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Effects of Histone Acetylation on Chromatin Structure

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Abstract The effect of histone acetylation was monitored on CHO chromatin structure, following the addition of 7 mM Na-butyrate to the cell culture medium. The properties of both control and hyperacetylated chromatins and nuclei were investigated by circular dichroism, ethidium bromide intercalation, differential scanning calorimetry, and affinity chromatography. Our results are compatible with modest but significant alterations in the various levels of chromatin organization, as a result of the charge neutralization of some lysine residues within the N-terminal region of the histonic octamer. Namely, large statistically significant differences do exist in the heat capacity thermograms of native nuclei, where unfolding into single nucleofilament of the highly packed native chromatin superfiber appears associated with acetylation; at the same time CD, EB, and affinity chromatography point to modest but consistent differences in the compactness of isolated nucleosomes and polynucleosomes. J. Cell. Biochem. 64:466–475.

Key words: histone acetylation; chromatin structure; circular dichroism; ethidium bromide intercalation; differential scanning calorimetry

Histone acetylations represent a posttranslational event that interests the α-amino groups of selected lysine residues in the core particle and results in the charge neutralization of few residues mainly in the N-terminal basic domain of the core histones H2A, H2B, H3, and H4 [Nicolini, 1986; Van Holde, 1988]. For this reason, acetylation has been associated to the weakening of the constraints applied by the core histone N-terminal tails to the nucleosomal DNA [Norton et al., 1989; Lee et al., 1993].

The effects of histone acetylation on the different levels of chromatin organization have been extensively analyzed and a number of experiments have been performed on chromatin samples acetylated both "in vivo" or "in vitro" [Riggs et al., 1977; Simpson, 1978; Bode et al., 1980].

In the first case, histones are acetylated in vivo by adding 7 mM Na-butyrate directly to the cell culture medium; Na-butyrate in fact has an inhibiting effect on the deacetylases causing in this way an accumulation of histones with high level of acetylation [Riggs et al., 1977]. In the second case histones after their dissociation in single proteins are acetylated with different procedures and then are associated again with DNA through an "in vitro reconstitution" system [Norton et al., 1989].

A few papers [Norton et al., 1989; Lutter et al., 1992] have shown that histone hyperacetylation has a "gyraselike" effect on the high orders of chromatin structure introducing a release of supercoiling in the nucleosome.

This loss of supercoiling has been quantified on "in vitro" reconstituted nucleosomes [Norton et al., 1989]; the authors from their experiences provide a model in which hyperacetylated histones release 0.2 negative supercoil per nucleosome in the topological domain. For this reason acetylation has been correlated with gene activation and transcription.

Different results have instead been obtained by determining "in vivo" the effect of histone acetylation on the linking number of minichromosomes; in this case in fact the change in supercoil is minimal [Lutter et al., 1992] and the correlation with transcription seems to be inconsistent. Therefore it is clear that acetylation has quite different effects "in vitro" and "in vivo."

Not many studies have been performed about the effect of acetylation on higher order chroma-

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tin structure and the results are often discording. J. Ausio, in fact, in a commentary entitled "Acetylation: An Unsolved Puzzle," underlines that in the absence of any other factor, a histone acetylation causing unfolding of either nucleosome or higher order chromatin structure has not been observed. Only there might be a partial loss of stability of the core particle in response to mechanical stress because of the removal of DNA-histone interactions at the level of the lysines residues [Ausio, 1992].

In order to assess the structural properties of chromatin in untreated and Na-butyrate treated CHO cells, we have here utilized the ethidium bromide intercalation, circular dichroism, differential scanning calorimetry, and affinity chromatography.

MATERIAL AND METHODS

Cell Culture

CHO-9 (Chinese hamster ovary) cells have been grown on Petri dishes in half D-MEM and half F12 medium supplemented with 10% FCS (Boehringer Mannheim, Sandhofer, Germany). Cells were allowed to reach confluence (control sample) or, following the procedure reported in Bode et al. [1980], were additioned for 20 h with 7 mM Na-butyrate in order to inhibit histone deacetylase (hyperacetylated sample) [Yau et al., 1982]. Alternatively cells were synchronized at the G1/S phase of the cell cycle adding to the culture medium 2 mM Hydroxyurea for 15 hours.

Nuclei have been extracted in 0.5% NP-40, washed twice in a hypotonic buffer (10 mM TRIS-HCl pH 7.4, 10 mM NaCl, 3 mM $MgCl_2$) to obtain a homogeneous suspension ready for digestion.

Chromatin Preparation

Chromatin has been extracted with two different procedures. The first procedure (cold water method) that gives rise to undigested chromatin [Nicolini et al., 1976] is obtained allowing the nuclei to swell for 1 h in ice cold distilled water additioned with 2 mM EDTA, pH 8 to inhibit proteolytic enzymes; after homogenization in a dounce (30 strokes) samples are centrifuged on a sucrose cushion 1.7 M for 80 min at 100,000*g* to divide chromatin from the broken membranes. After centrifugation chromatin pellet is gently resuspended in TE (10 mM Tris-HCl, 1 mM Na-EDTA, pH 8), and dyalized overnight versus TE at 4°C.

The second procedure (nuclease digested method) [Noll et al., 1976] utilizes instead a mild digestion with micrococcal nuclease causing a distribution of the chromatin fragments length. Nuclei, after dilution in solution A (0.3 M Sucrose, 0.05 M Tris-HCl, 0.025 M KCl, 0.005 M $MgCl_2$ 0.01 M $PhMSO_2F$, 0.001 M $CaCl_2$ pH 8) to a final concentration of 10^8 nuclei/ml, are digested with 10 U/ml Micrococcal Nuclease (Sigma Chem, Co. St. Louis, MO) for 45″ at 37°C. The reaction is stopped with 10 mM Na-EDTA. After a brief centrifugation nuclei are lysed as previously described, homogenized, and centrifuged at 2,000*g;* then the supernatant containing soluble chromatin is utilized for the subsequent experiments. All the solutions utilized for the extraction and measurements of treated bufferacetylated chromotin were additioned with 7 mM Na-Butyrate.

Preparation of Mononucleosomes and Hg-Affinity Chromatography

Mononucleosomes from CHO cells have been extracted according to the procedure of Allegra et al. [1987]. After digestion for 5 min at 37°C with 8 U/ml of micrococcal nuclease nuclei were centrifuged at 10,000*g* for 20 min. This procedure leading to preferential cleavage of active chromatin, avoids lysis of the nuclei, and yields a supernatant fraction comprised mainly of monomeric nucleosomes. Various mononucleosomal samples have been prepared from untreated cells, or treated with Na-butyrate or with hydroxyurea, as above described.

The supernatants obtained are applied one by one to an organomercurial agarose column (Affi-gel 501 Organomercurial Agarose Biorad) in order to isolate and separate the transcriptionally active mononucleosomes from the inactive ones [Allegra et al., 1987]. The gel matrix utilized in this affinity chromatography can retain free sulfhydryl groups through a covalent mercaptide bond to the Organomercurial function. The mononucleosomal particles transcriptionally active are subjected to an unfolding of their structure that affects the central domain of the nucleosome where the SH groups of the H3 are located; these groups are not accessible in the compactly beaded nucleosome of inactive chromatin, but become accessible as the nucleosome unfold during transcription [Chen and Allfrey, 1987]. This structural change

makes possible to separate the two classes of nucleosomes through affinity chromatography.

The nucleosome particles, resuspended in the suitable buffer (0.01 mM Tris-HCl pH 7.4, 0.01 mM NaCl, 0.003 M $MgCl_2$, 0.1 mM $PhMS0_2F$, 5 mM Na-EDTA) were loaded on a 1 × 6 cm gel column, with a flow rate of 1.5 ml/min. The elution buffer was 10 mM Tris-HCl pH 7.4, 10 mM NaCl, 3 mM $MgCL_2$, 0.1 mM $PhMSO_2F$, 5 mM Na-EDTA additioned with 10 mM Dithiothreitol. The OD profiles at 254 nm has been recorded for each sample. Also in these experiments all the solutions utilized for hyperacetilated samples were additioned with 7 mM Na-butyrate.

Circular Dichroism Spectroscopy

Chromatin extracted from both the samples, untreated and Na-butyrate treated cells, has been analyzed with a Jasco 710 spectropolarimeter interfaced to a personal computer, supplied with operative software for data acquisition, and elaboration. Circular dichroism measurements have been performed with a 1 cm path length quartz cuvette in nitrogen atmosphere at 25°C.

For all the measurements the concentration of DNA nucleotide residues was 0.75×10^{-4} M/nucleotide and was spectrophotometrically determined by the absorption at 260 using $\epsilon_{260} =$ 21,000 cm^2/g in 0.2% SDS [Augenlicht et al., 1974].

Different recordings have been performed as a function of increasing temperatures and concentrations of ethidium bromide. The ethidium bromide stock solution was 500 µg/ml (by Sigma Chem. Co. St. Louis, MO) and R values used in our experiments (molar ratio between dye and DNA concentration) ranged between 0 and 0.4.

In order to reduce the random error and the noise each acquired spectrum was the average of five repeated measures with a standard deviation of 5.5%. The other following parameters have been utilized: wavelength range 350–220 nm; step resolution 0.5 nm/data; scan speed 20 nm/min; band width 0.2 nm; sensitivity 10 mdeg; time constant 4 s; PMT voltage below 400 V [Vergani et al., 1994].

The resulting signal is expressed in terms of molar ellipticity (degrees × cm^2 $dmol^{-1}$).

Each molecule of ethidium bromide unwinds the DNA helix of 26° inducing a slight bending of the helix towards the major groove [Wang, 1974]. A positive signal can be detected in the circular dichroism spectrum at 308 nm when the molecules of the dye intercalate the DNA double helix; this signal increases with the increasing of the binding until saturation. Little agreement has been reached concerning the mechanism of binding. Some authors report a positive cooperation for the binding [Erard et al., 1979], while others report a negative cooperation [Wu et al., 1983].

DIFFERENTIAL SCANNING CALORIMETRY

Nuclei extracted from CHO cells as above described have been centrifuged at 12,000*g* for 45 min, then the pellet has been utilized for DSC analysis. DSC experiments have been performed on a Perkin-Elmer DSC-2C instrument with 75 µl aluminium capsules, in a temperature range between 310–410 K as previously described [Nicolini et al., 1988].

In order to improve the reproducibility and sensitivity of measurements an electronically interfaced computerized system was used [Diaspro and Nicolini, 1988].

The thermal scanning of the sample has been performed at a low rate (5 K min^{-1}), high sensitivity (0.1 mcal s^{-1}), and the pellet size was about 70 mg in each capsule. The corresponding baselines have been obtained by a new thermal scanning of the denatured sample and have been subtracted from the corresponding thermograms.

RESULTS

Thermal Denaturation

The denaturation profile of CHO nuclei is characterized by three main endothermic transitions in the temperature range 310–410 K (Fig. 1); the first one is centered at about 335 K, while the second and the third, which are dominant, are centered around the 356–358 K and 370 K respectively. A few similarities are evident between the thermograms of CHO and those of other cells analyzed in previous studies [Nicolini et al., 1989; Vergani et al., 1992; Balbi et al., 1988; van Holde, 1988]. A general agreement has been reached in the literature on the following assignment: the first transition is attributed to the melting of nuclear scaffold proteins (I transition); the second transition to the melting of the tertiary structure of DNA (mono and polynucleosomes) (II transition) and the third transition to the denaturation of the higher order structures of DNA (30 nm fiber or

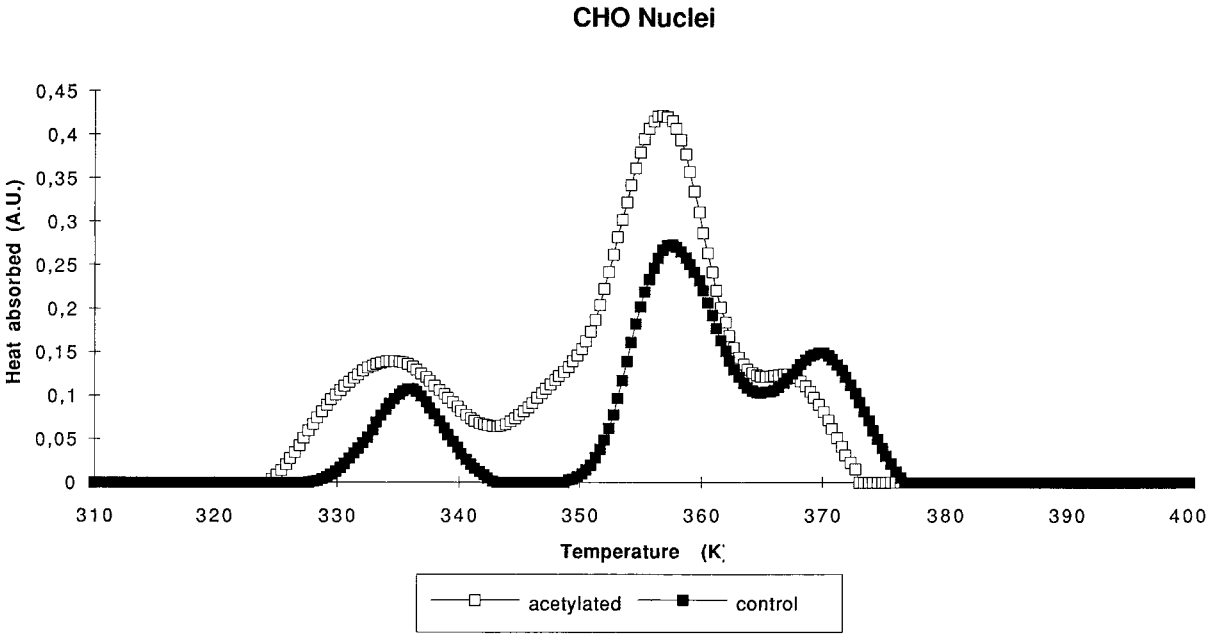

Fig. 1. Differential scanning calorimetry of nuclei isolated from CHO cells either untreated (■) and treated with 7 mM Na-butyrate (□): the profiles of relative heat capacity (arbitrary units) vs. temperature (K) are reported. Both nuclear samples have been isolated in Tris-HCl 0.01 M pH 7.4, NaCl 0.010 M, $MgCl_2$ 0.003 M.

TABLE I. Comparison Between the Relative Areas (%) of the Three Main Thermal Transitions Observed in Thermograms of CHO Nuclei and Thymocytes*

Sample	I Transition	II Transition	III Transition
CHO	22 ± 3.9	43 ± 11	27 ± 2
Thymocytes	8.61 ± 0.11	12.06 ± 3.7	79.33 ± 14.2

*The values of standard deviation are also reported for each transition.

TABLE II. Relative Area (%) of the Three Main Thermal Transitions Observed in Thermograms of CHO Nuclei as Function of the Treatment With Na-Butyrate*

CHO Sample	I Transition	II Transition	III Transition
Untreated	22 ± 3.9	43 ± 11	27 ± 2
Na-butyrate treated	25 ± 2	54 ± 16	18 ± 12

*The values of standard deviation are also reported.

more) (III transition). The thermograms of nuclei digested with micrococcal nuclease show in fact a complete disappearance of the III transition and an increase of enthalpy of the II one (data not shown).

It is interesting to observe that all the transitions appearing in the CHO nuclei thermograms are centered at lower temperatures with respect to calf thymocytes [Vergani et al., 1992]. For proteins (I transition) the temperature shifts from 345 K in thymocytes to 335 K in CHO; for DNA tertiary structure (II transition) the temperature shifts from 360–363 K to 358 K, while the higher order chromatin structure (III transition) denatures at 370 K instead of 375 K.

At the same time, CHO cells show an amount of chromatin-DNA organized in the 10 nm fiber more consistent than the chromatin organized in the higher order structures. As reported in Table I, in terms of relative peak area, the second transition is about the 43% of the total area in CHO thermograms, while in calf tymocytes is only 12%. On the other hand the relative area of the III transition is 27% in CHO and three-fold higher in tymocytes.

These calorimetric data can be correlated with the different metabolic activity of the cells analyzed. CHO in fact are characterized by an intense mitotic and synthetic activity in such a way that their chromatin has to be accessible for DNA transcription and duplication, therefore in a more relaxed conformation with respect to cells showing a reduced metabolic activity such as the calf tymocytes.

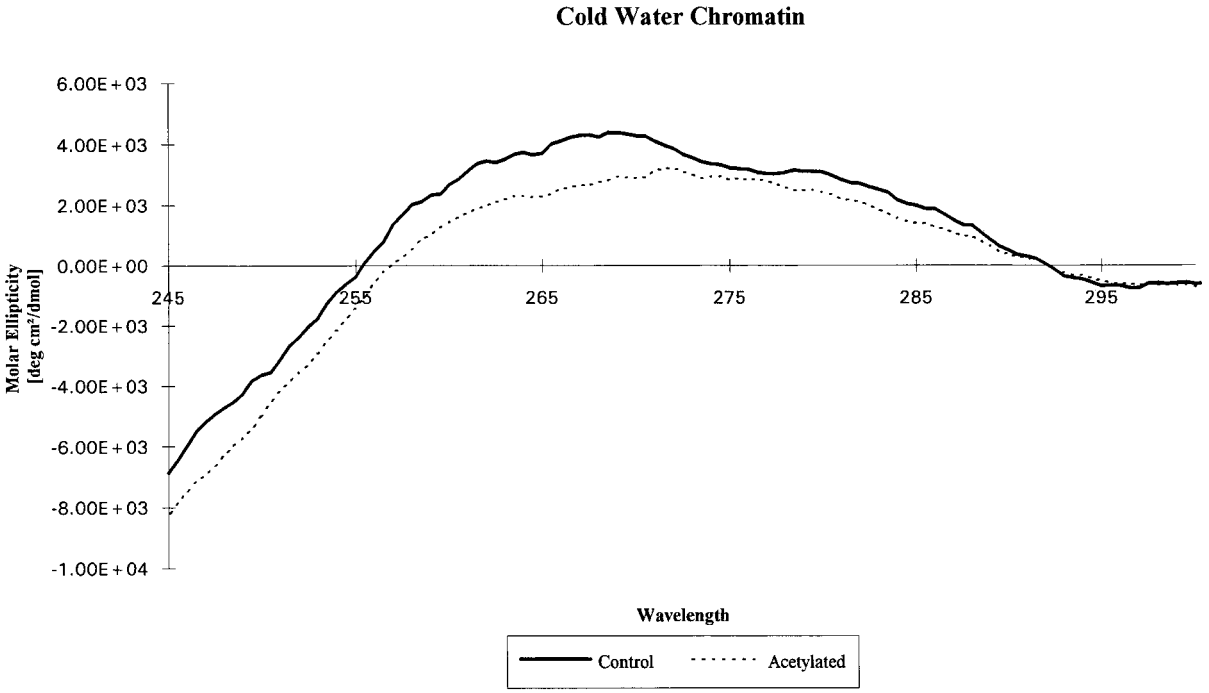

Fig. 2. Circular dichroism spectra of control and acetylated cold water chromatin for the 245–300 nm spectral region. The acquired spectra are normalized for the corresponding DNA concentration (as described in the text). The chromatin is analyzed in TE buffer, pH 8, under standard acquisition conditions. A standard deviation of 5.5% is measured.

This allows to interpret eventual differences in the thermal profile of CHO nuclei hyperacetylated by adding Na-butyrate in the cell culture medium, which are indeed present and statistically significant (Table II).

The thermal profile of Na-butyrate treated CHO nuclei is represented in Figure 1; the three main transitions observed in the untreated nuclei are always present but with a few interesting differences: the melting temperature of the II and III transition are shifted towards lower temperature and the relative areas of the same peaks point to a significant redistribution. Namely the II transition shifts of about 1 K around the 356 while the third shift from 370 K to 368 with an error of 1 K. Furthermore, in the same physiological conditions, Na-butyrate treated nuclei present a reduction of the III transition with a corresponding increase of the transition II. The peak associated with melting of the tertiary structure (II transition) becomes in fact higher and broader; this results evident also after the decomposition of the thermograms into gaussian components and the estimation of the area of the single peak, as shown in table II. It is interesting to point out the appearance in the treated sample of a shoulder at about 348 K, absent in the untreated counterpart, that can be assigned to the linker DNA denaturation [Balbi et al., 1989].

CD Spectroscopy

The CD analysis of CHO chromatin has been performed on samples extracted with the two procedures described in the section of the Methods. In Figures 2 and 3 the CD spectra of cold water chromatin and nuclease digested chromatin, either untreated or treated with Na-butyrate are reported.

It results evident how the different extraction procedures give rise to different CD spectra; in fact while the Cold Water chromatin presents two peaks in the DNA region, one at 268 nm the second at 284 nm, in the nuclease digested sample the second peak mostly disappears. In the same way, as resulting from Figure 2, the biellipticity characterizing the untreated sample disappears in the hyperacetylated cold water chromatin. No significative difference is shown in the spectrum of the nuclease digested sample between the untreated and the hyperacetylated chromatin. In order to better define the features of the chromatin in the

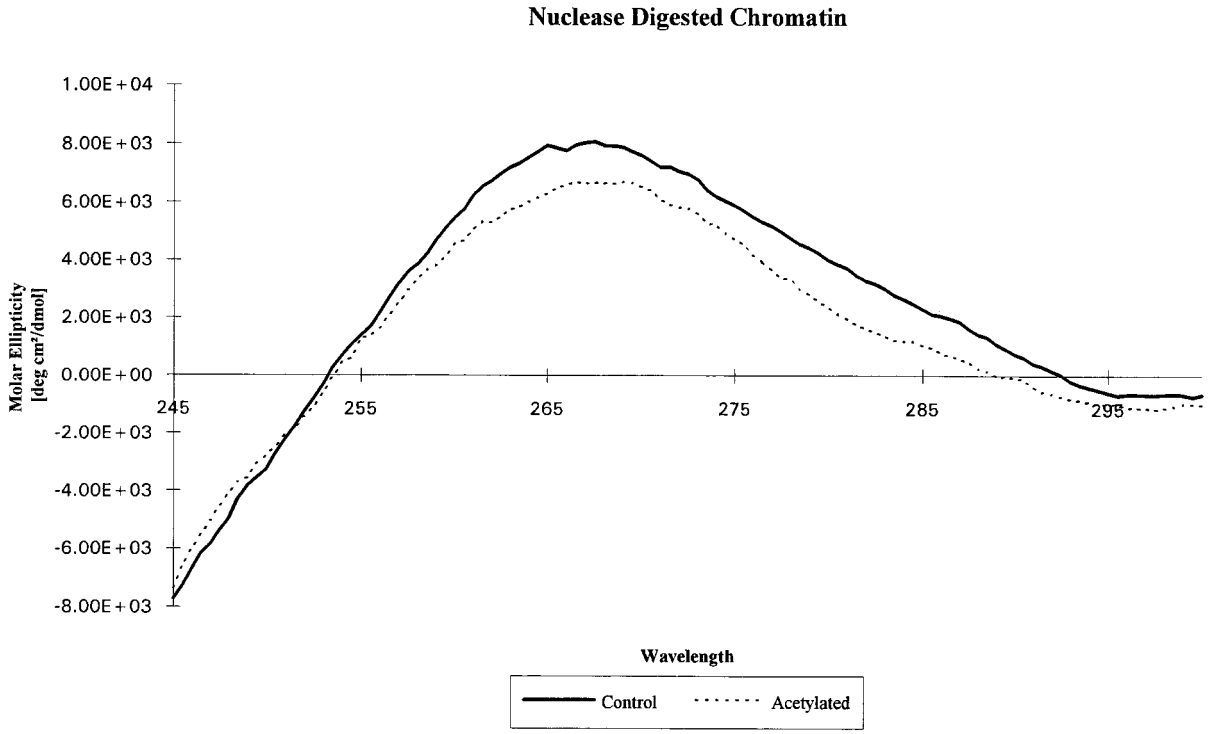

Fig. 3. Circular dichroism spectra of control and acetylated nuclease digested chromatin for the 245–300 nm spectral region. The acquired spectra are normalized for the corresponding DNA concentration (as described in the text). The chromatin is analyzed in TE buffer, pH 8, under standard acquisition conditions. A standard deviation of 5.5% is measured.

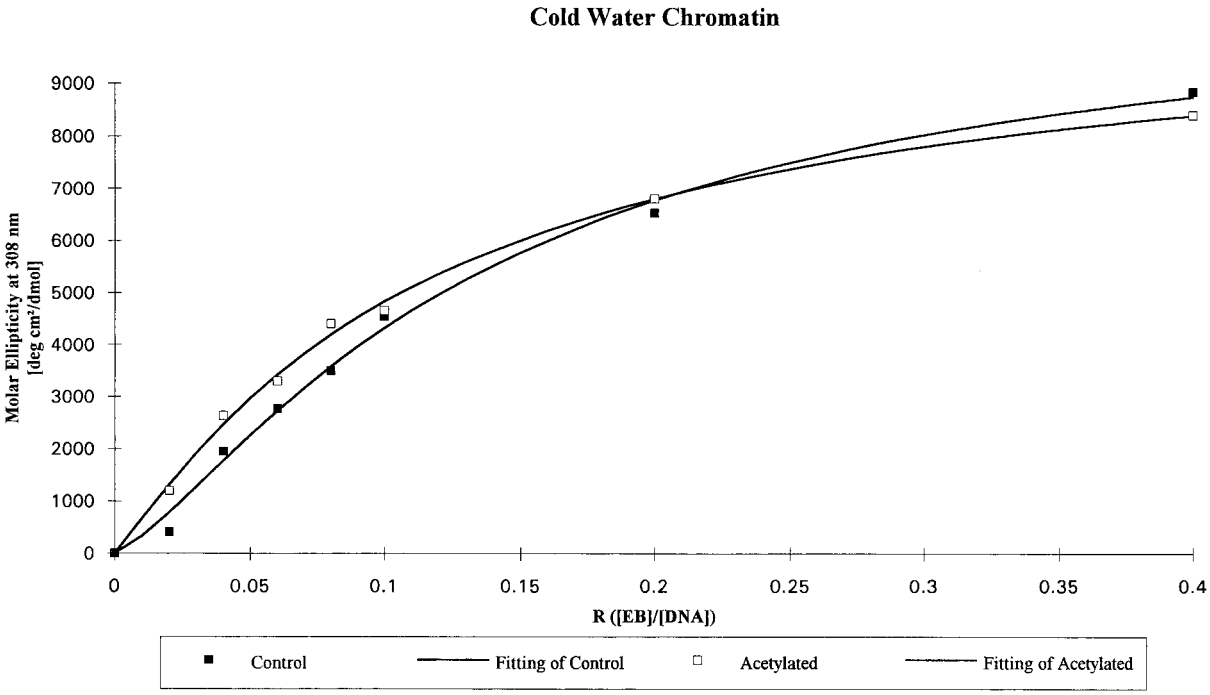

Fig. 4. Plots of molar ellipticity values at 308 nm (θ_{308}) vs. molar ratio R for cold water chromatin. The chromatin has been extracted either from untreated (■) or treated with Na-butyrate (□) CHO cells.

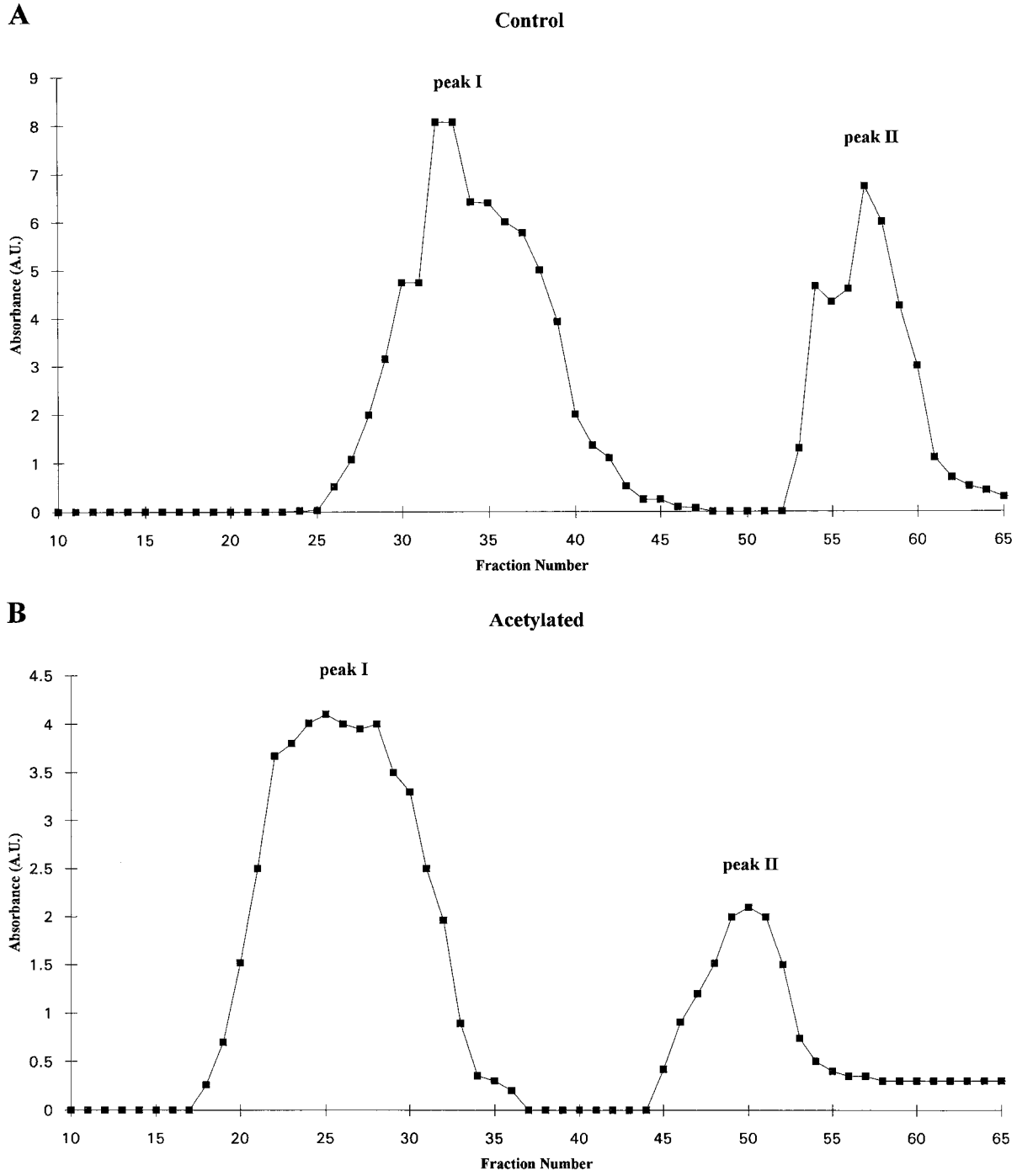

Figure 5

control and acetylated samples, we thought to get information from the behaviour of DNA-ethidium bromide interaction.

Ethidium bromide is an intercalative dye, supposed to be a useful probe to investigate the level of chromatin structure [Nicolini, 1986]. When ethidium bromide intercalates the DNA, in the CD spectrum a peak around the 308 nm becomes evident, related to the number of molecules bound per DNA-phosphate residue [Dalgleish et al., 1971]. However it is not clear how the higher orders of the chromatin structure can affect the binding of the dye to the DNA.

Figure 4 represents the plot of molar ellipticity values at 308 nm (θ_{308}) vs. R for cold water chromatin either control and Na-butyrate

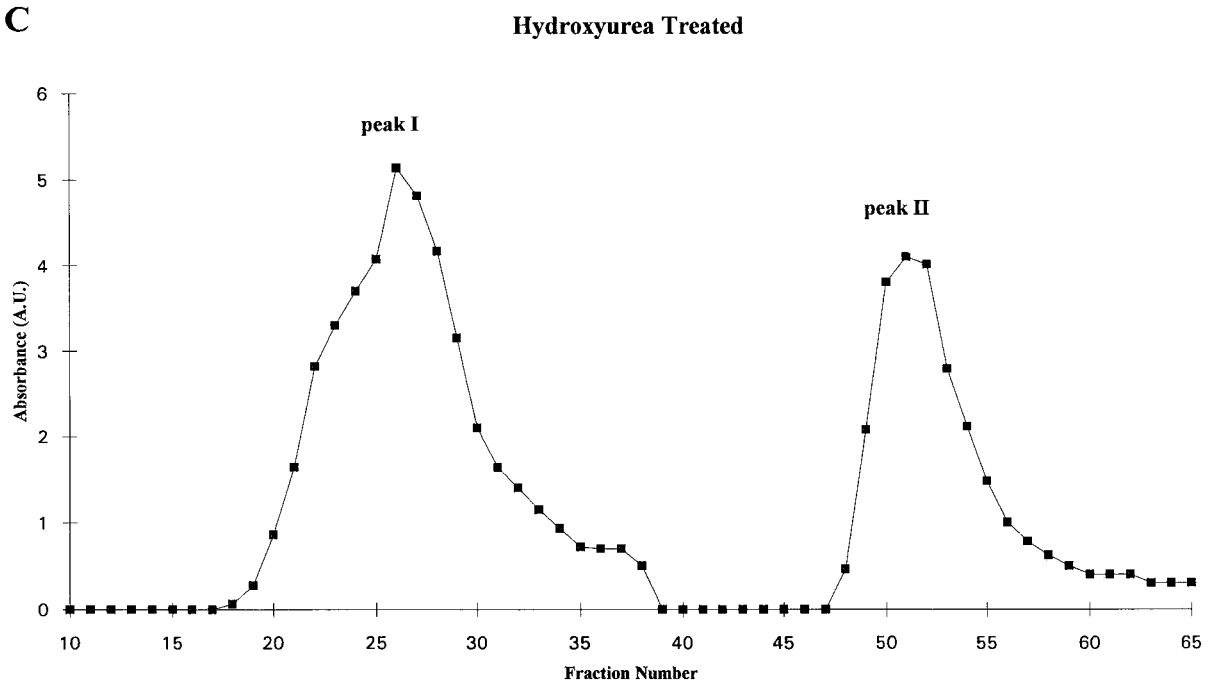

Fig. 5. Fractionation of mononucleosomes by Hg^{II}-affinity chromatography. After elution of the unbound nucleosome fraction (peak I) the Hg^{II}-bound mononucleosomes were displaced from the column with 10 mM DTT (peak II) as described in the text. **A:** Mononucleosomes extracted from untreated CHO cells. **B:** Mononucleosomes extracted from CHO cells treated with 7 mM Na-butyrate for 20 hours. **C:** Mononucleosomes extracted from CHO cells treated with 2 mM hydroxyurea for 15 h.

treated; for very low R (from 0B to 0.08) the untreated chromatin seems to have higher affinity for the dye with respect to the acetylated sample, while at saturating dye concentrations almost the same number of primary binding sites are present in the acetylated chromatin respect to the control. No significative differences are instead evident for nuclease digested chromatin (not shown) and mononucleosomes (data not shown) between the untreated and the Na-butyrate treated samples; mononucleosomes don't intercalate the dye at all as already reported [Vergani et al., 1994] also when they are hyperacetylated (data not shown).

Affinity Chromatography

Correlated with the above data we performed experiments of Hg^{II}-affinity chromatography in order to verify the changes in chromatin structure following histone acetylations. The procedure, described in Methods, can distinguish between the unfolded nucleosomes of transcriptionally active genes (in which the sulphydryl group of histone H3 are accessible for binding to Hg^{II}) and the compactly beaded nucleosomes of the transcriptionally inert DNA sequences (in which the H3 sulphydryl groups are not accessible) [Chen and Allfrey, 1987]. When the sample is loaded on the column, the mononucleosomes which do not expose the SH group flow through the column and can be immediately collected (peak I in the Fig. 5); the nucleosomes that are instead supposed to be in the actively transcribing conformation, bind the gel through their SH group and then are eluted with a suitable buffer giving rise to the second peak (peak II in Fig. 5). In Figure 5 the elution profiles are represented for untreated control (A), Na-butyrate treated (B), and hydroxyurea treated (C) samples. It's surprising to notice that the second peak, relative to the actively transcribing mononucleosomes, not only does not increase, but definitely decreases following Na-butyrate treatment. The same result was obtained in two different experiments.

Conversely hydroxyurea treated CHO, blocked in the late G1-S phase and characterized by a high level of transcription [Nicolini and Beltrame, 1982] consistently shows an increase in the second peak (Table III).

DISCUSSION

Among the multiple factors that can affect the higher order chromatin structure and func-

TABLE III. Percentual Absorbance (A_{254}) in Arbitrary Units of the Individual Peaks Appearing in the Chromatographic Profiles of the Mononucleosomes

Mononucleosomal sample	Peak I percentual absorbance (A.U.)	Peak II percentual absorbance (A.U.)	Ratio I/II peaks
Untreated	62	38	1.63
Na-butyrate treated	74	26	2.89
Hydroxyurea treated	61	39	1.51

tion, the physiological role of histone acetylation is a subject of interest. Our experiments have been therefore devoted to the study of the bulk chromatin in CHO cells, either untreated or treated with Na-butyrate.

Our results (Tables I, II, and III) are compatible with modest but significant alterations in the various levels of chromatin organization, as a result of the charge neutralization of some lysine residues within the N-terminal region of the histonic octamer.

The calorimetric data of native nuclei (Table I) shows indeed that the ratio between the III and the II peak of the thermograms is quite lower in the Na-butyrate treated nuclei (0.33) respect to the untreated (0.62), suggesting that histone acetylations tend to relax chromatin-DNA. Namely large statistically significant differences do exist in the heat capacity thermograms of native nuclei (Fig. 1), where the unfolding into a relaxed nucleofilament of the highly packed native chromatin superfiber appears associated with histone acetylation.

At the same time circular dichroism, ethidium bromide, and affinity chromatography point to quite modest but consistent increase in the compactness of isolated nucleosomes and polynucleosomes, which have been frequently associated with a change in the degree of supercoil [Nicolini, 1986].

The CD spectrum of the hyperacetylated chromatin in fact presents the loss of the biellipticity in the DNA region, earlier correlated to the DNA supercoil [Vergani et al., 1994]. In that case of linear lambda DNA the CD spectrum is characterized by the main peak at 272, while in circularized DNA, that is supercoiled, the peak was shifted around the 280 nm. Following this interpretation, the disappearance of the peak around the 270 nm in the cold water acetylated chromatin respect to the untreated (Fig. 2) could be ascribed a minor increase in the degree of supercoil which is furthermore associated with a minor increase in the EB affinity at low concentration (Fig. 4). This can be confirmed from the nuclease digested chromatin (Fig. 3) and from mononucleosome (data not shown) that lack, either in the untreated and in the Na-butyrate treated samples, the biellipticity of the DNA peak being instead centered at 265–270 nm; in both these cases in fact the constraints are disrupted by the enzymatic digestion.

The distribution of active and inactive mononucleosomes in untreated and Na-butyrate treated CHO, as results after separation on a Hg^{II}-cromatography, is compatible with the above findings on isolated chromatin suggesting that acetylation induces in CHO cells an increase in the fraction of folded nucleosomal particle without exposure of the sulphydril group of histone H3.

In summary, it can be inferred that the in vivo treatment with Na-butyrate, induces a significant release on the overall higher order superfolding of the native chromatin fiber into relaxed nucleofilament, while at the level of the single nucleosome appears to induce a surprising but consistent increase in the compactness and supercoil of nucleosomal DNA, which then would appear confirmed as the paradoxical prerequisite of gene activation [Nicolini, 1986].

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