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Magnesium Binding and Conformational Change of DNA in Chromatin†

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ABSTRACT: The structure of chromatin in the presence of Mg^{2+} ions was examined by circular dichroism and equilibrium dialysis. (1) Circular dichroism (CD) shows that above 260 nm the intensity of the spectrum of DNA in nucleoproteins decreases as the Mg^{2+} concentration increases. This change is an intrinsic characteristic of DNA since it is also observed in protein-free DNA and has been attributed to a change in the winding angle of base pairs around the DNA axis. Some structural elements of the DNA in the nucleosome core, therefore, are as movable as those of protein-free DNA. (2) The basic organization of H1-depleted chromatin, 146 base pairs (bp) of DNA wound around core histones and a residual 49 bp in the linker region in the repeating unit, is maintained both in the presence and in the absence of Mg^{2+} ions, as shown by the fact that the CD spectrum of H1-depleted chromatin has the same type of linear combination between the spectrum of protein-free DNA and that of the nucleosome core in 0.2 mM $MgCl_2$–10 mM triethanolamine (pH 7.8) as it has in 1 mM ethylenediaminetetraacetic acid–10 mM tris(hydroxymethyl)aminomethane (pH 7.8). (3) The ellipticity of chromatin shows a smaller decrease relative to the other nucleoproteins and protein-free DNA upon the addition of Mg^{2+} ions. Therefore, some structural elements of chromatin are apparently somewhat protected against the conformational change induced by these ions. The spectrum of chromatin becomes almost indistinguishable from that of H1-depleted chromatin in 0.2 mM $MgCl_2$. (4) The number of phosphate groups which do not experience interference with Mg^{2+} ion binding by core histones is calculated to be at least 2 per 10 phosphate groups in the nucleosome core. Therefore, it is suggested that these groups are located on the outer surface of the nucleosome core DNA and are easily attacked by DNase I. (5) Chromatin binds less Mg^{2+} ions than H1-depleted chromatin. This may be due to the effect of H1 itself or the formation of higher order structure of chromatin.

The magnesium ion is widely distributed in biological systems and plays an important role in many enzymatic activities, especially reactions involved in replication, transcription, and translation. Ribosomes, transfer RNA, and many enzymes involved in these processes lose their biological activity in the absence of this ion. Furthermore, it is known that the magnesium ion maintains the tertiary structure of transfer RNA (Lindahl et al., 1966; Lynch & Schimmel, 1974a; Quigley et al., 1978) and the folding of ribosomal particles (Weiss & Morris, 1973a,b; King et al., 1981). The stability of double-helical DNA against thermal denaturation is also greatly enhanced by magnesium ions. Thus, a great deal of data on magnesium binding to DNA, transfer RNA, and ribosomes has been reported (Edelman et al., 1960; Choi & Carr, 1967; Sander & T'so, 1971; Danchin, 1972; Clement et al., 1973; Lynch & Schimmel, 1974a,b; Reuben & Gabbay, 1975).

In the formation of a higher order structure and condensation of chromatin, the requirement and effectiveness of magnesium ions has already been reported by many researchers (Leake et al., 1972; Billett & Barry, 1974; Finch & Klug, 1976; Kiryanov et al., 1976; Sperling & Klug, 1977; Thoma et al., 1979; Langmore & Schutt, 1980; McGhee et al., 1980). It is generally accepted that histone H1 is responsible for the formation of this structural organization of chromatin in the presence of magnesium ions. Histone H1 plays a significant role in the condensation of chromatin (Bradbury et al., 1973; Billett & Barry, 1974; Osipova et al., 1980) and is necessary for the formation of the 300-Å-thick fibers of chromatin (Thoma et al., 1979; McGhee et al., 1980). The effect of magnesium ions on the reversible swelling and contraction of chromatin and the nuclei has been examined in relation to its transcriptional activity (Leake et al., 1972),

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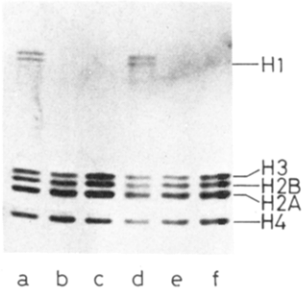

FIGURE 1: Gel electrophoresis of histones extracted from nucleoproteins. Chromatin (a), H1-depleted chromatin (b), nucleosome core (c) in 10 mM Tris (pH 7.8)–1 mM EDTA, chromatin (d), H1-depleted chromatin (e), and nucleosome core (f) left for 72 h in 10 mM TEACl (pH 7.8)–0.2 mM $MgCl_2$.

and the solubility of the chromatin fraction against the magnesium ion has a close relation to gene transcription. It has been reported that the Mg^{2+}-soluble fraction of chromatin obtained after brief digestion with DNase[1] II is enriched in actively transcribed DNA sequences (Billing & Bonner, 1972; Gottesfeld & Partington, 1977). Therefore, the interaction of magnesium ions with chromatin and changes in the secondary structure of DNA in chromatin induced by these ions are significant not only in the formation of the higher order structure but also in gene regulation, which seem to be closely related.

The structure of DNA in chromatin has been studied by circular dichroism (Cowman & Fasman, 1980; McClearly & Fasman, 1980; Watanabe & Iso, 1981), thermal denaturation experiments (Fulmer & Fasman, 1979), and binding experiments of ethidium bromide (Lawrence et al., 1976; Paoletti et al., 1977; Erard et al., 1979; Wu et al., 1980) or manganese (Clark & Felsenfeld, 1974; Girardet & Lawrence, 1979). In a previous paper, we analyzed the CD spectrum of nucleoproteins and showed that the spectrum of H1-depleted chromatin is representable by the linear combination of protein-free DNA and the nucleosome core above 260 nm (Watanabe & Iso, 1981). Here, we have studied by circular dichroism the conformational change of nucleoproteins induced by magnesium ions to learn whether their spectra maintained the same type of linear combination in the presence and absence of such ions. Quantitative analysis of binding isotherms of magnesium ions to nucleoproteins combined with the CD data clarified the structural organization of chromatin in the presence of these ions.

Materials and Methods

Isolation of Calf Thymus Nucleoproteins. Chromatin, H1-depleted chromatin, nucleosome cores, and protein-free DNA were isolated from calf thymus by the methods previously described (Watanabe & Iso, 1981) except that the chromatin was washed more than 3 times in 5 mM Tris-HCl (pH 7.8), 150 mM NaCl, and 1 mM EDTA after being isolated from the nuclei. The nucleosome core was eluted from a Sepharose 6B column (2.5 cm × 100 cm) equilibrated with 5 mM Tris-HCl (pH 7.8), 40 mM NaCl, and 0.2 mM EDTA. Little degradation of histones was detected after the dialysis experiment (Figure 1).

Equilibrium Dialysis Experiments. All of the dialysis experiments were carried out in TEACl (pH 7.8) at 4 °C. This buffer was used since it has been reported that the large cationic radius of the protonated triethanolamine reduces its binding to the negative phosphates on DNA so that it scarcely competes with divalent cations (Danchin, 1972; Schimmel, 1974). Actually, much more Mg^{2+} ion bound to the nucleoprotein in 10 mM TEACl than in 10 mM Tris-HCl (data not shown). Moreover, chromatin did not precipitate in 150 mM TEACl while it did in 150 mM Tris-HCl. Therefore, triethanolamine was used in our experiments as a buffer.

In order to avoid contamination with other metal ions, a stock solution of TEACl (500 mM) was passed over a Chelex-100 column and stored in plastic containers before use. Neither metal ions nor sodium ions were detected by atomic absorption in this buffer after elution from the column.

Visking tubes were treated as mentioned previously by Schreier & Schimmel (1974) in order to remove heavy metal ions.

The concentration of the nucleoproteins was varied between 150 μg/mL and 1 mg/mL. The nucleoproteins were extensively dialyzed against 10 mM TEACl before use. Equilibrium experiments were then carried out at 4 °C through dialysis (0.3 mL) against 500 mL of TEACl containing various concentrations of $MgCl_2$ between 1.25×10^{-6} and 3.0×10^{-4} M. The equilibration was obtained in 72 h by stirring the buffer outside the visking tubes with a magnetic stirrer. The time required for this was determined by monitoring the concentration of Mg^{2+} either in the visking tube containing the nucleoproteins or in a blank tube without nucleoprotein. Adsorption of Mg^{2+} to the visking tube was negligible compared to its concentration in the tube containing the nucleoproteins.

Since the amount of Mg^{2+} bound to the nucleoprotein was independent of the nucleoprotein used in our experiments (data not shown), the magnitude of the Gibbs–Donnan effect must be small. Theoretical calculation of the Gibbs–Donnan distribution ratio predicts no significant effect under the conditions used in our experiments. Therefore, no correction was made in our calculation of Mg^{2+} binding. The $s_{20,w}$ value of chromatin was 26 S right after the adjustment of the conditions to 0.2 mM $MgCl_2$ in 10 mM TEACl. This value was unchanged, and no increase in small DNA fragments was observed after leaving the nucleoproteins in the buffer containing 0.2 mM Mg^{2+} for 72 h.

Determination of Phosphorus and Mg^{2+} Concentrations. The phosphorus concentration of the nucleoproteins was calculated spectrophotometrically by using $\epsilon_{260} = 6600$ cm^{-1} M^{-1} (in nucleotide residues). The concentration of Mg^{2+} was determined by using a Shimadzu atomic absorption/flame emission spectrophotometer, AA-640-12. Samples were diluted with deionized and distilled water for atomic absorption and burned directly without removal of proteins or DNA. No difference was observed between concentrations determined from direct burning and those from which Mg^{2+} had first been extracted with trichloroacetic acid. This method has been successfully applied in the determination of Mg^{2+} bound to ribosomes (Edelman et al., 1960; Weiss & Morris, 1973). Expected values of Mg^{2+} concentration within experimental error were always obtained for the buffer outside the visking tubes, and control experiments demonstrated that triethanolamine (50 mM) did not interfere with the Mg^{2+} determination.

Circular Dichroism of Nucleoproteins. The CD spectra were recorded on a Jasco-J20 spectrophotometer using a 1.0-cm path-length cell at 25 °C. Nucleoproteins were dialyzed against 10 mM TEACl from which all of the divalent cations had been removed by passage though a Chelex-100

[1] Abbreviations: TEACl, triethanolamine hydrochloride; CD, circular dichroism; bp, base pairs; DNase, deoxyribonuclease; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; EDTA, ethylenediaminetetraacetic acid.

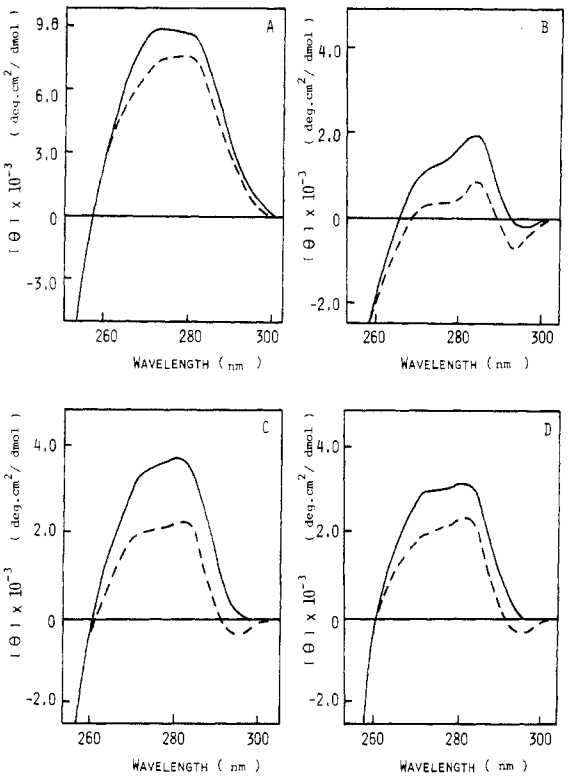

FIGURE 2: CD spectra of nucleoproteins at 0.2 mM EDTA (—) and 0.2 mM $MgCl_2$ (---) in 10 mM TEACl. (A) Protein-free DNA, (B) nucleosome core, (C) H1-depleted chromatin, and (D) chromatin.

column. Less than one Mg^{2+} ion per nucleosome remained in the nucleoprotein solution after dialysis. The CD spectra of nucleoproteins in the absence of divalent cations were measured in the presence of 0.2 mM EDTA; 12 μL of 100 mM EDTA was added to 6 mL of nucleoprotein solution. The Mg^{2+} concentration was adjusted by the addition of a small volume of 5×10^{-3} or 2.5×10^{-2} M $MgCl_2$ (16–240 μL added to 6 mL of nucleoprotein solution). The final concentration of Mg^{2+} in the nucleoprotein solution was determined by atomic absorption. Final concentration of nucleoproteins and light scattering effects were checked after the addition of Mg^{2+}. No large aggregation was observed in an analytical ultracentrifugation in 0.2 mM $MgCl_2$.

Results

Conformational Change of Nucleoproteins Induced by Mg^{2+}. Circular dichroism was measured in order to detect the Mg^{2+}-induced structural change of DNA in nucleoproteins. Both in the presence and in the absence of Mg^{2+}, protein-free DNA had higher ellipticity values above 260 nm than any of the nucleoproteins; the nucleosome core had the lowest values. Although the CD spectrum of H1-depleted chromatin exhibited higher ellipticity values above 260 nm than that of chromatin in the absence of Mg^{2+}, the spectrum of H1-depleted chromatin was indistinguishable from that of chromatin in the presence of Mg^{2+}. Addition of Mg^{2+} changed the CD spectra of nucleoproteins and protein-free DNA above 260 nm (Figures 2 and 3). On the other hand, ellipticity values below 260 nm were unchanged after the addition of Mg^{2+}. The constancy of the ellipticity at 245 nm, which is little affected by the addition of salts (Chan et al., 1979), confirms the CD spectrum change above 260 nm and eliminates the possibility of miscalculation of DNA concentration or a base-line shift during measurements.

A reproducible ellipticity maximum of the protein-free DNA was 8900 ± 300 deg·cm²/dmol at 275 nm in 10 mM

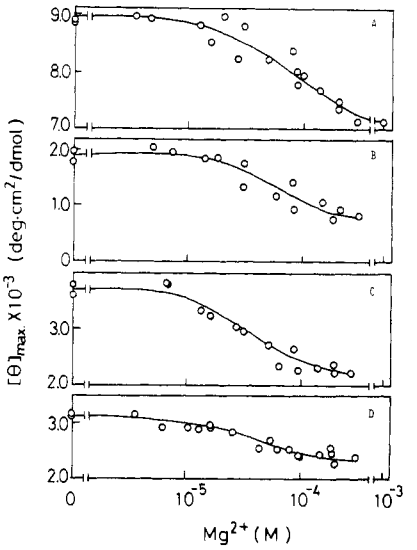

FIGURE 3: Ellipticity changes of nucleoproteins at their maximum with the addition of Mg^{2+}. (A) Protein-free DNA at 275 nm, (B) nucleosome core at 283 nm, (C) H1-depleted chromatin at 283 nm, and (D) chromatin at 283 nm. The curves are best fitted by eye.

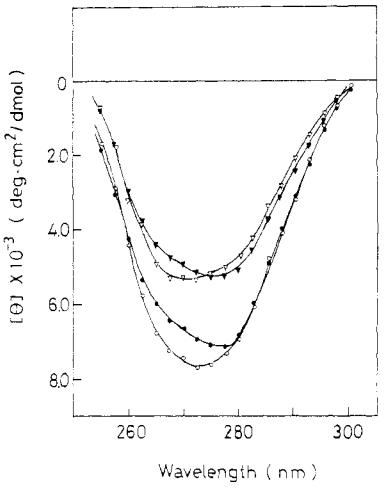

FIGURE 4: Difference spectra of nucleoproteins in 10 mM TEACl. Curves: Nucleosome core minus protein-free DNA in 0.2 mM EDTA (○) and 0.2 mM $MgCl_2$ (●); H1-depleted chromatin minus protein-free DNA in 0.2 mM EDTA (▽) and 0.2 mM $MgCl_2$ (▼).

TEACl–0.2 mM EDTA. The ellipticity at 275 nm started decreasing at a Mg^{2+} concentration around 1.0×10^{-5} M and reached 7200 ± 200 deg·cm²/dmol at 3.0×10^{-4} M Mg^{2+} (Figures 2A and 3A). The ellipticity value of −10 100 (SD = 520) deg·cm²/dmol at 245 nm, on the other hand, fluctuated within the experimental error.

The CD spectrum of the nucleosome core had an ellipticity maximum of 1900 ± 100 deg·cm²/dmol at 283 nm and a negative ellipticity of −300 ± 100 deg·cm²/dmol at 294 nm in 10 mM TEACl–0.2 mM EDTA (Figures 2B and 3B). At Mg^{2+} concentrations higher than 1.0×10^{-5} M, the positive ellipticity maximum was 850 ± 100 deg·cm²/dmol at 283 nm, and the negative ellipticity was −700 ± 100 deg·cm²/dmol at 294 nm. The ratio of $|[\theta]_{283}|/|[\theta]_{294}|$ in the CD spectrum of the nucleosome core changed from 6 ± 3 in 10 mM TEACl–0.2 mM EDTA to 1.2 ± 0.3 in 10 mM TEACl–0.2 mM $MgCl_2$. Ellipticity at 245 nm was invariably −12 100 (SD = 360) deg·cm²/dmol through all the Mg^{2+} concentrations used in our experiments. A difference of CD spectra between the protein-free DNA and the nucleosome core in 10 mM TEACl–0.2 mM EDTA was compared with that in 10 mM TEACl–0.2 mM $MgCl_2$ (Figure 4). The ellipticity values of

each difference spectrum differed from between 263 and 280 nm where the intensity of the difference spectrum in 0.2 mM $MgCl_2$ was a little smaller than that in 0.2 mM EDTA. However, an ellipticity change in the spectrum of the nucleosome core induced by Mg^{2+} reflected the ellipticity decrease of protein-free DNA with the addition of Mg^{2+}. With Mg^{2+} addition the DNA in the nucleosome core is able to change its conformation and was detectable with circular dichroism to almost the same extent as protein-free DNA.

An ellipticity decrease above 260 nm was also observed in H1-depleted chromatin when Mg^{2+} was added. The ellipticity maximum of H1-depleted chromatin was 3700 ± 200 deg·cm²/dmol at 283 nm in 10 mM TEACl–0.2 mM EDTA. On the other hand, in the presence of 0.2 mM Mg^{2+}, an ellipticity maximum of 2300 ± 150 deg·cm²/dmol was observed at the same 283 nm, and a small negative ellipticity appeared between 290 and 300 nm. Although the ellipticity decrease induced by Mg^{2+} ions started at 0.7×10^{-5} M Mg^{2+}, the ellipticity value of −11 600 (SD = 360) deg·cm²/dmol at 245 nm was constant. The difference in the CD spectrum between protein-free DNA and H1-depleted chromatin in 10 mM TEACl–0.2 mM EDTA was almost identical with that in 10 mM TEACl–0.2 mM Mg^{2+} but seemed to have a tendency to shift its ellipticity maximum to longer wavelengths (Figure 4). Consequently, this ellipticity decrease of H1-depleted chromatin caused by Mg^{2+} is the same effect as is observed in protein-free DNA and the nucleosome core. An intensity at 275 nm of the difference spectrum between H1-depleted chromatin and protein-free DNA in 10 mM TEACl–0.2 mM EDTA was 0.7 ± 0.05 of that between the nucleosome core and protein-free DNA (Figure 4). In 10 mM TEACl–0.2 mM $MgCl_2$, the intensity between H1-depleted chromatin and protein-free DNA at 275 nm corresponded to 0.73 ± 0.04 of that between the nucleosome core and the protein-free DNA. This ratio changed little before and after the addition of Mg^{2+} and did not contradict the ratio examined previously in 5 mM Tris-HCl–1 mM EDTA (Watanabe & Iso, 1981). In a case where the CD spectrum of H1-depleted chromatin is a linear combination of the spectra of protein-free DNA and the nucleosome core, this intensity ratio represents the ratio of the DNA in the core region of H1-depleted chromatin. Therefore, circular dichroism indicates that in H1-depleted chromatin, 146 ± 8 bp of DNA wound around core histones is maintained both in the absence and in the presence of Mg^{2+}.

The CD spectrum of the chromatin also showed a decrease in ellipticity above 260 nm with the addition of Mg^{2+}. The ellipticity of chromatin at 283 nm was 3100 ± 200 deg·cm²/dmol in 10 mM TEACl–0.2 mM EDTA while it was 2400 ± 200 deg·cm²/dmol in 10 mM TEACl–0.2 mM Mg^{2+}. This ellipticity decrease with the addition of Mg^{2+} was smaller than that of protein-free DNA, the nucleosome core, or H1-depleted chromatin. The CD spectrum of chromatin was almost identical with that of H1-depleted chromatin in the presence of 0.2 mM $MgCl_2$.

Mg^{2+} Binding Isotherm of Nucleoproteins and Protein-Free DNA. In order to clarify the effect of Mg^{2+} on the structure of the DNA in chromatin, the binding of Mg^{2+} to the various types of nucleoprotein was quantitatively compared. Scatchard plots (Scatchard, 1949; Scatchard et al., 1957) of Mg^{2+} binding to nucleoproteins and protein-free DNA in 10 mM TEACl are shown in Figure 5; here the results of two or three independent measurements are plotted. Protein-free DNA has the most and chromatin has the fewest binding sites for Mg^{2+} among the nucleoproteins examined. The nucleosome core binds Mg^{2+} ions slightly more extensively than chromatin, and

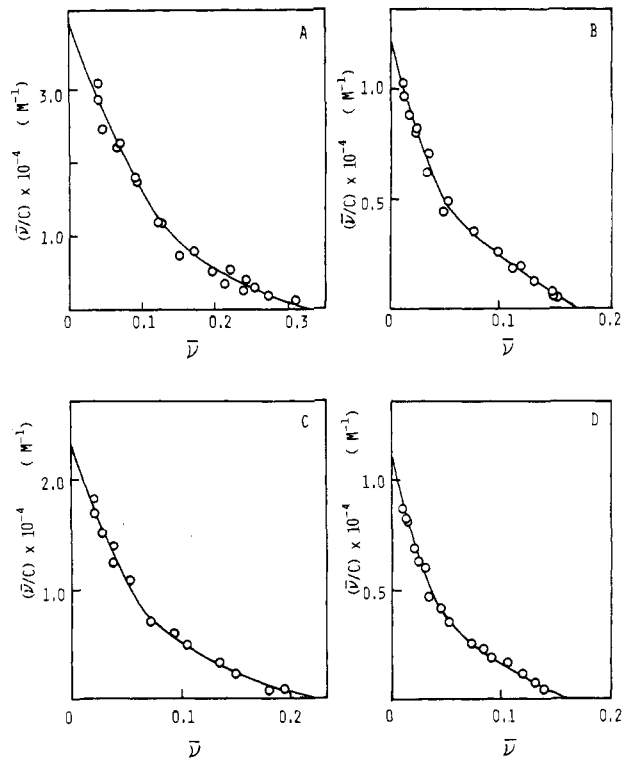

FIGURE 5: Scatchard plots of binding of Mg^{2+} ions to DNA and nucleoproteins in 10 mM TEACl. (A) Protein-free DNA, (B) nucleosome core, (C) H1-depleted chromatin, and (D) chromatin.

the amount of bound Mg^{2+} ions to H1-depleted chromatin is between that of the nucleosome core and that of protein-free DNA.

When each multiple binding site has an independent association constant (K_i), the number of binding sites (n_i) can be generally expressed by the following equation:

$$\bar{\nu} = \frac{n_i K_i C}{1 + K_i C} \quad (1)$$

where C is the concentration of free ligand and $\bar{\nu}$, the number of bound ligand molecules per macromolecule. For the simplest case of homogeneous binding sites the equation can be written as follows:

$$\bar{\nu}/C = K(n - \bar{\nu}) \quad (2)$$

The association constant (K) and the number of binding sites (n) can be obtained from a slope and an extrapolated value to the abscissa of the slope of plot $\bar{\nu}$ vs. $\bar{\nu}/C$ (Scatchard, 1949).

In 10 mM TEACl, the plots were curved, and their slope became less negative with increasing $\bar{\nu}$. A concave plot generally indicates that the binding sites are not independent but negatively interacting or heterogeneous. When a negative interaction occurs, a decrease in ligand binding affinity accompanies the binding of ligands to macromolecules. Negative interactions are marked whenever large numbers of ions are bound or where binding sites are in close proximity to each other. It is generally supposed that protein-free DNA has homogeneous binding sites for Mg^{2+} ions and that one Mg^{2+} ion binds to two phosphate groups electrostatically (Sander & T'so, 1971; Clement et al., 1973; Van Steenwinkel et al., 1981). Therefore, we assume that the curved Scatchard plot for protein-free DNA obtained here is not due to heterogeneous binding sites with different association constants but to negative interactions between bound Mg^{2+} ions. Since the association constant K can be expressed as $K = K_{DNA} \exp(-\omega \bar{\nu}_{DNA})$ in the case of negative interaction (Scatchard, 1949; Clement et al.,

Table I: Parameters for Magnesium Binding to Nucleoproteins in 10 mM TEACl, 4 °C

nucleoprotein	n_s	n_w	n_T	K_w (M^{-1})
protein-free DNA	0.33	0	0.33	0
nucleosome core	0.08	0.10	0.18	1.8×10^4
H1-depleted chromatin	0.15	0.08	0.23	1.6×10^4
chromatin	0.06	0.10	0.16	1.4×10^4

1973; Reuben & Gabbay, 1975), we revise eq 1 and 2 for protein-free DNA:

$$\bar{\nu}_{DNA} = \frac{nK_{DNA}C \exp(-\omega\bar{\nu}_{DNA})}{1 + K_{DNA}C \exp(-\omega\bar{\nu}_{DNA})} \quad (3)$$

$$\bar{\nu}_{DNA}/C = K_{DNA} \exp(-\omega\bar{\nu}_{DNA})(n - \bar{\nu}_{DNA}) \quad (4)$$

In the equation, ω is an average interaction parameter, n represents the average number of binding sites for Mg^{2+} ions per phosphate group, and K_{DNA} represents the intrinsic association constant for Mg^{2+} at an isolated DNA binding site. Each parameter was obtained from a simulation experiment using computer analysis. The average number of binding sites per phosphate group for protein-free DNA was determined to be 0.33 from the plot in 10 mM TEACl (Figure 5A). The n value of 0.33 obtained here compares fairly well with the expected value of 0.5 for the binding sites which was calculated from one Mg^{2+} ion per two phosphate groups of DNA. Therefore, the 0.33 value was assumed to indicate that all of the phosphate groups of DNA are equally available as Mg^{2+} binding sites. It is difficult to obtain this stoichiometric ratio from experiments of equilibrium dialysis due to the inaccuracy of the evaluation of $\bar{\nu}$ at higher concentrations of Mg^{2+} ions (Tanford, 1961). Values of $K_{DNA} = 1.3 \times 10^5$ M^{-1} and $\omega = 6.8$ were obtained from the simulation experiment. Various values for the association constant of Mg^{2+} ions to DNA have been reported previously ranging from 6.0×10^3 to 1.3×10^6 M^{-1} (Sander & T'so, 1971; Clement et al., 1973; Reuben & Gabbay, 1975).

The initial slope of the Scatchard plot for Mg^{2+} binding to each nucleoprotein was almost identical with that for protein-free DNA (Figure 5). We therefore assumed that some of the Mg^{2+} binding sites of nucleoproteins have the same association constant as that of protein-free DNA. Then, two kinds of binding sites were assumed for the nucleosome core. K_{DNA} and ω values obtained for protein-free DNA were used for the stronger binding sites. Residual binding sites were designated as weaker binding sites with an average association constant of K_w. This gives the equation for the nucleosome core as

$$\bar{\nu} = \frac{n_s K_{DNA}C \exp(-\omega\bar{\nu})}{1 + K_{DNA}C \exp(-\omega\bar{\nu})} + \frac{n_w K_w C}{1 + k_w C} \quad (5)$$

By successive approximations using computer analysis the Scatchard plots were simulated and the values of n_s, n_w (the average number of strong and weak binding sites per phosphate group), and K_w were obtained (Table I). $n_s + n_w$ was used as the value of n_T (the number of total binding sites per phosphate group in the nucleosome core), and this agreed well with the value obtained by extrapolation of the plot. From these values, the ratio among the number of phosphate groups shielded by core histones to those having weak binding sites and those having strong binding sites was calculated to be

$$n_c{:}n_w{:}n_s = 46{:}30{:}24$$

The 292 phosphate groups of DNA in the nucleosome core are classified into three kinds of phosphate groups: those covered by core histones (P_c), those with weak binding sites (P_w), and those having strong binding sites (P_s) (Figure 6). The number in category is

$$P_c = 133 \qquad P_w = 88 \qquad P_s = 71 \qquad \sum P = 292$$

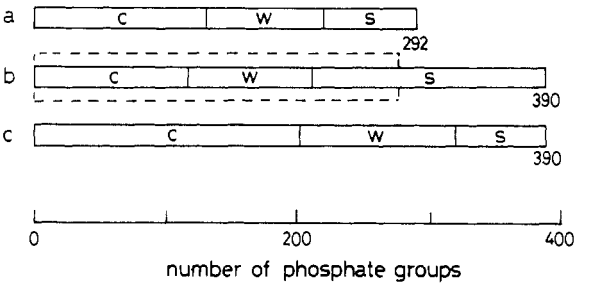

FIGURE 6: Schematic drawing of phosphate groups belonging to covered, weak and strong binding sites in the nucleoproteins. Columns: (a) 292 phosphate groups of DNA in the nucleosome core. (b) 390 phosphate groups of DNA in the H1-depleted chromatin. The region enclosed by the dashed rectangle represents 70% of the 390 phosphate groups. (c) 390 phosphate groups of DNA in chromatin. Rectangles denoted as c, w, and s represent the phosphate groups of DNA involved in the covered region and weak sites and strong binding sites in the nucleoproteins, respectively.

The core histones do not interfere with Mg^{2+} binding to the phosphate groups belonging to the strong binding site class. Therefore, about two phosphate groups per single turn of B-form DNA strand are free of this interference.

Equation 5 was also applied to H1-depleted chromatin and chromatin (Table I). The association constants of the weak binding sites of H1-depleted chromatin were consistent with that of the nucleosome core. Therefore, it seems that these weak sites in H1-depleted chromatin and chromatin exhibit the same kind of weak Mg^{2+} binding as observed in the nucleosome core. The weaker binding sites are possibly produced by the neighboring positively charged groups of core histones which interfere with Mg^{2+} binding to phosphate groups through electrostatic repulsion. Other parts of the DNA phosphate groups in nucleoproteins are covered by core histones. Phosphate groups in H1-depleted chromatin belonging to these different binding sites numbered

$$P_c = 118 \qquad P_w = 95 \qquad P_s = 177 \qquad \sum P = 390$$

When these values were compared with those of the nucleosome core, a difference in P_s value was great relative to the other sites (Figure 6). Thus, the phosphate groups of DNA in the H1-depleted chromatin can be divided into those from the core region and those from the linker region. In contrast, P_c and P_w show little difference between H1-depleted chromatin and nucleosome core. The relative increase in the P_s of H1-depleted chromatin suggests that many strong binding sites are in its linker region and that its core region has the P_w and the P_c groups. The structure of the core region in H1-depleted chromatin thus is probably almost the same as that of the nucleosome core, and the linker region does not interact with core histones. Since the difference in P_s between H1-depleted chromatin and nucleosome core is 106 phosphate groups, the length of the linker region is 53 bp assuming that all the additional P_s groups reside in that region. The residual 284 of 390 phosphate groups in the repeating unit of DNA must therefore be in the core region. In other words, Mg^{2+} binding experiments show that 142 bp of the DNA belong to the core region and 53 bp to the linker region of H1-depleted chromatin. The CD experiments have shown that the H1-depleted chromatin has a DNA length of about 140 bp in the core region and 55 bp in the linker region either in the presence or in the absence of Mg^{2+} ions. Here, the same results have been independently obtained by Mg^{2+} binding experiments.

The number of phosphate groups covered with histones in chromatin itself was

$$P_c = 201 \quad P_w = 118 \quad P_s = 71 \quad \sum P = 390$$

It is known that the core region of chromatin contains 166 bp of DNA which is wound two full turns around the histone core. Assuming that this has the same Mg^{2+} binding characteristics as the 146 bp DNA segment in the nucleosome core and core region of H1-depleted chromatin, the number of the phosphate groups belonging to each binding site can be calculated as

$$P_c = 151 \quad P_w = 101 \quad P_s = 80 \quad \sum P = 332$$

Subtracting these values from those for chromatin gives the following differences:

$$\Delta P_c = 50 \quad \Delta P_w = 17 \quad \Delta P_s = -9$$

On the other hand, chromatin contains histone H1 in addition to core histones. Since the net charge of H1 is +53, 53 phosphate groups can be covered by H1 in chromatin. Therefore, it seems that ΔP_c of 50 represents apparently the charge neutralization of phosphate groups in the presence of H1. The interference of H1 with the Mg^{2+} binding to peripheral phosphate groups will change P_s to P_w corresponding to $\Delta P_w = 17$.

From these values, the phosphate groups of DNA in chromatin are covered more than H1-depleted chromatin and nucleosome core. This is due to the presence of H1. The relatively small number of phosphate groups in the strong binding site suggests that protein-free DNA region, i.e., linker region, is short in chromatin.

Although triethanolamine ions compete with Mg^{2+} less than the other ions (see Materials and Methods), the binding affinity of Mg^{2+} to the nucleoproteins and protein-free DNA was shown to be strongly dependent upon triethanolamine concentration since the slope of the plots decreased in 50 mM TEACl relative to that in 10 mM TEACl (data not shown). Therefore, triethanolamine ions bind to DNA and compete with Mg^{2+} to some extent.

Discussion

The conformational change of both protein-free DNA and nucleoproteins induced by Mg^{2+} ions was detected by circular dichroism. It has been reported that the CD spectrum of protein-free DNA changes little with the addition of Mg^{2+} ions (Luck & Zimmer, 1972). We have shown, however, that the ellipticity values of protein-free DNA decrease above 260 nm as a function of increasing Mg^{2+} ion concentration in 10 mM TEACl (pH 7.8). In 0.2 mM $MgCl_2$ containing 10 mM TEACl, the ellipticity values of protein-free DNA and nucleoproteins are smaller than those in 10 mM TEACl–0.2 mM EDTA above 260 nm. It is known that the ellipticity of protein-free DNA above 260 nm decreases as a linear function of an increase in the winding angle of base pairs of DNA (Chan et al., 1979; Johnson et al., 1981). In general, the winding angle changes depending upon the cation species in the aqueous solution, the concentration of the cation, and the temperature (Wang, 1969, 1974; Anderson & Bauer, 1978). The Mg^{2+} ion is far more efficient in changing the winding angle of DNA than Na^+ ion (Wang, 1969; Anderson & Bauer, 1978). Therefore, the decrease in the ellipticity of protein-free DNA and the nucleoproteins above 260 nm induced by Mg^{2+} ions can be most simply explained by an increase in the winding angle of DNA and a corresponding decrease in the number of base pairs per turn.

The ellipticity change of protein-free DNA induced by Mg^{2+} almost parallels that of the nucleosome core except that the difference spectrum is skewed to longer wavelengths in the latter. Therefore, the DNA in the nucleosome core is able to change its structural parameters through Mg^{2+} binding, and the change is detectable with circular dichroism to almost the same extent as in the case of protein-free DNA. It has been reported that both grooves of DNA in the nucleosome core are nearly as accessible to the solvent as those in protein-free DNA (McGhee & Felsenfeld, 1979), and only 15% of the phosphates of the DNA termini of nucleosome core are involved in intimate charge–charge interactions with histones (McGhee & Felsenfeld, 1980). Furthermore, the core histones appear to shield phosphate groups of DNA asymmetrically on one side of the surface of the DNA double helix along all its length inside the nucleosome core while the external side is almost free (Mirzabekov, 1980). We conclude that some structural elements of the DNA in the nucleosome core are as movable as those of protein-free DNA.

On the other hand, the ellipticity decrease induced by Mg^{2+} ions is smaller in chromatin than in protein-free DNA and other chromatin-derived nucleoproteins. This indicates that these ions have only a slight effect on the structure of chromatin because of its protection against the conformational change induced by Mg^{2+} ions. Histone H1 itself or a higher order structure of chromatin formed by H1 conserves DNA conformation in the core region of chromatin in the presence of these ions. It is known that H1 stabilizes the structure of chromatin against changes in ionic strength (Fulmer & Fasman, 1979; Osipova et al., 1980; Watanabe & Iso, 1981). Otherwise, the formation of a higher order of structure of chromatin in $MgCl_2$ could produce new spectra and compensate for the ellipticity decrease by Mg^{2+} ions.

It has been reported that about 40–60% of the total phosphate groups of DNA are covered with histones in chromatin (Leake et al., 1972; Schmidt et al., 1972; Clark & Felsenfeld, 1974; Jacobs et al., 1976) and about 40% in the nucleosome core (Girardet & Lawrence, 1979). The Mg^{2+} binding results obtained here for both chromatin and the nucleosome core are in this range. On the other hand, McGhee & Felsenfeld (1980) have shown that 15% of the phosphates of the DNA termini of nucleosome core are involved in the intimate charge–charge interactions with core histones. X-ray diffraction results (Finch et al., 1981) and nanosecond fluorescence anisotropy decay measurements (Ashikawa et al., 1983) have also shown that the DNA is not firmly fixed to the core histones all along the superhelical path in the nucleosome core and fixed regions are those which are less susceptible to DNase I digestion. Therefore, most of the covered region against Mg^{2+} in the nucleosome core may be concentrated on the protected sites against nuclease digestion [Figure 8b of Finch et al. (1977)].

By the equilibrium dialysis experiments, Mg^{2+} binding sites in chromatin were decreased by 83 phosphate groups relative to the sites in H1-depleted chromatin. It is impossible to attribute all of this coverage solely to the electrostatic interaction of H1 since H1 has only 53 basic amino acid residues. One of the possible explanations is that some of the phosphate groups which are free in H1-depleted chromatin interact with core histones additionally. It has been reported that the N-terminals of histone H2A and H2B are free and those of histone H3 and H4 are weakly bound in the nucleosome core (Gary et al., 1978). Furthermore, an extended interaction of histone H3 with DNA beyond 146 bp of the core DNA is observed in H1-containing nucleosomes which have 165 or 175 bp of DNA (Mirzabekov, 1980). Therefore, it is probable that the N-terminals of core histones interact with more than 146

bp of DNA in chromatin to form two full turns of DNA around the core histones. However, since the entire major groove of DNA in chromatin is solvent accessible, the interactions with histones are competitively available to Mg^{2+}, and the quantitative difference in the magnitudes of the binding shown by the Scatchard plots may be affected by this heterogeneity.

Electron microscopic observation has revealed that a higher order structure of chromatin emerges in the presence of either 0.2 mM Mg^{2+} or 60 mM Na^+ (Thoma et al., 1979). It is known that the binding of Mg^{2+} ions to DNA is competitively inhibited by Na^+, K^+, and tris(hydroxymethyl)aminomethane ions (Schmidt et al., 1972) although Mg^{2+} ion binds to DNA much more efficiently than other cations. The structure of DNA in chromatin is more resistant to the conformational change induced by Mg^{2+} ions than the other chromatin-derived nucleoproteins. Therefore, the conformation of nucleosome structure of chromatin is preserved when the higher order structure is formed by the addition of Mg^{2+} ions. Modification of histones in chromatin makes it possible to change its conformation to one suitable for biological functions. For example, the DNA may be able to change its conformation more easily for replication when the binding of H1 to chromatin becomes weak through phosphorylation during M and S phases. In transcription, most of the nucleosomes containing hyperacetylated histones are involved in the Mg^{2+}-soluble chromatin fraction which is enriched in transcriptionally active genes (Perry & Chalkley, 1981). The acetylation of histones is considered to weaken their binding to DNA and to increase the structural freedom of DNA. Moreover, the conformation of the nucleosome that is necessary for the biological functions may be related to divalent cations and histone modifications. Therefore, knowledge of the conformational changes and Mg^{2+} binding properties of chromatin will be essential to understanding the mechanism of chromatin function.

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Nature of DNA Repair Synthesis Resistant to Inhibitors of Polymerase α in Human Cells†

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ABSTRACT: Arabinocytidine and aphidicolin are inhibitors of α-DNA polymerase that have been shown to affect both normal DNA replication and repair synthesis in mammalian cells. In contradiction to the prevalent hypothesis that these inhibitors merely slow the polymerization rate at incision sites near lesions, our results suggest that the repair synthesis resistant to inhibitors is mediated by a separate pathway. Repair synthesis in contact-inhibited human cells following UV irradiation was inhibited 75–80% by arabinocytidine or aphidicolin, and most of the repair patches were not ligated into parental DNA, as judged by an enzymatic assay. However, the patches were not demonstrably shorter than those in untreated cells. Even following low-UV doses at which no inhibition of repair synthesis by the inhibitors was observed, a majority of the patches were not ligated. DNA polymerase β is implicated in this alternate pathway, both by the known specificity of the inhibitors and by evidence from their sensitivity to S1 nuclease that the patches arise from displacement synthesis. The unligated patches are not degraded in vivo and eventually become ligated into parental DNA, very slowly in the presence of inhibitors but much more rapidly following their removal. Thus, under conditions of α-polymerase inhibition, a limited number of normal length repair patches are made, apparently by displacement synthesis, leaving displaced strands that remain substantially undegraded.

Most types of cells can remove damage from their DNA by the process of excision repair (ER),[1] whereby a short stretch of the DNA strand containing the damage is removed and replaced by new DNA, synthesized by using the undamaged complementary strand as template (Hanawalt et al., 1979). This process appears to be responsible for much of the resistance of both prokaryotic and eukaryotic cells to killing by short-wavelength ultraviolet light and a number of chemical damaging agents. Our understanding of ER in *Escherichia coli* is relatively well advanced because of the ease with which both genetic manipulations and biochemical studies may be done with this organism. Although we understand well the broad outlines and overall features of the process in mammalian cells, many of its details at the molecular level remain obscure. Chemicals that inhibit specific molecular processes can offer an alternative to genetic manipulation, and in recent years, inhibitors of DNA synthesis have been used to obtain information about the roles of the various eukaryotic DNA polymerases in ER and to facilitate studies of the incision step in that process (Collins & Johnson, 1984).

The frequencies of incision breaks in the cellular DNA remain small even after high UV doses, presumably because the rate of incision is much slower than the rates of the subsequent steps. Many investigators have used inhibitors of DNA synthesis (usually a combination of hydroxyurea and arabinocytidine) to retard completion of repair events, conditions under which the frequency of DNA single-strand breaks is increased (Collins & Johnson, 1984; Ben-Hur & Ben-Ishai, 1971; Hiss & Preston, 1977; Collins, 1977; Erixon & Ahnström, 1979). When we began this study, it had been reported that these conditions also reduced DNA repair synthesis in UV-irradiated human cells (Dunn & Regan, 1979). Subsequently, a number of investigators have shown that at least in contact-inhibited cells, ara-C or aphidicolin alone can inhibit repair synthesis, although not totally (Johnson et al., 1982; Snyder & Regan, 1982; van Zeeland et al., 1982).

The prevailing hypothesis to explain these effects is that incision proceeds normally (at least to a limited extent) in the presence of the inhibitors but that at each incision site the rate of repair synthesis is so diminished that a single-strand interruption remains, either because a gap made by prior exonuclease action remains or because ligation of a patch cannot take place until it reaches a certain size. We set about to test

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[1] Abbreviations: apc, aphidicolin; ara-C, arabinocytidine (1-β-D-arabinofuranosylcytosine); ER, excision repair; HU, hydroxyurea; mtDNA, mitochondrial DNA; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid; Cl_3CCOOH, trichloroacetic acid; FdUrd, fluorodeoxyuridine; BrdUrd, bromodeoxyuridine; BrUra, bromouracil.

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