very broad and included peaks 1, 2 and 3 from intact chromatin.

These experiments suggest that NHP are able to modify the stability of DNA. In the absence of histones some parts of DNA are stabilized some other are destabilized. The binding of NHP to DNA explains the peak 1 observed in intact chromatin, in which the DNA is less stable than pure DNA.

Effect of urea on chromatin thermal denaturation profile : Urea at a 5 M concentration extracts most of the NHP (43,44). The melting profile of the remaining chromatin presents at least three major peaks (Fig.6), one corresponding to free DNA (at 65°C), one to nucleosomes (at about 75°C) and an intermediary peak. These data are difficult to interprete since it has been shown that urea causes some destabilization of DNA
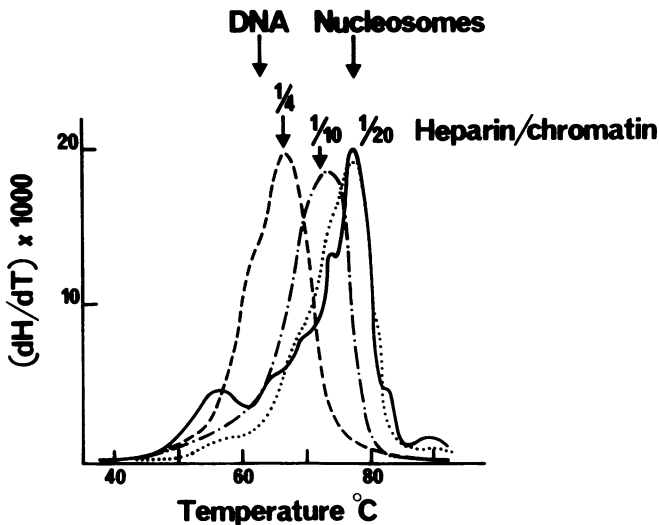

FIGURE 5 : Effect of heparin on the thermal denaturation of chromatin. Chromatin was treated by increasing amounts of heparin as described by Kitzis et al. (28). The chromatin pellets were collected by centrifugation and solubilized in 2 mM NaCl, 0.1 mM EDTA. The heparin chromatin ratios (w/w) were : 1/4 (---) ; 1/10 (—•—) and 1/20 (• • •). Untreated native chromatin (——).
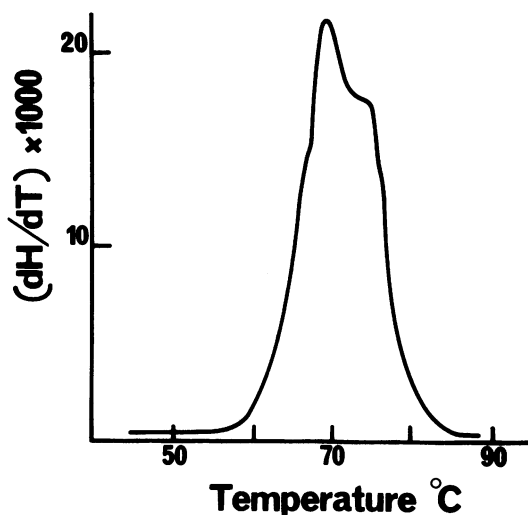
FIGURE 6 : Effect of 5 M urea on the thermal denaturation of chromatin. Chromatin was treated with 5 M urea in a Tris-EDTA-β -mercapto ethanol buffer (pH 8.0). The chromatin pellet was collected by centrifugation and solubilized with 2 mM NaCl, 0.1 mM EDTA.

complexed with histones, without increase in the fraction of histone-free DNA and alters the periodic structure of chromatin (45-47). However, there is no peak at a temperature lower 63°C indicating the absence of destabilization of the DNA.

Thermal denaturation profile of DNA-NHP reconstituted complex : Another approach to studying the effect of NHP on DNA is to measure the thermal denaturation of reconstituted DNA-NHP complex. NHP extracted from chromatin with 1 M NaCl and free from histones were added to free DNA at a 1:1 ratio (see "Methods"). The reconstitution was performed by a stepwise lowering of the ionic strength from 2 M to 2 mM NaCl. The melting profile is shown in Fig.7. In addition to a major peak at about 66°C, corresponding to free DNA-melting, we observed 2 peaks at the lower temperature side with maxima at approximately 54°C and 60°C, corresponding to peak 1 of chromatin. Another peak gives a maximum at 73°C. These data confirm the results obtained after treatment of chromatin with heparin : some NHP destabilize whereas, some other stabilize DNA structure. NHP could be responsible for the occurence of peak 1 at a lower temperature.
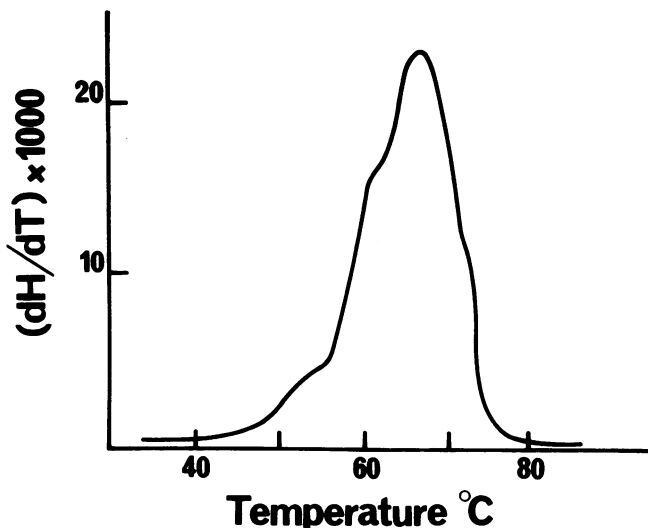
FIGURE 7 : Thermal denaturation profile of reconstituted NHP-DNA complex. NHP free from histones were mixed with DNA at a 1:1 ratio in 2 M NaCl. The NaCl concentration was lowered stepwise to 2 mM NaCl.

DISCUSSION

In the present investigation, an attempt is made to reach some understanding of the role of NHP to the overall structure of chromatin. Despite of the complexicity of the first derivative melting profile of NHP rich chromatin, six separate transitions are easily resolved for which the following main conclusions should be considered :

1) At temperature higher than that of free DNA melting several transitions can be observed indicating a stabilizing effect.

2) A small but appreciable fraction of chromatin DNA melts at temperatures well below that of infinite DNA helices or of free DNA in mononucleosome preparations. This reflects a destabilization of the DNA native structure.

The method of chromatin preparation used in the present studies, based on sucrose gradient centrifugation and in the absence of shearing, insures chromatin integrity close to the native state.

The analysis of these results of complex derivative melting profile of NHP rich chromatin is facilitated by correlation with the results obtained on nucleosomes, with reconstituted complexes of DNA with NHP and with selectively extracted chromatin.

I - The stabilizing effect on DNA : The majority of thermal transitions

of chromatin occurs at temperatures higher or equal to that of free DNA.
The melting of free DNA helices and of free DNA in nucleosomes preparations
occur at a temperature of about 64°C (see Fig.2 and Fig.3) under our
experimental conditions. The thermal transitions of nucleosomes occurs at
temperatures of about 74.5°C, which allows a clear assignment of the major
peak of chromatin (Fig.1) at 78°C and probably of its small satellite at
74°C to the nucleosome melting. In fact, an increase in the melting
temperature was observed with the size of oligomers (73.5°C for monomer,
79°C for pentamer, see ref. 20). The two peaks at lower temperatures,
i.e. at 64°C and at 71°C, can be possibly assigned to the contribution
of free DNA. The peak at 64°C corresponds quite well to the melting of
free DNA helices (see Fig.1). One may expect that the peaks at 64°C and
71°C will correspond to the thermal transitions of free DNA between
nucleosomes. In fact, the free DNA melt of T_M 63°C is derived from very
long helix. The unbound DNA in chromatin could well be of shorter type
helix which in the free state would melt at lower T_M. The effect of
stabilization of DNA by the presence of nucleosomes will be reflected in
the increase of melting temperature (as pointed out by Subirana (13) and
Staynov (48)). It is to be noted that the peak at 71°C is quite broad
which may correspond to the heterogeneity of length distribution of these
DNA cooperative units.

At a temperature of 82°C one observes a separate transition which
corresponds to a DNA-Protein complex. This pronounced stabilization effect
is outside the temperature zone of nucleosomes melting. It must correspond
to a tightly bound DNA-Protein complex. Such a tightly bound NHP protein
fraction was observed recently even under strongly dissociating conditions :
2 M NaCl and 5 M urea (44). It was found that the tightly bound protein
consists of one major high molecular weight protein in a relatively
appreciable concentration.

II – The destabilizing effect on DNA : One of the most interesting results
of the present investigation is the observation of the existence of a small
part of chromatin characterized by a thermal transition occuring at
temperatures below that of the melting of free DNA, i.e. at about 56°C.
This is established in a series of reconstitution experiments of DNA and
NHP complexes and in experiments involving selective extraction of
chromatin with heparin. In both types of experiments, one observes a
thermal transition below the melting temperature of free DNA.

There is a good correlation between the present results on chromatin

thermal transitions and some data on isolated NHP. A class of non-histone chromosomal proteins was extracted from rat liver, which interacts optimally with A-T rich and single-stranded DNA (44). Similarly proteins isolated from calf thymus appear analogous to previously characterized prokaryotic DNA-unwinding proteins, but which may bind to double stranded DNA (49,50). One may expect that the observed lowering of the free DNA melting temperature (see Fig.2) is a result of formation of loops, due to the opening of the double helix by fraction of NHP.

In allmost all previous investigations on chromatin thermal transitions only the stabilization of DNA structure by complex formation with histones and other proteins was reported. The use of a very sensitive differential method of investigation of thermal denaturation and the choice of NHP-rich chromatin allowed us to observe the contribution of a particular melting-in protein. Current studies of more quantitative character will allow to understand the possible role of NHP in mononucleosomes and in chromatin.

It is generally accepted that some of the NHP may have an important role in the regulation of gene expression. The mechanism of action of these proteins may involve a modification of DNA stability. It is highly probable that a destabilization of DNA helical structure is a prerequisite for gene activation, whereas the stabilization of DNA and the loss of its conformational flexibility may be involved in the negative control of gene expression.

In conclusion, the demonstration of the existence of NHP fractions that respectively stabilize and destabilize DNA structure seems to be promising for the understanding of the role of chromatin non-histone proteins in the modulation of gene activity.

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