width of the minor groove (Drew, 1984; Lomonossoff et al., 1981; Drew & Travers, 1981; Suck & Oefner, 1986), the observed sequence-dependent affinities of **5** are probably due to significant differences in local DNA structure associated with sites of type $(G\cdot C)_3(A\cdot T)$.

Comparison of the results of this study with those involving the prototype series of lexitropsins (Lown et al., 1986a) suggests that the reduction of charge and/or alteration of one of the van der Waals contacts between the ligand and DNA has a significant effect on binding specificity. Studies currently in progress are designed to separate the contributions that each of these factors may have in controlling specificity.

Registry No. **2**, 23999-81-7; **3**, 109528-35-0; **4**, 109528-36-1; **5**, 109528-37-2; [[1-methyl-4-[(1-methyl-4-aminoimidazol-2-yl)-carboxamido]pyrrol-2-yl]carboxamido]propionamidine hydrochloride, 109528-38-3; formylimidazole, 3197-61-3; [[1-methyl-4-[(1-methyl-4-aminopyrrol-2-yl)carboxamido]imidazol-2-yl]carboxamido]propionamidine hydrochloride, 109528-39-4; [[1-methyl-4-[(1-methyl-4-aminoimidazol-2-yl)carboxamido]imidazol-2-yl]-carboxamido]propionamidine hydrochloride, 109528-40-7.

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A Reexamination of the Reported B → Z DNA Transition in Nucleosomes Reconstituted with Poly(dG-m^{5}dC)·Poly(dG-m^{5}dC)†

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ABSTRACT: Polynucleosomes with poly(dG-m^{5}dC)·poly(dG-m^{5}dC) have been reconstituted, and well-defined nucleosome core particles from these have been prepared. Upon addition of $MgCl_2$ to the levels used to induce the B to Z transition in this highly methylated DNA, significant changes in the circular dichroism spectrum are observed in solutions of these particles. However, such core particles also exhibit a noticeable instability when compared to chicken erythrocyte core particles under the same conditions. The change in circular dichroism can be entirely accounted for on the assumption that only free nucleotide, released by core particle dissociation, undergoes the B → Z transition. Therefore, no evidence has been found for "Z nucleosomes" in these solutions. In fact, the histone–DNA interaction in the nucleosome seems to partially inhibit the B to Z transition of the DNA. The analysis of our results is consistent with a model in which all of the DNA that remains bound to the histone octamer retains the B form.

Since of discovery of the left-handed conformation of DNA by analysis of poly(dG·dC) synthetic polymers in the presence of solutions of high ionic strength (Pohl & Jovin, 1972) and by X-ray crystallography (Wang et al., 1979), several attempts have been made to ascertain the biological relevance of this DNA form in vivo [see Rich et al. (1984) for a review]. At the structural level, the existence in eukaryote organisms of this left-handed DNA (also called Z DNA), as opposed to the canonical right-handed B form of DNA, would obviously be expected to introduce topological changes and constraints in those regions of chromatin associated with it. The first question thus arising is how the B to Z DNA transition (in those sequences with potential for such a transition) may affect the most elementary subunit of chromatin—the nucleosome.

Most of the studies undertaken to answer this question have taken advantage of the fact that poly(dG-m^{5}dC) can be induced to undergo the B to Z transition under very mild con-

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ditions, such as in the presence of millimolar amounts of divalent ions (Behe & Felsenfeld, 1981). Such conditions in turn may be easily achieved under in vivo situations.

The reconstitution studies carried out so far using poly(dG-m^{5}dC) and histones are in clear contradiction. On the one hand, Nickol et al. (1982) were not able to reconstitute nucleosomes on Z DNA formed in the presence of Co$(NH_3)_6^{3+}$. Furthermore, when they attempted to induce poly(dG-m^{5}dC) nucleosomes to undergo the B to Z transition by heating in 0.075 mM $MgCl_2$, only aggregated products were formed. In contrast, Miller et al. (1985, 1986), using the same DNA polymer and a nucleosome-assembling factor, have reported not only reconstitution of Z DNA containing nucleosomes but also induction of the B to Z transition in poly(dG-m^{5}dC) nucleosomes. Furthermore, it has been stated (Miller et al., 1986) that the assembly of DNA into histone octamers not only allows for that transition but in fact also facilitates it.

The transition from B to Z DNA, promoted by methylation for instance, may play an important regulatory role in vivo. It is therefore important to clearly understand how such dynamic behavior may affect the structure of chromatin. In this study we have reconstituted and isolated B DNA nucleosomes from poly(dG-m^{5}dC) and chicken erythrocyte histone octamers. The well-defined nucleosome core particles obtained have been further subjected to conditions where the free methylated polynucleotide is induced to go from its B to its Z conformation and the stability of the nucleosome cores under these conditions has been analyzed.

Materials and Methods

Reagents. Poly(dG-m^{5}dC)·poly(dG-m^{5}dC) with an average length of ~1900 base pairs (bp) was purchased from Pharmacia (Milwaukee, WI). Chicken erythrocyte histone octamers were obtained from chicken erythrocyte nucleosome core particles after adsorption of the DNA on hydroxylapatite as described by Simon and Felsenfeld (1979).

Polynucleosome Reconstitution and Nucleosome Isolation. Polynucleosome reconstitution was carried out by salt gradient dialysis (Tachell & van Holde, 1977). In this case, 1 volume of poly(dG-m^{5}dC)·poly(dG-m^{5}dC) at ≃1.3 mg/mL in 2 M NaCl and 10 mM tris(hydroxymethyl)aminomethane hydrochloride (Tris-HCl) (pH 8.0) was mixed with 1 volume of histone octamers at ≃0.9 mg/mL in the same buffer, and the final concentration of DNA in the mixture was adjusted to 0.35 mg/mL by addition of 2 M NaCl and Tris-HCl (pH 8.0) buffer. After incubation in the cold room for 4–6 h, the mixture was dialyzed in Spectrapor 3 (Spectrum Medical Industries, Inc., Los Angeles, CA) against 1.5 M NaCl for 4 h, 1.0 M NaCl for 8 h, 0.75 M NaCl for 4 h, 0.5 M NaCl for 6 h, and finally 50 mM Tris-HCl (pH 8.0) for 12 h. In all cases, the buffer was 10 mM Tris-HCl (pH 8.0), and the whole process was carried out at 4 °C. The final concentration of the DNA at the end was approximately half that at the beginning (i.e., ~0.18 mg/mL) as a consequence of dilution during dialysis. The sample thus obtained was concentrated in a Centricon 30 (Amicon Corp., Danvers, MA) up to ≃1 mg/mL of DNA. The polynucleosome sample was brought to 40 mM NaCl and 2 mM $CaCl_2$ and was finally digested for 6–8 min with micrococcal nuclease (Worthington) at ≃250 units/mg of DNA. The appropriate time was chosen from an analytical time course of digestion carried out as described by Lutter (1978). The digestion was stopped by addition of a small volume of 200 mM ethylenediaminetetraacetic acid (EDTA) (pH 8.0) to a final concentration of 10 mM on ice, and the sample was then loaded on top of a 5–20% sucrose gradient containing 50 mM NaCl and 10 mM Tris-HCl (pH 8.0) and was centrifuged for 18 h at 33000 rpm at 4 °C in an Beckman SW40 rotor. The fractions containing the core particles were then pooled, dialyzed against 50 mM Tris-HCl (pH 8.0), and concentrated in a Centricon 30.

Determination of Concentration. Histone and polynucleotide concentrations were measured spectrophotometrically in a Varian 2200 spectrophotometer by using E_{230} = 4.2 cm^2 mg^{-1} for the histone octamer (Stein, 1979) and E_{260} = 21.1 cm^2 mg^{-1} for poly(dG-m^{5}dC)·poly(dG-m^{5}dC). This latter value was calculated on the basis of ϵ_{260} = 7100 cm^{-1} M^{-1} measured for poly(dG-m^{5}dC)·poly(dG-m^{5}dC) (Pohl & Jovin, 1972).

Circular Dichroism. Circular dichroism experiments were carried out in a Jasco J-41 spectropolarimeter at 23 °C in thermostated cells of 1-cm path length. The starting A_{260} of the sample in all cases was ≃0.4. The spectra were recorded at a scale expansion of 1 mdeg/cm with a time constant of 4 s and at 5 nm/cm at a scan rate of 2.5 nm/min. The different concentrations of $MgCl_2$ were achieved by addition of known small amounts of 100 mM $MgCl_2$ to the starting sample (in 50 mM Tris-HCl, pH 8.0). The addition was carried out slowly under vigorous stirring.

Gel Electrophoresis. Gel electrophoresis of core particles was carried out in 4% polyacrylamide gels as described by Yager and van Holde (1984). The samples were dissolved in 1 volume of sample buffer with or without $MgCl_2$ added. Three sample buffers were used: (a) 40 mM Tris-HCl, 20 mM sodium acetate, 1 mM EDTA, and 20% glycerol; (b) the same as (a) but containing 20% sucrose instead of glycerol; (c) the same as (a) but containing also 0.3% bromophenol blue and 0.4% sodium dodecyl sulfate (SDS).

To evaluate the amount of nucleosome that had dissociated into free DNA, i.e., to calibrate the gel in order to evaluate the amount of free DNA present in a given sample, a mixture containing aliquots of all the samples being analyzed was mixed 1:1 (v/v) with sample buffer c and warmed to 37 °C for 5–10 min. Then different known amounts of that sample were loaded onto the gel. Usually six samples were loaded, covering the range of 5–50% free DNA.

Analytical Ultracentrifuge Analysis. Sedimentation velocity experiments were carried out in a Beckman Model E analytical ultracentrifuge. Samples with an $OD_{260} \leq 0.2$ were run in 3-cm double sector cells in an AN-K rotor. Samples at OD_{260} = 0.6–0.8 were run in a 1-cm double sector cell in an AN-F rotor. All experiments were performed at 18–20 °C and at 40000 rpm. The scans were recorded at 265 nm. To evaluate the relative amounts of free DNA and that of the remaining undissociated particles, the heights of the boundaries were corrected for the radial dilution effect.

Results

We have reconstituted nucleosome core particles by using poly(dG-m^{5}dC)·poly(dG-m^{5}dC), chicken erythrocyte histone octamers, and a stepwise dialysis salt gradient. Figure 1A,B shows the fractionation of the reconstitute in a sucrose gradient after digestion with micrococcal nuclease. As can be seen there, the nucleosome core particles sediment in a sharp symmetrical peak. Figure 1C shows the size homogeneity of the polynucleotide associated with these core particles, which corresponds to 146 ± 1 bp. It should be noted that the reconstituted nucleosomes, as prepared, show very little contamination with free DNA or larger oligomers. On the gel shown in Figure 1B, free DNA would run at the point indicated by the arrow. However, when nucleosomes are stored at low concentration in 50 mM Tris-HCl (pH 8.0), a slight

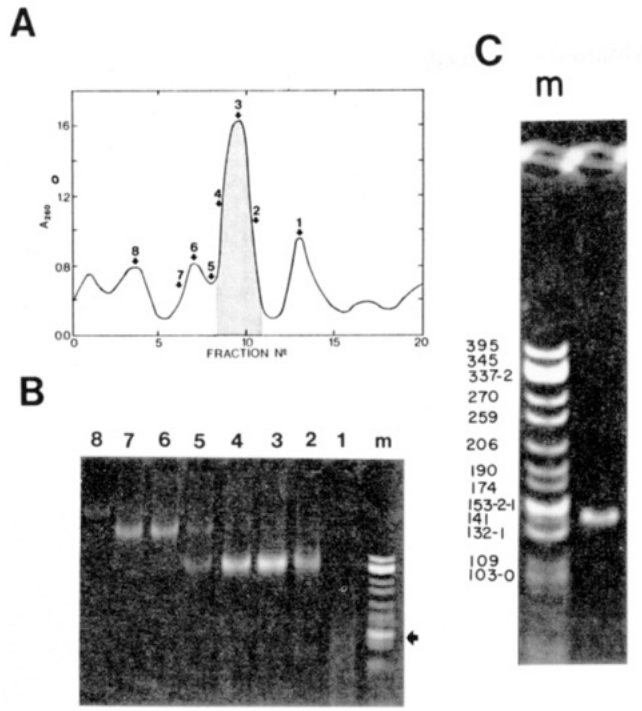

FIGURE 1: (A) Sucrose gradient fractionation of the reconstituted poly(dG-m^{5}dC)·poly(dG-m^{5}dC)–chicken erythrocyte histone complexes after digestion with micrococcal nuclease. The gradient was linear from 5% to 20% sucrose in 10 mM Tris-HCl (pH 8.0) and 50 mM NaCl buffer. The sample fractionation was carried out at 4 °C at 33000 rpm for 18 h in a Beckman SW40 rotor. The direction of the sedimentation is from right to left. (B) Four percent polyacrylamide native gel electrophoretogram of the fractions of the gradient marked with numbered arrows in (A). The arrow indicates where the free DNA would migrate [see (C)]. (C) Electrophoretic analysis of the piece of poly(dG-m^{5}dC)·poly(dG-m^{5}dC) polymer from the reconstituted nucleosome core particles corresponding to the shaded region of the gradient in (A). (m = pBr322 cut with *Cfo*1 used for size calibration).

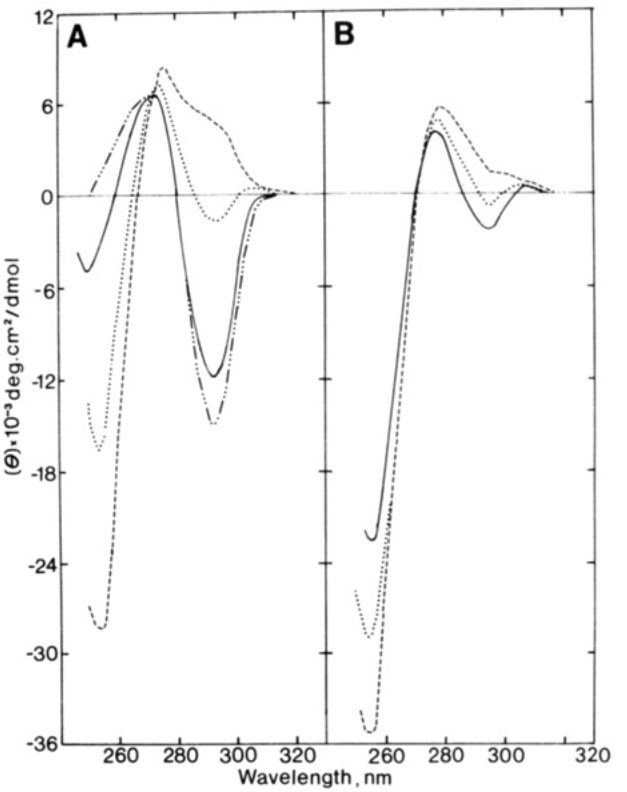

FIGURE 2: (A) Circular dichroism spectra from poly(dG-m^{5}dC)·poly(dG-m^{5}dC) in 50 mM Tris-HCl (pH 8.0) buffer containing (---) 0 mM $MgCl_2$, (···) 1 mM $MgCl_2$, (—) 3 mM $MgCl_2$, and (–·–·–) 5 mM $MgCl_2$. (B) Circular dichroism spectra of the poly(dG-m^{5}dC)·poly(dG-m^{5}dC)–histone erythrocyte nucleosomes in 50 mM Tris-HCl (pH 8.0) buffer containing (---) 0 mM $MgCl_2$, (···) 1 mM $MgCl_2$, and (—) 3 mM $MgCl_2$. The molar ellipticity ([θ]) was calculated on the basis of DNA nucleotide residue concentration.

amount of dissociation occurs (see Figure 3 and below).

Following Miller et al. (1986), we have tried to induce these particles to undergo the right- to left-handed transition by addition of $MgCl_2$ in millimolar amounts. The results are shown in Figure 2. This figure compares the effect of magnesium chloride on the free poly(dG-m^{5}dC)·poly(dG-m^{5}dC) before reconstitution and after being complexed to histones as nucleosome core particles. As can be seen, there is an inversion of the nucleosome CD band at about 290 nm, as is observed with free DNA. However, the band is much less intense. Three explanations are possible for the observed spectra: (1) the nucleosomes have undergone the B → Z transition, but the intensity of the Z nucleosome CD spectrum in this region, like that for B nucleosomes, is lower than for free DNA; (2) only a portion of the DNA on each nucleosome can undergo the B → Z transition; (3) a fraction of the nucleosomes has dissociated, and only this free DNA has undergone the B → Z transition.

Figure 3A shows the scans from a 4% polyacrylamide native gel comparing poly(dG-m^{5}dC)·poly(dG-m^{5}dC) reconstituted nucleosomes before and after addition of $MgCl_2$ to 3 mM. It is very clear from these two scans that these nucleosome cores exhibit a noticeable increase in the amount of core size free DNA upon addition of magnesium. Since free DNA binds ethidium much more strongly than does nucleosomal DNA (McMurray & van Holde, 1986), such scans give the impression of a larger fraction of free DNA than is actually present. Therefore, we have used the method shown in Figure 3B to quantitate the amount of dissociated DNA from the gel scans. When the poly(dG-m^{5}dC)·poly(dG-m^{5}dC) reconstituted cores in 3 mM $MgCl_2$ were analyzed in the analytical ultracentrifuge (see Figure 3C), the sample showed two boundaries. The sedimentation coefficient of the slower boundary was found to be 5.4 S, corresponding to the protein-free dissociated 146-bp polynucleotide (Ausio et al., 1984). Both the analytical ultracentrifuge and the gel electrophoresis gave the same value in the presence of 3 mM $MgCl_2$: 32 ± 2% dissociated DNA. In the case of no added magnesium, the amount of free DNA in the buffer used (50 mM Tris-HCl, pH 8.0) was found to be 8–10% at the concentrations used in these experiments.

The sedimentation coefficient of the remaining undissociated nucleosomes was 10.8 S. This value is indistinguishable from the value measured for chicken erythrocyte nucleosome particles under exactly the same conditions. Furthermore, the amount of free DNA observed for chicken erythrocyte nucleosomes by the same technique was ~6% and remained constant over the range of $MgCl_2$ concentrations analyzed here (data not shown).

All this experimental evidence clearly points toward the third possibility mentioned above to account for the apparent B → Z transition exhibited by the poly(dG-m^{5}dC)·poly(dG-m^{5}dC) reconstituted nucleosome core particles. In other words, we suggest that the B to Z transition induced by the magnesium destabilizes the interaction of the polynucleotide with the core histones. Such behavior is reflected in the increase in the amount of free polynucleotide under these circumstances. In the most stringent version of this hypothesis, we propose that *only* the DNA that is dissociated is capable of freely undergoing the transition. To check this hypothesis, we have added the CD spectrum of poly(dG-m^{5}dC)·poly(dG-m^{5}dC) in 3 mM

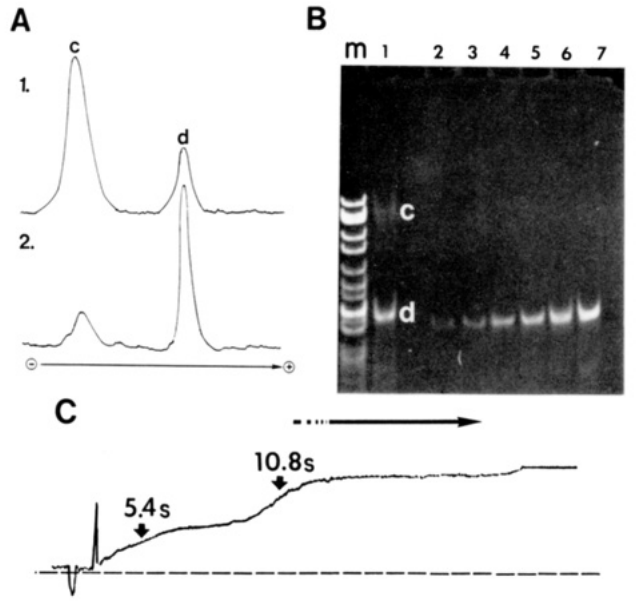

FIGURE 3: (A) Laser scanner tracings of poly(dG-m^{5}dC)·poly(dG-m^{5}dC) reconstituted nucleosomes in the absence (1) or in the presence (2) of 3 mM $MgCl_2$ when analyzed in a 4% polyacrylamide native gel (c = core particle; d = free DNA). Gels were stained by ethidium, which binds more strongly to free DNA than to nucleosomes. Therefore, the scans suggest more dissociation than was actually present. (B) Four percent polyacrylamide gel electrophoretogram of reconstituted nucleosome particles in 3 mM $MgCl_2$ (lane 1) and the DNA standards used to calibrate the amount of free DNA (lanes 2–7). As described under Materials and Methods, these latter lanes were loaded with the same sample as in lane 1 but mixed with sample buffer c. Different amounts of such a resulting mixture were loaded on the gel representing the following percentages of DNA present in lane 1: lane 2, 5%; lane 3, 10%; lane 4, 20%; land 5, 30%; lane 6, 40%; lane 7, 50% (m is the DNA marker series). (C) Sedimentation velocity profile of the poly(dG-m^{5}dC)·poly(dG-m^{5}dC) reconstituted nucleosomes in 50 mM Tris-HCl, pH 8.0, in the presence of 3 mM $MgCl_2$ at 19 °C and at 40 000 rpm. The horizontal arrow indicates the direction of the sedimentation.

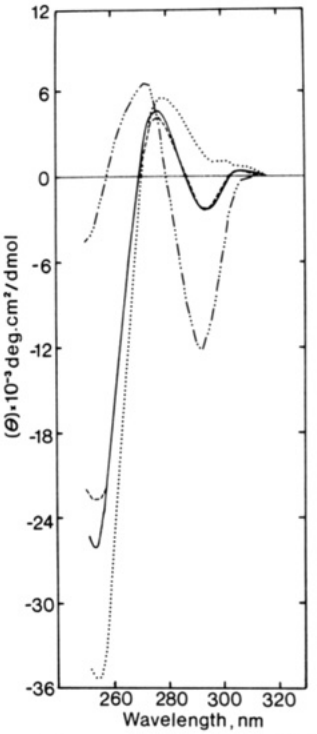

FIGURE 4: Circular dichroism spectra of poly(dG-m^{5}dC)·poly(dG-m^{5}dC) (–···–) in 50 mM Tris-HCl, pH 8.0, and 3 mM $MgCl_2$ and of poly(dG-m^{5}dC)·poly(dG-m^{5}dC) reconstituted nucleosomes (···) in 50 mM Tris-HCl, pH 8.0. After the latter spectrum was corrected for 10% free DNA [i.e., subtracting 10% of the spectrum for poly(dG-m^{5}dC)·poly(dG-m^{5}dC) in 0 mM $MgCl_2$], the two spectra were added in different ratios until the spectrum (—) that best matches the experimental one for reconstituted nucleosomes in 3 mM $MgCl_2$ (- - -) was obtained. This iterative procedure was carried out on the assumption that only the free DNA would be able to freely undergo the B → Z transition. On this basis the best fitting spectrum (—) was found for 31% free DNA.

$MgCl_2$ to that of the B-form reconstituted nucleosomes with the same polymer but in the absence of magnesium. This latter spectrum was corrected for the 10% free DNA present in this sample. Different ratios of both spectra were added until the best fitting spectrum to that observed for the poly(dG-m^{5}dC)·poly(dG-m^{5}dC)-reconstituted nucleosomes in the presence of 3 mM $MgCl_2$ was obtained. The results are shown in Figure 4. The best fit was found for 31% of poly(dG-m^{5}dC)·poly(dG-m^{5}dC) in the Z-form and 69% of poly(dG-m^{5}dC)·poly(dG-m^{5}dC) in the B-form nucleosome core particles. The value of 31% found for the poly(dG-m^{5}dC)·poly(dG-m^{5}dC) in the Z form is in very good agreement with the experimentally observed value (32 ± 2%) found for the free dissociated polymer in the presence of 3 mM $MgCl_2$, corroborating our previous hypothesis.

Discussion

In 1982, Nickol and co-workers analyzed, for the first time, the effect of the B → Z transition in poly(dG-m^{5}dC)·poly(dG-m^{5}dC) on nucleosome formation. With their experimental approach, they concluded that when the B form of that methylated polynucleotide was bound in a nucleosome core, it was resistant to conversion to the Z form. Furthermore, they also noticed that when the solvent conditions were changed so as to attempt to induce their poly(dG-m^{5}dC)·poly(dG-m^{5}dC) B-form reconstituted nucleosomes to undergo the B to Z transition, a protein-free polymer fraction was converted to the Z form at 50 °C. In contrast, the polymer bound in the nucleosome structure did not give any evidence of the B → Z transformation (Nickol et al., 1982). In apparent disagreement with these initial observations are those of Miller et al. (1986). Using a protein trout testis assembly factor, they reconstituted poly(dG-m^{5}dC)·poly(dG-m^{5}dC) nucleosome particles. From their analysis of the behavior of these particles upon changing the solvent conditions (addition of $MgCl_2$ to 3 mM), they concluded that the B to Z transition did not only occur on the particles but was also facilitated by the nucleosome assembly.

In this paper we have been concerned only with the possibility of inducing the B to Z transition in the DNA polymer once it is associated with the histones in the nucleosome core. We have also checked for changes in nucleosome stability accompanying such a process. We did not feel that the above-mentioned discrepancies could be explained merely on the basis of the slightly different experimental conditions used in the two cases (Nickol et al., 1982; Miller et al., 1985, 1986).

We have not used any assembly factor to reconstitute B-form nucleosomes with the poly(dG-m^{5}dC)·poly(dG-m^{5}dC) polymer. Rather, we have used a simple salt gradient dialysis, which has proven in the past to give excellent reconstitution when the more conventional calf thymus DNA was used (Tatchell & van Holde, 1977). As a matter of fact, it had been shown previously (Nickol et al., 1982) that poly(dG-m^{5}dC)·poly(dG-m^{5}dC) in the B form is able to reconstitute normal nucleosomes with DNase I digestion patterns closely resembling those of the native chromatin core particles, and this is fully confirmed by our results. The nucleosomes obtained after reconstitution by dialysis were very homogeneous in DNA size and had a sedimentation coefficient of 10.8 S,

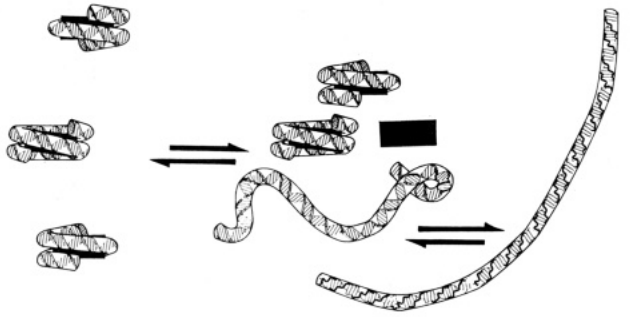

FIGURE 5: Proposed model to explain the apparent B → Z transition of poly(dG-m^{5}dC)·poly(dG-m^{5}dC) reconstituted nucleosomes in the presence of 3 mM $MgCl_2$.

and their mobility in native 4% polyacrylamide gel was the same as that observed for native chicken core particles.

When we attempted to induce the B to Z transition in such particles by addition of $MgCl_2$ under exactly the same experimental conditions as described by Miller et al. (1986), the most noticeable phenomenon was an apparent loss of stability of the particle as reflected by an enhanced DNA dissociation. This was observed to exactly the same extent both in 4% polyacrylamide gels (see Figures 3A and 3B) and also in the analytical ultracentrifuge (see Figure 3C). Indeed, a careful examination of the sedimentation velocity profiles reported by Miller et al. (1986) under the same conditions suggests to us the presence of large amounts of free DNA in their $MgCl_2$-treated samples; this was referred to as a "background of species sedimenting at less than 10.8 S". We attributed the changes in CD accompanying the titration of poly(dG-m^{5}dC)·poly(dG-m^{5}dC)-reconstituted nucleosomes as due entirely to the free DNA produced, since the spectra of these particles in 3 mM $MgCl_2$ can be completely accounted for by appropriately adding the CD spectra of B-type core particles and free Z DNA. Thus, there is no necessity to postulate Z nucleosomes to account for the data. Furthermore, we do not think that the nucleosome structure enhances the B to Z transition as proposed by Miller et al. (1986), but on the contrary, and in agreement with Nickol et al. (1982), we find that it prevents it, since the amounts of polymer in the Z conformation are much lower in this latter case as is shown by a comparison of the CD spectra (Figure 2).

On this basis, we propose a model for the $MgCl_2$ titration, which is shown in Figure 5. In an attempt to determine whether the influence of the Mg^{2+} ion is mainly in the first or in the second step of such a model, was also analyzed native core particles obtained from chicken erythrocytes, in the presence of different amounts of $MgCl_2$. In the range of $MgCl_2$ concentrations used to induce the B to Z transition in poly(dG-m^{5}dC)·poly(dG-m^{5}dC), we were unable to detect any significant increase in the degree of DNA dissociation. It seems therefore clear that the influence of the magnesium ions lies mainly in the second step of the model proposed in Figure 5. In other words, the B to Z transition in the free polynucleotide is the major driving force for the loss of stability of the nucleosome particle. Such observations are not surprisingly at all; on the contrary, they reconcile very well with the well-known fact that, by undergoing the B to Z transition, DNA becomes much stiffer. The persistence length of poly(dG-dC)·poly(dG-dC) in the Z form, in high salt, was found to be 208 nm (Thomas & Bloomfield, 1983), a value to be compared with that of 93.8 nm found for the same polymer in the B form at low salt or with that of 33 nm for the linear *Col*E1 DNA (Ausio et al., 1983) under the same high salt concentrations used to induce the Z form in poly(dG-dC)·poly(dG-dC). It is hard to imagine how this enormous loss of flexibility of the DNA molecule could be consistent with an enhanced affinity for nucleosome assembly.

Finally, our data also reconcile better with the in vivo observations. The transcriptional enhancers in SV40 contain regions able to undergo the B to Z transition. They are found in the SV40 control region, which is usually depleted of nucleosomes (Rich et al., 1984).

Registry No. Poly(dG-m^{5}dC), 51853-63-5.

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