Ethidium Bromide Intercalation and Chromatin Structure: A Spectropolarimetric Analysis†

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ABSTRACT: Native chromatin has been characterized at different ionic strengths, according to different methods of preparation, by means of circular dichroism and ethidium bromide intercalation. For a more precise interpretation of these results, a series of analogous experiments on phagic DNA, as a function of its circularization, and on mononucleosomes have been performed. Results are discussed here in terms of high order chromatin structure, DNA supercoiling, and topological constraints.

It is a common notion that chromatin represents a dynamic system undergoing continuous modifications of its structure in response to the metabolic alterations of the cell (Nicolini, 1983, 1991; Van Holde, 1990; Hansen & Ausio, 1992). Probably the folding/unfolding of the chromatin fiber between the 30-nm form (higher order structure) and the less condensed one (nucleosomal filament) represents the most important of these dynamic processes.

The fundamental repeating unit of chromatin is the nucleosome which consists of about 145 bp of DNA wrapped in 1.8 superhelical turns around the histone core. The diameter of this left-handed superhelix is 8.6 nm, 4 times wider than DNA double helix itself (Travers & Klug, 1987). Several papers (Germand et al., 1975; Richet et al., 1986; Giaever & Wang, 1988; Clark & Felsenfeld, 1991) have shown that these superhelical turns are associated with DNA supercoiling in eukaryotic cells and suggest that the primary role of this supercoiling is to facilitate the formation of a wrapped and functionally active structure—the nucleosome (Okba et al., 1992)—and to control gene expression (Nicolini, 1991; Nicolini et al., 1991).

Some further considerations are necessary for a more precise definition of supercoiling (Nash, 1990; Brotherton et al., 1989; Van Holde, 1990): in a closed DNA molecule, the linking number defines the number of times the two strands are interwound. The difference in the linking number between a topologically closed DNA molecule and the corresponding relaxed one determines the degree of supercoiling. Two kinds of processes contribute to the linking number: the first one (twist) describes the intertwining of the double-helix strands around their common axis, while the second one (writhe) describes the path of the helical axis itself. In a relaxed DNA molecule, twist is equal to that expected for the B form of DNA and writhe is close to zero. In the negatively supercoiled DNA, the molecule is instead under tension; therefore, the processes reducing the difference in either twist or writhe will be favored (for example, DNA wrapping into a higher order structure).

It is generally considered that DNA supercoiling has strong effects on the interactions between DNA and other molecules (Sen & Crothers, 1986; Giaever & Wang, 1988), namely, the intercalating molecules.

Over the years, a large number of studies have been performed in order to understand the mechanism and the effects of drug intercalation inside the double helix of DNA. One of the most important intercalating molecules is represented by ethidium bromide, a polycyclic aromatic dye which has been employed in several kinds of studies (Parodi et al., 1974; McMurray et al., 1991a,b). It is now accepted that the intercalation of one molecule of ethidium bromide unwinds the DNA helix by an angle of 26° (Wang, 1974; James, 1980) and displays an affinity constant of 10^4–10^6 M^{-1} depending on the buffer ionic strength.

Little is known about the effects of this binding at the level of the higher order chromatin structure. Previous papers (Nicolini & Kendall, 1977; Nicolini et al., 1977; Sasi et al., 1982; Wilson et al., 1986) suggested that ethidium bromide intercalation can be used to investigate the level of chromatin structure. Several open questions still remain: How does the level of chromatin high order structure alter the accessibility of DNA to ethidium bromide binding and how is DNA supercoiling influenced by the method of chromatin isolation from intact cells?

Recent biophysical studies (Nicolini et al., 1989a,b; Diaspro et al., 1991) pointed to the artifactual nature of the soluble chromatin prepared by limited nuclease digestion (Noll, 1976) in comparison to the preparation using hypotonic swelling of nuclei (Nicolini et al., 1975a, 1989a,b) which appears to preserve the native chromatin structure.

With regard to the above problems, we decided to conduct a study of native chromatin isolated by these two procedures in correlation with the analysis of phage DNA in two different conformations: the linear form and the circular one. A large number of previous studies (Bauer, 1980; Wang, 1990) have shown that in a topologically closed system (like circular DNA) the ethidium bromide intercalation at first determines a decrease of the twist and the writhe of the double helix until the writhe becomes null for a critical concentration (υ) of intercalated dye. When the intercalated ethidium bromide increases over the critical value, the writhe begins to increase again but in the opposite direction. Therefore, the final result is the formation of a superhelix with opposite writhe with respect to the initial one.

For this kind of analysis, we used circular dichroism spectroscopy, which is strictly sensitive to the structural properties of chiral molecules (Nicolini, 1986; Clark et al., 1991; Gray et al., 1992), together with ethidium bromide

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binding: the EB intercalation in fact has been shown to affect in a complex manner the CD spectrum of DNA in the 220–300-nm region (Parodi et al., 1974).

EXPERIMENTAL PROCEDURES

Lambda DNA Circularization. DNA supplied by Gibco-BRL (Gibco, Bethesdsa, MD) was heated to 65 °C for 10 min and quickly cooled in ice in order to restore the linear form and remove the linear aggregates and the circular structures.

As previously described (Nicolini et al., 1991), circularization of phagic DNA was carried out by incubation at 37 °C in the presence of T4 DNA ligase (Gibco); 1 unit of enzyme/mg of DNA was incubated in 1 × ligation buffer [10 mM Tris-HCl, 1 mM DTT, 10 mM $MgCl_2$, 1 mM ATP, and 1% poly(ethylene glycol)] for 2 h.

Chromatin Preparation. Calf thymocytes were prepared as previously reported (Vergani et al., 1992): 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 centrifugation (4 min at 300*g*), the pellet was washed in PBS; at last the nuclei were extracted by brief incubation (3 min at 4 °C) with 0.1% Triton X-100.

The nuclear pellet washed with 10 volumes of 0.15 M NaCl/0.01 M Tris-HCl, pH 8, was then resuspended for 1 h in cold distilled water. Finally the nuclei were lysed by homogenization in a Dounce homogenizer, and the chromatin was purified by centrifugation through 1.7 M sucrose at 100000*g* for 80 min. The chromatin pellet was gently resuspended in low (0.01 M Tris-HCl/0.001 M EDTA, pH 8) or physiological (0.15 M NaCl in TE) ionic strength buffer. All these procedures were carried out at 4 °C. We refer to this chromatin preparation as the cold water method (Nicolini & Baserga, 1975a; Nicolini et al., 1989a,b).

Chromatin was also extracted from nuclei after limited nuclease digestion as described by Noll et al. (1976). Nuclei (5 × 10^8 nuclei/mL), prepared as described above, were resuspended in buffer A (0.3 M sucrose, 0.05 M Tris-HCl, 0.025 M KCl, 0.005 M $MgCl_2$, 0.001 M $PhMSO_2F$, and 0.001 M $CaCl_2$, pH 8) and digested with 15 units/mL micrococcal nuclease (Sigma Chemical Co., St Louis, MO) for 30 s at 37 °C. The reaction was stopped by the addition of EDTA to a final concentration of 10 mM. After a brief centrifugation (5 min at 4000*g*), the digested nuclei were lysed as previously described and centrifuged for 2 min at 4000*g*: the supernatant contains the soluble chromatin while the pellet contains the unsoluble fraction. We refer to this procedure as the nuclease digested method.

Before the CD measurements, all the chromatin samples were dialyzed overnight at 4 °C versus a suitable buffer.

Oligonucleosome Preparation. Oligonucleosomes were prepared by prolonged nuclease digestion (15 min with 15 units/mL enzyme) in the same conditions as above.

Mononucleosome Preparation. Nuclei (50 OD_{260} units) were resuspended in buffer A′ (10 mM Tris-HCl, pH 7.4, 3 mM $MgCl_2$, and 10 mM NaCl) and digested for 2 min at 37 °C with 50 units/mL micrococcal nuclease. The reaction was stopped by the addition of EDTA (final concentration 10 mM), and the nuclei were centrifuged at 10000*g* for 20 min. This procedure avoids the lysis of nuclei and yields a supernatant fraction comprised mainly of monomeric nucleosomes lacking in histone H1 (Allegra et al., 1987).

Gel Electrophoresis. Calf thymus DNA was extracted from chromatin samples according to the standard procedure (Sambrook et al., 1989), and its molecular weight was determined by an electrophoretic run on an 0.8% agarose gel in Tris–borate buffer (pH 8) containing 1 g/mL ethidium bromide.

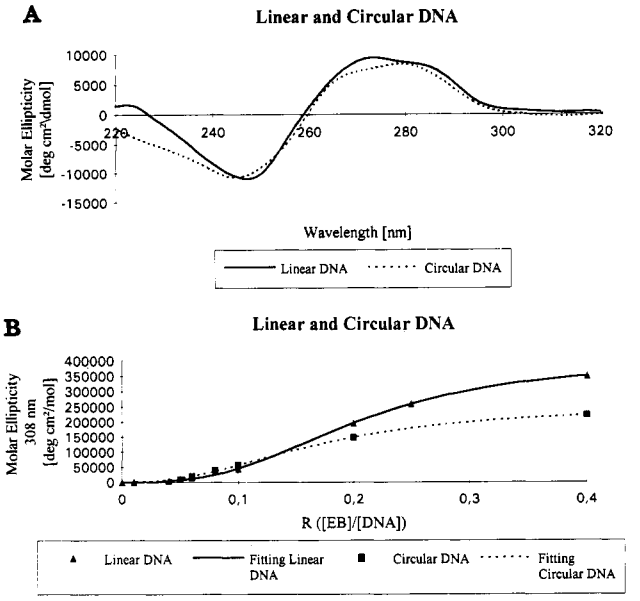

FIGURE 1: (A) Circular dichroism spectra of linear versus circular DNA: the acquired spectra are normalized for the corresponding DNA concentration (7.5 × 10^{-5} M) calculated by spectrophotometric methods (as described in the text). Both samples are analyzed in TE buffer, pH 8, under standard acquisition conditions (standard deviation of the measurement is 5.5%). (B) Molar ellipticity plots of molar ellipticity values at 308 nm (θ_{308}) versus R (ratio between total ethidium bromide concentration and DNA concentration) for linear and circular DNA. The markers represent the experimental values, and the lines represent the corresponding curves fitting these data.

Table 1[a]

source of DNA	a	b	c	r^2	N
linear DNA	24828	0.0086	3.121	1.00	9
circular DNA	15351	0.0117	2.448	0.96	10
cold water chromatin	13223	0.0829	1.901	0.99	7
nuclease-digested chromatin	11415	0.0590	1.571	1.00	8

[a] This table reports the parameter values of the sigmoidal equation $y = ax^c/(b + x^c)$ which has been employed to best-fit (by minimizing the root mean square error) the experimental data (θ_{308}) recorded at increasing ethidium bromide concentrations (R). r^2 is the correlation coefficient that represents a measure of the goodness of fit, and N is the number of experimental data points.

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

Circular dichroism measurements were made in a nitrogen atmosphere at 25 °C in a 1-cm path-length quartz cell, in 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 have been used in our experiments: time constant, 4 s; scanning speed, 20 nm/min; bandwidth, 0.2 nm; sensitivity, 20 mdeg, step resolution, 0.5 nm; PMT voltage, below 400 V (Nicolini & Baserga, 1975b). The acquired signal is usually expressed in terms of molar ellipticity (degrees centimeter squared per decimole).

For all the measurements, the DNA concentration of the samples was kept constant (7.5 × 10^{-5} M); it was spectrophotometrically determined by using for nucleosomal DNA

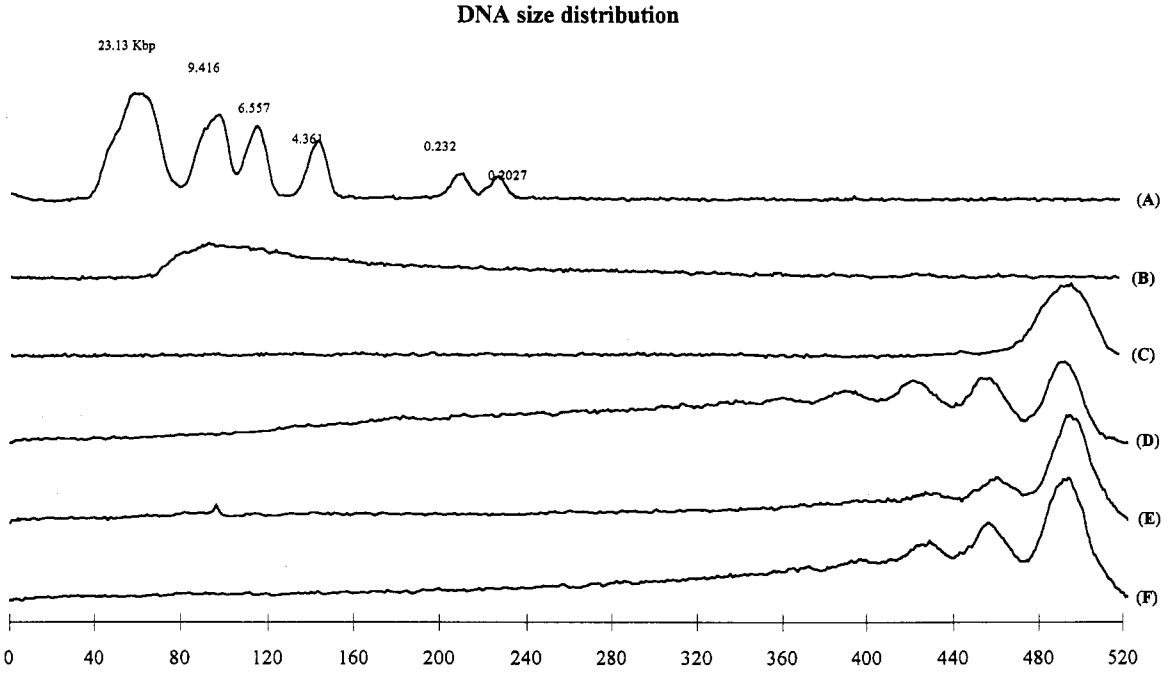

FIGURE 2: Densitometric profiles obtained by electropohoretic run of DNA fragments extracted by the following samples: (A) DNA markers; (B) cold water chromatin; (C) mononucleosomes; (D) nuclease-digested chromatin prepared by 1-min incubation with enzyme; (E) nuclease-digested chromatin prepared by 10-min incubation with enzyme; (F) nuclease-digested chromatin prepared by 15-min incubation with enzyme.

an extinction coefficient of 21 000 M^{-1} cm^{-1} in 0.2% sodium dodecyl sulfate buffer (Augenlicht et al., 1974).

The ethidium bromide solution was 1 mg/mL, and the concentration was determined using a molar extinction coefficient of 5600 M^{-1} cm^{-1} at 480 nm (Waring, 1965).

RESULTS

Linear and Circular Lambda DNA. The typical CD spectrum of DNA (Figure 1A) shows a positive band with a maximum at 272 nm and a symmetric negative band with a maximum at 246 nm.

At the level of the CD spectrum, comparison between the linear DNA and the corresponding circular form shows an interesting difference: in the circularized sample, the maximum of the positive band moves from 272 to 280 nm, displaying a red shift with respect to the linear DNA spectrum. The appearance of this new maximum following DNA circularization could be associated with a change in DNA twist and/or writhe. A further difference between the linear and the circular DNA spectrum is the position of the crossover that occurs at 257 nm for the linear DNA and shifts to 260 nm for the circularized one.

Ethidium bromide intercalation brings about the appearance of a positive ellipticity signal with a maximum at 308 nm, which is known (Dalgleish et al., 1971) to be related to the number of ethidium bromide molecules bound per DNA phosphate residue (r). At saturation, the value of the molar ellipticity signal at 308 nm has been shown to be directly related to the number of primary binding sites on DNA (Le Pecq & Paoletti, 1967; Williams et al., 1972; Nicolini & Baserga, 1975b). In all cases, the experimental values at 308 nm are best fitted by a sigmoidal function whose parameters are reported in Table 1: the cooperative effect of ethidium bromide binding to DNA is therefore confirmed, with the highest degree of cooperativity apparent in linear DNA.

The plot of θ_{308} versus R (ratio between ethidium bromide and DNA concentrations) reassuringly shows (Figure 1B) that circular DNA has a reduced number of primary binding sites and a significantly larger affinity, for low dye concentrations, with respect to the linear form: this last result is compatible with the well-known presence of negative supercoiling in a closed DNA molecule.

Chromatin. CD spectra have been obtained at low ionic strength (TE buffer: 10 mM Tris and 1 mM EDTA with added electrolytes) for native chromatin prepared by the above two procedures: cold water and nuclease digestion (Nicolini & Baserga, 1975a,b; Nicolini et al., 1991). The molecular weight of the DNA fragments extracted from these two preparations has been checked by an electrophoretic run on an agarose gel (Figure 2).

Both chromatin preparations show at low ionic strength (Figure 3A, top) the typical positive band with two different maxima, one at 272 nm and one at 284 nm; this positive biellipticity is characteristic of the chromatin CD spectrum, with respect to the free DNA one, and brings about the appearance of the well-known signal decrease in the positive band associated with the presence of chromosomal proteins.

A significant difference between the two different chromatin samples appears at wavelengths higher than 300 nm, where the cold water chromatin shows a positive ellipticity signal (Nicolini et al., 1977). This difference occurring outside its absorption band is related to the differential scattering of polarized light which is present in chromatin prepared by the cold water procedure but is lacking in the sample prepared by nuclease digestion (Figure 3A, bottom). As previously shown (Nicolini & Kendall, 1977), we assumed that the CD signal in the region above 305 nm was entirely due to scattering and can estimate the CD contribution from the scattering artifacts by best-fitting the experimental data between 305 and 360 nm with the exponential function $A + B^{-4}$ and by extrapolating it backward into the absorptive region. At each wavelength in the 200–300-nm range, the subtraction of the estimated value from the experimental one supplies the true molar ellipiticity properly corrected for the scattering contribution (Nicolini & Kendall, 1977).

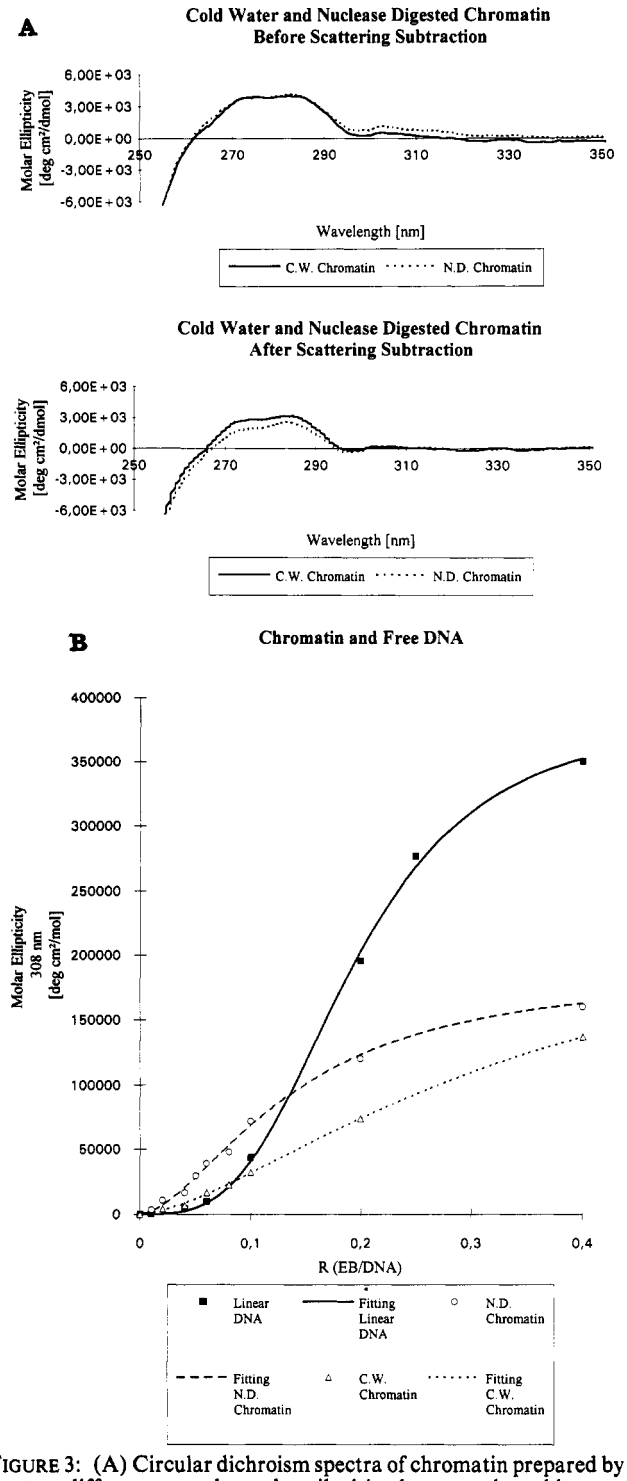

FIGURE 3: (A) Circular dichroism spectra of chromatin prepared by the two different procedures described in the text: the cold water method and the limited nuclease digestion method. (Top) CD chromatin spectra before scattering subtraction; (bottom) same CD chromatin spectra after scattering subtraction. The scattering subtraction has been performed by using the $A + B^{-4}$ exponential equation (where A and B represent the experimental parameters): $r^2_{CW} = 0.83$ and $r^2_{ND} = 0.90$ are the nonlinear correlation coefficients obtained by the two different chromatin preparations (cold water and nuclease-digested chromatin, respectively); for both of them, the number of experimental data points is 111. (B) Plots of molar ellipticity values at 308 nm (θ_{308}) versus increasing R values for linear DNA and for chromatin samples prepared either by cold water or by nuclease digestion procedures. The markers represent the experimental values, and the lines represent the corresponding curves fitting these data. The experimental values have not been corrected for scattering.

When both samples are exposed to increasing ethidium bromide concentrations, the resulting plots of θ_{308} versus R

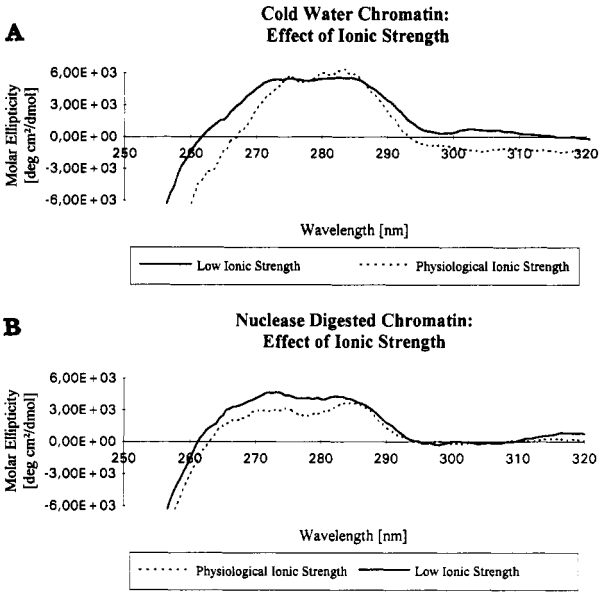

FIGURE 4: Circular dichroism spectra of chromatin prepared by the two different procedures previously described as a function of ionic strength: (A) cold water chromatin at low and physiological ionic strengths; (B) nuclease-digested chromatin in the same conditions. The acquired spectra are normalized for the corresponding DNA concentration (standard deviation of the measurement is 5.5%) but have not been corrected for the scattering.

(Figure 3B) show that at low ethidium bromide concentrations both chromatins, either cold water (partly) or nuclease-digested, appear accompanied by a higher affinity for the dye with respect to free DNA. When both chromatin preparations are resuspended in TE buffer containing 0.15 M NaCl, the CD spectrum displays in the 250–300-nm range a pronounced effect in the nuclease-digested chromatin while the effect is reduced in the cold water one (Figure 4).

Nuclease-digested chromatins of decreasing lengths, as monitored by agarose gel electrophoresis (Figure 2), were prepared by increasing the period of incubation with enzyme (namely 1, 10, and 15 min): they show differences in their CD spectra (Figure 5) mostly in the 220–250-nm region, reflecting chromosomal protein structure, with the corresponding percentage of primary binding sites for ethidium bromide remaining invariant (data not shown).

Mononucleosomes. The CD spectrum of mononucleosomes, prepared as described under Experimental Procedures, has been acquired in their preparation buffer between 200 and 360 nm (Figure 6). The length of DNA extracted from our mononucleosomal sample has been checked by electrophoresis and measures less than 200 bp and therefore lacks histone H1 (Figure 2). As previously shown (Allegra et al., 1987), the electrophoretic profiles of proteins extracted from our mononucleosome preparation point to a lack of histone H1 (data not shown).

Comparison between mononucleosomes and chromatin shows an interesting difference at the level of their CD spectra: while the chromatin spectrum shows, as seen previously, two different maxima (one at 272 nm and one at 284 nm) in the positive band, the mononucleosome sample is instead characterized by only one maximum at 272 nm, resembling the free DNA in linear form.

Furthermore, it shows a lack of any differential light scattering outside the absorption band above 310 nm (Figure 6) and a lack of ethidium bromide intercalation: the mononucleosomes appear unable to bind this dye at any concentration, as shown in Figure 7B by the zero CD signal

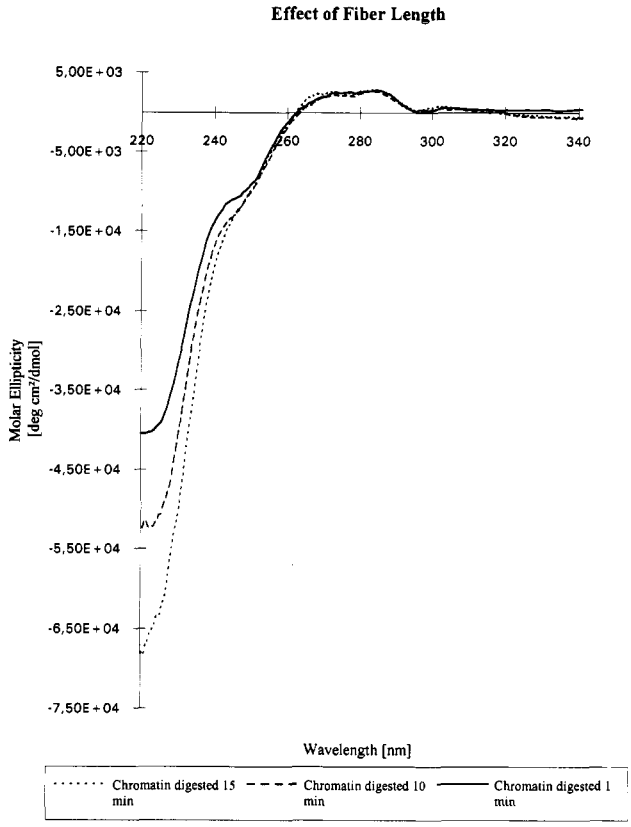

FIGURE 5: Circular dichroism spectra of limited nuclease-digested chromatin as a function of fiber length: the samples, prepared by different digestion times (namely, 1, 10, and 15 min), are normalized for the corresponding DNA concentration. All the samples are analyzed in TE buffer, pH 8, under standard acquisition conditions (standard deviation of the measurement is 5.5%).

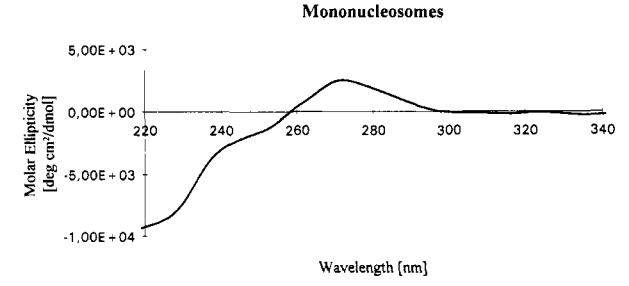

FIGURE 6: Circular dichroism spectrum of mononucleosomes prepared as described in the text. The acquired spectrum is normalized for the corresponding DNA concentration. The sample is analyzed in buffer A′ under standard acquisition conditions (standard deviation of the measurement is 5.5%).

at 308 nm for the EB–mononucleosome complexes and by the invariance of the CD spectra in the 250–300-nm region (pointing to the lack of any DNA unfolding which had been earlier reported upon EB intercalation for other mononucleosome preparations). At the same time, free DNA and chromatin display a strong CD signal corresponding to the EB absorption band (Figure 7A,C) in the 300–320-nm range.

The existence of a hidden negative band, in the 285–292-nm region, showing an identical amplitude with respect to the positive band at 308–315 nm was previously deduced from the CD spectra of the DNA–EB complex (Parodi et al., 1974). It is indeed only after the correction of this hidden band that the true DNA structure can be estimated as a function of EB intercalation (Figure 8): when the θ_{282} values are plotted versus R, after subtraction of the corresponding molar ellipticity values at 318 nm (Parodi et al., 1974), the linear region of the curve represents a measure of the direct proportionality between the normalized EB concentration and the corresponding ellipticity signal. The parameters which have been obtained by linear regression fitting of these data give quantitative information about the characteristics of binding: in particular, the R value corresponding to the loss of linearity can be related to the number of primary binding sites in DNA.

DISCUSSION

The differences between CD spectra of linear and circular DNA could really be explainable in terms of DNA supercoiling: following DNA circularization, supercoiling indeed becomes apparent together with a change in twist and writhe parameters. The appearance of a maximum over 280 nm in the circular DNA spectrum is probably due to these changes. It is interesting that the maximum at 284 nm is also present in chromatin spectra but not in mononucleosome spectra: we can therefore deduce that both circularization and nucleosomal superhelical organization cause similar modifications in the DNA structure. In fact, the prolonged nuclease digestion employed for preparing the mononucleosomal sample is responsible for the lack of histone H1 and the consequent loss of negative DNA supercoiling at this level of nucleosomal organization. These results are in accordance with previously published data that pointed to nucleosomal arrangement as the principal factor responsible for eukaryotic DNA supercoiling (Richet et al., 1986; Clark & Felsenfeld, 1991; Okba et al., 1992).

The similarities between circular and nucleosomal DNA are also detectable at their ethidium bromide binding level: in fact, at low R values, the circularized DNA shows a greater affinity for ethidium bromide with respect to the linear one (Figure 1B), which is quite similar to the DNA arranged in the nucleosomal structure (Figure 3B). This different affinity for ethidium bromide can be attributed to DNA supercoiling which is present in DNA molecules fixed by topological constraints (like circular DNA and nucleosomal DNA) but is absent in linear DNA. The similarity between circularized free DNA and nucleosomal DNA results is also evident when the number of primary binding sites is evaluated: as reported in Table 2, the number of binding sites per nucleotide, n, is between 0.2 and 0.25 for linear DNA and decreases to 0.1–0.12 for circular DNA; a similar n value has been found for nuclease-digested and cold water chromatin. This result points out that circularization and nucleosomal arrangement of the DNA molecule produce the same effect in topological terms. This interpretation can be explained in different ways: one of these is that the core particle DNA is accessible to the intercalating ligand as well as the linker DNA, but alternative interpretations could be considered (for example, a different level of superhelical density in these two DNA samples).

The disappearance of the maximum at 284 nm in mononucleosomes deprived of H1 histone can therefore be interpreted as a loss of DNA supercoiling. Moreover, the lack of any dye intercalation in the mononucleosomal sample points to the DNA phosphates completely bound to the histone proteins, as recently suggested by ^{31}P NMR experiments (Nicolini et al., 1993).

When chromatin (nuclease digested or cold water) is resuspended in a buffer at physiological ionic strength, the molar ellipticity increase in the negative band of the spectrum can be explained in terms of an increased content of helix in the histone secondary structure when the ionic strength is raised to a physiological level. This result matches perfectly

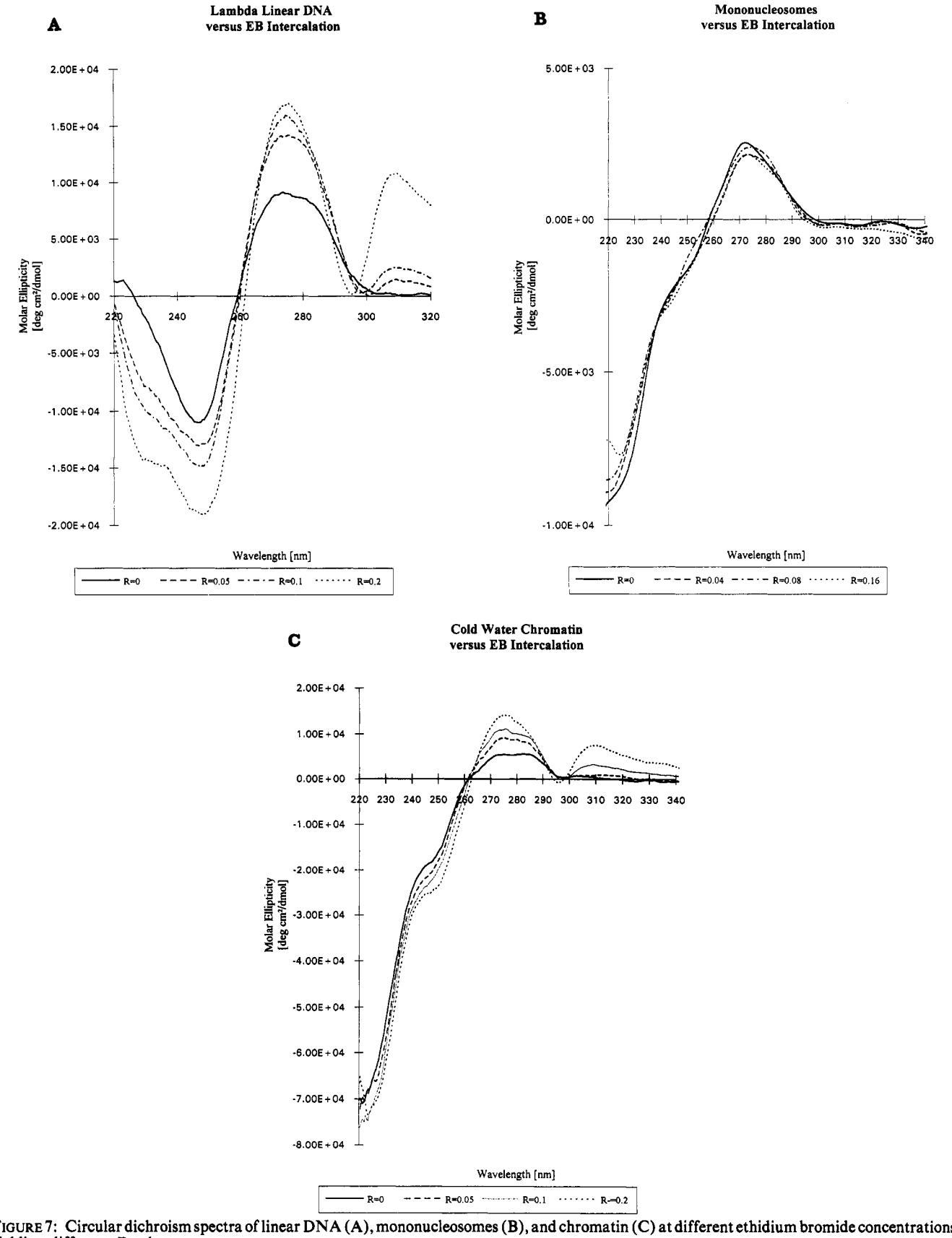

FIGURE 7: Circular dichroism spectra of linear DNA (A), mononucleosomes (B), and chromatin (C) at different ethidium bromide concentrations yielding different R values.

with the data previously obtained by differential scanning calorimetry measures (Nicolini et al., 1989a): at physiological ionic strength, chromatin actually shows an increase of enthalpy related to nuclear protein melting in accordance with more structured histones.

In the positive region of the CD spectrum, both chromatins show a sharper biellipticity at physiological ionic strength, with respect to the same sample at low ionic strength, that could be attributed to some alterations of the secondary structure of DNA, perhaps at the level of its twist or writhe.

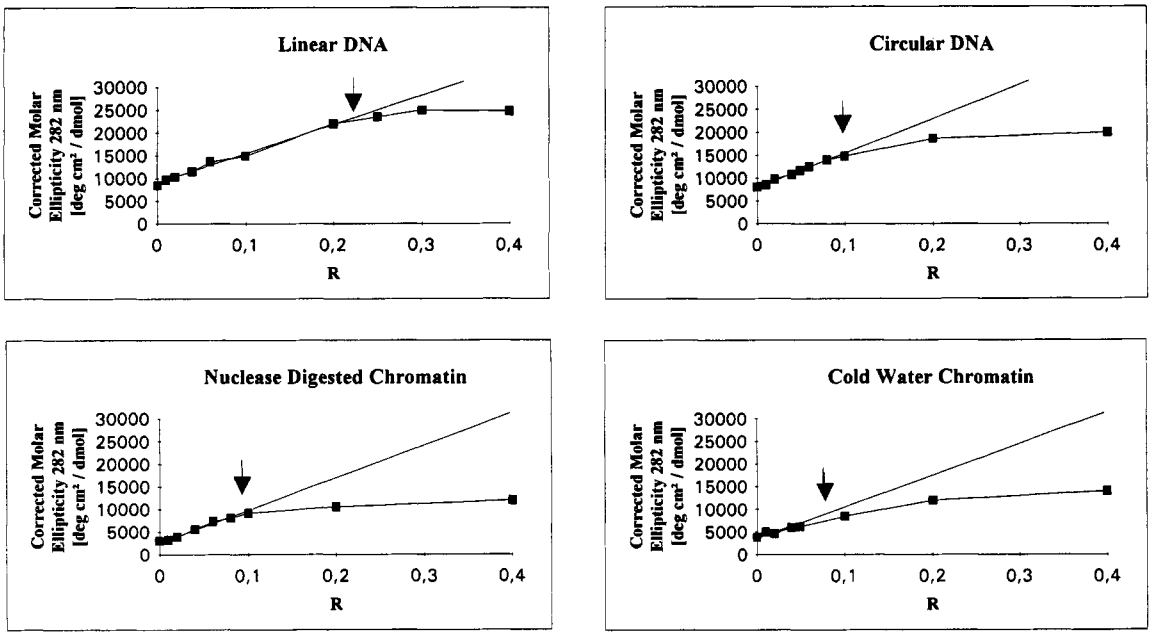

FIGURE 8: Plots of molar ellipticity values at 282 nm (θ_{282}) versus R for linear and circular DNA, for nuclease-digested and cold water chromatin. As described in the text, the ellipticity values at 282 nm are corrected for the hidden negative band (corresponding to subtraction for the corresponding 318-nm values) but not for the scattering.

Table 2: Number of Binding Sites per Nucleotide, n, for Different DNA and Chromatin–DNA Samples[a]

source of DNA	n
linear DNA	0.20–0.25
circular DNA	0.10–0.13
cold water chromatin	0.08–0.10
nuclease-digested chromatin	0.09–0.11

[a] These values have been evaluated by plotting the θ_{282} values (properly corrected for the hidden band) versus R, as described in detail in the text.

As previously reported (Sasi et al., 1982), the depression of the peak at 278 nm probably reflects a change in DNA wrapping around the histonic core; therefore the enhancement of molar ellipticity values at 284 nm could be explained in terms of DNA supercoiling increasing when the nucleosomal filaments are arranged in a higher order structure. It is interesting to note that the effect of increasing ionic strength, at the level of chromatin CD spectrum, can explain the differences between the cold water and nuclease-digested samples. On nuclease-digested chromatin, an increase of ionic strength brings about a reduction of the CD signal in the 260–280-nm region and leaves nearly unaffected the chromatin prepared by the cold water method.

The above CD data and the difference in ethidium bromide affinity between chromatin prepared by the cold water method and that prepared by the nuclease-digested one point to a difference in their higher order structure [as earlier reported by Nicolini et al. (1989b) and Diaspro et al. (1991)] but not in the degree of DNA supercoiling. While the first procedure partially preserves the chromatin higher order structure, the second misses it.

These results confirm the data previously obtained by different biophysical techniques such as differential scanning calorimetry and circular intensity differential scattering (Nicolini et al., 1989a,b; Diaspro et al., 1991; Vergani et al., 1992).

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