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Formation of Z-DNA in negatively supercoiled plasmids is sensitive to small changes in salt concentration within the physiological range

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Negative supercoiling of the plasmid pBR322 with or without an insert of $(dG\text{-}dC)_n$ induces the formation of Z-DNA as measured by the binding of antibodies specific for Z-DNA. Increasing the concentration of Na^+ (or K^+) is shown to inhibit the B to Z-DNA conversion. This may be due to the effect of the cation on the B-Z junction. Using the data for B to Z-DNA conversion of the $(dG\text{-}dC)_n$ inserts, we have estimated the free energy change per base pair as well as the energy of the B-Z junction. In pBR322, a 14-bp segment [CACGGGTGCGCATG] is believed to form Z-DNA at bacterial negative superhelical densities under salt conditions which are similar to those found *in vivo*.

Key words: antibody binding/antibody-DNA cross-linking/B-Z junction/free energy of Z-DNA/supercoiling

Introduction

Z-DNA is a left-handed conformation of the double helix which is favored in sequences containing alternating purine and pyrimidine residues (Wang *et al.*, 1979). Unlike B-DNA, Z-DNA is strongly immunogenic and can be used to produce both polyclonal (Lafer *et al.*, 1981; Malfoy and Leng, 1981; Nordheim *et al.*, 1981) and monoclonal (Möller *et al.*, 1982) antibodies which react specifically with Z-DNA. The polymer poly(dG-dC) is known to form Z-DNA in solution at elevated concentrations of NaCl (Pohl and Jovin, 1972; Thamann *et al.*, 1981), at lower concentrations of magnesium chloride (Pohl and Jovin, 1972; Behe and Felsenfeld, 1981; van de Sande and Jovin, 1982), or submillimolar concentrations of magnesium chloride (van de Sande *et al.*, 1982). An easy way to stabilize Z-DNA is to brominate this polymer in a 4 M salt solution (Lafer *et al.*, 1981). At a low level of bromination the polymer remains in the Z-DNA conformation in physiological salt solution. Other methods known to stabilize Z-DNA include methylation of the C5 position of cytosine in poly(dG-dC) (Behe and Felsenfeld, 1981), adding low concentrations of polyamines (Behe and Felsenfeld, 1981) and reaction with platinum derivatives (Malfoy and Leng, 1981). Z-DNA can also be stabilized through the presence of proteins found in the nucleus of *Drosophila* cells (Nordheim *et al.*, 1982a) or through the presence of negative supercoiling (Peck *et al.*, 1982; Singleton *et al.*, 1982; Nordheim *et al.*, 1982b).

Most covalently closed, circular DNA molecules from natural sources are negatively supercoiled, i.e., underwound (reviewed by Bauer, 1978). In plasmids, the degree of negative supercoiling can be measured directly and described by the number of negative supercoils per plasmid (negative linking difference, $-\tau$) or as negative superhelical density (negative specific linking difference, $-\sigma$). Supercoiled DNA has a higher free energy than relaxed DNA, and the free energy increases as the square of the number of negative superhelical turns (Bauer and Vinograd, 1970; Hsieh and Wang, 1975; Depew and Wang, 1975; Pulleyblank *et al.*, 1975). Any process which reduces the superhelical density, e.g., formation of left-handed Z-DNA, thus has a favorable free energy. The formation of left-handed DNA occurs in plasmids which carry cloned segments of $(dG\text{-}dC)_n$ (Klysik *et al.*, 1981; Peck *et al.*, 1982; Singleton *et al.*, 1982; Nordheim *et al.*, 1982b). Upon increasing the negative superhelical density, the conversion of these cloned segments to Z-DNA has been measured using changes in electrophoretic mobility (Peck *et al.*, 1982; Singleton *et al.*, 1982), ultracentrifugation (Peck *et al.*, 1982) or the ability to bind antibodies against Z-DNA as detected in filter binding experiments (Nordheim *et al.*, 1982b). In addition, it has been possible to show that the plasmid pBR322 itself forms Z-DNA at elevated negative superhelical density (Nordheim *et al.*, 1982b). In these experiments, Z-DNA formation was monitored by the binding of Z-DNA antibodies to pBR322 with increasing negative superhelical density. The binding was measured by the ability of the antibody to retain the plasmid on a nitrocellulose filter in a solution containing ~300 mM sodium ions. These experiments showed that the midpoint for the formation of Z-DNA occurred at a negative superhelical density of 0.09. However, this figure is somewhat above the negative superhelical density which is found in pBR322 when this plasmid is isolated from the bacterial cell. Therefore, we have studied the binding of the antibody to negatively supercoiled pBR322 in solutions in which the sodium concentration varied. The amount of Z-DNA detectable in the plasmid increased rather markedly as the sodium ion concentration decreased. The amount of Z-DNA detectable in plasmids containing cloned inserts of alternating $(dG\text{-}dC)_n$ also shows a salt dependence, but the effect is less pronounced. A similar observation has been made by Singleton *et al.* (1982), who measured the formation of Z-DNA in cloned inserts by changes in electrophoretic mobility. Here we report that when pBR322 is assayed at a superhelical density which is found in bacteria, Z-DNA can be detected when the assay is carried out under physiological salt conditions. Additional experiments identify more fully the region of DNA which is forming the left-handed segment. The conclusion is that there is a natural sequence of nucleotides in pBR322 which forms Z-DNA under conditions which are similar to those found inside bacteria.

Results

Effect of NaCl

A number of experiments were carried out to detect the presence of Z-DNA in negatively supercoiled pBR322 at different concentrations of NaCl. As described in Materials and methods, the negative superhelical density of pBR322 can be adjusted by topoisomerase I relaxation in the presence of controlled amounts of ethidium bromide. The number of negative supercoils induced by this treatment was measured by agarose gel electrophoresis and, as has been found previously,

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a number of topoisomers were seen which reflect the Gaussian distribution of energy levels clustering around a mean. The values of the negative superhelical density determined in this way correspond to those existing under the conditions of topoisomerase relaxation (200 mM NaCl, 10 mM Tris-HCl, pH 8.0, 0.1 mM EDTA, 25°C). A preparation was made containing an average of 31.7 negative superhelical turns, which corresponds to a negative superhelical density of 0.076. The presence of Z-DNA was assayed by adding varying amounts of anti-Z-DNA antibody to the plasmid DNA and then filtering it through nitrocellulose. If the plasmid contains a Z-DNA segment, the antibody binds to it and the protein-DNA complex is retained on the filter. Figure 1 shows the retention of negative superhelical pBR322 as a function of increasing concentration of antibody in solutions of varying NaCl concentrations. At 50 mM NaCl the percentage binding rises sharply and plateaus at ~85% binding. The binding is not greater than that since the preparations often have 10–20% of plasmids which are nicked and therefore cannot be supercoiled. As the concentration of NaCl in the assay medium was increased, progressively smaller amounts of plasmid were trapped on the filter. Finally, at 300 mM NaCl there was no detectable binding. Figure 1 is in agreement with the experiments reported previously in which all of the assays were carried out at a sodium concentration of ~300 mM (Nordheim *et al.*, 1982b).

These results seem somewhat paradoxical at first. It is known, for example, that poly(dG-dC) is stabilized in the Z conformation at elevated concentrations of NaCl (Pohl and Jovin, 1972; Thamann *et al.*, 1981; Behe and Felsenfeld, 1981). The midpoint for the conversion from B-DNA to Z-DNA, is 2.7 M NaCl. Here, on going from 50 mM to 300 mM NaCl, we appear to decrease steadily the amount of Z-DNA which is present, as measured by the ability of the antibody to bind to the negatively supercoiled plasmid. Clearly the effect of sodium in Figure 1 must reflect some other process than the stabilization of Z-DNA due to elevated concentrations of sodium.

We tested whether the combination of the antibody with Z-DNA is sensitive to NaCl concentration. In the experiments shown in Figure 2, the binding of Z-DNA, as well as B-DNA, to antibodies against Z-DNA is given for three different concentrations of NaCl, i.e., 50, 150 and 300 mM. The solid curve represents the binding of the antibody to brominated poly(dG-dC) which exists as Z-DNA at all of the measured salt concentrations. There is no appreciable difference in the binding of the antibody to Z-DNA at these three different NaCl concentrations. Increasing amounts of antibody rapidly bring about a rise in the amount of Z-DNA which is retained on the filter until virtually all is retained at the highest antibody concentration (500 nM). The dashed line at the bottom represents the antibody binding to poly(dG-dC) (B-DNA) at these three different salt concentrations. It can be seen that there is no binding present in 150 and 300 mM NaCl, and ~10% binding in 50 mM NaCl. The latter is probably associated with a non-specific interaction of the positive charges on the antibody molecule with the negatively charged phosphates of DNA in a solution with low concentration of cations. When unspecific IgG antibodies are used, a similar increase in binding is seen at 50 mM NaCl but there is no binding apparent at 150 or 300 mM NaCl (results not shown). Similar results are also found for the binding to brominated poly(dG-dC) (Z-DNA) using the IgG antibodies isolated from

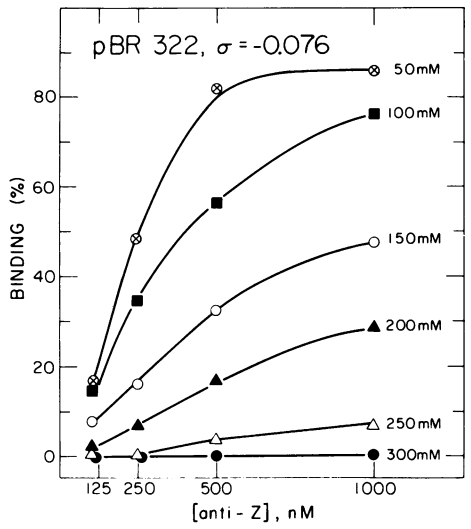

Fig. 1. The binding of increasing amounts of anti-Z-DNA specific antibodies to negatively supercoiled pBR322 at a negative superhelical density ($-\sigma$) of 0.076 is shown for six NaCl concentrations in the incubation medium: (⊗) 50 mM; (■) 100 mM; (○) 150 mM; (▲) 200 mM; (△) 250 mM (●) and 300 mM. Binding is measured by trapping plasmid-antibody complexes on nitrocellulose filters.

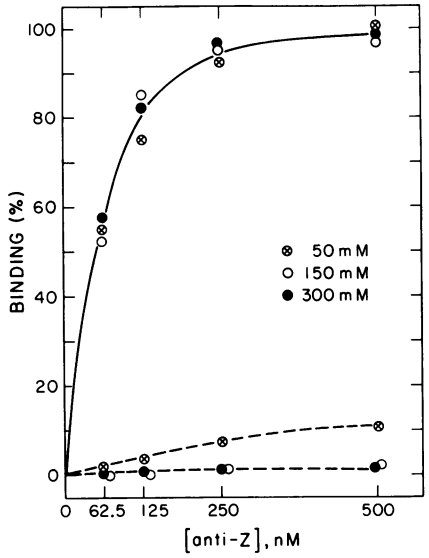

Fig. 2. The binding of DNA by increasing amounts of anti-Z-DNA specific antibodies to [^{3}H]poly(dG-dC) (B-DNA, dotted line) and [^{3}H]Br-poly-(dG-dC) (Z-DNA, solid line). These polymers are described by Lafer *et al.* (1981). Data are presented for three different NaCl concentrations in the incubation medium: (⊗) 50 mM; (○) 150 mM and (●) 300 mM.

preimmune serum. These experiments show that the observed dependence of plasmid binding on salt concentration is not a consequence of effects on antibody-DNA interactions. This dependence must reflect some other interaction.

Effect of changing negative superhelical density

We next studied the effect of NaCl on the binding of the anti-Z antibody to pBR322 plasmids as a function of the negative superhelical density. In Figure 3, the percentage of filter binding at one antibody concentration (1000 nM) is plotted as a function of NaCl concentration for plasmids with different negative superhelical densities ranging from 0.025 up to 0.113. At the highest negative superhelical density,

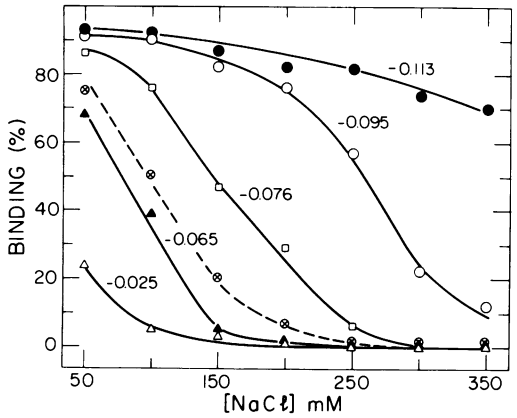

Fig. 3. The percentage binding of negatively supercoiled pBR322 plasmids to anti-Z-DNA antibodies at an antibody concentration of 1000 nM is plotted as a function of the NaCl concentration present in the incubation medium. The numbers refer to the measured negative superhelical densities ($-\sigma$) of the plasmids. The dotted line corresponds to the binding of the antibody to pBR322 at the superhelical density found in plasmids isolated directly from *E. coli*.

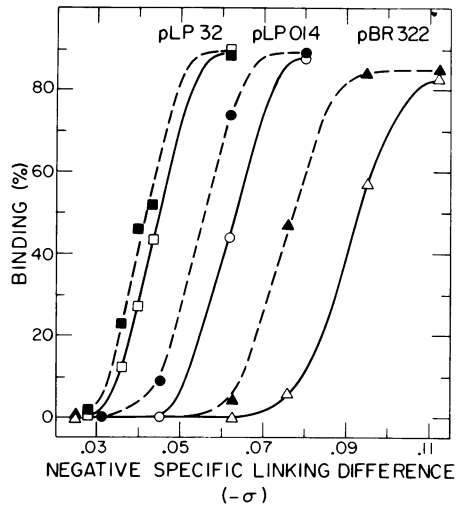

Fig. 4. The percentage binding of negatively supercoiled pBR322, pLP014 and pLP32 plasmids to anti-Z antibodies is plotted as a function of the negative specific linking difference (negative superhelical density) ($-\sigma$). The values were determined at an antibody concentration of 1000 nM, except in the case of pLP32 where an antibody concentration of 100 nM was adequate to produce complete retention of the plasmid on the filters, as noted previously (Nordheim *et al.*, 1982b). Solid lines binding at 250 mM Na^+, dashed lines binding at 150 mM Na^+.

changing the sodium concentration from 50 to 350 mM has only a small effect in decreasing the percentage of plasmids bound to the nitrocellulose filter. However, at a slightly reduced negative superhelical density of 0.095, increasing the NaCl concentration had a rather strong effect in that the binding was almost abolished at 350 mM NaCl. The results plotted at a density of -0.076 are the same as those shown in Figure 1 in which no Z-DNA was detected by antibody binding when assayed at 300 mM NaCl.

The dashed line in Figure 3 represents the binding of the antibody to a preparation of pBR322 isolated directly from the bacterial cell. Bacterial superhelical density characteristically has a broader Gaussian distribution of topoisomers than is seen in preparations in which the negative superhelical density is adjusted through the use of ethidium bromide and

Table I. Estimated energetics of the B to Z transition in negatively supercoiled plasmids[a]

	250 mM NaCl			150 mM NaCl		
	$-\sigma(-\tau)$	ΔG_{BZ}	ΔG_J	$-\sigma(-\tau)$	ΔG_{BZ}	ΔG_J
pLP32						
$(CG/GC)_{16}$	0.044 (18.4)			0.040 (16.9)		
		0.45	6.1		0.45	5.0
pLP014	0.062 (25.8)			0.055 (22.9)		
$(CG/GC)_7$						

$-\sigma$: negative superhelical density at the midpoint of the transition.
$-\tau$: number of negative superhelical turns at the midpoint of the transition.
ΔG_{BZ}: free energy change per GC base pair in going from B to Z-DNA, kcal per base pair.
ΔG_J: free energy change at the B-Z junction; kcal per junction.
[a]The values of $-\sigma$ used in these calculations correspond in all cases to those measured under topoisomerase relaxation conditions (25°C, 200 mM Na, 10 mM Tris-HCl, 0.1 mM EDTA, pH 8.0). No correction was applied for the effect of the ionic strength on the negative superhelical density, since according to Anderson and Bauer (1978), the actual values of $-\sigma$ would be modified only slightly, approximately ± 0.0005 in going from 200 mM NaCl to either 150 or 250 mM.

topoisomerase I. Nonetheless, the general form of the curve is similar and 75% of the plasmids are retained by the antibody on the nitrocellulose filter when assayed in 50 mM NaCl. The amount retained decreases steadily when assayed at higher NaCl concentrations and no retention is visible at 250 mM NaCl.

pBR322 carrying inserts of either 14 (pLP014) or 32 bp (pLP32) of alternating (dG-dC) residues formed Z-DNA at lower negative superhelical densities than pBR322 (Nordheim *et al.*, 1982b). Figure 4 plots the percentage binding of these plasmids plus pBR322 as measured at 1000 nM antibody concentration as a function of negative superhelical density ($-\sigma$). The results are presented for the binding at 250 mM Na^+ (solid lines) and at 150 mM Na^+ (dashed lines). In agreement with the results reported previously (Nordheim *et al.*, 1982b), the plasmid with the 32-bp insert forms Z-DNA at the lowest negative superhelical density, while pBR322 forms Z-DNA at the highest negative superhelical density. There are rather striking effects when the NaCl concentration is lowered from 250 to 150 mM. The midpoint of the transition for pBR322 is near -0.09 in 250 mM NaCl, and close to -0.075 in 150 mM NaCl. The shift in the curve for pLP014 is less pronounced than in pBR322 on going from 250 mM to 150 mM NaCl. Similarly, a smaller change is registered for pLP32. These data show that the magnitude of the salt effects appears to be related to the magnitude of the required negative superhelical density with a greater change occurring at the negative superhelical density for pBR322.

Negatively supercoiled plasmids are underwound and the excess free energy (ΔG_τ) increases as the square of the number of negative superhelical turns (Bauer and Vinograd, 1970; Hsieh and Wang, 1975; Depew and Wang, 1975; Pulleyblank *et al.*, 1975). When Z-DNA is formed, it reduces the negative superhelical strain of the plasmid. In the experiments of Figure 4, we have determined the midpoints of the B to Z-DNA transitions for inserts containing 14 (pLP014) and 32 (pLP32) base pairs of alternating C and G residues at both 250 mM and 150 mM NaCl (Table I). It has previously been shown that these inserts form Z-DNA (Nordheim *et al.*,

1982b). The midpoint values correspond to those measured when the CG inserts were in the B form. The conversion of such inserts to the Z-form changes the values of τ by +5.7 superhelical turns (for the 32-bp insert) and +2.5 superhelical turns (for the 14-bp insert). If we assume that the midpoint of the transition represents the point at which 50% of the inserts are in the Z form, the values of τ would be changed by +5.7/2 superhelical turns (for the 32-bp insert) and +2.5/2 superhelical turns (for the 14-bp insert). Taking into account the previous assumption, we can express the free energy of supercoiling at the midpoint of the transition as equal to the free energy of Z-DNA formation. According to Nordheim *et al.* (1982b), the free energy balance at this point can be expressed by:

$$\frac{400RT}{10.5}(-\sigma) = \Delta G_{BZ} + 2\Delta G_J/n \quad (1)$$

where the terms on the right correspond to the free energy of Z-DNA formation for a segment of Z-DNA containing n base pairs and two B-Z junctions. ΔG_{BZ} and ΔG_J are the free energies for forming Z-DNA per base pair and per junction, respectively. When the values of σ in Table I are corrected for the convesion to the Z form, we can then estimate the two unknowns in (1). Table I also lists the values estimated for ΔG_{BZ} and ΔG_J at both 250 mM and 150 mM NaCl. It is interesting that the change in free energy per GC base pair in going from B to Z-DNA remains constant at 0.45 kcal, but the free energy of the junction changes somewhat from 6.1 kcal in 250 mM to 5.0 kcal in 150 mM NaCl. A similar value of the change in free energy per base pair was estimated previously at a sodium ion concentration of ~300 mM and a value of 7.1 kcal was obtained for the free energy of the junction (Nordheim *et al.*, 1982b). It is important to note that these calculations are only approximate and are subject to many assumptions. For example, we assume that the antibody binding curve is a good indication of the B-Z equilibrium, and that the junction energy can be approximated by a single term. Further assumptions are made about the number of bases forming Z-DNA and the manner in which the free energy of supercoiling is calculated. However, these calculations seem to suggest that the free energy changes associated with the B-Z junctions may be sensitive to the ionic environment.

Z-DNA under ionic conditions close to physiological

Most cells accumulate potassium almost to the exclusion of sodium. Intracellular concentrations of potassium range from 150 to 200 mM in *Escherichia coli* and mammalian muscle to ~30 mM in fresh water invertebrates (Metzer, 1977). Thus, it is interesting to study the presence of Z-DNA at physiological concentrations of potassium. An experiment similar to that described in Figure 3 for pBR322 at bacterial superhelical density was performed, but KCl was used instead of NaCl in the assay medium. The same salt dependence was observed but a slightly higher percentage of binding was found when sodium was replaced by potassium (data not shown).

Magnesium is the major divalent cation found in cells. Most of the intracellular magnesium is bound to macromolecules and soluble compounds such as ATP and ADP. The intracellular concentration of free magnesium is reported to be in the range 1–5 mM (Lusk *et al.*, 1968; Damadian, 1971). We also studied the effect of magnesium on the formation of Z-DNA in superhelical pBR322. The addition of 1–5 mM of magnesium showed no significant effect upon

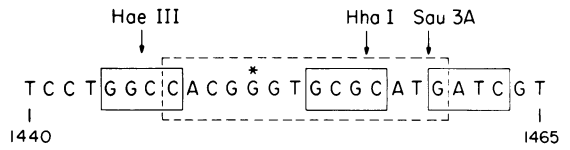

Fig. 5. The nucleotide sequences of pBR322 from residues 1440 to 1465 (Sutcliffe, 1979). The solid boxes correspond to the recognition sites for *Hae*III, *Hha*I and *Sau*3A. Enclosed in the dashed box is a 14-bp sequence of alternating purine and pyrimidine residues with one base pair (G*) out of alternation. This corresponds to the proposed attachment site of the anti-Z-DNA specific antibody (Nordheim *et al.*, 1982b).

the amount of Z-DNA formed at 100 mM NaCl in pBR322 at bacterial superhelical density, as detected by antibody binding. Higher concentrations of magnesium alone seem to be effective in inhibiting the B to Z transition, as the binding of the antibody is completely inhibited at 25 mM $MgCl_2$. Moncay and Kellenberger (1981) have recently reported higher concentrations of total Mg^{2+} in *E. coli*; however, they have not determined the concentration of free magnesium. The results of the experiments described generally indicate that a significant amount of Z-DNA is present in pBR322 at bacterial superhelical density under ionic conditions similar to those found *in vivo*.

Characterization of the Z-DNA segment in pBR322

Evidence was presented previously which suggested that, in pBR322, a 14-bp segment (1447–1460) of alternating purine-pyrimidine residues with one base pair out of alternation was forming Z-DNA (Nordheim *et al.*, 1982b). The sequence of this segment (Sutcliffe, 1979) is shown in Figure 5 with boxes outlining the recognition sequences for three different restriction endonucleases. In the previous experiments it was shown that the *Hae*III restriction site in Figure 5 was blocked by the presence of the antibody (Nordheim *et al.*, 1982b). In these experiments, the antibody was cross-linked to the Z-DNA using glutaraldehyde. Following this, the DNA was cut with *Hae*III and the digestion mixture was passed through a nitrocellulose filter. The *Hae*III fragments on either side of the cleavage position in Figure 5 were stoichiometrically reduced when the filtrate was analyzed on a polyacrylamide gel. This indicated that DNA containing two *Hae*III segments was cross-linked to the antibody and thus retained on the filter.

Similar experiments were performed involving the two additional restriction endonucleases, *Hha*I and *Sau*3A, which cleave at the positions indicated in Figure 5. The result of the *Hha*I digestion experiment is illustrated in Figure 6. Negatively supercoiled pBR322 was cleaved by *Hha*I restriction endonuclease either in the absence or in the presence of anti-Z-DNA antibodies which had been cross-linked to the DNA with glutaraldehyde. The digestion mixture was passed through a nitrocellulose filter and the filtrate was analyzed on an acrylamide gel. The *Hha*I restriction site at position 1456 shown in Figure 5 cleaves the DNA to form fragment 8 (190 bp) and fragment 27 (36 bp). As shown in Figure 6, in the presence of antibody the intensity of fragment 8 is reduced to ~30% of the value that it had in the absence of antibody. Fragment number 27 is not detected in this type of gel. These results support the interpretation that binding the antibody to negatively supercoiled pBR322 blocked the *Hha*I cleavage site at position 1456 shown in Figure 5.

Similar experiments were carried out using *Sau*3A. The

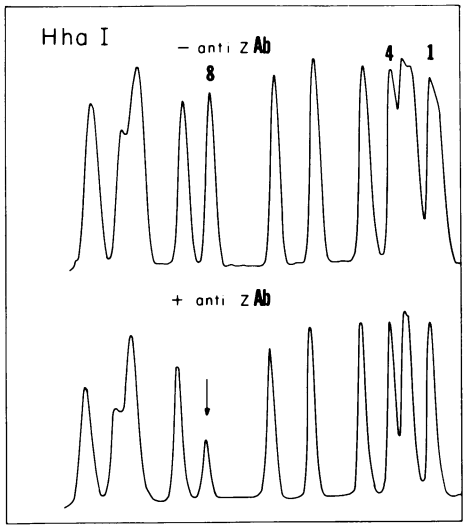

Fig. 6. Negative supercoiled pBR322 was treated with *Hha*I either in the absence or in the presence of anti-Z-DNA antibodies. Both samples were treated with glutaraldehyde which cross-linked the antibody to the DNA where it was present. The restricted plasmids were passed through a nitrocellulose filter and the filtrate analyzed in a 10% polyacrylamide gel. The densitometer tracing of the electrophoretically separated *Hha*I fragments are shown. It is evident that the intensity of *Hha*I fragment 8 is considerably reduced after cross-linking of the antibody to pBR322. Electrophoretic migration was from right to left.

presence of antibody produces a reduction in the intensity of the *Sau*3A restriction fragments numbers 4 and 8, which are the two fragments flanking the *Sau*3A site at position 1459 (results not shown). These experiments plus the previous results with *Hae*III cleavage support the proposal that Z-DNA was formed within the 14-bp sequence shown in Figure 5 (Nordheim *et al.*, 1982b).

Discussion

This paper deals with the effect of ions on the B to Z transition of segments of negatively supercoiled plasmids. Increase in sodium concentration from 50 to 350 mM results in reduced Z-DNA formation within the plasmid, as assayed by binding of Z-DNA specific antibodies. As seen in Figure 3, this effect is most pronounced in plasmids at lower negative superhelical densities. At negative superhelical densities >0.1, the increased ion concentrations appear to have a smaller effect. The control experiments in Figure 2 show that the observed salt effect is not a consequence of ionic influences on antigen-antibody interactions with either Z-DNA or B-DNA. Furthermore, there are not many protein-DNA interactions which happen to be salt-sensitive in this concentration range. For example, no salt effect was detected when core histones were used to retain the plasmids on the nitrocellulose filters (data not shown). Also, the fact that the combination with the antibody is not markedly inhibited with high superhelical densities (Figure 3) rules out other possible interpretations such as, for example, that the higher sodium concentrations affect the filterability of the antibody-plasmid complexes. In addition, measuring changes in electrophoretic mobility, Singleton *et al.* (1982) have reported a similar salt effect, thus ruling out the possibilty that the effect of sodium is on the Z-DNA-antibody interaction.

It is known that the superhelical density depends on the ionic strength (Hinton and Bode, 1975; Anderson and Bauer, 1978; Bauer, 1978). A value of 4.47×10^{-3} has been determined for $\Delta\sigma/-\Delta \log [Na^+]$ over the ionic strength range 50–300 mM (Anderson and Bauer, 1978; Bauer, 1978). Using this value, the actual change in superhelical density on going from 50 to 300 mM NaCl is −0.0035. Therefore, increasing the ionic strength actually produces a small increase in the negative superhelical density (i.e., σ has a higher negative value), thus facilitiating the formation of Z-DNA. Since we observed inhibition of Z-formation upon increasing the concentration of salt, this effect cannot be accounted for by the salt effects on negative supercoiling.

The salt effect also appears to have a certain generality since the potassium ion shows an effect which is qualitatively similar to that observed with sodium even though there are some small differences in the exact amount of inhibition. The fact that the magnesium ion seems to be very effective in inhibiting the B to Z conversion emphasizes the point that the charge and, in particular the nature, of the ion is important in determining the magnitude of the inhibitory effect.

There are other indications of the generality of the reaction. For example, the amount of Z-DNA detected by antibody binding in a pBR322 derivative containing a $d(CA/GT)_{32}$ insert also shows a dependence on sodium concentration similar to that shown above (Nordheim and Rich, 1983). Likewise, negative supercoiled SV40 viral DNA shows a similar dependence on sodium concentration (Nordheim and Rich, in preparation).

What is likely to be the cause of the marked sensitivity to cations in the formation of Z-DNA in negatively supercoiled plasmids? Figure 1 shows that negatively supercoiled pBR322 has a strong salt effect while Figure 2 shows no salt effect for either linear Z-DNA or B-DNA. One major difference between these two sets of data is that the negatively supercoiled plasmids have junctions between B- and Z-DNA, whereas the linear polymers of either B- or Z-DNA in Figure 2 do not have any junctions. Could the ion effect be important at the B-Z junction? For example, is it possible that on beginning to form a B-Z junction an ion complexing site is created into which the cation binds, thereby stabilizing the intermediate conformation? For the segment to flip all the way and form Z-DNA that intermediate conformation has to be broken to form the final B-Z junction. If an explanation of this type were valid, one would expect that increasing the concentration of cations would increase the stability of the postulated intermediate conformation. An explanation of this type would also suggest that if one has a large enough negative superhelical density, then the plasmid would have a high enough driving force to push through this postulated intermediate structure.

Some support for an interpretation of this type may be found in the data presented in Table I. The change in free energy per junction (ΔG_J) for the B to Z transition of $(dG\text{-}dC)_n$ sequences is calculated at different concentrations of NaCl and it is found to be sensitive to changes in ionic environment. The calculation of the junction energy is not likely to be very precise because it involves the difference between two large numbers; nonetheless, it suggests that the junction energy may change with salt concentration. This makes it reasonable to consider that the B-Z junction may be involved in the explanation of the sensitivity to NaCl described above.

What is striking about the effect of salt is that higher sodium concentrations in the molar range are known to stabilize Z-DNA (Pohl and Jovin, 1972; Thamann *et al.*, 1981). As mentioned above, the effect of salt on supercoiling is also in

the opposite direction since increased salt results in a small increase in negative superhelical density (i.e., σ is more negative) (Hinton and Bode, 1975; Anderson and Bauer, 1978; Bauer, 1978) which would tend to push towards increasing Z-DNA formation.

Very little is known about the B-Z junction. Singleton *et al.* (1982) have reported that B-Z junctions appear to have some sensitivity to a single-stranded S1 nuclease. If this is a general characteristic of B-Z junctions it is not impossible that these could be associated with ions in a manner that inhibited their formation. However, it is clear that the data available at present do not allow a satisfactory explanation for the effect of cations in inhibiting the conversion of B-DNA to Z-DNA in plasmids.

An interesting feature of the salt effect is the comparison of the different plasmids shown in Figure 4. The change in the negative superhelical density which is required to flip a segment from the B conformation to the Z conformation as a function of changing salt environments seems to depend upon the negative superhelical density at which the conversion takes place. In the plasmid pLP32 with a long stretch of alternating CG sequences, changing from 150 to 250 mM Na^+ has only a small effect on the superhelical density required to make this transition. On the other hand, pBR322, which requires a higher negative superhelical density in order to convert a segment to the Z conformation, is much more sensitive to NaCl. It suggests that the inhibitory effect, possibly due to sodium, on the B-Z junction is important when the segment which is flipping to the Z conformation requires a greater energy; in those segments which require less energy to flip from Z- to B-DNA the inhibitory influence of the junction is correspondingly reduced.

The data presented in Figure 3 dealing with the effect of NaCl on the amount of Z-DNA detected in antibody binding in pBR322 as well as the experiments with KCl and $MgCl_2$ strongly suggest that pBR322 contains some Z-DNA segments *in vivo*. However, they show that small changes in salt concentration in the physiological range appear to influence the amount of Z-DNA which is formed. The access of ions to plasmids may be somehow controlled *in vivo*, and polyamines may be especially important here. In addition, proteins which bind to B-DNA and to Z-DNA (Nordheim *et al.*, 1982a) are likely to influence the balance of ionic factors.

The experiments shown in Figures 5 and 6 together with the data described by Nordheim *et al.* (1982b) suggest that the 14-bp segment included in the dotted line of Figure 5 is involved in forming Z-DNA to generate the specific antibody binding site. These experiments do not give a direct measure of the length of the Z-DNA region. However, a plausible interpretation is that the entire 14-bp segment may be forming Z-DNA.

DNA is a polyanion and it is well known that cations influence the behavior of polyanions. Our observations suggest that cations in low concentrations may play a special role, perhaps at the B-Z junction, in controlling the formation of Z-DNA. Cations also play a role in stabilizing Z-DNA once it is formed. Further experiments are required before we can fully understand the mechanism of ionic interactions with B- and Z-DNA.

Materials and methods

Plasmids and DNA preparation

Plasmids pLP32 and pLP014 were constructed as described previously (Peck *et al.*, 1981). For the antibody binding experiments the plasmids were radioactively labeled. The plasmids were transformed into a thymidine requiring strain D41-TL3. *In vivo* labeling was carried out by growing the plasmid-containing bacteria in minimal medium in the presence of 1 μCi/ml of [^{3}H]-thymidine (New England Nuclear) (Nordheim *et al.*, 1982b). Plasmids were then prepared according to Clewell and Helinski (1970).

The plasmids were supercoiled by topoisomerase I relaxation in the presence of controlled amounts of ethidium bromide as described previously (Peck *et al.*, 1981; Nordheim *et al.*, 1982b). The superhelical densities of the resultant plasmids were determined by agarose gel electrophoresis in the presence of different amounts of chloroquine, as described before (Peck *et al.*, 1981; Nordheim *et al.*, 1982b), using the band-counting method of Keller (1975). The midpoint of the Gaussian distribution of topoisomers was determined from the densitometer tracing of the gel, and the actual superhelical density (σ) was determined from the measured midpoint of the Gaussian distribution by the use of the relationship $\sigma = \tau(4362/10.5)$.

Antibody-plasmid complexes

Affinity-purified antibodies against Z-DNA were prepared as described previously (Lafer *et al.*, 1981; Nordheim *et al.*, 1981). Formation of antibody-plasmid complexes was determined by nitrocellulose filter binding assays. In these experiments the antibody was mixed with the plasmid in 5 mM Tris-HCl, 1 mM EDTA (pH 8.0) buffers containing different amounts of NaCl, ranging from 50 to 350 mM NaCl. Incubation was carried out at room temperature for 1 h in a volume of 100 μl (30 min incubation produced the same level of antibody binding). 0.2 mg of DNA was used per assay and the antibody was serially diluted to varying concentrations. Millipore type HA 0.45 μm filters were used to retain the antibody-plasmid complexes. Filters were dried and counted in a scintillation counter. The level of background binding was determined by adding a rabbit IgG preparation at the highest concentration used with the anti-Z antibody.

Antibody-plasmid cross-linking

Antibody-plasmid cross-linking was carried out as described previously (Nordheim *et al.*, 1982b). 20 μg antibody were mixed with 2 μg of plasmid in 50 mM triethanolamine (pH 7.9), 100 mM NaCl buffer. Incubation was carried out at 37°C for 45 min, and then the solution was made 0.1% in glutaraldehyde. Cross-linking was carried out at 37°C for 30 min. Free glutaraldehyde and unbound antibody were removed by passage of the reaction mixture through a Sepharose 2B column equilibrated with 6.6 mM Tris-HCl (pH 7.5), 6.6 mM $MgCl_2$, 6.6 mM dithiothreitol, 6.6 mM NaCl. Fractions containing the plasmid-antibody complex were pooled and then incubated with *Hae*III, *Hha*I or *Sau*3A restriction endonucleases at a concentration of 5 units/μg of DNA. Incubation was carried out at 37°C for 2 h. The restriction digest was then passed through a Millipore nitrocellulose filter (Millex-HA, 0.45 μm) and the eluate was precipitated with ethanol. The precipitates were analyzed either on 6% or 10% polyacrylamide gels in Tris/borate/ EDTA buffer (90 mM Tris-HCl, 90 mM boric acid, 2.5 mM EDTA, pH = 8.3). Gels were stained with ethidium bromide (0.5 μg/ml) and photographed under u.v. light.

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