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Ethidium bromide intercalation and chromatin structure: A thermal analysis

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Abstract

Differential Scanning Calorimetry has been performed in the temperature range 310 K–410 K on intact thymocytes and physiologically isolated chromatin following Ethidium bromide intercalation. Native thymocytes exhibited four main thermal transitions (at 339 K, 347 K, 362 K and 375 K) that were assigned to the melting of different cellular components. At increasing dye concentrations an enthalpy redistribution became evident between the thermal transition at 362 K related to the melting of nucleosome organized in the 10 nm filament, and the transition at 375 K related to the melting of nucleosome organized in the 30 nm (or more) fiber. In correlation with increasing concentrations of Ethidium bromide, the disappearance and the subsequent reappearance of the highest temperature transition seem to be related to the unwrapping and subsequent wrapping of the chromatin fiber. Under similar condition, free DNA and digested chromatin do not show any enthalpy redistribution in their calorimetric profiles following Ethidium bromide intercalation. On the contrary, physiologically isolated chromatin displayed similar enthalpy redistribution between transitions assigned to chromatin DNA melting. An interesting difference appeared in the calorimetric profile of isolated chromatin with respect to the in situ material after chromatin extraction. In fact, a transition at 354 K, probably related to the melting of linker DNA became apparent (the transition at 362 K was assigned to the melting of DNA around the core particle). Selective digestions with different enzymes (micrococcal nuclease, proteinase K and DNase I) were carried out on thymocytes to verify the assignment of the main thermal transitions. In order to clarify the nature of the high temperature transitions native thymocytes were treated with topoisomerase I that removes superhelical turns from topologically closed DNA molecules. A comparison of calorimetric data with thermal denaturation profiles obtained by spectropolarimetric measurements on physiologically isolated chromatin gave further confirmation to the peak assignment by distinguishing the thermal transitions related to protein denaturation from the ones assigned to chromatin-DNA. © 1997 Elsevier Science B.V.

Keywords: Chromatin Structure; Circular dichroism; DSC; Ethidium bromide intercalation; Thermal Denaturation

1. Introduction

A large number of studies have been performed by using Ethidium bromide intercalation as a probe for chromatin-DNA structure [1–7]. It is well-known that the intercalating binding of Ethidium bromide to a DNA molecule initially produces an unwrapping of the DNA double helix by an angle of 26° [8] until the helix completely unfolds at a critical dye concentration (v). When Ethidium bromide concentration further increases, the DNA double helix begins to wrap again but in the opposite direction; therefore the final result is a helix with an opposite spiral [9,10].

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In order to study the effects of ethidium bromide intercalation on high order chromatin-DNA structures we utilized Differential Scanning Calorimetry on both intact cells and isolated chromatin. This technique has been used successfully in a large number of studies investigating the chromatin-DNA structure and its modulations as a function of cell-cycle progression, ionic strength conditions and fixative effects [3,8,11–17]. The advantage of this techniques in comparison to the spectroscopic methods usually employed for structural investigation of chromatin, is its ability of studying samples in both heterogenous suspension or in pellet form. This non-invasive tool was therefore useful for investigating high order chromatin structures directly inside the cells: in fact chromatin may be damaged easily or lost by extraction procedures.

Following the results recently published by other groups [3,18,19] we carried out a series of selective digestions of thymocytes with micrococcal nuclease, proteinase K and Dnase I in order to verify with calf thymocytes the assignments previously carried out on rat hepatocytes [12,14].

Native thymocytes were treated with topoisomerase I. This enzyme is able to relax the negative and positive DNA supercoiling by a transient breakage and rejoining of phosphodiester bond. The effects of the removal of superhelical turns have been observed in calorimetric profiles of native thymocytes and used to clarify the nature of high temperature transitions.

For a more convincing interpretation of the calorimetric data obtained on intact thymocytes and isolated chromatin we decided to compare them with the thermal denaturation profiles obtained on native chromatin by using CD spectroscopy that can furnish complementary information.

2. Experimental

2.1. Cell isolation

Intact cells were isolated from calf thymocytes by the usual procedure [17]: small pieces of thymus were homogenized in a Homogenization Buffer (0.8% NaCl dissolved in 0.1 M Tris-HCl pH 7.2) and filtered through a steel-mesh. After the first brief centrifugation (4 min at 300 × g) the resulting pellet was washed in a PBS (Dulbecco's Phosphate Saline) buffer containing 2 mM EDTA and 2 mM $MgCl_2$. All these procedures were carried out at 4°C in order to preserve the physiological organisation of the cells.

2.2. Chromatin preparation

After centrifugation (4 min at 300 × g) the cellular pellet was washed in PBS; the nuclei were extracted by brief incubation (3 min at 4°C) with 0.1% Triton X 100. The nuclear pellet was washed with 10 vol. of 0.15 M NaCl–0.01 M Tris-HCl pH 8 and resuspended for 1 h in cold 2 mM EDTA buffer. Finally the nuclei were lysed by homogenization in a Dounce homogenizer and Chromatin was purified by centrifugation through 1.7 M sucrose at 10^3 × g for 80 min. All these procedures were carried out at 4°C. We refer to this chromatin preparation as Cold Water method [7,12,20].

2.3. Enzymatic digestions

DNase digestion was carried out on native thymocytes resuspended in a PBS buffer (with 5 mM $MgCl_2$) 10^8 cells/ml were then incubated for 90 min with 500 U ml^{-1} of DNase I at 37°C.

Nuclease digestion was performed on native thymocytes resuspended in a PBS buffer(with 5 mM $MgCl_2$) 10^8 cells/ml were then incubated for 90 min with 15 U ml^{-1} of micrococcal nuclease at 37°C.

Protease digestion was carried out on native thymocytes resuspended in a PBS buffer (with 5 mM $MgCl_2$) 10^8 cells/ml incubated for 30 min with 1 mg ml^{-1} of proteinase K at 37°C.

The enzyme was purchased from Sigma (Milan, Italy). Topoisomerase digestion was carried out on native thymocytes resuspended in the reaction buffer (50 mM Tris-HCl pH 7.5, 50 mM KCl, 10 mM $MgCl_2$, 0.5 mM DTT, Na_2EDTA, 30 g ml^{-1} BSA). 10^8 cells/ml were incubated for 30 min with 500 U ml^{-1} of topoisomerase I (Gibco BRL, MD) at 37°C. Each of the above four reactions was stopped by adding Na_2EDTA to a find concentration of 10 mM.

2.4. Ethidium bromide Staining

The native thymocytes were divided into different tubes so that each sample contained 10^8 cells, corre-

sponding to 1 mg of DNA per tube. A value of 10 pg of DNA per cell was obtained.

A 1 mg ml^{-1} Ethidium bromide solution was used and its concentration was determined by using a molar extinction coefficient of 5600 at 480 nm [21]. After brief centrifugation the samples (intact cells or physiologically isolated chromatin) were resuspended in 1 ml of PBS containing the required concentration of Ethidium bromide. All samples were incubated at 4°C for 10 min and then centrifuged to eliminate the free Ethidium bromide. It was assumed that at 4°C the rate of EB dissociation was quite slow and therefore do not effectively change the initial EB/DNA ratio.

In order to obtain a pellet suitable for calorimetric analysis, each sample was centrifuged for 30 min at 5000 × g and finally loaded in the aluminium capsule.

2.5. Differential Scanning Calorimetry

DSC experiments were performed on a Perkin Elmer DSC-2C (Perkin-Elmer, Norwalk, CT) using a temperature range between 310 K and 410 K and 75 μl aluminium capsules [12].

A computerized system interfaced to the Calorimeter allowed good reproducibility and sensitivity of the signal acquisition, background subtraction and data display [14]. During scanning the following parameters were used; low scanning rate (5 K min^{-1}), high sensitivity (0.1 mcal s^{-1}) and sample size (60 mg). After each measurement, the corresponding baseline was acquired by performing a new scan on the denatured sample in order to check the irreversibility of the thermal transitions under analysis.

Finally the interpolated baseline was subtracted from the raw acquired Scan in order to obtain the correct thermal profile that allowed a quantitative interpretation of the thermal transitions. Deconvolution of the heat capacity profiles into gaussian components was carried out by least square fitting of the acquired data.

2.6. Circular Dichroism

Molar Elipticity measurements of chromatin and DNA samples were made on a Jasco 710 spectropolarimeter interfaced to a computer supplied with operative software for data acquisition and elaboration.

Circular dichroism measurements were made in a nitrogen atmosphere in a 1 cm path length quartz cell, with a wavelength range between 360 and 220 nm. In order to reduce random error and noise, each acquired spectrum was the average of five different measurements (standard deviation<5.5%).

The following parameters were used: time constant 4 s, scanning speed 20 nm min^{-1}, band width 0.2 nm, sensitivity 20 m degree, step resolution 0.5 nm and PMT voltage below 400 V [20]. The acquired signal was expressed as Molar ellipticity (degrees cm^2/decimoles).

For all the measurements the DNA concentration of samples was kept constant (7.5×10^{-5} M). It was spectrophotometrically determined by using an extinction coefficient of 21 000 cm^2 g^{-1} for nucleosomal DNA in 0.2% sodium dodecyl sulfate buffer [22]. A PTC-343 Jasco system was used to control the temperature of the quartz cell which increased at a predeterminated rate (100 K h^{-1}) by using a Peltier thermostatic cell holder.

2.7. Quantification of the nuclear DNA

For calf thymocytes the average DNA concentration per single cell was calculated by using the Munro procedure. This spectrophotometric method utilizes the precipitation of DNA following the addition of perchloric acid to the DNA solution [23].

3. Results

As previously reported [17] the calorimetric profiles of native thymocytes at physiological conditions (without Ethidium bromide) show (Fig. 1 A) four main thermal transitions centered at about 338 K (0 Transition) 347 K (I Transition), 362 K (II Transition) and 375 K (III Transition). These endotherms, marked with Roman numerals, have been previously assigned [2,8,11–14,16–19,27,28] as follows: Transition 0 to the melting of membranes and debris, Transition I to nuclear proteins, Transition II to nucleosome organized in 10 nm filament and Transition III to nucleosome organized in higher order structures (corresponding to the 30 nm or more condensed fibers). When these scans were deconvoluted into their gaussian components Transition III resulted in two

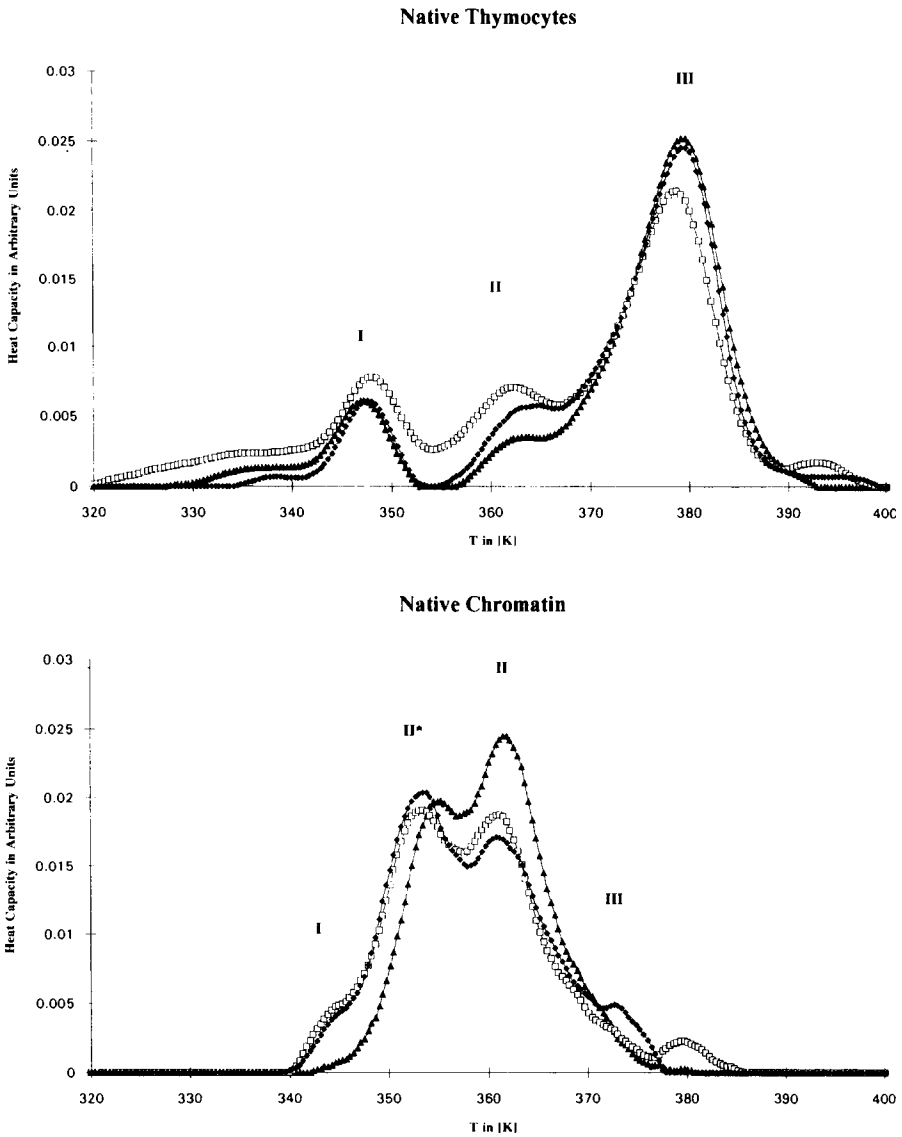

Fig. 1. Profiles of heat capacity (arbitrary units) versus temperature (K) for three different samples of native calf thymocytes(A) and isolated chromatin (B) prepared as described in the experimental procedures paragraph.

components centered at 372 and 380 K that are referred to as III_a and III_b these components are probably related to the melting of differently condensed chromatin fibres,as previously reported by other authors [19]. For a more immediate appreciation of the results, the gaussian deconvolution of the scans are not shown in the figures. The calculated area and enthalpy values are reported.

When Ethidium bromide was added to calf thymocytes (Fig. 2) a reduction of Transition III was observed at low dye concentrations. For all the data the Ethidium bromide concentration is reported as the ratio (R) between dye and DNA (namely phosphate residues) concentration [7,20,21,24–26]. The reduction of Transition III was not clearly evident at very low dye concentrations until (R=0.08) and became

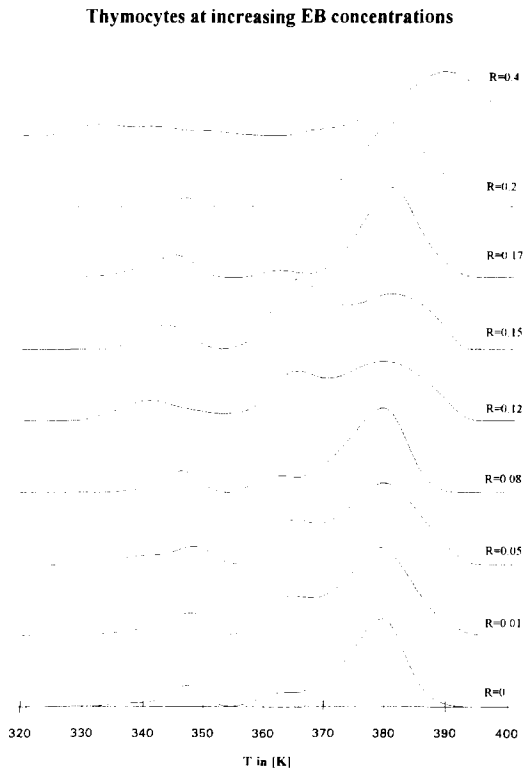

Fig. 2. Profiles of heat capacity (arbitrary units) versus temperature (K) for calf thymocytes at increasing Ethidium bromide concentrations: R=0; R=0.01; R=0.05; R=0.08; R=0.12; R=0.15; R=0.17; R=0.4.

more evident at increasing dye concentrations (from R=0.08 to R=0.12),. When the Ethidium bromide concentration was further increased, Transition III continued to decrease until it completely disappeared at a critical dye concentration (between R=0.15 and R=0.17). Further addition of Ethidium bromide (R=0.2) caused a reappearance of Transition III and a disappearance of Transition II. For higher EB concentrations (R=0.4) a broadening of this last peak became apparent together with a shift of Transition III_b towards higher temperatures (from 380 to 386 K).

For each main endotherm exhibited by thymocytes the Relative Peak Area is reported in Fig. 3 as a function of EB intercalation. The corresponding Relative Melting Enthalpy is reported in Table 1. The Enthalpy of Transition I did not change significantly following Ethidium bromide intercalation. However it is interesting to point out that for this transition a shift towards lower temperatures (from 347 to 341 K) became evident with higher Ethidium bromide concentrations (Fig. 2). On the contrary, under analogous conditions, the II and the III transitions showed significant and opposite alterations of their enthalpy. For low dye concentrations (R between 0.01 and 0.1) the enthalpies changed slightly, for higher R values (R above 0.1) the EB effects were quite evident. Finally for R=0.15 the II transition showed an increase of its Relative Melting Enthalpy, while the III transition

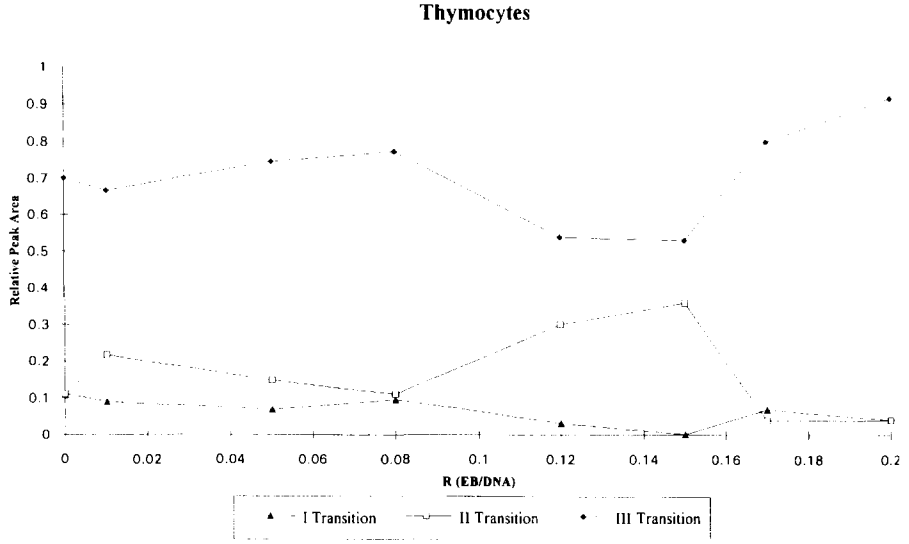

Fig. 3. Estimation of Relative Peak Area at increasing EB concentrations for the main thermal transitions appearing in thymocytes calorimetric profiles shown in Fig. 2.

Table 1
Relative Melting Enthalpy (Kcal mol^{-1}) of the main thermal transitions appearing in thymocytes thermograms at different Ethidium bromide concentrations: the I Transition is assigned to the melting of nuclear proteins, the II Transition to nucleosome organized in higher order structures. Transition III has been deconvoluted into two gaussian components that in native thymocytes are centred at 372 and 380 K(here referred to as III_a and III_b).

R	Thermal Transitions			
(EB/DNA)	I 341–348 K	II 362–365 K	III_a 370–376 K	III_b 380–386 K
0.000	5.55	3.36	11.13	28.70
0.010	7.44	18.25	27.35	28.68
0.050	6.76	14.50	17.55	55.44
0.080	8.73	10.09	21.15	49.64
0.120	2.76	25.65	26.51	19.54
0.150	0.05	26.18	19.91	18.66
0.175	4.48	2.50	1.60	50.12
0.200	2.27	2.18	0.00	51.77
0.400	2.15	1.33	3.56	36.78

The following Standard Deviations have been calculated for the three main transitions: 1.3%(I Transition), 31.8% (II Transition) and 18.4 % (III Transition).

exhibited a drastic reduction. Further observations on the Ethidiun bromide binding can be noted at higher dye concentration (R=0.2 and R=0.4) where the II transition completely disappeared causing a reappearance of the III transition.

In order to better understand the nature of the structural changes observed on in situ chromatin after Ethidiun bromide intercalation, analogous measurements were carried out on physiologically isolated chromatin.

There were some differences between the DSC scans of isolated chromatin and intact thymocytes (Fig. 1 A and B). First of all the procedure of chromatin isolation provided a partial loss of the high order chromatin structure which was revealed by an enthalpy reduction of the Transition III that was only evident as a shoulder in the last peak of the scan. It is also interesting to observe that this shoulder corresponds to Transition III_a, transition III_b being completely lost after chromatin extraction (see Table 2.) Secondly a reduction of the Transition I was apparent probably due to the loss of non-chromosomal proteins following chromatin extraction. Thirdly the transition II, that in native thymocytes was centered at 362 K, was split in chromatin into two different transitions centered respectively at 354 K (Transition II^*) and 362 K(Transition II). Transition II^* could be ascribed to the melting of the linker DNA.

After Ethidium Bromide intercalation, chromatin changed its calorimetric profile (Fig. 4 and Table 2). At low dye concentrations (R ranging from 0 to 0.125) Transition III showed no significant alteration increasing the Ethidium bromide concentrations. An enthalpy redistribution between Transition III completely disappeared. At this point when EB concentration is still increasing (R=0.2 and R=0.4) a further reduction of Transition II appeared together with a corresponding increase of the III one. These results are consistent with the data previously obtained for in situ chromatin. The only difference was a less evident change of Transition III after EB intercalation in isolated chro-

Table 2
Relative Melting Enthalpy (Kcal mol^{-1}) of the main thermal transitions appearing in chromatin thermograms at different Ethidium bromide concentrations. In comparison with thymocytes, chromatin shows an additional thermal transition at 354 K which is probably related to the melting of linker DNA, while the Transition III_b is absent and appears only at R=0.4.

R	Thermal Transitions				
EB/DNA	I (341–348 K)	II^* (350–355 K)	II (362–365 K)	III_a (370–375 K)	III_b (380–386 K)
0.00	1.61	17.90	23.53	6.91	0.00
0.05	1.25	6.71	29.14	3.91	0.00
0.08	2.04	0.00	23.91	4.35	0.00
0.10	0.00	8.18	8.66	1.59	0.00
0.150	0.00	10.92	4.14	1.42	0.00
0.200	0.00	2.30	7.94	8.90	2.06
0.400	0.00	0.06	8.27	10.01	2.41

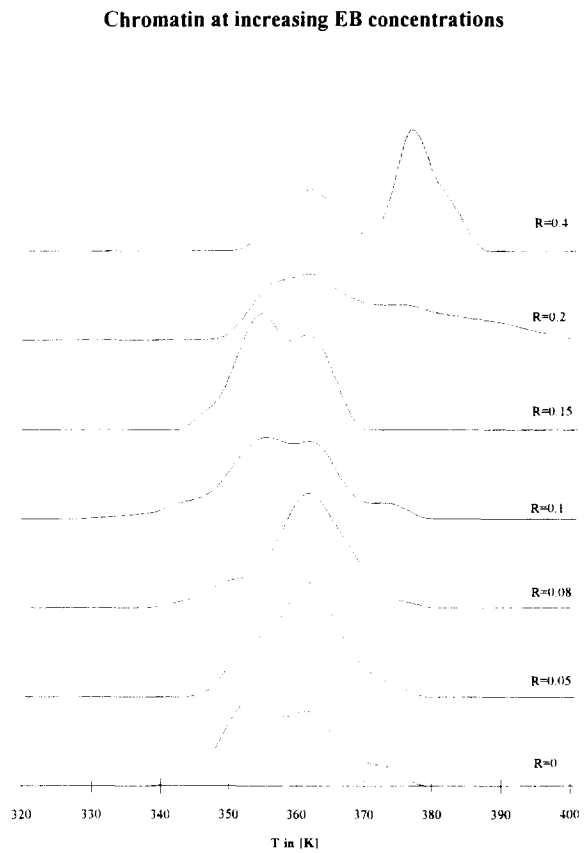

Fig. 4. Profiles of heat capacity (arbitary units) versus temperature (K) for isolated chromatin at increasing Ethidium bromide concentrations: R=0; R=0.05; R=0.08; R=0.1; R=0.15; R=0.2; R=0.4.

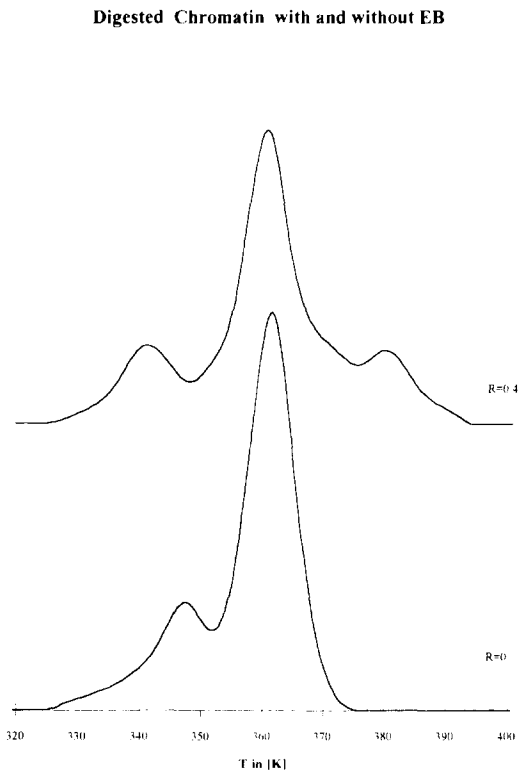

Fig. 5. Profiles of heat capacity (arbitrary units) versus temperature (K) for chromatin digested with nuclease with and without Ethidium bromide (R=0; R=0.4)

matin because this transition was already poor when the dye was absent. Similar experiments were carried out on digested chromatin (Fig. 5) and calf thymus DNA(Fig. 6) in order to clarify the kind of structural changes appearing in chromatin (isolated and in situ) when EB intercalates DNA. No significant enthalpy reduction was observed for both free DNA (R=0.05 and R=0.2) and digested chromatin (R=0 and R=0.4) at increasing EB concentrations. It is interesting to point out that in physiologically isolated chromatin a pronounced enthalpy redistribution became apparent between Transitions II* and II. At low EB concentrations the enthalpy of Transition II* (centered at 354 K) decreased until it disappeared at R values ranging from 0.05 and 0.1. For higher EB concentrations this transition started to increase until it becomes more pronounced than Transition II (centered at 362 K). Finally these two peaks merged in a single broad transition (R=0.2 and R=0.4).

In order to verify the peak assignment proposed here, we carried out a series of selective digestions using micrococcal nuclease, DNase I and proteinase K. The effects of these digestions are reported in Fig. 7. While the digestion with micrococcal nuclease produced a disappearance of Transition III without changing Transition I ,the digestion, with DNase I induced both a loss of Transition III and a minor shift of Transitions II and I to lower temperatures (the melting temperatures of these two transitions moved respectively to 357 and 343 K). When thymocytes were treated with proteinase K their scans exhibited a disappearance of Transition I (which confirms its assignment to protein melting) together with a loss of Transition III.

Fig. 8 shows the Molar Ellipticity values at 272 nm and 224 nm acquired for native chromatin at increas-

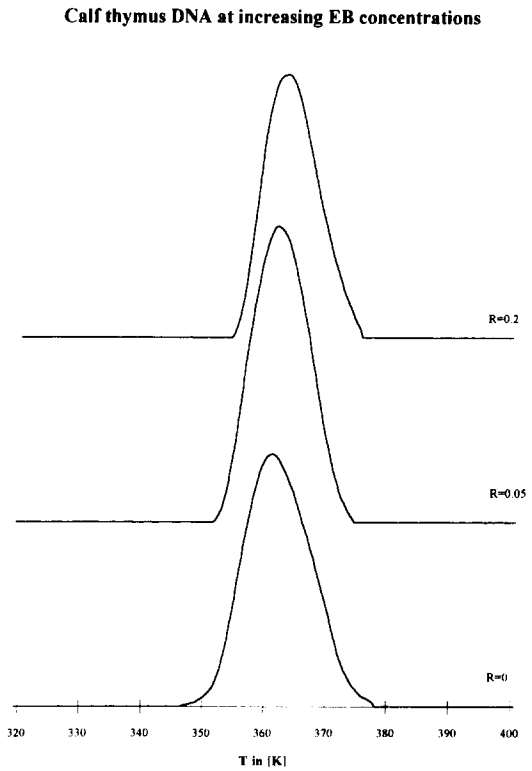

Fig. 6. Profiles of heat capacity (arbitrary units) versus temperature (K) for calf thymus DNA at increasing Ethidium bromide concentrations; R=0; R=0.05; R=0.2.

ing temperatures. It is well-known that circular dichroism of chromatin gives a characteristic ellipticity spectrum with the positive region (above 258 nm) related to the DNA component and the negative one (below 230 nm) mostly related to protein components of chromatin. Therefore the Molar Ellipticity values at 272 nm and 224 nm (corresponding to the absorption band of DNA and proteins respectively) can be considered as indicative parameters for investigating the structural changes of these two chromatin components.

When native thymocytes were treated with topoisomerase I, as described in detail in Material and Methods, their scan (Fig. 9) showed significant and reproducible alterations that can be related to changes in geometrical or topological parameters of DNA. Fig. 9 show three scans of native thymocytes . Each of these were at a saturating concentration of EB (R=0.2) but differently treated. While reference thymocytes were measured without incubation at 37°C, control thymocytes were incubated at 37°C for 30 min without enzymes and sample thymocytes were incubated at 37°C for 30 min with 500 U ml^{-1} of topoisomerase I. As previously described reference thymocytes at EB saturation (R=0.2)exhibited the increase of Transition III_b together with the disappearance of Transition II. When the same sample were

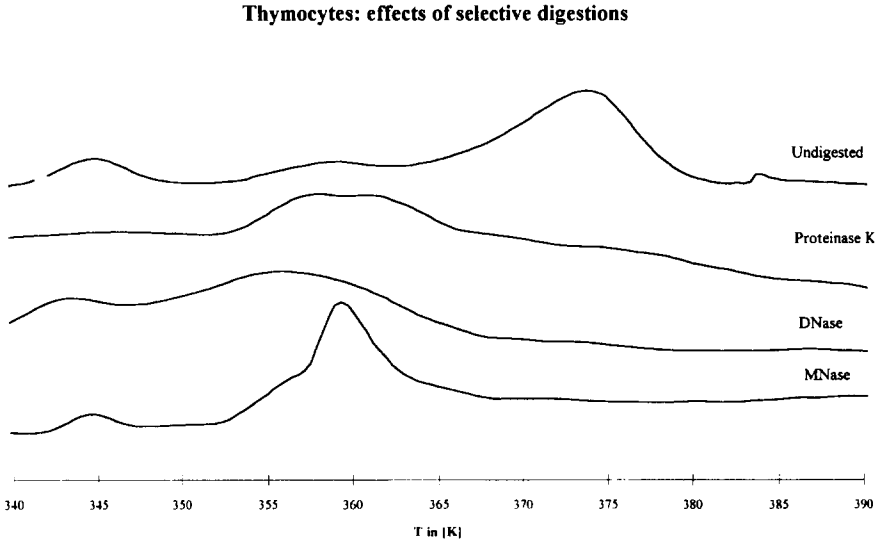

Fig. 7. Profiles of heat capacity (arbitrary units) versus temperature (K) for native thymocytes and thymocytes digested with proteinase K, DNase I and micrococcal nuclease.

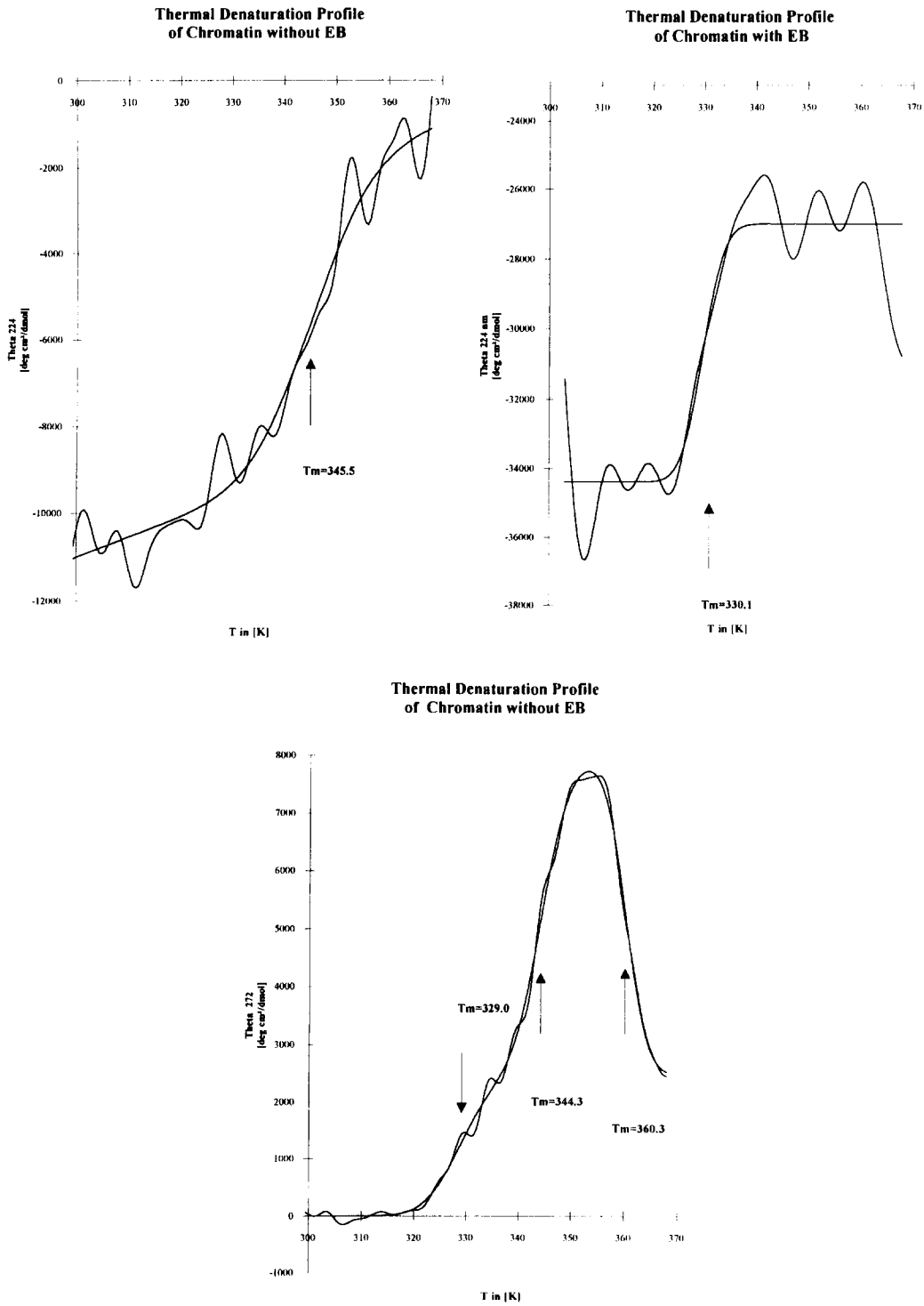

Fig. 8. Plots of Molar Ellipticity values at 224 nm (θ_{224} acquired for native cold water chromatin at increasing temperatures(from 300 to 370 K).(A) isolated chromatin without EB; (B) isolated chromatin with EB (R=0.4). Plots of Molar Ellipticity values at 272 nm (θ_{272}) acquired for native cold water chromatin without EB (C).Samples have been analyzed in TE buffer pH 8 under standard acquisition conditions (standard deviation of the measurements is<5.5 %)

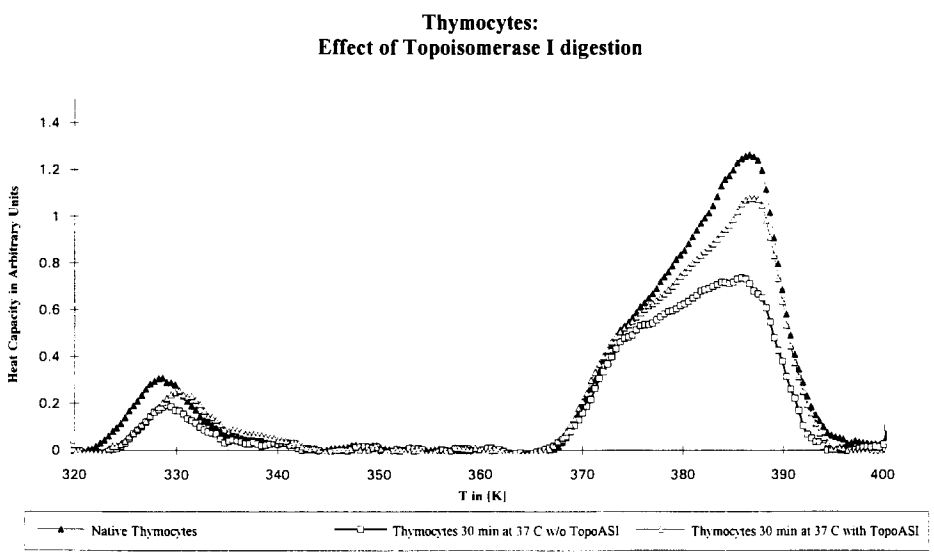

Fig. 9. Profiles of heat capacity (arbitrary units) versus temperature (K) for calf thymocytes at saturating concentration EB (R=0.2) but differently treated. (Reference): thymocytes measured without incubation at 37°C for 30 min;(Control): thymocytes incubated at 37°C min without enzyme;(Sample): thymocytes incubated at 37°C min for 30 min with 500 U ml^{-1} of topoisomerase I.

incubated at 37°C for 30 min a significant reduction of transition III_b was observed as a consequence of the activity of the endogenous nucleases. When the sample was incubated at 37°C for 30 min with topoisomerase I less evident decrease in Transition was observed III_b. The reduction of negative and positive supercoiling produced by this enzyme gives rise to an increase of the most condensed chromatin structure and topoisomerase I is therefore able to balance the loss of this structure produced by the activity of endogenous nucleases. It is also interesting to note that both EB intercalation and topoisomerase digestion produce similar effects at a level of chromatin higher order structure by increasing the melting enthalpy of Transition III_b.

4. Discussion

The transitions previously described for HeLa cells [3,11] and for hepatocytes [14,18,27] were evident also for thymocytes (Fig. 1 A) and for chromatin (Fig. 1 B) as reported previously [17,28]. With respect to rat hepatocytes, thymus cells display a significant reduction in the low temperature (338 K) transition that has been previously assigned to the melting of non-chromsomal components of the cells [14,15]. The lack of this peak can be ascribed to the low membrane and RNA constituents in thymocytes.

The experiments of selective digestions carried on intact thymocytes by using different enzymes confirm the peak assignment reported above (Fig. 7). When proteins are removed by proteinase K digestion a disappearance of Transition I can be observed together with a loss of Transition III. The latter was due to a loss of highly condensed chromatin following extraction. The disappearance of Transition I after the removal of the proteins confirms our hypothesis that non-chromosomal proteins melt at 347 K. This interpretation is supported by the spectropolarimetric data acquired on native chromatin at increasing temperatures (Fig. 8). These data show a major change of Θ_{224} values at around 347 K. Indeed at these wavelengths, the CD spectrum contribution is related to proteins

More interestingly effects were produced in the thermal denaturation of thymocytes by two different enzymatic digestions of DNA (Fig. 7)while MNase induces a simple loss of Transition III without affecting the two other transitions, DNase I gave rise to both a loss of Transition III and a shift of the two other ones towards lower temperatures. This effect can be explained by the different activities of these two

enzymes. The double strand cleavage of DNA helix induced by MNase gave rise only to a loss of higher order structure of chromatin (disappearance of Transition III) but the single strand nachs produced by DNase I also cause a destabilization of the DNA filament that can be seen as a shift in the corresponding Transition II to lower temperatures.

The results reported in the paper show that increasing Ethidium bromide concentrations bring about significant modifications of the thermal profiles of both thymocytes and isolated chromatin, in accordance with parallel experiments of circular dichroism carried on similar samples [7].

In particular as shown in Fig. 2 and Fig. 3 an enthalpy redistribution between the III and the II transitions becomes evident when the dye intercalates chromatin-DNA fibres in situ. The disappearance of Transition III would appear to correspond to the complete unwrapping of the chromatin fiber following the binding of a critical concentration (υ) of Ethidium bromide. The chromatin fiber involved in this structural change can be identified either in the solenoid or in other superhelical structures recently proposed [28,29]. Under the same conditions nuclease digested chromatin and free DNA do not display any enthalpy redistribution (Fig. 5 and Fig. 6) probably as a consequence of the lack of the topological constraints which are present in situ and in physiologically isolated chromatin.

The similarity between the effects of EB intercalation on in situ and isolated chromatin (Fig. 4), confirms our previous data pointing out that the physiological method of preparation (Cold Water procedure) is able to maintain, at least partially, the topological constraints that are present in situ. In fact, parallel experiments performed on free DNA and digested chromatin (Fig. 5 and Fig. 6) displayed a lack of enthalpy redistribution after EB intercalation

It is very interesting to emphasize for isolated chromatin the appearance of a new thermal transition at 354 K (Transition II^*) that we ascribe to the melting of linker DNA (Fig. 1B). The lack of this transition in native thymocytes can, probably be explained as follows. In a cellular system with a closely packed genome the transition related to the melting of the linker DNA can be observed only when the chromatin is in a more relaxed status, as it occurs in the isolated fibres. The observation that this additional transition changes significantly following EB intercalation (Table 2 and Fig. 4) could represent a confirmation of its assignment to the melting of linker DNA. An additional enthalpy redistribution between the II^* and II transitions is in fact observed when EB intercalates DNA. It is well known that at low R values, linker DNA shows a higher affinity for EB in comparison with the core DNA [4,5]. Therefore the enthalpy redistribution observed between Transition II (at 362 K) and Transition II^* (at 354 K) can be explained in terms of a specificity of EB binding for linker DNA which changes its conformation after dye intercalation . For the saturating EB concentration, a rearrangement in nucleosomal (linker and core) DNA conformation can be proposed in order to explain the appearance of a single broad transition in place of the two transitions characteristic of the native chromatin.

In order to clarify the nature of Transition III_a and III_b, that are significantly affected by EB intercalation, native thymocytes were digested with a topological enzyme (topoisomerase I). This enzyme is able to relax the negative and positive supercoiling by changing the Linking Number (Lk) of a topologically closed DNA molecule. Because of the relationship [9,30–32] between this topological parameter (Lk)and the geometrical parameters Twist (Tw) and Writhe (Wr) (in fact Lk=Tw+Wr), when Lk is modified this change has to be balanced by related modifications of the other two parameters. When EB intercalates a negatively supercoiled DNA this binding produces a progressive reduction of Twist and an opposite increase of Writhe from its negative value (Lk in fact is not modified) until it becomes null at a critical dye concentration (υ). When EB concentration further increases, Writhe starts to assume positive values while Twist decreases progressively.

A comparison between the effects of EB intercalation and topoisomerase activity (Fig. 9) shows that each of them produces an increase in melting enthalpy of Transition III_b. Therefore, we could assign it to the denaturation of chromatin fiber with high Writhe value (this parameter being increased by each of these treatments). This result is very interesting because it differentiates chromatin fibers with different negative supercoiling on the basis of their melting temperatures directly inside the nucleus. In future this possibility can be used to investigate the DNA supercoiling of

chromatin in relation to its role in regulating the gene transcription by using chromatin fibres in situ.

Some considerations have to be made about the thermal shifts exhibited by the I (towards lower temperatures) and the III (towards higher temperatures) transitions after EB intercalation (Fig. 2.) These shifts can be explained in terms of the thermodynamic stability changes of the related cell components [15]. In detail, at saturating Ethidium bromide concentrations (R>0.2) nuclear proteins seem to be destabilized, while the new higher order chromatin structure resulting from the dye binding appears to be stabilized with respect to the physiological one. In order to verify that the transition appearing at low temperature (332 K) is due to proteins which resulted in being destabilized by EB intercalation we then performed a spectroscopic measurement of thermal denaturation on isolated chromatin at R=0.4 (Fig. 8c). When this result is compared with the one obtained by native chromatin (without EB)we indeed see a shift of melting temperature after EB intercalation towards a lower temperature(from 34.5 to 330.1 K).

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