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Articles

Differences in the Condensation of Chromatin by Individual Subfractions of Histone H1: Implications for the Role of H1° in the Structural Organization of Chromatin†

Christian Marion,*,‡ Joëlle Roche,§ Bernard Roux,‡ and Claude Gorka§

Laboratoire de Physico-Chimie Biologique, LBTM-CNRS (LP 5421), Université Claude Bernard, 69622 Villeurbanne Cedex, France, and Laboratoire de Biologie Moléculaire du Cycle Cellulaire, Département de Recherche Fondamentale CENG 85X, 38041 Grenoble Cedex, France

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ABSTRACT: The effectiveness of histone H1 subfractions H1-1 and H1° in inducing the ordered condensation of chromatin was examined by thermal denaturation, circular dichroism, electric birefringence, orientation mechanism, and orientational relaxation time measurements. Soluble rat liver chromatin was stripped of H1 by dissociation in 500 mM NaCl and long fragments of chromatin were subsequently reassociated with purified individual H1 subfractions for ratios of 1 and 2 mol of H1 per nucleosome. H1 subfractions behave differently with respect to their interactions with DNA in chromatin: although the orientation mechanisms of reconstituted chromatins are identical, H1° induces a less efficient protection of DNA than H1-1, as shown by nuclease digestion and by the length of free extended linker DNA determined by electric birefringence. This corresponds to a more extended structure of H1°-reconstituted chromatin as judged by the value of relaxation time. One can imagine that the replacement of H1 by H1° leads to a different structure or stability of the chromatin, confering a certain degree of flexibility of this region. This may be related to the functional role of H1° in DNA replication or transcription and may explain metabolic and evolutionary differences among H1 subfractions as recently suggested by Lennox [Lennox, R. W. (1984) *J. Biol. Chem. 259*, 669-672]. The extent of condensation when H1-depleted chromatin is overloaded with histones is probably a function of the electrostatic interactions between the basic C-terminal tails of histones and chromatin. Electric birefringence also reveals differences between native and reconstituted chromatins that are overlooked by several other criteria.

It is now widely accepted that histone H1 is involved in the maintenance and modulation of higher order structure in chromatin (Littau et al., 1965; Bradbury et al., 1973; Billett & Barry, 1974; Noll & Kornberg, 1977; Thoma et al., 1979). In fact, the class of lysine-rich histone H1 found in most eucaryotic cells is the most variable of the five histone classes. It shows both tissue and species specificity (Bustin & Cole, 1968; Kinkade, 1969; Panyim & Chalkley, 1969), and this heterogeneity in the primary structure of H1 subfractions may play a role in the degree of chromatin condensation (Huang & Cole, 1984). Despite their common structural organization, previous studies have suggested that H1 histones differ among themselves with respect to their interactions with DNA (Gorka & Lawrence, 1979; Welch & Cole, 1979, 1980; Liao & Cole, 1981a,b). It is therefore conceivable that the structural diversity of H1 subfractions is related to their potential for functional diversity. Among H1 histones, the fraction H1° could be involved in cell proliferation (Pehrson & Cole, 1980) or rather in terminal differentiation (Gjerset et al., 1982). When H1° is present, it replaces H1 on the linker region of

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‡Laboratoire de Physico-Chimie Biologique.
§Laboratoire de Biologie Moléculaire du Cycle Cellulaire.

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chromatin (Smith & Johns, 1980), and it has been suggested that it is associated with chromatin having an increased resistance to nuclease attack (Gorka & Lawrence, 1979; Gjerset et al., 1981; Roche et al., 1985a). A recent study has however suggested that H1° might be considered as belonging to a family of proteins distinct from the H1 family (Smith et al., 1984) in agreement with Pehrson & Cole (1981).

To understand how the subfractions of histone H1, particularly H1°, affect chromatin structure, the histones were purified, and their interactions with H1-depleted chromatin with a variety of techniques were studied (Biard-Roche et al., 1982; Roche et al., 1984).

We report here circular dichroism, thermal denaturation, and electric birefringence studies, at low ionic strength, on the structure of chromatin reconstituted with various H1 subtypes (both at the native one and at two molecules per nucleosome). From measurements on nucleosomal DNA (Roux et al., 1979; Marion & Roux, 1980a) and oligonucleosomes (Marion & Roux, 1980b; Marion, 1984), the length of free extended linker DNA can be estimated, and we used this advantage of the ability of electric birefringence to reveal details about the interactions of H1-1 and H1° fractions with DNA. Recall that electric birefringence is a method of considerable potential utility because of its high sensitivity not only to the size and shape of particles but also to the orientation in the electric field of a few unfolded or/and nonprotected DNA segments in the fibers. While circular dichroism, thermal denaturation, and orientation mechanisms studies did not show any difference, steady-state birefringence and orientational relaxation time measurements showed an extended structure due to the presence of H1°.

Materials and Methods

Preparation of Chromatin and H1-Depleted Chromatin. Native chromatin was prepared as described (Biard-Roche et al., 1982). Rat liver nuclei, prepared as described by Hewish & Burgoyne (1973), were gently digested by micrococcal nuclease (Worthington) in order to obtain long chromatin fragments (20 units of DNase/10^8 nuclei, for 90 s at 37 °C). High-order oligomers were further purified on a 5–30% isokinetic sucrose gradient in 10 mM Tris-HCl[1] (pH 7.4) containing 0.2 mM EDTA. Fractions containing oligomers higher than 10 nucleosomes were collected, and aliquots were analyzed by gel electrophoresis for protein and DNA content. H1-stripped chromatin was obtained by increasing the ionic strength of lysis medium to 500 mM NaCl, and fractionation of the oligonucleosomes was performed on a sucrose gradient containing 500 mM NaCl.

Preparation of H1 Subfractions. Procedures for histone H1 extraction and purification of H1 subfractions were described previously (Biard-Roche et al., 1982). Briefly, histone H1 was extracted from frozen bovine liver by 5% perchloric acid and precipitated by 20% trichloracetic acid. Fractionation of this extract by gel filtration chromatography (Bio-Gel P-100) completely separated histone H1° from H1-1 and H1-2, which were collected as a single peak. H1-1 was further purified by ion-exchange chromatography (Bio-Rex 70). The purity of H1 subfractions as well as the absence of their proteolytic digestion during the reconstitution process was checked on heavily loaded SDS/polyacrylamide gels (Laemmli, 1970).

[1] Abbreviations: SDS, sodium dodecyl sulfate; TEACl, triethanolamine chloride; CD, circular dichroism; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; EDTA, ethylenediaminetetraacetic acid.

Reconstitution Experiments. Purified H1-1 and H1° at 1 mg/mL in 10 mM Tris-HCl (pH 7.4) were rapidly added with thorough mixing to H1-depleted chromatin (A_{260} = 2) in Tris buffer with 500 mM NaCl and allowed to reassociate at 4 °C (Biard-Roche et al., 1982). The reconstituted samples were then dialyzed against 1 mM sodium phosphate buffer (pH 7.4) and 0.2 mM EDTA. The concentration of the histone solution was determined by the method of Lowry.

The molar ratio of histone to nucleosome in reconstituted mixtures was determined by taking one molecule of H1 as 21 500 daltons and nucleosomal DNA (200 base pairs) as 132 000 daltons. The ratios of H1/nucleosome used in this study were 1 and 2. In the case of the reconstitution with the mixture of the two purified histones H1-1 and H1°, the proportions used were 1:1. All the solutions contained 0.5 mM phenylmethanesulfonyl fluoride as protease inhibitor.

Characterization of Chromatin Samples. Kinetic analysis of the digestion of native, stripped, and reconstituted chromatin samples by micrococcal nuclease was used to control the reassociation of H1 subfractions to H1-depleted chromatin. Samples were digested for 20 min at 4 °C in 1 mM TEA, pH 7.4, 1 mM NaCl, and 0.1 mM $CaCl_2$ with nuclease at a ratio of 60 units/A_{260}. DNA was analyzed on horizontal 2.5% agarose gels (Modak et al., 1980). Electron microscopy of chromatin samples was performed as previously described (Biard-Roche et al., 1982).

Thermal Denaturation. Chromatin samples at A_{260} (25 °C) ~ 0.8–1.0 were thermally denatured in a Gilford 250 spectrophotometer connected to a thermoprogrammer 2527 equipped with a reference compensator 2535 for thermal-expansion correction. Samples were placed in quartz cuvettes with 1-cm path length, carefully degassed, and sealed with a Teflon stopper. The heating rate was 1 °C/min. For convenience in interpretation, the data were processed by determining the relative hyperchromicity defined as

$$h(t) = (A^t_{260} - A^{25}_{260})/(A^{95}_{260} - A^{25}_{260}) \quad (1)$$

where A^t_{260} is the absorbance at wavelength 260 nm and temperature t and A^{25}_{260} and A^{95}_{260} are the absorbances at 25 °C (the base temperature) and 95 °C, respectively. The transition midpoint t_m is defined as the temperature of maximum dh/dT for each thermal transition.

Circular Dichroism Analysis. Circular dichroism measurements were made at room temperature with a Jobin and Yvon Mark IV dichrograph in cuvettes of 1-cm path length and chromatin solutions at A_{260} ~ 0.8–1.0. Concentrations of DNA nucleotide residue were determined by absorption at 260 nm with ϵ_{260} = 6600 cm^{-1} mol^{-1} nucleotide. The results are presented in terms of molar ellipticity (deg cm^2 $dmol^{-1}$) (error ±300).

Electric Birefringence Measurements. Electric birefringence was measured on an apparatus built in our laboratory and described elsewhere (Bernengo et al., 1973; Marion et al., 1984). Briefly, the light source was an He–Ne (λ = 632.8 nm, Spectraphysics Model 120) laser powered by a highly stabilized high-voltage supply. Light beam variations were converted to electric signals by a low-noise solid-state photodetector followed by a specially designed amplifier. The signal intensity is directly proportional to the birefringence. Single rectangular and reverse electric pulses having amplitudes up to 1200 and 200 V, respectively, were applied across a 0.5-cm interelectrode cell. The pulse duration was about 0.5–2 ms, and the transition times of the apparatus were about 50 ns. The birefringence signals displayed on a storage oscilloscope were sampled on a transient recorder and then transferred to an XY plotter. Samples of 1.5 mL containing 0.8 A_{260} were

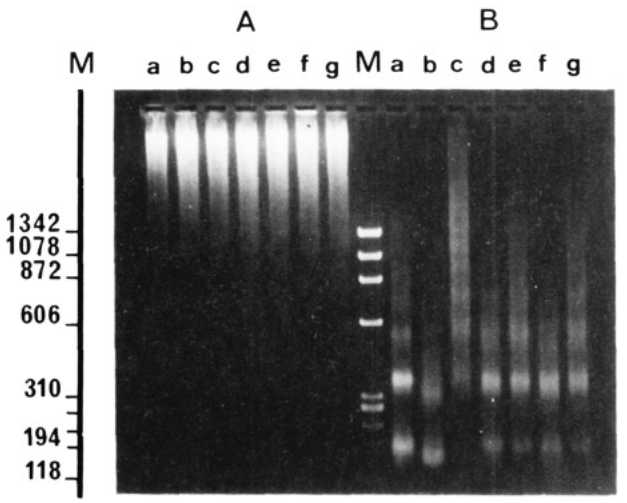

FIGURE 1: DNA analysis on 2.5% agarose horizontal slab gel of nuclease-digested chromatin (60 units of DNase/A_{260}, 4 °C): (A) zero time of digestion; (B) 20 min of digestion. Analysis of native chromatin (a), of H1-depleted chromatin (b), and of reconstituted chromatin with 2 mol of H1-1 (c), 1 mol of H1-1 (d), 2 mol of H1° (e), 1 mol of H1° (f), and H1-1 + H1° (1/1) (g). (M) *Hae*III digest of RF ϕX 174 used as a size marker. The length of the fragments is indicated in base pairs.

used, and the birefringence results have been expressed in terms of the specific birefringence ($\Delta n/A_{260}$).

Procedures for measurements on chromatin solutions and calculations were essentially as previously described (Marion & Roux, 1978; Roux et al., 1979; Marion, 1984; Chauvin et al., 1985).

From a large number of measurements with chromatin solutions originating from one stock solution, a standard deviation has been calculated pertaining to a single determination of each of the electrooptical parameters. These standard deviations are 6–8% for the steady-state birefringence and the Kerr constant and 8–10% for the relaxation time.

Measurements on native, depleted, and reconstituted samples have been repeated on four different chromatin preparations at an interval of several months. So, we are confident in the overall effect reported here.

RESULTS

Reconstitution Studies. The experimental conditions for the characterization of native and reconstituted chromatin were identical with those described previously (Biard-Roche et al., 1982; Roche et al., 1984). The size of the polynucleosomes has been found to be higher than 15 nucleosomes by electrophoresis studies. Due to the high electric fields required to measure electric birefringence, low ionic strength buffer must be used. The measurements were performed in 1 mM sodium phosphate, pH 7.4, and 0.2 mM EDTA.

The fidelity of reconstitution was ascertained by digestion with micrococcal nuclease and electron microscopy. In low ionic strength (1 mM TEACl, pH 7.4), the results of kinetics of digestion were similar to those observed previously in a higher ionic strength (Biard-Roche et al., 1982; Roche et al., 1984). Following removal of histone H1, the 168-bp barrier of digestion disappeared, and reassociation of H1 subfractions to H1-depleted chromatin with molar ratios of 1 and 2 H1 per nucleosome restored the 168-bp pause (Figure 1). The intensity of the bands of mono- and dinucleosome observed in the gels after digestion of native, H1-depleted, and reassociated chromatin had shown that the hierarchy between the samples in their ability to be resistant to nuclease attack is as follows: chromatin reassociated with H1-1 (2 mol) > native chromatin

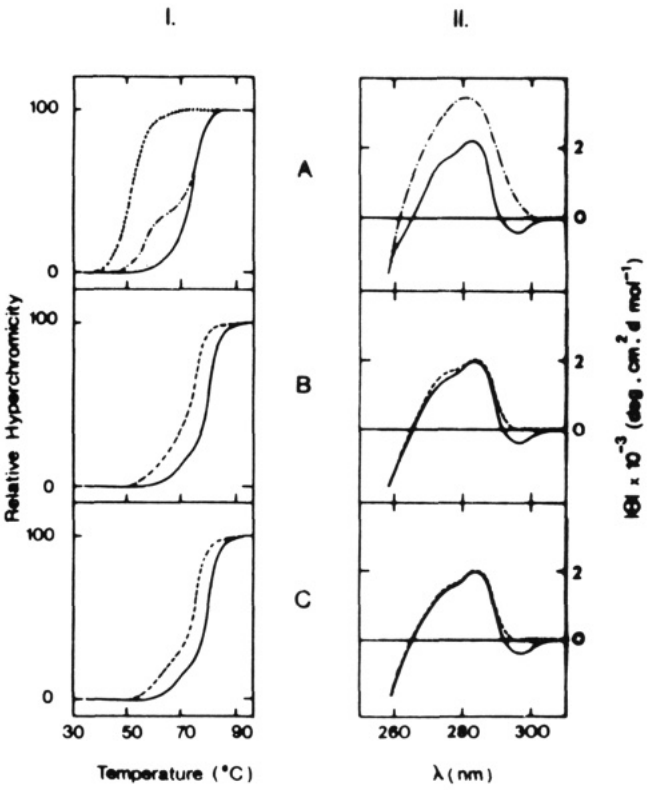

FIGURE 2: Thermal denaturation profiles (I) and circular dichroism spectra (II) of DNA and chromatin in the presence of 1 mM sodium phosphate buffer, pH 7.4, and 0.2 mM EDTA. Analysis of (A) DNA (···), native chromatin (—), and H1-depleted chromatin (–·–). (B) Chromatin reconstituted with H1-1: (···) 1 mol/nucleosome; (—) 2 mol/nucleosome. (C) Chromatin reconstituted with H1°: (···) 1 mol/nucleosome; (—) 2 mol/nucleosome.

= chromatin reassociated with H1-1 (1 mol) or with H1° (2 mol) or with H1-1 (1 mol) plus H1° (1 mol) > chromatin reassociated with H1° (1 mol). On the other hand, the ability of reconstituted chromatin samples to undergo condensation in 80 mM NaCl was verified by electron microscopy (data not shown).

Thermal Denaturation Analysis. Figure 2 shows the melting profiles of various chromatin samples. The melting curve of chromatin is mainly monophasic with a melting point (t_m) at 76 °C. Stripped chromatin displays a typic biphasic melting profile with t_{m1} = 55 °C and t_{m2} = 74 °C. We must note that the melting of the linker corresponding to the lower transition occurred at a higher temperature than that of DNA in the corresponding buffer, about 5 °C. When H1° is reassociated to stripped chromatin, the first transition is still present in the range 60–75 °C but with a smaller amplitude. The samples reconstituted with H1-1 recover a mainly monophasic profile very similar to that of native chromatin. These results show the same hierarchy in stabilization of the DNA as that observed in the protection of DNA against nuclease attack. They indicate, namely, that H1° is less efficient in this process than H1-1 since a small part of the DNA has a lower thermal stability than that of the chromatin reconstituted with H1-1, even with a saturating amount of H1°.

What is striking is the higher melting point of the second transition in the samples reconstituted with 2 mol of H1-1 or H1° (79.5 °C as compared to 76 °C in native chromatin), which implies a greater stabilization of the corresponding DNA.

Circular Dichroism Analysis. Conformational evaluation by CD of native and reconstituted chromatin can be seen in Figure 2. Native chromatin produces a CD spectrum with

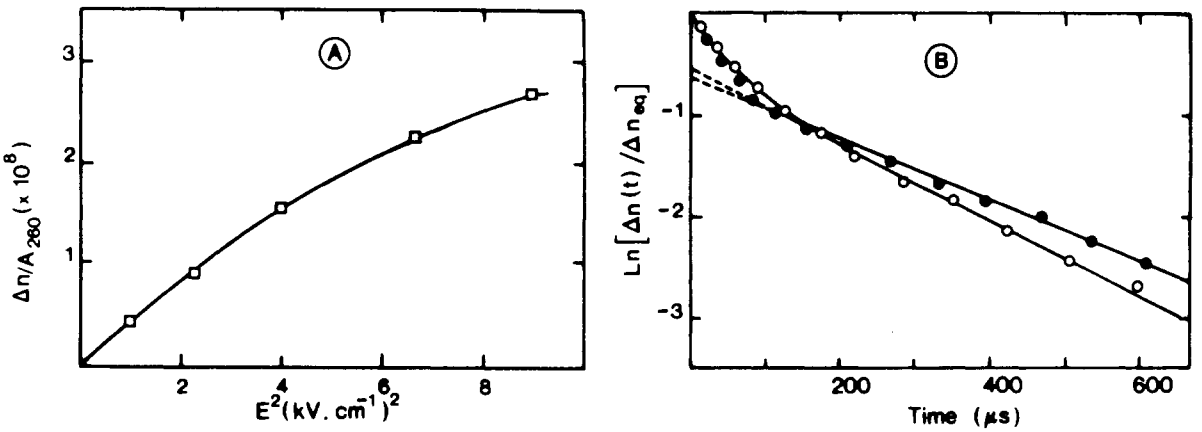

FIGURE 3: Influence of the electric field on the specific birefringence (A) and relaxation times (B) of native chromatin. Measurements were made in 1 mM sodium phosphate buffer, pH 7.4, and 0.2 mM EDTA. The specific Kerr constant is determined from the slope in (A). The semilogarithmic birefringence decays are shown for electric fields applied of 800 V cm^{-1} (○) and 1000 V cm^{-1} (●). The contribution a_2 to the total birefringence of the longest relaxation time τ_2 is calculated by extrapolating the linear part of the plot (ln a_2).

the characteristic negative peak around 296 nm and a maximal positive ellipticity at 282 nm of 2200 ± 300 deg cm^2 dmol^{-1} (De Murcia et al., 1978). The removal of histone H1 results in an increase of $|\theta|_{282}$ to 3200 deg cm^2 dmol^{-1}; the shoulder at 274 nm increases to 2750 deg cm^2 dmol^{-1} while the crossover point has shifted by 5–6 nm. Within the experimental uncertainties of our measurements, the H1-1- and H1°-reassociated samples yield identical CD spectra that are substantially like the native chromatin spectrum in the spectral region 260–320 nm. However, the negative peak exhibited by native chromatin is only recovered when the subfractions are reassociated to H1-depleted chromatin with a molar ratio H1/nucleosome of 2.

Steady-State Electric Birefringence Studies. As previously reported (Marion et al., 1982, 1983a,b; Roche et al., 1984; Chauvin et al. 1985), native chromatin exhibits a positive birefringence. No measurable changes in specific birefringence are detected when the sample absorbance A_{260} varies from 0.8 to 2.4 or when the sample is exposed to repeated applications of the electric fields.

The field-strength dependence of the birefringence reported in Figure 3A shows that the Kerr's law is followed up to 1200 V cm^{-1}. The value of the specific Kerr constant is 0.14 ± 0.02 esu cgs. The release of H1 from native chromatin has a drastic effect on the birefringence oscillograms; the sign of the birefringence is reversed, becoming negative, as we have reported for purified oligonucleosomes (Marion & Roux, 1980b; Marion, 1984). The field-strength dependence of the birefringence is shown in Figure 4. Contrary to native chomatin, Kerr's law is obeyed only at very low fields. The Kerr constant determined can be used to estimate the number n of nucleosomes in the chains and the length of free DNA. Assuming values of -7.15×10^{-2} esu cgs (Marion, 1984) and -1.75×10^{-3} esu cgs (Marion & Roux, 1980a) for the Kerr constant per H1-depleted mononucleosome and per free DNA base pair, respectively, we deduce a mean number of 22 nucleosomes per fragment and each nucleosome contains a length of free extended DNA of 40 base pairs.

Contrary to the results obtained on reconstituted samples in 10 mM Tris buffer (Roche et al., 1984), the characteristic positive birefringence is never observed for reconstituted samples in the presence of phosphate buffer. This discrepancy arises from a field-induced internal deformation or/and segmental orientation in the polynucleosome fibers, which are more flexible in phosphate buffer (Lee & Crothers, 1982). However, the use of such a low ionic strength allows the detection of small but reproducible differences in the ability of H1 subfractions to condense stripped chromatin. The amplitude of the negative signal is a function of the amount and the nature of the reassociated fraction; the lowest amplitude of the negative signal is observed for samples reconstituted with 2 mol of H1-1, the highest for H1-depleted chromatin. Between these two extremes, the hierarchy is the following: for 2 mol, H1-1 < H1-1 + H1° < H1°; for 1 mol, H1-1 < H1° < stripped chromatin.

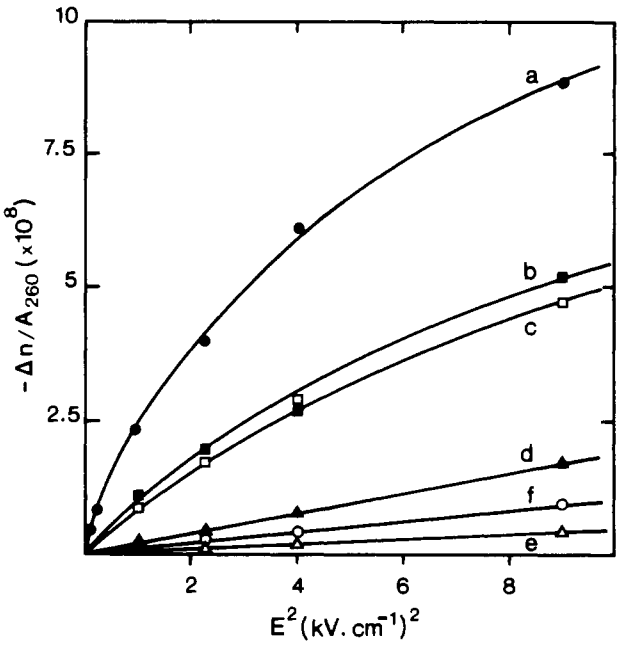

FIGURE 4: Influence of the electric field on steady-state-specific birefringences of H1-depleted and reconstituted chromatin. Measurements were made in 1 mM sodium phosphate buffer (pH 7.4) and 0.2 mM EDTA. (a) H1-depleted chromatin; (b and c) chromatins reconstituted with 1 mol of H1/nucleosome; (b) H1°; (c) H1-1; (d–f) chromatins reconstituted with 2 mol of H1/nucleosome; (d) H1°; (e) H1-1; (f) H1-1 + H1° (1/1).

Figure 4 shows the field dependence of Δn for these samples. Kerr's law ($\Delta n \sim E^2$) is followed at sufficiently low fields, and the Kerr constant determined from these samples allows the calculation of the number of DNA base pairs left free after the reassociation process (Table I). In this case, the number of free base pairs in sample reconstituted with 1 mol of H1°/nucleosome is found significantly and repeatedly higher than in samples reassociated with 1 mol of H1-1 (13 base pairs and 10 base pairs, respectively).

Table I: Estimation of Lengths of Extended Free Linker DNA[a]

chromatin	H1/nucleosome (molar ratio)	specific Kerr constant (esu cgs)[b]	extended free DNA (base pairs)	extended free linker DNA per nucleosome (base pairs)
H1 depleted		−1.550	885 ± 50	40 ± 3
H1° reconstituted	1	−0.515	295 ± 20	13 ± 2
H1-1 reconstituted	1	−0.410	235 ± 20	10 ± 2
H1° reconstituted	2	−0.055	30 ± 5	
H1-1 reconstituted	2	−0.016	10 ± 3	
H1° + H1-1 reconstituted	2	−0.030	17 ● 3	

[a] Measurements are performed in 1 mM sodium phosphate buffer, pH 7.4, and 0.2 mM EDTA. [b] The values are given as ±6–8% standard deviations.

Table II: Analysis of Birefringence Decays of Chromatin Samples[a]

applied electric field (V cm^{-1})	native chromatin			H1-depleted chromatin			H1°-reconstituted chromatin			H1-1-reconstituted chromatin		
	τ_1 (μs)	τ_2 (μs)	a_2	τ_1 (μs)	τ_2 (μs)	a_2	τ_1 (μs)	τ_2 (μs)	a_2	τ_1 (μs)	τ_2 (μs)	a_2
1000	105	240	0.61	90	520	0.59	90	400	0.49	80	300	0.49
1500	90	280	0.55	80	540	0.45	53	400	0.41	38	290	0.49
2000	70	260	0.45	70	520	0.39	42	440	0.32	25	300	0.41
3000				30	500	0.33	30	440	0.29	25	330	0.31

[a] Measurement are performed in 1 mM sodium phosphate buffer, pH 7.4, and 0.2 mM EDTA. The molar ratio H1/nucleosome in reconstituted chromatin is 1. Birefringence decay kinetics are fitted with two exponential terms. τ_1 and τ_2 are respectively the short and long relaxation times. a_2 is the contribution of the slow relaxation process to the total birefringence. The values of relaxation times are given as ±8–10% standard deviations.

Relaxation Time Measurements. For native chromatin the decay of the birefringence is not monoexponential and can be fitted with a minimum of two exponential terms, with values for the short relaxation time of the order of 80–100 μs, which decreases with increasing the field strength (Table II). The value of the slower relaxation time, which represents the rotation of the whole chromatin particles (here called τ_2), is about 240–280 μs. The relative amplitude a_2 of this component decreased with increasing the electric field although the value of τ_2 is independent of the field strength. τ_2 so contributes ~45% of the birefringence at 2000 V cm^{-1} and ~61% at 1000 V cm^{-1} (Figure 3B). This variation of a_2 with the field indicates a deformation of the chromatin when the applied force is increased. Hence, although the chromatin is maintained in a compact state, as shown by the lower values of both Kerr's constant and relaxation times (Chauvin et al., 1985), it does not prevent local flexibility, allowing distorsion of the structure by the electric fields (Lee & Crothers, 1982). Within the limits of the experimental error, there is no detectable dependence of τ_2 on chromatin concentration. Stripped chromatin exhibits birefringence decay curves multiexponentially, giving relaxation times in the order of 500 μs (Table II), and we notice that, as for native chromatin under identical field strength conditions, stripped chromatin exhibits decreases of τ_1 and a_2 as the field increases.

Figure 5 shows the birefringence decays measured for reconstituted samples. It is noticeable that the general shapes of the signals are different when 2 mol of H1 are reassociated to nucleosome as compared to samples reassociated with 1 mol of H1 or H1-depleted chromatin. In these last cases, samples do not exhibit a unimodal decay even at the lowest fields at which the relaxation times can be measured accurately. From the data given in Figure 5, two relaxation times can be calculated (Table II) showing the presence of a long relaxation time (τ_2), which is found field-independent. As for native and stripped chromatin, the contribution of this relaxation process to the total birefringence decreases as the field increases. At the same time the relaxation time τ_1 of the faster decay process decreased strongly. τ_2 is significantly and repeatedly found longer in samples reconstituted with 1 mol of H1° than in samples reconstituted with 1 mol of H1-1. This finding is consistent with a more flexible structure in the presence of H1° histone.

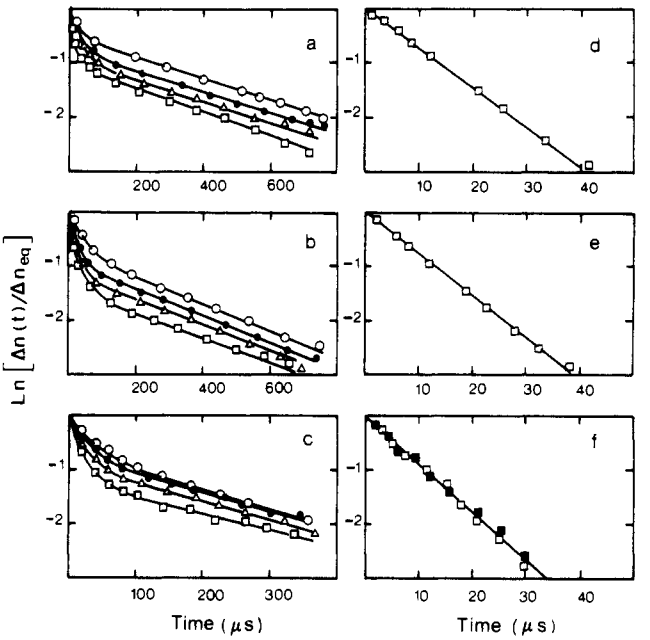

FIGURE 5: Influence of the electric field on the relaxation times of H1-depleted and reconstituted chromatin. Letters stand for the same samples as in the legend of Figure 3. The field strengths used were respectively 1000 (○), 1500 (●), 2000 (△), 3000 (□), and 4500 V cm^{-1} (■).

Chromatin reconstituted with a molar ratio H1/nucleosome of 2 shows perfect monoexponential birefringence decays (Figure 5d–f). We observe that the remaining birefringence can be measured down to about 6% of the steady-state value for all the curves. This implies that the particles contributing to the orientation effect are monodisperse. Because of the small signal obtained, we cannot measure accurately relaxation times below about 2000 V cm^{-1}, and hence, we are forced to compare the effects of H1 subfractions at higher fields. The values of τ, identical for the different chromatin samples, are about of 12–14 μs. This dramatic decrease in the relaxation times reflects the behavior in an electric field of chromatin

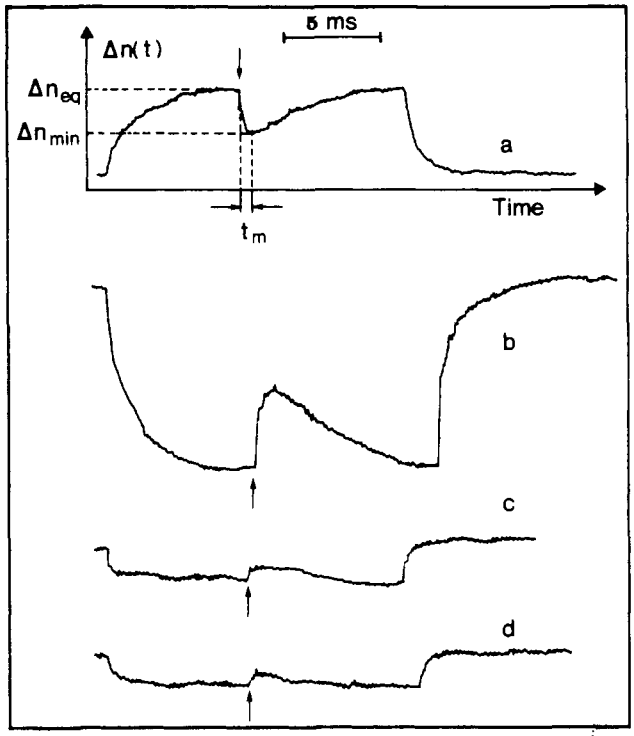

FIGURE 6: Transient electric birefringence under bipolar pulses. Native chromatin (a); H1-depleted chromatin (b); chromatin reconstituted with 1 mol/nucleosome of H1° (c) and H1-1 (d). The field strength was 300 V cm^{-1} in 1 mM sodium phosphate buffer (pH 7.4) and 0.2 mM EDTA. The arrows show the field reversal.

rigidly maintained in a very compact conformation. The higher compacted structure of reconstituted chromatin is then very similar to that recently observed for native chromatin in phosphate buffer containing 60 mM NaCl (Chauvin et al., 1985).

Reversing Pulse Experiments. Under bipolar pulses, a decrease in the birefringence signal can be observed when the field is reversed. Figure 6 shows the variation of Δn as a function of time when a reversing electric pulse is applied. The depth of the birefringence minimum with respect to the steady-state value allows the ratio of the permanent to the induced dipole, r, to be calculated. Because of the low birefringence value and of experimental limitations, we are not yet able to perform the experiments with chromatin reconstituted with a molar ratio H1/nucleosome of 2. For native chromatin, we found $r = 0.8$. This value is similar to that previously determined either by the ratio of the rise over decay areas or by using theoretical rising functions (Fredericq & Houssier, 1973) for oligomers containing more than seven nucleosomes (Marion, 1984). We observed a smaller amplitude of the depth of the minimum for both stripped ($r = 0.65$) and reconstituted chromatin for which no significant differences are observed ($r = 0.45$–0.50). Since in no case r exceeded 1, the orientation mechanism may be considered to be mainly of the induced dipole type.

We cannot distinguish between the variations of permanent and induced dipole moments, which may cause the decrease of r when H1 subfractions are added to H1-depleted chromatin. However, our results clearly show that the reconstituted chromatins exhibit identical orientation mechanisms. It is therefore reasonable to think that H1-1 and H1° bind to H1-depleted chromatin in a similar way.

From the values of t_m (Figure 6), rotary diffusion constants θ can be calculated; within the limits of experimental accuracy, relaxation times are of the order of 400–600 μs, which are in satisfactory agreement with the values determined for decay curves.

Discussion

In this paper we have demonstrated that H1 subfractions differ with respect to their interactions with DNA in chromatin. More specifically, H1° is shown to induce a more extended structure than H1-1. This has been achieved by studying reconstitutes of H1-depleted chromatin with various H1 subtypes by a variety of techniques. Reconstitutions were carried out at both one and two H1's per nucleosome. In order to characterize the properties of the reconstituted complexes and to assess the role of H1 subfractions, we have particularly used the electric birefringence technique, and three criteria have been considered: (1) the orientation of the complexes and the presence of extended DNA were compared by the sign and the amplitude of the steady-state birefringence; (2) conformational properties were compared by orientational relaxation times; (3) the orientation mechanisms of the particles were investigated by the bipolar pulse technique. At the same time, the extent and the efficiency of DNA protection were respectively analyzed by micrococcal nuclease digestion, thermal denaturation, and circular dichroism.

It should be mentioned at the outset that the aim of the work presented here was not to study chromatin reconstitution. Since on one hand H1 was removed by 0.5 M NaCl treatment (Marion et al., 1983a; M. F. Hacques and C. Marion, unpublished results) and on the other hand the reassociation was done with subfractions, we did not expect to obtain reconstitutes exhibiting all of the physical properties of native chromatin.

We had however shown previously that the H1 subfractions H1-1 and H1° are able individually to reconstitute chromatin bearing several similarities to native chromatin (Biard-Roche et al., 1982) as reported in reconstitution studies with H1 (Allan et al., 1980b; Thoma & Koller, 1981; Klingholz & Strätling, 1982; Kaplan et al., 1984), H5 (Allan et al. 1980a; Kaplan et al., 1984), H1 + H5 (Fulmer & Fasman, 1979; Allan et al., 1981; Thoma et al., 1983), phosphorylated H1 (Kaplan et al., 1984), and different fragments of H1 and H5 (Thoma et al., 1983). Although circular dichroism, thermal denaturation, and nuclease digestion studies at low ionic strength tend to confirm this fidelity of reconstitution and fail to detect differences between reconstitutes and native chromatin, differences are observed with the more sensitive method of electric birefringence. Briefly, even with saturating amount of histones, reconstituted chromatin displays local morphological imperfections probably due to irreversible changes in DNA–protein interactions (M. F. Hacques and C. Marion, unpublished results). A nonperfect charge neutralization of DNA is observed. This failure of chromatin to reassociate may be due either to the binding of the C-terminal tail of H1 to an unfavorable place, preventing the globular region from finding site (Thoma et al., 1983), or to a nonreversible dissociation of basic core histone tails during the preparation of H1-stripped chromatin (Marion et al., 1983a,b).

Efficiency of H1 Subfractions in Condensation of Chromatin. By nuclease digestion and thermal denaturation, small differences have been shown between the protection and the stability that the two H1 subfractions confer to DNA, H1° being the less efficient in this process. This significant destabilization of chromatin caused by H1° compared to H1-1 was however not sufficiently large to be detectable by circular dichroism. On the contrary, electric birefringence revealed differences between H1-1 and H1° in their ability to condense stripped chromatin and recover the properties of native

chromatin. Indeed, the electric properties of the reconstituted samples are dependent both upon the stoichiometry and upon the nature of histone added.

When a molar ratio of 1 is used, we observe from the results summarized in Tables I and II that if about 67% of the linker DNA is reassociated to H1°, this interaction is not followed by significant conformational changes. We only note a slight decrease of the longest relaxation time without appreciable decrease of chromatin flexibility. When chromatin is reconstituted with H1-1, the amount of bound DNA is 75% (Table I) and the values of relaxation times are very close to that of native chromatin. The difference, then, between native whole chromatin and H1-1-reconstituted chromatin is the difference in the length of extended naked DNA.

From these results, one can conclude that with native stoichiometry a structural alteration is induced in chromatin due to the presence of H1° as compared to H1-1. With H1° the length of free extended DNA is longer, about three bp of DNA per nucleosome (Table I), and the conformation appears more extended as shown by the longer relaxation times (Table II), as compared to samples reconstituted with H1-1.

When more than the stoichiometric amount of H1 is bound to chromatin, a significant destabilization of chromatin was also caused by H1° compared to H1-1, as shown by the length of free DNA (Table I). On the other hand, the excess of histone introduces additional condensation since a drastic transition from an extended to a compact form is observed in the presence of two molecules of H1 per nucleosome. First, the relaxation time dramatically decreases from 300–400 to 12–14 μs, indicating the formation of a short compact structure more condensed than the native fibers. Second, the character of the birefringence kinetics changes drastically since the decay becomes monoexponential, reflecting the rigidity of chromatin fibers practically without internal motion. This highly compact structure exhibits an orientational relaxation behavior very close to that observed for native chromatin condensed by NaCl (Chauvin et al., 1985). We can therefore assume that reconstituted chromatin behaves hydrodynamically as cylinders (McGhee et al., 1980; Lee et al., 1981), and for a particle 30 nm in diameter, a relaxation time of 13 μs corresponds to an axial ratio of 1.5 (Lee et al., 1981). With the solenoid parameters of six nucleosomes per turn and 11-nm pitch (Finch & Klug, 1976), reconstituted chromatin would contain a mean number of nucleosomes of about 24. This is in good agreement with the mean number of 22 nucleosomes determined above from the Kerr constant of H1-depleted chromatin. This agreement is particularly interesting because the values are obtained from independent measurements, the one by using the Kerr constant, which depends on electrical and optical properties of particles, the other by using the rotational diffusion constant, which depends only on the shape and dimensions of particles.

Structural Role of H1° in the Organization of Chromatin. The differences observed in the properties of chomatin samples reconstituted with various H1 subfractions could be due to a specific mode of interaction of each subfraction with the linker DNA. These differences could be due to differences in the binding strength of histones to DNA or to the DNA lengths protected by the terminal regions of histones (specific interactions or not). Our results only clearly show a weaker protection of DNA by H1°: specific interactions could be explained by amino acid composition and/or different accessibility to binding sites of the two proteins. Histone H1 subfractions are conformationally homologous, which suggests that their interactions with H1-depleted chromatin are basically identical. The results obtained in reverse-pulse experiments showing similar orientation mechanisms for reconstituted chromatins agree with this fact.

On the other hand, Smith et al. (1984) have suggested that H1° and H5 belong to the same family, distinct from the H1 family. However H1° and H5 do show variation in the structure of their N- and C-terminal tail regions, and this fact may explain the apparent discrepancy between our results showing a weaker protection of DNA by H1° and the hypothesis of Cary et al. (1981). Indeed, they proposed that the globular part of H5 may bind more strongly to DNA than that of the corresponding region of H1 due to the larger arginine content of H5. Moreover, since the interactions between H5 and chromatin are found to be different from that of H1 (Thoma et al., 1983; Thomas & Rees, 1983), it is not surprising that the electrooptical behaviors of H1-1- and H1°-reassociated samples are different. The results of nuclease digestion experiments suggest however that the globular parts of the H1 subfraction are well positioned. They appear to be bound to the same specific site on the nucleosome, sealing off two turns of DNA (Smith & Johns, 1980). Since H1° complexes are more sensitive than H1-1 complexes to nuclease attack and the electric properties of native chromatin are less well recovered even with two molecules per nucleosome, their C-terminal tails, which bind more strongly to H1-depleted chromatin than the globular and N-terminal regions (Thoma et al., 1983), could be good candidates for this process. One has also to keep in mind that when native chromatin is depleted of H1, the H1-1 subfraction dissociates at a slightly higher NaCl concentration than the other subfractions (Thoma & Koller, 1981).

In conclusion, one can imagine that when in the cell nucleus H1° replaces H1 or a part of H1, the regions of chromatin that contains H1° may have a different structure or stability than those containing H1, due either to a less efficient binding of H1° to DNA or to a smaller neutralization of the DNA negative charges. This decrease of electrostatic interaction of histone with DNA confers a certain degree of flexibility to chromatin fibers, allowing H1° to play its functional role in replication or transcription. It is indeed not unreasonable to propose that, in response to local variations of ionic concentrations, the presence of H1° would permit the association with some other proteins, leading to a chromatin more resistant to nuclease attack (Gorka & Lawrence, 1979; Roche et al., 1985a) in relation to the physiological properties of this protein (Gjerset et al., 1982; Chabanas et al., 1983; Roche et al., 1985b). Our results are then additional arguments for Lennox (1984) to explain metabolic and evolutionary differences among H1 subfractions.

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