Supercoiling and Left-handed Z-DNA

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For about 20 years now it has been known that DNA in cells of living organisms can exist in the form of double-stranded, covalently closed, circular molecules (Cairns 1963; Weil and Vinograd 1963). The covalently closed nature of the DNA sugar-phosphate backbone in such a ring results in topological linkage of the complementary DNA strands that are helically coiled around each other. The underlying topological constraint applies not only for closed circular DNA (plasmids) but also for linear DNA segments, the ends of which are fixed relative to one another. In a circular DNA duplex containing a single-chain scission, the degree of intertwining between the two DNA strands is determined by the size of the circular molecule and the resulting number of double-helical turns. Upon closure of the single-chain scission, the two DNA strands are linked as many times as there are helical turns, and this linking number (α^0) is a constant as long as the circle remains covalently closed. This circular DNA is referred to as the *relaxed* form. If at the time of ring closure the double-helical molecule deviated from its relaxed form, a supercoiled circle is generated. Helically underwound or overwound states at the time of ring closure result in negatively or positively supercoiled circles, respectively. All circular DNAs isolated from natural sources exist in the negatively supercoiled state.

There has been considerable study of the phenomenon of supercoiling, and the discovery of topoisomerases that act to modify the degree of supercoiling has given emphasis to the fact that this is likely to be a biological phenomenon used by nature in a significant way. By thermodynamic criteria, supercoiled DNA is in a higher free-energy state relative to the relaxed form; consequently, any process that decreases the degree of supercoiling is energetically favored in such a system (Bauer and Vinograd 1970; Wang 1980, 1982). In a negatively supercoiled circle, such mechanisms include intercalation of dyes, local DNA denaturation, and formation of cruciform structures (Gellert et al. 1979; Mizuuchi et al. 1982). The discovery of a left-handed, double-helical form of DNA—Z-DNA (Wang et al. 1979)—added additional significance to the phenomenon of superhelicity. Conformational transitions from right-handed B-DNA to left-handed Z-DNA must be favored in a negatively supercoiled DNA circle. Conceptually, the formation of one left-handed, double-helical turn (12 bp/turn) out of 1.2 right-handed helical turns (10.5 bp/turn) results in the removal of two negative superhelical turns. This is schematically illustrated in Figure 1, which shows a supercoiled plasmid containing three negative superhelical turns when it is entirely in the form of B-DNA. It is shown to be in equilibrium with another conformer that has approximately one right-handed, double-helical turn flipped into one left-handed turn. This B→Z transition is associated with the removal of two negative superhelical turns.

Salt-induced Transition of B-DNA to Z-DNA in Plasmids

Initial experiments dealing with the conversion of B-DNA to Z-DNA in closed circular plasmids were carried out with plasmids that contained inserts of alternating C and G residues (Klysik et al. 1981; Peck et al. 1982). These experiments used the fact that in solutions containing high molar concentrations of NaCl or lower concentrations of cations such as cobalt hexamine, Z-DNA is the stable form of poly(dG-dC) (Pohl and Jovin 1972; Behe and Felsenfeld 1981).

Peck et al. (1982) studied two plasmids in their experiments: pBR322 (Bolivar et al. 1977), which contains 4362 bp (Sutcliff 1979), and a closely related plasmid, pLP32, which had inserted 32 bp of alternating C and G sequences. Quantitative experiments were carried out to determine the relation between sedimentation constant and the number of superhelical turns in the plasmid. Sedimentation analyses using pLP32 demon-

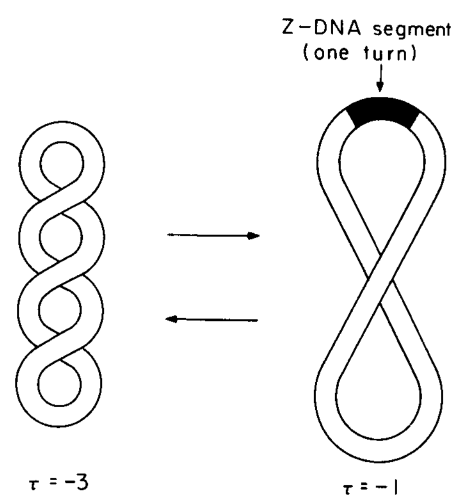

Figure 1. Schematic diagram illustrating the effect on supercoiling of converting a segment of a plasmid from B-DNA to Z-DNA. In this diagram a 12-bp segment of B-DNA has converted to Z-DNA, and it has resulted in a loss of two superhelical turns.

strated a sharp change in sedimentation constant upon increasing NaCl concentration from 2 M to 3.5 M. A change in the average number of superhelical turns of 5.6 was calculated. If 32 bp of B-DNA were converted to Z-DNA, the expected change in supercoiling would be 5.7. Thus, the quantitative agreement between the change in sedimentation constant and the known geometries of B-DNA and Z-DNA made it possible to infer that that entire segment of alternating CG residues had converted to Z-DNA in 3.5 M NaCl. The quantitative agreement in these experiments is important because an alternative conformational change into a cruciform structure is theoretically possible due to the self-complementarity of the inserted, alternating C and G residues. However, formation of a cruciform structure would have removed only four superhelical turns.

B→Z Conversions in Plasmids Induced by Negative Supercoiling

The expectation that the conversion of a right-handed, double-helical segment to a left-handed one is energetically favored in a negatively supercoiled plasmid was recently put to test (Nordheim et al. 1982; Peck et al. 1982). Peck et al. (1982) introduced into the plasmid pLP32 (containing 32 bp of alternating CG sequence) a selected critical superhelical density and studied its rate of migration in agarose gel electrophoresis in the presence and absence of the intercalating drug chloroquine. The rate of migration in agarose gel electrophoresis is a direct measure of the superhelical density of a plasmid preparation.

Intercalators are generally planar molecules that have an affinity for inserting themselves between the planar base pairs in B-DNA. In B-DNA the twist per base pair along the double helix is 34°. When the intercalator ethidium bromide is inserted between two adjoining base pairs, the twist per base pair is reduced by 26° (Wang 1974). Thus, the effect of the addition of intercalators to DNA is to produce an unwinding. For a closed circular plasmid, the introduction of a planar intercalator results in a reduction of the negative superhelicity of the plasmid, i.e., it becomes more positively supercoiled. Likewise, subsequent removal of the drug has just the opposite effect in that it allows the plasmid to assume its original, negative superhelical density.

These properties of intercalating molecules can be used to demonstrate the conversion of B-DNA to Z-DNA in negatively supercoiled plasmids. In general, topoisomers exist in a distribution that can be visualized in agarose gel electrophoresis. In Figure 2 the relative electrophoretic migration velocities of pBR322 and pLP32 are compared at a given superhelical density in the presence (Fig. 2A) and absence (Fig. 2B) of chloroquine (450 nM). A pBR322 preparation with 15.5 negative superhelical turns (negative-linking difference) is run in the two outer lanes, whereas pLP32 migrates in the center panels. In the presence of chloroquine (Fig. 2A), it can be seen that the topoisomers of pLP32 migrate more rapidly than those of pBR322. If, how-

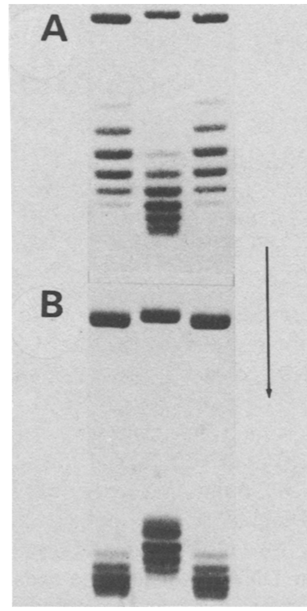

Figure 2. Agarose gel electrophoretic patterns of plasmids pBR322 and pLP32. The outer two lanes contain pBR322, and the center lane contains pLP32. (*A*) In addition to the electrophoresis buffer, the gel contains 450 nM chloroquine. (*B*) Plasmids are run in the same buffer as *A* but in the absence of chloroquine. The pBR322 used in these runs has a linking difference of −15.5.

ever, the identical DNA preparations are electrophoresed in the absence of chloroquine (Fig. 2B), the relative mobilities are reversed, with pLP32 migrating more slowly than the pBR322 preparation. Removal of the intercalator chloroquine allowed both plasmids to assume an equally increased state of superhelical density. This higher superhelical density, in turn, induced the flipping of the CG segment of pLP32 to a left-handed double helix, which resulted in removal of superhelical turns. No such flipping occurred in pBR322 itself, so that a change in the relative mobilities of migration can be observed. This experiment illustrates in a qualitative way that changes in the superhelical density and associated changes in the hydrodynamic features of a plasmid can be used to monitor conformational transitions in the helical hand of right-handed B-DNA segments.

Antibodies Can Identify Z-DNA Segments in Supercoiled Plasmids

Unlike B-DNA, Z-DNA is highly immunogenic. When it is injected into rabbits or mice, it stimulates the production in a high titer of antibodies that have been shown to be highly specific for the Z-DNA conformations and will not react with B-DNA or a variety of other single- or double-stranded nucleic acid conformations (Lafer et al. 1981). Antibodies have been used to study the conversion from B-DNA to Z-DNA in supercoiled plasmids upon changing their negative superhelical density (Nordheim et al. 1982). The negative superhelical density of closed circular DNA plasmids can be modi-

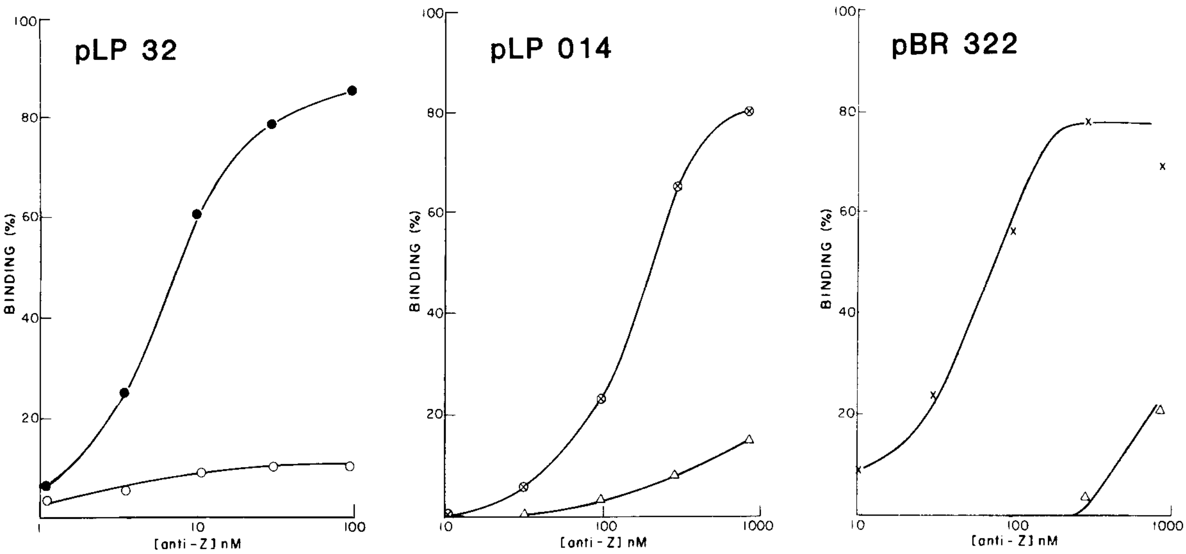

Figure 3. Binding of radioactive plasmids pLP32, pLP014, and pBR322 to nitrocellulose filters in the presence of antibodies specific for Z-DNA is shown as a function of antibody concentration. The plasmids have the following number of negative superhelical turns: pLP32: ● 26.2, (○) 15.1; pLP014: (⊗) 35.0, (Δ) 27.0; pBR322: (×) 46.0, (Δ) 35.5. These numbers indicate the center of the Gaussian distribution of topoisomers for each of the plasmid preparations. The antibody binds the DNA only in the negatively supercoiled plasmids in the ranges of superhelical densities indicated, but it does not bind the relaxed plasmid.

fied in vitro using the enzyme topoisomerase I. Topoisomerases have the ability to change the linking number of covalently closed, circular DNA. Topoisomerase I relaxation reactions can be performed in the presence of different concentrations of intercalating dyes like ethidium bromide. After inactivation of the enzyme and removal of the ethidium bromide, plasmid preparations are generated in which the degree of supercoiling is proportional to the concentration of intercalator present during the enzyme reaction. The negative superhelical density generated can be measured by agarose gel electrophoresis, producing patterns similar to those described in Figure 2. By having an overlapping series of topoisomers, the band-counting method (Keller 1975) allows determination of the center of the Gaussian distribution for any particular topoisomer preparation.

Radioactively labeled plasmid DNAs of different superhelical densities were allowed to react with anti-Z-DNA antibody. Antibody-antigen recognition was detected in a nitrocellulose-filter-binding assay. This assay depends on the inability of proteins or protein-DNA complexes to pass through the filter, whereas uncomplexed plasmid DNA is not retained.

The results of a quantitative study are shown in the curves of Figure 3. Here it can be seen that supercoiling the plasmid pLP32 to an average of 26.2 negative superhelical turns results in the retention of 80% of the plasmid on the filter paper when 96 nM of anti-Z-DNA antibody are added (Fig. 3). In another plasmid preparation in which the negative supercoiling had an average value of 15.1, less than 10% was retained on the filter. Furthermore, the completely relaxed plasmid was not retained at all. A number of measurements were made with plasmid preparations of negative linking differences between 15 and 26, and the results were intermediate between those shown in the figure.

Can shorter segments be driven into left-handedness upon negative supercoiling? In Figure 3, plasmid pLP014 is shown in which there are 14 bp of alternating cytosine and guanine residues cloned into pBR322. It can be seen that 35 negative superhelical coils are required to retain 80% of the plasmids on the filter, in contrast to the lower number needed for pLP32. It should be noted in this case that the concentration of antibodies required is tenfold over that needed for pLP32. With 27 negative superhelical turns, only slightly more than 10% is retained on the filter, and again the totally relaxed plasmid is not retained at all. These experiments suggest that both the 32-bp insert and the 14-bp insert are capable of forming Z-DNA; however, a larger number of negative superhelical turns must be introduced into the plasmid with the smaller insert in order for it to bind to the filter.

In these experiments the maximum binding was observed to be 80%. This is because almost 20% of the plasmids were found to be nicked and accordingly could not be supercoiled. It is interesting to note that a larger number of negative superhelical turns are required in order to convert the shorter segment of alternating CG residues into Z-DNA. Pohl and Jovin (1972) have shown that the conversion of B-DNA to Z-DNA is cooperative, suggesting that the generation of a B-Z junction is energetically unfavorable. The flipping of an internal B-DNA segment of any length to the Z-form creates two B-Z junctions; thus, it is expected that for an internal segment of increasing length that converts from the B to the Z form, the junction free-energy term assumes a decreasing proportion of the total free energy that is required to effect the transition. The driving force provided by the reduction in the degree of supercoiling accompanying the B→Z transition increases with the length of the segment flipped: the longer the length of the segment flipped, the larger the change in the number of superhelical turns, and the larger the free energy gained. Thus, it is reasonable that at a given negative superhelical density, longer alternating CG inserts undergo the B→Z transition more readily than shorter ones.

In control experiments with the parent plasmid pBR322 itself, no antibody binding could be detected up to a linking difference of −33; further supercoiling, however, resulted in the generation of anti-Z antibody recognition. In Figure 3 it can be seen that 80% of the pBR322 plasmids are retained when there are 46 negative superhelical turns in the plasmid. With 35 negative superhelical turns, about 20% of the plasmids are maintained. The data in Figure 3 thus show that different segments of DNA in the various plasmids are forming Z-DNA as a function of increasing amounts of negative superhelical turns.

Figure 4 presents data summarizing the binding of the three plasmids pLP32, pLP014, and pBR322 as a function of negative superhelical density (or negative specific linking difference, $-\sigma$). If a plasmid in the relaxed state has 400 helical turns of DNA and that plasmid has 20 negative superhelical turns, the negative superhelical density is 0.05 (the number of superhelical turns divided by the total number of helical turns in the relaxed plasmid). The percentage of binding is measured for all of these plasmids at an antibody concentration of 860 nM. These results show in greater detail the conversions illustrated in Figure 3. The midpoint of these curves for the retention of the plasmid occurs at a negative superhelical density close to 0.05 for pLP32, 0.07 for pLP014, and at 0.09 for pBR322 itself. These numbers are of interest because they fall within the range of negative superhelical densities observed in biological systems (Bauer 1978). We can infer from the data presented in Figure 4 that there may exist in DNA a variety of segments of DNA that, depending on their sequence, may have different proclivities for forming Z-DNA as a function of increasing negative superhelical density.

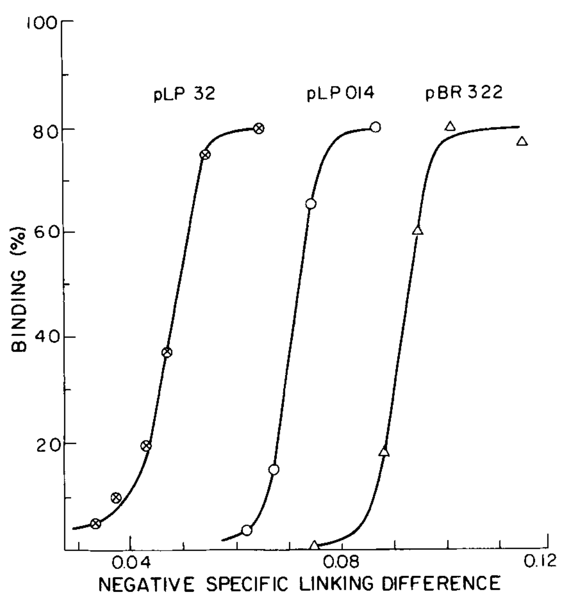

Figure 4. Results of experiments similar to those shown in Fig. 3 are illustrated by plotting the percentage of negatively supercoiled plasmids that are bound to nitrocellulose filters by antibodies as a function of negative specific linking difference ($-\sigma$; negative superhelical density). The data shown are all obtained at an antibody concentration of 860 nM. It can be seen that the plasmids with larger inserts are retained on the filter by antibody at lower negative superhelical densities than those for plasmids with shorter inserts or for pBR322 without inserts.

It is possible to visualize the complex of antibody with negative superhelical plasmids by electron microscopy (Fig. 5). A quantitative study of the distribution of antibodies on negatively supercoiled plasmids has shown that there is one antibody found per plasmid on pLP32 when it has been negatively supercoiled such that it shows retention in the filter-binding assay. However, the same statistical count reveals that in the absence of negative supercoiling, few antibodies were found associated with relaxed plasmids.

Identification of DNA Sequences in Negatively Supercoiled Plasmids Bound to Antibodies

The experiments shown in Figures 3 and 4 strongly suggest that the antibody is probably combining with the inserted segments of alternating C and G that are capable of forming Z-DNA when they are negatively supercoiled. However, the experiments involving pBR322 leave us wondering which sequences in that plasmid the antibody may be binding to. In an attempt to solve that problem, a modification of the filter-binding experiment was carried out (Nordheim et al. 1982). In this experiment the plasmid pLP32 was negatively supercoiled and the antibody was added to it. Following this, the antibody was cross-linked to the nucleic acid through the addition of 0.1% glutaraldehyde. The supercoiled plasmid–antibody complex was then digested with the enzyme *Hae*III, which cleaves the sequence GGCC and produces 22 fragments of DNA. Following complete cleavage by *Hae*III, the fragments are then passed through the nitrocellulose filter. Fragments to which the antibody is attached will be removed from the mixture and the other fragments will filter through. The presence of the fragments in the filtrate was then determined by carrying out acrylamide electrophoresis of the DNA. The *Hae*III digestion pattern of pLP32 has been well characterized, and the results of this experiment showed that more than 80% of the fragment containing the insert was removed from the filtrate (Fig. 6A,B). All other fragments of the *Hae*III digest were recovered quantitatively as visualized in the electrophoresis pattern. This experiment strongly suggests that it was the CG insert that had formed Z-DNA in pLP32.

The results of the equivalent experiment using pBR322 is shown in Figure 6 C and D. In lane C the *Hae*III digestion pattern of pBR322 itself is shown after glutaraldehyde treatment in the absence of antibody. Lane D shows the digestion pattern obtained in the presence of the antibody. It can be seen that two of the

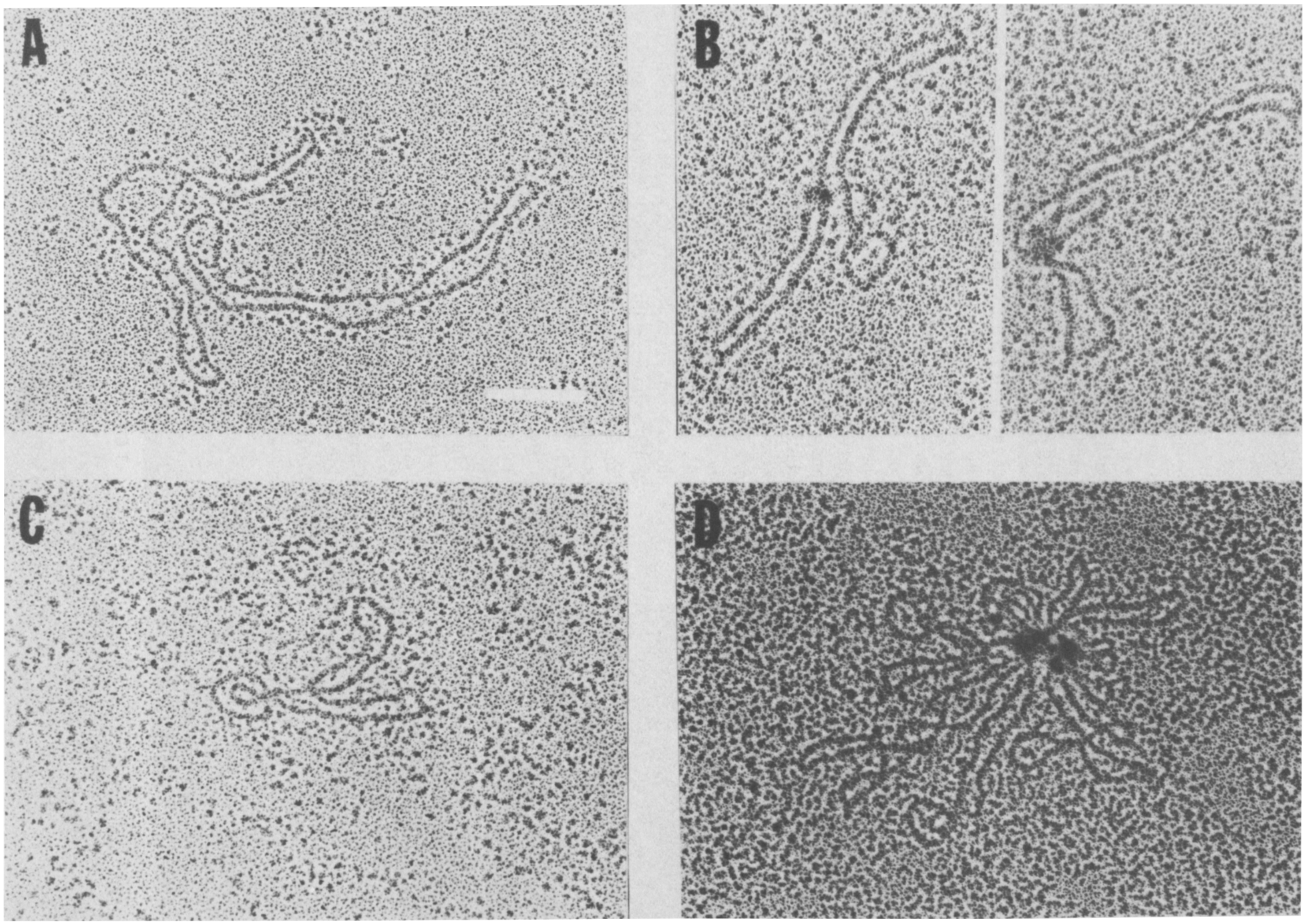

Figure 5. Electron micrographs of pLP32 containing a 32-bp insert of alternating CG residues. The plasmids were mixed with antibody and visualized with an electron microscope, using the cytochrome-*c* spreading technique. (*A*) Relaxed pLP32 plasmid not bound by antibodies; (*B*) negatively supercoiled pLP32 plasmids ($\sigma = -26.2$) with only one antibody attached per DNA circle; (*C*) pBR322 of low superhelical density ($\sigma \cong -18$); (*D*) highly coiled pBR322 ($\sigma = -41$).

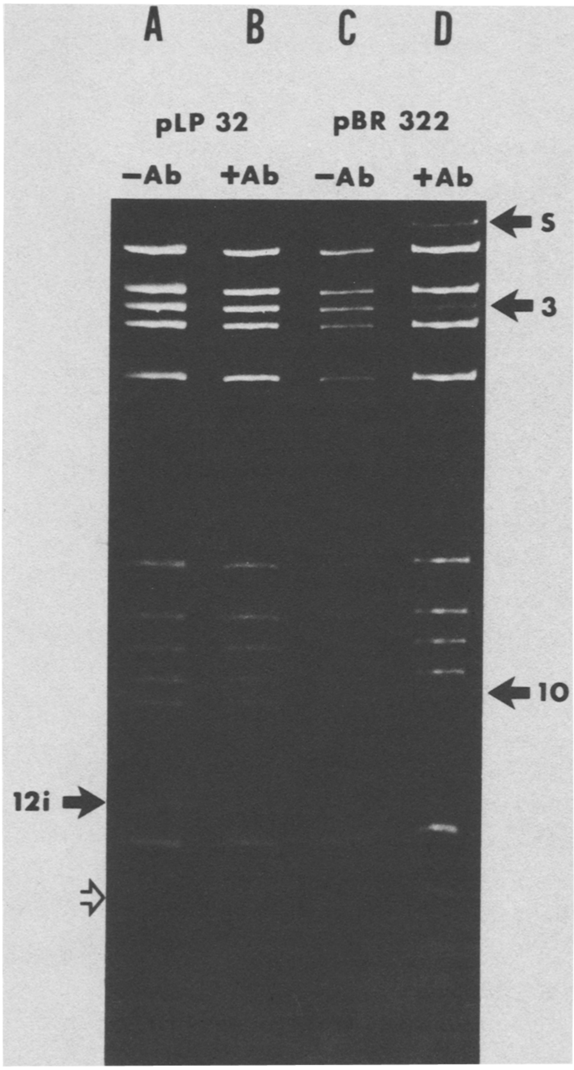

Figure 6. Polyacrylamide gel electrophoresis of *Hae*III digestion fragments of negatively supercoiled pLP32 (*A,B*) and pBR322 (*C,D*) plasmids. Negatively supercoiled plasmids were divided into two aliquots, and anti-Z-DNA antibodies were added to one. Following this, both samples were cross-linked with 0.1% glutaraldehyde and digested extensively with *Hae*III restriction endonuclease. The material was then passed through a nitrocellulose filter, and the filtrate was analyzed by polyacrylamide gel electrophoresis. (*A,C*) *Hae*III digestion patterns of pLP32 and pBR322, respectively, in the absence of the antibody; (*B,D*) electrophoretic patterns of *Hae*III fragments that passed through the filter following cross-linking to the antibody. It can be seen that there is a reduced intensity of fragment 12i of pLP32. With pBR322, fragments 3 and 10 are reduced, and a new fragment, S, appears in the pattern (Nordheim et al. 1982).

bands, 3 and 10, are reduced in intensity compared with the bands found in the pBR322 digestion pattern. Furthermore, a densitometer scan of these electrophoretic patterns reveals that the reduction in these two bands is stoichiometric. At the same time, a new band, labeled S, is seen at the top of the gel. From its position, it can be shown that band S has a length in nucleotides that is approximately the sum of band 3 and band 10. Furthermore, fragments 3 and 10 are neighboring fragments on either side of the *Hae*III recognition site at nucleotide 1445.

Z-DNA is strongly favored by alternating purine-pyrimidine sequences (Wang et al. 1979). The reason for this rests with the fact that every other residue along the Z-DNA polynucleotide chain has a *syn* conformation of the base relative to the sugar. Although *syn* conformations can form with purine residues without any energy loss, there is a bias in favor of the *anti* conformation for pyrimidines, and an energy penalty is associated with the *syn* conformation of pyrimidines. A computer analysis of the nucleotide sequence of pBR322 has been carried out to determine the length of the tracks containing alternating purine and pyrimidine sequences. This search reveals that there is 1 sequence containing 10 bp of alternating purines and pyrimidines, 8 sequences containing 9 residues, 6 containing 8 residues, and 11 containing 7 residues. One of the sites containing 9 bp of alternating purine-pyrimidine sequence is located close to the *Hae*III site at nucleotide 1445 (Fig. 7). Furthermore, a stretch containing 14 bp of alternating purine-pyrimidines can be formed with one nucleotide out of phase found at guanine 1451. This 14-bp sequence includes the last C of the *Hae*III site. The data shown in Figure 6 are consistent with the interpretation that on negatively supercoiling pBR322, a 14-bp segment of alternating purine-pyrimidines with one mismatch, or a 9-bp section of alternating purine-pyrimidines within this segment, is in the Z structure, and the antibody molecule combines with that site to block the action of the *Hae*III enzyme at site 1445. This accounts for the stoichiometric reduction in fragments 3 and 10 in Figure 6D. The presence of band S in Figure 6 with an approximate length of fragments 3 and 10 in the *Hae*III digest suggests that some of the cross-linking may have been reversed during the filtration process.

In a survey of the other segments of alternating purines and pyrimidines in pBR322, we looked for segments that are longer than the 14-bp segment shown in Figure 7 containing one nucleotide out of phase. There are no other segments longer than the one at position 1447, and there is one other segment found in *Hae*III fragment 2 at position 2315 that contains a 14-bp segment with one nucleotide out of phase. At present, it

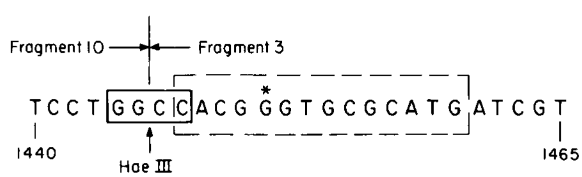

Figure 7. Nucleotide sequence of pBR322 near the *Hae*III site at residue 1445. The solid box encloses the *Hae*III site. Fragment 3 and fragment 10 are contiguous. The dashed line encloses a 14-bp fragment containing alternating purine and pyrimidine residues in which there is 1 residue out of phase (*). The segment enclosed with the dashed line is believed to have formed a Z-DNA fragment to which the antibody binds.

is not clear why the site near 1447 is preferred at this negative superhelical density, but these observations open the way to further studies of additonal segments of pBR322 that may form Z-DNA at even higher negative superhelical densities.

Superhelical Density and Z-DNA

The degree of supercoiling in a plasmid can be expressed as the linking difference τ (Vinograd and Lebowitz 1966) in the form

$$\tau = \alpha - \alpha^0 \tag{1}$$

where α represents the linking number of a covalently closed double helix and α^0 represents the linking number of the same DNA when it is relaxed. The superhelical density can then be expressed as the specific linking difference σ:

$$\sigma = (\alpha - \alpha^0)/\alpha^0 \tag{2}$$

The linking number α is invariable in a covalently closed duplex circle. Changes in the form of the helix associated with a B→Z conformational transition changes the magnitude of the linking difference τ, which means that the reference standard for the relaxed DNA (α^0) must have changed.

The degree of supercoiling of a circular DNA profoundly affects its hydrodynamic and thermodynamic characteristics, as mentioned above. From a thermodynamic point of view, supercoiled DNA is in a higher free-energy state relative to the relaxed form. This can be demonstrated experimentally by the fact that nicking of the plasmid results in its immediate return to a relaxed conformation. The excess free energy due to supercoiling has been shown to be proportional to the square of the linking difference $\alpha - \alpha^0$ (Bauer and Vinograd 1970; Depew and Wang 1975; Hsieh and Wang 1975; Pulleyblank et al. 1975). Thus, with a higher level of negative superhelical coiling, processes will be favored that reduce the magnitude of the linking difference. A number of processes are capable of reducing the negative superhelical coiling. One of them, mentioned above, is the addition of a dye molecule, which changes the unwinding angle of DNA and reduces its negative supercoiling. Other processes of this type include binding to proteins such as RNA polymerase or histones to form nucleosomes, or the disruption of a segment of DNA to form a cruciform. However, one of the most effective methods for reducing negative superhelical density is by twisting the double helix in the opposite direction to form Z-DNA. It has been estimated that the change in the free energy associated with flipping a 30-bp insert from B-DNA to Z-DNA results in a change of −30 kcal at a negative superhelical density of 0.05 (Wang 1982).

If we wish to treat this conversion process in a quantitative way, it is relatively easy to modify Equation 1 by considering the reference standard for the linking number of the relaxed state α^0 as composed of two parts:

$$\alpha^0 = \alpha^0_R + \alpha^0_L \tag{3}$$

In this formulation, α^0_R is the linking number of the unstressed right-handed DNA segments. α^0_L is applied to the left-handed segments only and refers to a "virtually" relaxed left-handed state, which has 12 bp per helical turn. Because the helix is left-handed, α^0_L will always be a negative number. Equation 1 can now be expressed as:

$$\tau = \alpha - (\alpha^0_R + \alpha^0_L). \tag{4}$$

For example, if 12 bp of pBR322 were to convert from right-handed to left-handed DNA, then α^0_R would be decreased by slightly more than 1, and α^0_L would have a value of −1. Thus, the linking difference $\alpha - \alpha^0$ has changed slightly greater than 2, and this would be registered by a reduction in negative superhelical turns by slightly more than 2 (see Fig. 1). This relationship may be useful in the analysis of a variety of naturally occurring DNA sequences in which the supercoiling changes can be measured with increasing, negative superhelical density. These changes can be used in a quantitative way to interpret the total number of bases involved in forming Z-DNA if that is the only process taking place. However, it must be borne in mind that other processes can also reduce negative supercoiling, so it is important to demonstrate that all of the changes are due to Z-DNA formation.

The experiments described above show that negatively supercoiled plasmids in the physiological range of negative superhelical densities can convert segments into Z-DNA. Negative superhelical DNA is not confined to covalently closed circles of DNA, but it is believed that the chromosomes in prokaryotic cells as well as eukaryotic cells are found in domains of DNA with local topological constraints. These experiments on supercoiling and Z-DNA formation may also have some relevance to an understanding of the more complex systems in eukaryotic genomes in which DNA is organized into nucleosomes and higher-order structures. The hypothesis that segments of genomes may form Z-DNA (Nordheim et al. 1981) introduces a new element into our thinking about the behavior of DNA in vivo, and this may enable us ultimately to understand some of the molecular mechanisms associated with the various biological activities carried out by DNA in living systems.

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