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Effect of the B–Z transition in poly(dG-m^{5}dC)·poly(dG-m^{5}dC) on nucleosome formation

(Z DNA/chromatin/histones/methylation)

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ABSTRACT **We have studied the properties of complexes formed between histones and the methylated synthetic polydeoxynucleotide poly(dG-m^{5}dC)·poly(dG-m^{5}dC). This polymer undergoes the transition from B DNA to left-handed Z DNA at moderate ionic strength. When the polymer is in the Z form it will bind histones, but nucleosomes are not detected. When the polymer in the B form is combined with equimolar quantities of the four core histones and digested with micrococcal nuclease, particles are formed which behave in all respects as normal nucleosome cores. When these core particles are placed in solvents that would result in conversion of the protein-free polymer to the Z form, no transition is observed. The formation of a nucleosome core particle thus stabilizes the B form, whereas the presence of the Z form prevents nucleosome formation. The results suggest that if Z DNA is present in eukaryotic nuclei, it will serve to disrupt the normal chromatin structure.**

Double-stranded polydeoxynucleotides composed of alternating guanine and cytosine residues are capable of undergoing a transition from the right-handed B form to a left-handed "Z DNA" structure under appropriate conditions (1–5). We have recently shown (2) that methylation of the cytosine residues in the alternating G-C polymer has a dramatic effect on the B–Z transition in solution, lowering the transition point from very high concentrations of salt (e.g., 0.7 M $MgCl_2$) into the physiological range (e.g., 50 mM NaCl/0.6 mM $MgCl_2$). This is particularly interesting because the sequence C-G is the principal site of cytosine methylation in eukaryotic cells and because modulation of the level of methylation at these sites is correlated with gene expression (6).

It has been suggested by Wang *et al.* (7) that Z DNA, or the B–Z transition, may play a structural or regulatory role *in vivo*. Consideration of a possible role for Z DNA in eukaryotic nuclei raises the question of the effect of the Z conformation on chromatin structure. Do histones bind to Z DNA, and do they form nucleosomes?

In this paper, we describe experiments that address these questions. We have taken advantage of the fact that the methylated polymer poly(dG-m^{5}dC)·poly(dG-m^{5}dC) undergoes the B–Z transition at moderate salt concentrations, well below the ionic strength at which histones dissociate from DNA. This makes it possible to study the binding of histones to both the B and Z forms of the polymer. We first show that nucleosome core particles can be isolated from complexes of histones with the B form of the polymer, and we compare the properties of these particles with the properties of particles made with the unmethylated polymer poly(dG-dC)·poly(dG-dC). We then examine the effect of conversion to the Z form on the interaction of the methylated polymer with histones. We show that, although histones bind to Z DNA under appropriate solvent conditions, the properties of the complex are not those of a nucleosome. Furthermore, the formation of core particles greatly stabilizes the B form relative to the Z form.

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MATERIALS AND METHODS

Reagents. Poly(dG-dC)·poly(dG-dC), phage T4 polynucleotide kinase, and micrococcal nuclease were obtained from P-L Biochemicals, and DNase I (bovine pancreatic DNase) was from Worthington. Poly(dG-m^{5}dC)·poly(dG-m^{5}dC) was synthesized as described (2). Uniformly labeled synthetic polymers were obtained by supplementing normal synthetic reactions with [α-^{32}P]dGTP (New England Nuclear). Chicken core histones (H2a, H2b, H3, and H4) used in the reconstitutions were isolated from adult erythrocytes by hydroxylapatite chromatography (8).

Association of Synthetic Polymers with Core Histones. Because the B–Z transition of the poly(dG-m^{5}dC)·poly(dG-m^{5}dC) polymer is very sensitive to ionic conditions in the range normally used for reconstitution, it was necessary to select reconstitution solvents that would maintain the desired form of the polymer, as monitored by circular dichroism. For this reason histones were complexed to the synthetic polymers (uniformly labeled or unlabeled) at a concentration of 0.2 mg/ml by direct mixing (9) in solutions of 10 mM Tris·HCl, pH 8.0/0.2 M NaCl containing either 0.55 mM $Co(NH_3)_6^{3+}$ or 0.1 mM EDTA for the Z and B forms, respectively. Before addition of histones, conversion to the Z form of the methylated polymer (where desired) was accelerated by incubation in reconstitution buffer at 50°C for 10 min. Reconstitutions were allowed to continue for 12 hr at 37°C with shaking. Optimal results were obtained at a histone-to-DNA ratio of 0.8 g/g for both methylated and unmethylated polymer.

Nuclease Digestion. For micrococcal nuclease digestion of reconstituted complexes containing the B form of poly(dG-dC)·poly(dG-dC) and poly(dG-m^{5}dC)·poly(dG-m^{5}dC), the samples were diluted to 10 mM Tris·HCl, pH 8.0/50 mM NaCl/0.25 mM $CaCl_2$. After digestion at room temperature for 10 min with various amounts of enzyme, the reactions were stopped by addition of both EGTA and EDTA to 1 mM. The DNA was deproteinized by proteinase K (100 μg/ml) and extraction with phenol, and it was analyzed on nondenaturing 5% polyacrylamide gels in Tris/borate/EDTA buffer (10).

For comparative micrococcal nuclease digestion of the B and Z forms of the methylated polymer reconstituted complexes, the samples were diluted to 10 mM Tris·HCl, pH 8.0/50 mM NaCl/1.5 mM $CaCl_2$ in the presence or absence of 0.125 mM $Co(NH_3)_6^{3+}$ for the Z and B forms, respectively. Digestions were performed at 37°C, and the DNA was extracted and analyzed on nondenaturing polyacrylamide gels.

For mapping the sites of DNase I cutting, methylated and unmethylated core particles were treated with kinase, mixed with unlabeled chicken erythrocyte monomers, and isolated on

isokinetic sucrose gradients (5–26.9% sucrose in 10 mM Tris·HCl, pH 8.0/50 mM NaCl/0.1 mM EDTA). The resulting monomer peak was digested at 37°C with DNase I (5 units/ml) after addition of both $CaCl_2$ and $MgCl_2$ to 0.5 mM. Digestion was concluded by addition of both EDTA and EGTA to 2 mM; the DNA was extracted, glyoxylated, and analyzed on denaturing gels (11). All gels were autoradiographed by using Kodak XAR film and Du Pont Lightning Plus intensifying screens.

Thermal Denaturation Measurements. Methylated and unmethylated core particles were dialyzed extensively against buffer containing 0.2 mM NaOH, 0.22 mM cacodylic acid, 0.005 mM Na_2EDTA, final pH 7.0. A Cary model 219 spectrophotometer attached to a Digital Equipment (Marlboro, MA) LSI-11 microcomputer was used to monitor and process all absorption measurements.

RESULTS

Reconstitution of Histone Complexes Containing Poly(dG-dC)·Poly(dG-dC) and Poly(dG-m^{5}dC)·Poly(dG-m^{5}dC) in the B Form. Simpson and Kunzler (12) have shown that poly(dG-dC)·poly(dG-dC) is capable of folding around core histones to form a well-defined chromatin-like structure. We show here that poly(dG-m^{5}dC)·poly(dG-m^{5}dC) in the B form can form nucleosomes upon interaction with the inner histones, as judged by four criteria: micrococcal nuclease digestion, DNase I digestion, sedimentation velocity, and thermal denaturation analysis. We also compare the properties of these particles with those of particles formed with the unmethylated polymer poly(dG-dC)·poly(dG-dC).

Uniformly labeled methylated and unmethylated polymers were maintained in the B form and reconstituted with core histones. The effect of micrococcal nuclease digestion on these reconstituted complexes is shown in Fig. 1. In both digestions distinct bands diagnostic of core particle formation are generated. The prominent monomer bands are approximately 145 base pairs long; with increasing enzyme concentration discrete submonomer fragments appear, and they are equivalent for the two synthetic chromatins. For preparative purposes the reaction was stopped at the point that produced the maximal yield of core particles. Analysis of these core particles by analytical ultracentrifugation in 0.1 M NaCl solvent yielded $s_{20,w}$ values of 10.6 S and 11.1 S for poly(dG-dC)·poly(dG-dC) and poly(dG-m^{5}dC)·poly(dG-m^{5}dC), respectively.

DNase I digestion sites on 5′-end-labeled core particles of both poly(dG-dC)·poly(dG-dC) and poly(dG-m^{5}dC)·poly(dG-m^{5}dC) are shown in the autoradiogram of Fig. 2*A*. Both copolymer digests, when glyoxylated and analyzed on denaturing gels, have discrete bands at multiples of two nucleotides. Superimposed on these patterns, which reflect enzymatic preference for alternate nucleotides, are prominent modulations showing preferential sites for DNase I cutting at periodicities of about 10 nucleotides. The scan of the autoradiogram (Fig. 2 *B* and *C*) helps to show that, within the resolution of the gel, the most susceptible cutting sites are at bands 2, 4, 5, and 9. Some cutting occurs at bands 5 and 7, but sites 3 and 8 are relatively insensitive to DNase I digestion. The relative frequency of cutting at each band does not appear to change with longer times of digestion, nor is there any major difference between the patterns of the two synthetic polymers. The fact that these DNase I patterns closely resemble those of native chromatin core particles (13) is consistent with the ability of histones to form nucleosomes with both B form poly(dG-dC)·poly(dG-dC) and B form poly(dG-m^{5}dC)·poly(dG-m^{5}dC). The DNase I cutting pattern reported here is different from that of Simpson and Kunzler

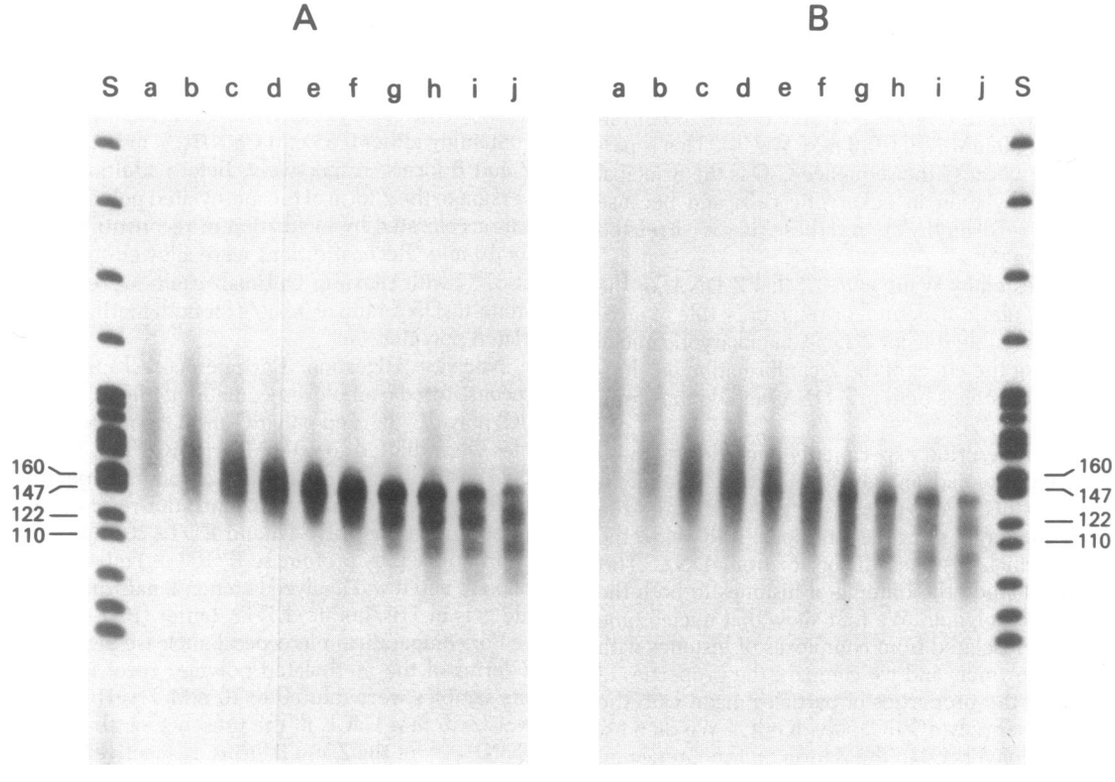

FIG. 1. Autoradiograms of micrococcal nuclease digestion of reconstituted complexes of histones with B form polymers. Poly(dG-m^{5}dC)·poly(dG-m^{5}dC) (*A*) and poly(dG-dC)·poly(dG-dC) (*B*) were digested with micrococcal nuclease at 0, 1, 10, 25, 50, 100, 250, 1000, and 2500 units/ml (lanes a–j). End-labeled fragments of a *Hpa* II digest of pBR322 are standards, with lengths given in base pairs (lane S). Analysis was on a 5% polyacrylamide (20:1 monomer/crosslinker) nondenaturing gel.

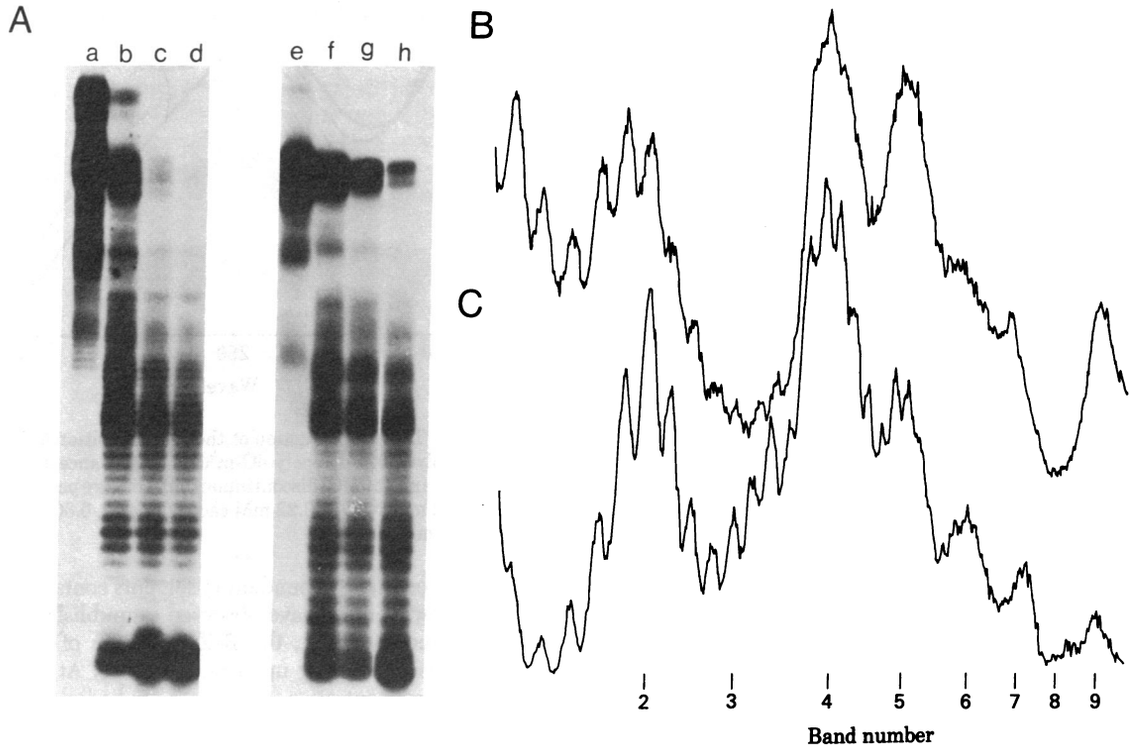

FIG. 2. DNase I digestion of 5′-end ^{32}P-labeled core particles containing histones and alternating copolymer. (*A*) Autoradiogram of 8% polyacrylamide/7 M urea gel of glyoxylated products of DNase I digestion. Core particles containing poly(dG-m^{5}dC)·poly(dG-m^{5}dC) (lanes a–d) and poly(dG-dC)·poly(dG-dC) (lanes e–h) were digested with DNase I for 0 (lanes a and e), 0.5 (lanes b and f), 1.0 (lanes c and g), and 2.5 (lanes d and h) min. (*B*) Densitometer tracing of DNase I digestion pattern of poly(dG-dC)·poly(dG-dC) core particles shown in *A*, lane g. (*C*) Densitometer tracing of DNase I digestion pattern of poly(dG-m^{5}dC)·poly(dG-m^{5}dC) core particles shown in *A*, lane c. Band numbers corresponding to a periodicity of ≈10 bases are provided for reference.

(12), who reported band 6 cut more frequently than bands 4 and 5, and low susceptibility at band 2.

Further evidence that the B form of the methylated as well as the unmethylated alternating copolymer can form nucleosome-like structures is provided by the thermal denaturation studies of Fig. 3. Under the solvent conditions used, naked poly(dG-dC)·poly(dG-dC) melts with a sharp transition at 67°C; the methylated polymer has a higher melting temperature (73°C). Histones interacting with these polymers to form core particles increase the polymer thermal stability sufficiently that complete denaturation of the cores cannot be obtained. At the highest temperatures (≈98°C) the hyperchromicity of the monomer containing poly(dG-dC)·poly(dG-dC) is about 47% of the expected hyperchromicity for complete denaturation. Similar results have been reported earlier (12), with a transition to 30% of maximal hyperchromicity at 85°C. For monomers containing poly(dG-m^{5}dC)·poly(dG-m^{5}dC), which melts at an even higher temperature, only 35% of the maximal possible hyperchromicity is obtained at 98°C. It has been shown that there is a discrete first step in thermal denaturation of nucleosome core DNA, which involves liberation of the terminal regions of the DNA from the nucleosome surface followed by strand separation (14). Although no separate first step is obvious in the meltings shown in Fig. 3, it is reasonable to suppose that a similar process of melting the ends is occurring in these synthetic core particles and that most or all of the observed hyperchromicity reflects that process.

Reconstitution with Poly(dG-m^{5}dC)·Poly(dG-m^{5}dC) in the Z Form. The above results show that the methylated polymer in the B form readily folds into nucleosomes. We next tested the ability of core histones to interact with this same polymer after it had been converted to the Z form by $Co(NH_3)_6^{3+}$. Reconstitution was carried out with uniformly labeled Z form polymer (and with a B form control); the products were digested with micrococcal nuclease and the DNA was analyzed on a nondenaturing gel, as shown in Fig. 4. Whereas the B form reconstituted complex yields defined bands that approach monomer length (145 base pairs) with increasing times of digestion, the Z form reconstituted complex shows no sharp, distinct bands as intermediates at any time during the digestion, although we have sometimes observed a more polydisperse, higher molecular weight protected component. Digestion of the naked Z polymer under identical conditions (Fig. 4, lanes m–p) shows that the enzyme is capable of acting on the Z form in the presence of $Co(NH_3)_6^{3+}$. The histones are presumably interacting with the polymer to form an ill-defined complex that has no internal nucleosomal architecture capable of being detected by micrococcal nuclease. We have attempted to isolate this histone–polymer complex on isokinetic sucrose gradients, but it pellets under conditions in which the B form complex sediments as a normal 11S monomer, and the Z form histone-free DNA sediments normally.

We also investigated the effect of nucleosome formation on the B–Z transition. Nucleosome core particles were made as described above by reconstitution of core histones with the B form of poly(dG-m^{5}dC)·poly(dG-m^{5}dC), followed by micrococcal nuclease digestion. The solvent conditions were then changed (final conditions: between 0.075 and 0.2 mM $MgCl_2$, 0.2 mM sodium cacodylate at pH 7.0, 0.005 mM Na_2EDTA) so that the Z form would normally be stable. Under these conditions, a protein-free polymer sample converted to the Z form with a half-time of ≈1 min at 50°C. In contrast, the polymer

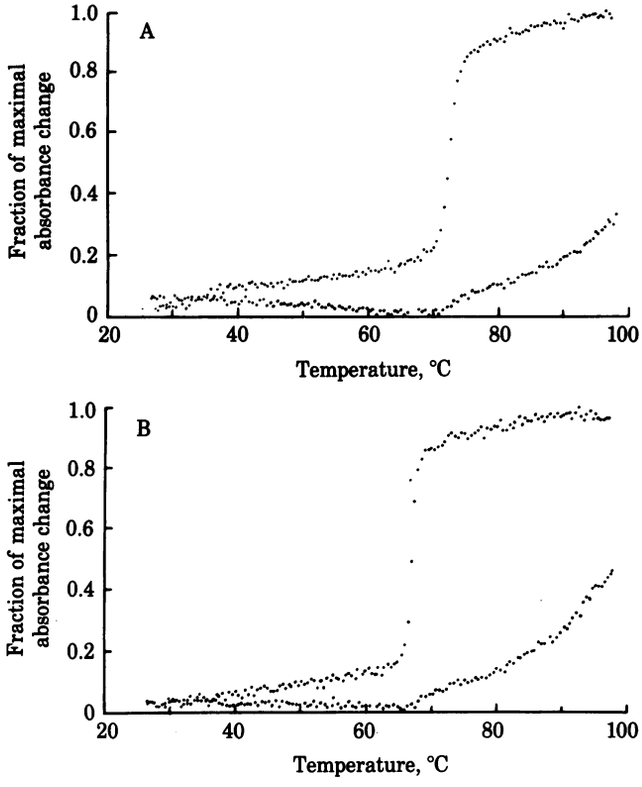

FIG. 3. Thermal denaturation profiles of both free polymers and histone-reconstituted core particles. The upper curves are for the free polymers. (*A*) Poly(dG-m^{5}dC)·poly(dG-m^{5}dC). (*B*) Poly(dG-dC)·poly(dG-dC).

bound in the nucleosome structure gave no evidence of conversion to the Z form (as judged by changes in absorption spectrum) after 24 hr at either room temperature or 50°C.

The transition of protein-free G-C polymers from B to Z form

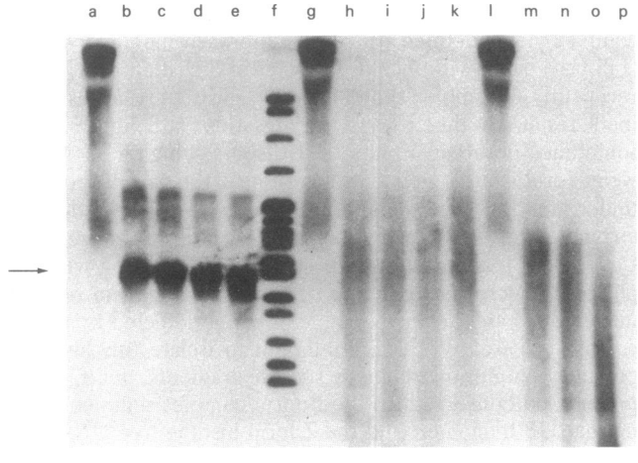

FIG. 4. Micrococcal nuclease digestion of histone-complexed poly(dG-m^{5}dC)·poly(dG-m^{5}dC) in B and Z forms. B form histone reconstituted complex was digested with micrococcal nuclease at 5 units/ml for 0, 10, 20, 50, or 100 min (lanes a–e), whereas the corresponding Z form digestion involved micrococcal nuclease at 250 units/ml with the same time points (lanes g–k). Histone-free poly(dG-m^{5}dC)·poly(dG-m^{5}dC) in the Z form was digested with micrococcal nuclease at 250 units/ml for 0, 10, 20, 50, or 100 min (lanes l–p). Samples were analyzed on a 5% polyacrylamide gel (20:1); an equal amount of radioactivity (25,000 cpm) was analyzed in each lane. The arrow designates the mobility of a 145-base-pair DNA fragment. End-labeled fragments of a *Hpa* II digest of pBR322 were run in lane f.

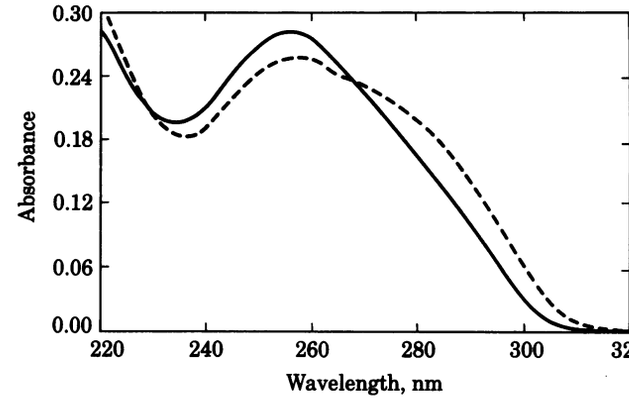

FIG. 5. Thermal induction of the B–Z transition of histone-reconstituted poly(dG-m^{5}dC)·poly(dG-m^{5}dC). Absorbance spectrum at 25°C (solid line) and at 95°C (discontinuous line) of core particles in the presence of 0.2 mM NaOH, 0.22 mM cacodylic acid, 0.005 mM Na_2EDTA, and 0.075 mM $MgCl_2$.

is not temperature dependent in solvents containing only NaCl (1, 2). However, we have observed (unpublished data) that in the presence of Mg^{2+}, the B–Z transition of the methylated polymer is dependent upon temperature. At appropriate divalent ion concentration, the Z form can be induced by heating. We observe similar behavior in the case of the synthetic core particles described above. In 0.075 mM $MgCl_2$/0.2 mM sodium cacodylate, pH 7.0, the Z form of protein-free poly(dG-m^{5}dC)·poly(dG-m^{5}dC) is stable at room temperature. As noted above, the B form is stable in this solvent over a period of 24 hr in core particles maintained at 50°C. However, the B–Z transition can be induced in core particles containing this polymer by raising the temperature above 60°C. The absorption spectra at 25°C and 95°C are shown in Fig. 5. If the sample is cooled to room temperature after heating to 95°C, the spectrum remains that of the Z form. Although no light scattering is observed at 320 nm at either 95°C or room temperature, sedimentation in the analytical centrifuge, carried out in the same solvent, reveals that the particles are now aggregated, with values of $s_{20,w}$ greater than 40 S.

DISCUSSION

Nucleosome core particles can be reconstructed by using DNA from almost any source. Bacterial DNA is as satisfactory as DNA from eukaryotes (15); it is even possible to use the glucosylated DNA of T4 bacteriophage (unpublished data). Some synthetic two-stranded polydeoxynucleotides, such as the alternating polymers poly(dA-dT)·poly(dA-dT) and poly(dG-dC)·poly(dG-dC), also are capable of forming nucleosome core particles (12, 16). On the other hand, it has not so far proven possible to construct nucleosomes by using the nonalternating polymers poly(dA)·poly(dT) or poly(dG)·poly(dC) (12). Double-stranded RNA is also ineffective in forming nucleosomes (17).

In this paper we have examined the properties of histone complexes with the alternating methylated polydeoxynucleotide poly(dG-m^{5}dC)·poly(dG-m^{5}dC). As shown in Fig. 1, micrococcal nuclease digestion of high molecular weight B form poly(dG-m^{5}dC)·poly(dG-m^{5}dC) complexed to histones results in discrete intermediates containing about 145 base pairs of the polymer. The nucleoprotein product of this digestion sediments at 11 S and, when end-labeled with ^{32}P and digested with DNase I, yields polynucleotide fragments with size and abundance distributions characteristic of normal nucleosome core particles (Fig. 2). Furthermore, the thermal denaturation properties of the particles (Fig. 3) are those expected for nucleosome cores

containing a duplex of high thermostability.

These studies have also given us an opportunity to examine the effect of methylation on nucleosome formation. The data shown in Figs. 1–3 reveal no significant differences between core particles containing the methylated and unmethylated polymers. Direct competition experiments (unpublished data) between 145-base-pair B form poly(dG-dC)·poly(dG-dC) and poly(dG-m^5dC)·poly(dG-m^5dC) for the binding of core histones also suggest that there is at most a 2-fold difference in affinity. Thus the presence of methyl groups in these B form model compounds has little effect on either the structure or the thermodynamic stability of the resulting core particle.

The major difference between the two polymers lies in the relative ease with which the methylated polymer can be converted to the Z form. We have taken advantage of this behavior to study the interactions of Z form poly(dG-m^5dC)·poly(dG-m^5dC) with core histones under moderate ionic strength conditions. The interactions are quite different from those with the B form. Micrococcal nuclease digestion of complexes of the Z form with histones does not produce protected fragments 145 base pairs in length (Fig. 4), though larger, polydisperse fragments are sometimes observed during digestion. There is no question that the histones are bound in some way, because the rate of nuclease digestion in the presence of histones is slower by an order of magnitude than the rate with Z DNA alone.

The fact that we are unable to form a nucleosome-like particle with Z DNA is not definitive proof that there exist no conditions under which such a particle could be formed. Our second approach to the problem was therefore to start with a core particle containing the B form of the methylated polymer and to alter ionic conditions so that the Z form would normally be stable. We found that when the B form of poly(dG-m^5dC)·poly(dG-m^5dC) is bound in a nucleosome core, it is resistant to conversion to the Z form. Under ionic conditions such that the naked polymer would rapidly be converted to the Z form, the core particle DNA remains in the B form over periods of 24 hr.

It is reasonable to suppose that under such conditions the B form would be stable indefinitely, but we cannot yet rule out the possibility that this stability is the result of a kinetic barrier, rather than the reflection of an altered equilibrium. As noted above, the B–Z equilibrium is temperature dependent in solvents containing Mg^{2+}, so that the conversion (Fig. 5) of nucleosome-bound DNA to the Z form at higher temperatures (above 60°C under our solvent conditions) may very well reflect a temperature-dependent change in the free energy of that process. We have not yet been successful in characterizing the histone–DNA complex produced by heating, because it has a strong tendency to aggregate, like all of the complexes we have observed between histones and Z DNA; no molecule containing 145 base pairs of Z DNA and an octamer of histones has yet been detected.

The inability of Z DNA to form a nucleosome-like structure is perhaps not surprising. Although the principal forces holding B DNA to the nucleosome surface are electrostatic in nature, there is evidence that the interaction is not simply a matter of maximal neutralization of negative charges on DNA by positive charges on the histones. At least in the case of the 25 base pairs at the termini of core DNA, the stabilizing interactions appear to involve only a small fraction of the total number of charges (18). This suggests that the spacing between charges on DNA may be a critical factor in determining the stability of the structure. Similar suggestions have been made concerning the apparent absence of nucleosome formation with double-stranded RNA (17) and with the nonalternating polymer poly(dA)·poly(dT) (19), neither of which has the same number of base pairs per turn in solution as random sequence B DNA. This does not necessarily mean that histones will not bind to DNA of unusual conformation, but such binding might well require distortion or even dissociation of the core histone octamer.

In attempting to draw conclusions about the possible role of Z DNA sequences in chromatin structure it is important to keep in mind that long runs of alternating C-G sequence have not yet been found in the typical eukaryotic genome. This does not rule out the possibility that relatively short stretches of alternating C-G, or other kinds of alternating purine-pyrimidine sequences, could take on Z DNA-like conformations. Assuming that regions with such conformation exist, our data provide information about the way in which they are likely to interact with histones. These results show that DNA that is capable of undergoing the B–Z transition can form nucleosomes when in the B form. When nucleosomes are formed, their presence inhibits the transition. Finally, if DNA is already in the Z form, it will bind histones, but the complex tends to be insoluble, and digestion with micrococcal nuclease leads to a polydisperse pattern of protected DNA rather than to formation of fragments of the discrete size characteristic of nucleosomes.

Our results support a model of the nucleosome in which stability is determined by details of DNA conformation and charge distribution on the DNA surface. Our observations also suggest that if there are regions within the eukaryotic genome containing Z DNA, then in their neighborhood the regular structure of chromatin would almost certainly be disrupted.

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