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An Assessment of the Z-DNA Forming Potential of Alternating dA-dT Stretches in Supercoiled Plasmids†

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Received October 11, 1985; Revised Manuscript Received February 10, 1986

ABSTRACT: The ability of a stretch of alternating dA-dT to adopt the left-handed Z form has been assessed by examining the behavior of the sequence $d(CG)_6(TA)_4(CG)_6$ contained in the plasmid pBR322. The structural transition occurring within this sequence as a function of negative superhelicity was analyzed by several methods, including (1) the supercoiling-dependent unwinding of the insert as determined by two-dimensional gel electrophoresis, (2) the binding of anti-Z-DNA antibodies to the insert, (3) the sensitivity of the sequence to a single strand specific endonuclease, and (4) the sensitivity of the insert to digestion by a restriction endonuclease that cuts within the $d(CG)_6$ segments when in the right-handed form. These studies have shown that in negatively supercoiled DNA the two $d(CG)_6$ portions of the insert adopt the Z form, while the central $d(TA)_4$ segment forms an underwound structure with a helical repeat that is best approximated as being intermediate between the B form and the Z form. A statistical mechanical treatment of the unwinding of the insert as a function of negative superhelicity provides an estimate of the minimum free energy required to convert an A-T bp from the B form to the Z form, as well as the free energy associated with the conversion of an A-T bp from the B form to the unwound form. These results strongly indicate that Z DNA is an unfavored structural alternative for stretches of $d(AT)_n$ in negatively supercoiled DNA.

It is now apparent that duplex DNA can assume a variety of conformations that differ by degree from the canonical B form. Since these altered structures could act as focal points for important genetic processes, it is of interest to determine which DNA sequences are the most adept at undergoing structural change. The most dramatic and certainly the best characterized structural transition known to date is the conversion of right-handed B DNA to the left-handed Z form [see Rich et al. (1984) for review]. In Z DNA, the bases along each strand of the helix remain Watson and Crick base-paired but alternate regularly between the syn and anti conformation.

Although the rules governing which DNA sequences can adopt the Z form are only partially understood, it is clear that certain sequences can flip to the Z form far more readily than others. Stretches of alternating $d(CG)_n$ and $d(CA)_n$ have been shown to adopt the Z form in negatively supercoiled plasmids under physiological conditions (Singleton et al., 1982; Peck & Wang, 1983; Haniford & Pulleyblank, 1983; Nordheim et al., 1982). The strong Z-forming potential of these alternating purine–pyrimidine stretches has been explained empirically by the observation that purine nucleotides assume the syn conformation more easily than pyrimidine nucleotides (Haschemeyer & Rich, 1967). This observation implies that Z DNA will be formed most stably in an alternating purine–

† This research was supported by grants from the National Institutes of Health, the American Cancer Society, the National Aeronautics and Space Administration, the Office of Naval Research, and the National Science Foundation. M.J.E. was supported by a fellowship from the Medical Research Council of Canada. J.F. was supported by Damon Runyon-Walter Winchell Cancer Fund Postdoctoral Fellowship DRG-585. R.J.K. was supported by funds from the Undergraduate Research Opportunities Program at MIT.
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0006-2960/86/0425-3648$01.50/0

pyrimidine arrangement of nucleotides in which all of the purines are in syn while the pyrimidines remain in the anti conformation.

Recently, however, the positive correlation between Z-forming potential and purine–pyrimidine alternation has become less clear. Several studies from this laboratory have shown directly that purine–pyrimidine alternation is not an absolute requirement for Z-DNA formation (Wang et al., 1985; Feigon et al., 1985). In addition, it appears that this correlation cannot be extended to include the alternating sequence $d(AT)_n$. While conditions have been reported that promote the formation of Z DNA in the polymers $d(CG)_n$·$d(CG)_n$ and $d(CA)_n$·$d(TG)_n$ (Pohl & Jovin, 1972; McIntosh et al., 1983; Taboury & Taillandier, 1985), similar conditions are not sufficient to induce $d(TA)_n$·$d(TA)_n$ to form Z DNA (Vorlickova et al., 1980). Several groups have also shown, for instance, that long stretches of $d(AT)_n$ contained in plasmids extrude to form cruciforms in response to negative supercoiling rather than undergo the transition to Z DNA (Haniford & Pulleyblank, 1985; Panyutin et al., 1985; Greaves et al., 1985).

In this work, we examine as a function of negative superhelicity the ability of a short stretch of alternating dA-dT to adopt the Z form when flanked by strong Z-forming sequences contained in a plasmid. Since statistical mechanical methods exist for quantifying the extent of the transition as a function of the free energy change associated with negative supercoiling, it is possible to estimate the minimum free-energy change associated with the conversion of an A-T bp from the B form to the Z form. Here, we demonstrate that although Z DNA induces a structural transition in the adjacent $d(AT)_n$ segment, this structure does not predominate as Z DNA.

Materials and Methods

Plasmid Construction and Manipulation. The self-complementary DNA 32-mer 5′-$d(CG)_6(TA)_4(CG)_6$ was synthesized by the phosphoramidite method. The single-stranded DNA was annealed to the duplex form and was cloned into the unique *Pvu*II site of pBR322. A plasmid, pZ64, containing the correct insert sequence, as determined by the method of Maxam and Gilbert (1980), was isolated and used in all subsequent experiments.

Topoisomers of pZ64 ranging from the relaxed configuration to a linking difference of about −30 turns were prepared and electrophoresed in two dimensions on agarose gels precisely as described (Ellison et al., 1985). Plasmids labeled with [^{3}H]thymidine for use in antibody filter binding assays were prepared as previously described (Nordheim et al., 1982).

Nitrocellulose Filter Binding Assays. One-milliliter samples of TBE buffer [90 mM tris(hydroxymethyl)aminomethane (Tris)–borate, pH 8.3, 2.5 mM ethylenediaminetetraacetic acid (EDTA)] containing radioactive plasmid DNA (0.5 μg/mL, 10 000 cpm/μg) and varying concentrations of a polyclonal preparation of anti-Z-DNA antibody (Lafer et al., 1981) (specific concentration 0–2.0 μg/mL final) were incubated for 30 min at room temperature. The samples were then filtered through nitrocellulose filters (Millipore, type HA, 2.5 cm in diameter) under vacuum and washed twice with 1 mL each of TBE buffer. Filters were dried, and the radioactivity retained on the filters was determined by scintillation counting [in Beta Fluor (National Diagnosis)]. Filters were prepared prior to use by being soaked in 0.3 M NaOH for 10 min followed by three washes with distilled water.

Mung Bean Nuclease Digestion of pZ64. Aliquots containing 10 μg each of the plasmid pZ64 in 100 μL of 100 mM NaCl, 10 mM Tris (pH 7.5), and 2 mM $ZnCl_2$ were digested with 40 units of mung bean nuclease (New England Biolabs) for varying lengths of time at room temperature. Under these conditions, 1 h of incubation is required to convert approximately 5% of the supercoiled closed circular form to the nicked circular form. The reaction was terminated by phenol extraction, followed by ethanol precipitation.

Results

Experimental Approach. In assessing the Z-DNA forming potential of $d(AT)_n$ relative to other sequences, we have taken advantage of the fact that the energy derived from negative supercoiling in plasmids can be partitioned into the stabilization of locally underwound structures such as Z DNA. Negative supercoiling is a property of plasmids that arises when the linking number α is lower than the theoretical linking number of the relaxed plasmid $\alpha_0°$. The linking number in this context is defined as the number of times one strand is wound around the other along the helix axis. Changes in the linking number produce corresponding changes in the duplex twist of the plasmid (Tw) and its axial writhe (Wr). These two properties of a closed circular DNA duplex are related to changes in linkage in the following way (Wang et al., 1983; Fuller, 1971):

$$(\alpha - \alpha_0°) = \Delta Tw + \Delta Wr \quad (1)$$

While it is not possible at the present time to determine how differences in the linking number are distributed between the ΔTw and ΔWr, it is nonetheless true that topoisomers that vary in $\alpha - \alpha_0°$ also vary in the writhe component. Since the degree of writhe determines the hydrodynamic properties of each topoisomer, it is possible to separate topoisomers that differ in $\alpha - \alpha_0°$ electrophoretically. It is apparent from eq 1 that when a segment of a negatively supercoiled plasmid flips from the right-handed form to the left-handed form, the increase in the magnitude of negative duplex twist produced by this change must be balanced by a corresponding decrease in the magnitude of the writhe component at some fixed value of $\alpha - \alpha_0°$. This shift manifests itself as a retardation of the electrophoretic mobility of topoisomers that have undergone a transition to an underwound form relative to a plasmid with the same value of $\alpha - \alpha_0°$ but in which the transition has not occurred. The retardation of topoisomers on an electrophoretic gel provides a direct measure of the amount of unwinding occurring in a particular sequence as function of $\alpha - \alpha_0°$, provided that the transition occurs over a range of topoisomers that can be resolved electrophoretically. The free energy of supercoiling ΔG_τ is related to $\alpha - \alpha_0°$ by the expression (Pulleyblank et al., 1975; Depew & Wang, 1975)

$$\Delta G_\tau = K(\alpha - \alpha_0°)^2 \quad (2)$$

where the constant K depends both on the temperature and on the size of the plasmid under consideration. From electrophoretic gels then, it is not only possible to obtain information about the magnitude of the transition occurring in a selected sequence, but it is also possible to determine the energetic requirement associated with such a change.

The approach outlined above forms the basis for assessing the Z-forming potential of a stretch of $d(TA)_n$. In this work the structural behavior of the sequence $dG(CG)_6(TA)_4(CG)_6C$, contained in the *Pvu*II site of the plasmid pBR322, was examined as a function of $\alpha - \alpha_0°$ (the *Pvu*II site contributes a G to the 5′ end and a C to the 3′ end of the insert). This sequence was chosen for several reasons. Long stretches of $d(TA)_n$ have been observed to form cruciforms in negative supercoiled plasmids (Haniford & Pulleyblank, 1985; Panyutin et al., 1985; Greaves et al., 1985); therefore, it was necessary to confine $d(TA)_n$ to a length short enough that cruciform

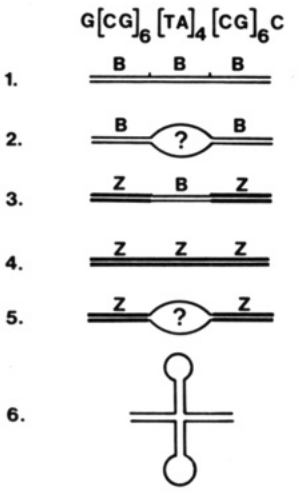

FIGURE 1: Possible conformational states of the plasmid insert dG-$(CG)_6(TA)_4(CG)_6C$ that might be induced upon negative supercoiling. In (2) and (5), the $d(TA)_4$ stretch is postulated to adopt an unwound form of unknown structure. In (6), the entire sequence is incorporated into a cruciform.

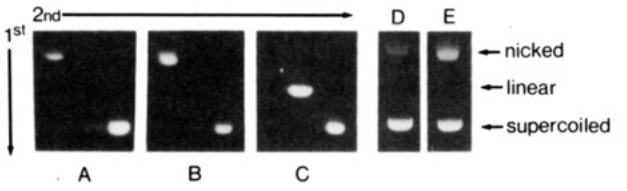

FIGURE 2: Sensitivity of pZ64 to *Bss*HII and mung bean nuclease: pZ64 (A–C); pBR322 (D and E). The superhelical density of both plasmids was estimated to be approximately –0.06. (A and D) Untreated; (B) digested with mung bean nuclease as described; (C and E) digested with *Bss*HII (8 units/μg of DNA, 16 h at room temperature in mung bean nuclease buffer plus 1 mM $MgCl_2$, final concentration). In the case of panels A–C, samples were electrophoresed on a submerged 1.0% agarose gel in two dimensions [first dimension, Tris–borate, 90 mM, pH 8.3; second dimension, same as first plus ethidium bromide (0.5 μg/mL final)]. Panels D and E were electrophoresed only in the first dimension.

extrusion would be unlikely. The transition from B DNA to Z DNA in short sequences, however, is less favored than in longer sequences and occurs at values of $\alpha - \alpha_0°$ that are not easily resolved by gel electrophoresis (Peck & Wang, 1983). In addition, the magnitude of the transition is small, thereby increasing the error of the measurement. These problems were overcome by situating $d(TA)_4$ between the two strong Z-forming blocks $d(CG)_6$. Since the energetic characteristics of $d(CG)_n$ have been previously determined under conditions identical with those employed here, the effect of these portions of the insert can be thermodynamically separated from the energetic behavior of $d(TA)_4$.

The $d(CG)_6$ Blocks of the Insert $dG(CG)_6(TA)_4(CG)_6C$ Form Z DNA. While the presence of alternating $d(CG)_n$ sequences in the test sequence $d(CG)_6(TA)_4(CG)_6$ should impart a strong tendency for this sequence to adopt the Z form, both the palindromic nature of the sequence and the presence of the $(TA)_4$ block open up the possibility that other structures such as cruciforms might prevail under the influence of negative supercoiling. Some of these structural alternatives have been schematically represented in Figure 1.

In order to test whether or not the insert contained in pZ64 forms a cruciform in negatively supercoiled plasmids, an experiment was performed similar to the one described recently by Haniford and Pulleyblank (1985). These workers have observed that single nicks introduced into the loop of the cruciform by single strand specific nucleases fail to cause relaxation of the plasmid. This phenomenon is a direct consequence of the topological isolation of the scission from the helical axis of the rest of the plasmid. Nicks located at the loops of cruciforms can be detected by first separating the supercoiled plasmid from the nicked plasmid electrophoretically. Those supercoiled molecules containing nicks that are uniquely situated at the cruciform loop can then be resolved from covalently closed plasmid by electrophoresis in a second dimension under conditions that remove the cruciform (such as the addition of ethidium bromide).

The results from such an experiment are shown in Figure 2. Here, pZ64 at a superhelical density of –0.06 was treated with mung bean nuclease until approximately 50% of the molecules contained nicks as observed from electrophoresis of the sample in the first dimension (Figure 2B). At this level of nicking, a substantial proportion of the plasmid migrating in the supercoiled position can be expected to contain trapped scissions pending cruciform formation. Electrophoresis of this material in the second dimension, in the presence of ethidium bromide, failed to reveal a band coincident with the position of relaxed, nicked circular DNA as would be expected if cruciform formation had occurred.

In another experiment, negatively supercoiled pZ64 was treated with the restriction endonuclease *Bss*HII. This enzyme cleaves the recognition sequence 5′-GCGCGC-3′ only when it is in the right-handed helical conformation. The unique targets for *Bss*HII in pZ64 are the two $d(CG)_6$ segments of the test insert. Cleavage of negatively supercoiled pZ64 with this enzyme would provide strong evidence that these segments remain right-handed. The insert of pZ64 is a perfect palindrome 38 base pairs in length (including the 6 base pairs contributed by the *Pvu*II cloning site). Extrusion of this sequence into a cruciform would produce two stems 17 base pairs in length that contain the recognition sites for *Bss*HII. Under the conditions of ionic strength employed here, these stems would assume the B conformation and therefore should be cleavable by *Bss*HII. Evidence that such stem structures do in fact serve as substrates for *Bss*HII under the conditions used here has come from our observation that hairpins of $d(CG)_n$ upon ligation to form dumbell-like structures are readily accessible to cleavage by this enzyme (unpublished). The results presented in Figure 2C, however, demonstrate that a substantial proportion of negatively supercoiled pZ64 resists cleavage when treated exhaustively with *Bss*HII. By comparison, the nicked circular DNA present in the reaction mixture is converted entirely to the linear form.

It is clear from a comparison of untreated pZ64 (Figure 2A) and the *Bss*HII treated sample (figure 2C) that a fraction of supercoiled pZ64 is cleaved. It should be emphasized, however, that a significant amount of nicking occurs also in pBR322 treated with *Bss*HII (Figure 2D,E). In view of the fact that pBR322 does not contain the recognition site for *Bss*HII, much of the reduction in supercoiled pZ64 resulting from the *Bss*HII treatment must arise from nonspecific nicking of the plasmid followed by its subsequent linearization.

The results presented above are inconsistent with the idea that cruciform formation occurs within the test sequence examined here. The insensitivity of this insert to *Bss*HII cleavage further argues that both $d(CG)_6$ segments adopt an altered conformation, consistent with the formation of Z DNA.

Conformations of the test sequence that do not involve, at least in part, the formation of Z DNA can be further ruled out on the basis of the two experiments described below. In the first experiment, the ability of the plasmid containing the test sequence (pZ64) to react with anti-Z-DNA antibodies was compared with the parental vector pBR322, as well as with

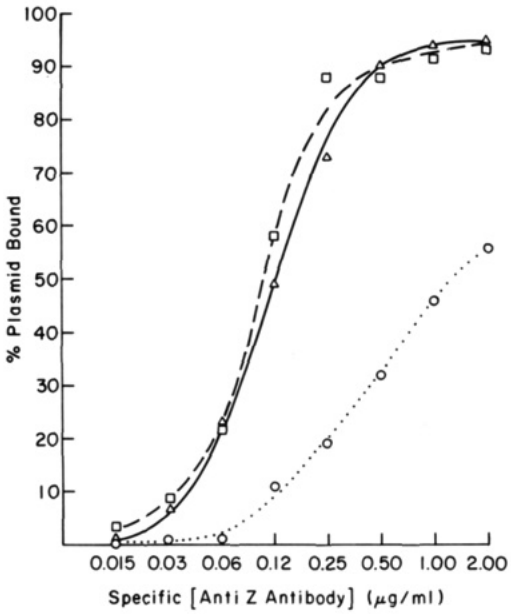

FIGURE 3: Anti-Z-DNA antibody binding to plasmids. Shown here is the percentage of supercoiled plasmid retained on nitrocellulose filters in the presence of different concentrations of a polyclonal preparation of anti-Z-DNA antibody. pBR322 (no insert) (○); pZ64 (containing [$dG(CG)_6(TA)_4(CG)_6C$]) (△); pLP32 (containing [$d(CG)_{16}$]) (□). Estimated values of $\alpha - \alpha_0°$ for these plasmids range from −25 to −30 under the conditions used, corresponding to superhelical densities of −0.06 to −0.07.

pBR322 containing the alternating sequence $d(CG)_{16}$ (pLP32) (Peck et al., 1982). The superhelix density of all three plasmids was estimated to be −0.06, well above the superhelix density required to flip the $d(CG)_{16}$ insert of pLP32 under similar conditions (Peck & Wang, 1983). It can be seen from Figure 3 that pZ64 and pLP32 show virtually identical antibody-binding characteristics as a function of antibody concentration. In comparison, pBR322 requires 10 times more antibody to bind 50% of the input DNA. These results demonstrate that a significant portion of the test sequence of pZ64 exists in the Z form at this level of superhelicity.

The extent of Z-DNA formation in the insert of pZ64 can be measured with greater precision by two-dimensional gel electrophoresis. Shown in Figure 4 is the pattern produced when topoisomers of pZ64 ranging from the relaxed configuration to more negative values of $\alpha - \alpha_0°$ are electrophoresed on an agarose gel. Electrophoresis in the second dimension in the presence of the intercalator chloroquine permits the resolution of topoisomers that have undergone the transition from those that have not. In the first dimension, the mobility of topoisomers increases as $\alpha - \alpha_0°$ deviates from $\alpha_0°$ (defined experimentally as the apex at the top of the gel).

The onset of the transition occurs at a negative linking difference of 15.6 and appears to be complete by 19.6. At a negative linking difference of 19.6, slightly less than six turns of the test sequence have been removed on the basis of the retardation of the mobility of this topoisomer relative to those that have not yet undergone the transition. This value is two full turns greater than that expected for the formation of a cruciform within the 32-bp insert plus the base pairs of the *Pvu*II site, which extend the palindrome to 38 base pairs in length. The formation of Z DNA has been previously shown to result in the loss of 0.18 turn/bp in addition to 0.4 turn for each B–Z junction formed (Peck & Wang, 1983). On the basis of these values, the degree of unwinding observed for pZ64 can only be reconciled with the transition of both blocks of $d(CG)_6$ to Z-form DNA.

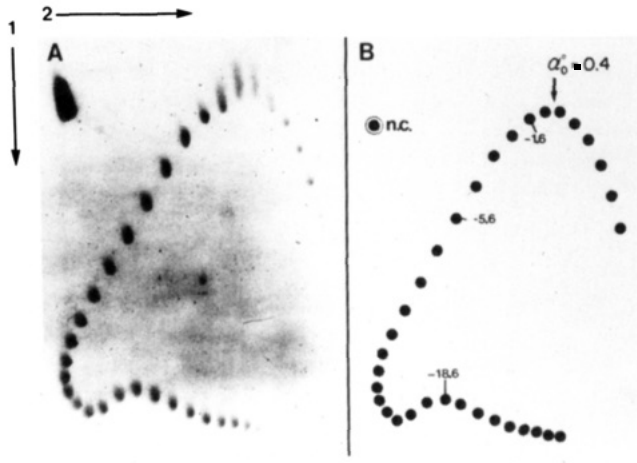

FIGURE 4: Supercoiling-induced structural transition in the plasmid insert $dG(CG)_6(TA)_4(CG)_6C$ observed by the 2-D gel electrophoresis of plasmid topoisomers. Panel A is a photograph of the actual gel. A schematic of the photograph is shown in panel B. Nicked circular DNA is indicated by "n.c.". Also shown is the linking difference $-(\alpha - \alpha_0°)$ for selected topoisomers. In this case, $\alpha_0°$ has been determined to be −0.4. The transition is considered to be essentially complete at $\alpha - \alpha_0° = -19.6$. Gels were run at room temperature in TBE buffer (90 mM Tris–borate, 2.5 mM EDTA, pH 8.3).

The structural state of the $d(TA)_4$ segement, however, is less clear. Shown in Figure 1 are three possible conformations of the test sequence in which the $d(CG)_6$ segments assume the Z form. The $(TA)_4$ segment is shown either in the B form (Figure 1.3), in the Z form (Figure 1.4), or in a structure with no net twist (Figure 1.5). The theoretical change in twist calculated for each of these structures is 6.3, 6.9, and 6.3, respectively. Although the experimental unwinding is closer to that expected if the $d(TA)_4$ segment were not in the Z form, a more detailed description of the behavior of this region was obtained by subjecting the data to statistical mechanical analysis.

Statistical Mechanical Analysis of the Supercoiling-Induced Transition in $dG(CG)_6(TA)_4(CG)_6C$. An analysis based upon statistical mechanical principles has been previously used to determine the free-energy change for the B–Z transition (Peck & Wang, 1983; Vologodskii & Frank-Kamenetskii, 1984). In this approach, each dinucleotide of the chain was assumed to adopt either the B form or the Z form, with the restriction that chain propagation of the Z form results in a single contiguous stretch of Z-form dinucleotides (a "zipper" model). However, this type of treatment can be readily extended to conformational states other than the B form and the Z form. In our analysis of the supercoiling-induced transition of $dG(CG)_6(TA)_4(CG)_6C$, we have used different models to describe structural alternatives for the $d(TA)_4$ segment.

The configuration partition function for a chain in which each element exists in one of two possible conformational states is given explicitly by the series expansion:

$$Q = 1 + \sum_{i=1}^{n}\sum_{k=1}^{n}\sigma\left(\prod_{j=i}^{k}s_j\right)\exp\left[(-K/RT)\left[\alpha - \alpha_0° - \left(\sum_{j=i}^{k}a_j\right) - 2b\right]^2\right] \quad (3)$$

Here, s_j represents the equilibrium constant for transition between the two possible conformational states for the jth element of the chain. The a_j term represents the change in twist associated with the conformational transition of each element, and b is the unwinding associated with an external junction with B-form DNA. σ is a nucleation parameter that

encompasses the free energy required for formation of the junctions. K is the proportionality constant for the free energy of supercoiling (see eq 2), which is taken to be $1100RT/N$ for a closed circular DNA in which N exceeds approximately 2000 bp (Pulleyblank et al., 1975; Depew & Wang, 1975).

The average value of the change in twist, the quantity that is measured experimentally as a function of plasmid linking difference, is then calculated as a product over the probability sum:

$$\langle \Delta Tw \rangle = Q^{-1}\left(\sum_{i=1}^{n}\sum_{k=1}^{n}\left[\left(\sum_{j=i}^{k} a_j\right) + b\right]\sigma\left(\prod_{j=i}^{k} s_j\right)\exp\left[\left(-K/RT\right) \times \left[\alpha - \alpha_0^\circ - \left(\sum_{j=i}^{k} a_j\right) - 2b\right]^2\right]\right) \quad (4)$$

By use of this expression for the average change in twist, the free-energy change associated with a given alteration in a reference sequence, such as $d(CG)_n$, may be determined by varying the appropriate s_j term(s) until the best agreement with the experimental data is obtained.

All three models for the structural transition in the $dG(CG)_6(TA)_4(CG)_6C$ insert have in common the assumption that all nucleotides in the two $d(CG)_6$ blocks exist in either the B form or the Z form, with a free energy of transition of 0.33 kcal/mol of bp. In addition, all models share the same value for the nucleation parameter [$\sigma = \exp(-10.0/RT)$] and the junctional unwinding ($b = -0.4$ turn). The values of these three parameters were previously determined experimentally under conditions identical with those used here (Peck & Wang, 1983). These values have also been shown to be valid for describing the behavior of $d(CG)_n$ tracts cloned into the *Pvu*II site of pBR322 as in the case of pZ64 (Ellison et al., 1985).

Here we analyze three different models that differ in the conformation states allowed to the dinucleotides of the $d(TA)_4$ segment but in which the $d(CG)_6$ segments are ultimately allowed to adopt the Z form. In model 1, all dinucleotides of the insert sequence, including those of the $d(TA)_4$ segment, can adopt either the B form or the Z form, with nucleation occurring only once in each of the partially Z-form states of the chain. Therefore, the value of the a_j terms (the change in twist) for the eight A-T base pairs is $-(1/10.5 + 1/12)$, identical with that for the G-C base pairs in all models. The s_j value (related to the energy change for the transition) for the eight A-T base pairs was then varied until the best agreement with the experimental data was obtained. Shown in Figure 5A are three plots of the change in twist observed in the insert sequence as a function of plasmid linking difference calculated from model 1. These curves represent the fits of the data obtained by varying only the energy-related s_j term for the A-T base pairs. However, it is evident from Figure 5A that none of these curves adequately matches the experimental data. It appears that the total change in twist observed with the insert is significantly lower than that predicted by model 1, indicating that a significant fraction of the A-T base pairs are not adopting the Z form but rather some structure that retains more positive twist than Z DNA.

The second model is one in which the $d(TA)_4$ segment is constrained to the B form, and the two $d(CG)_6$ blocks undergo independent structural transitions to the Z form, coupled only through the free energy of supercoiling, ΔG_τ. This situation is described by a product of the partition functions for the two $d(CG)_6$ blocks alone, neglecting any contribution from the $d(TA)_4$ segment. All possible combinatorial states of the two chains are enumerated, and the product of the associated Boltzmann factor and the equilibrium constant related to the residual free energy of supercoiling defines the fractional

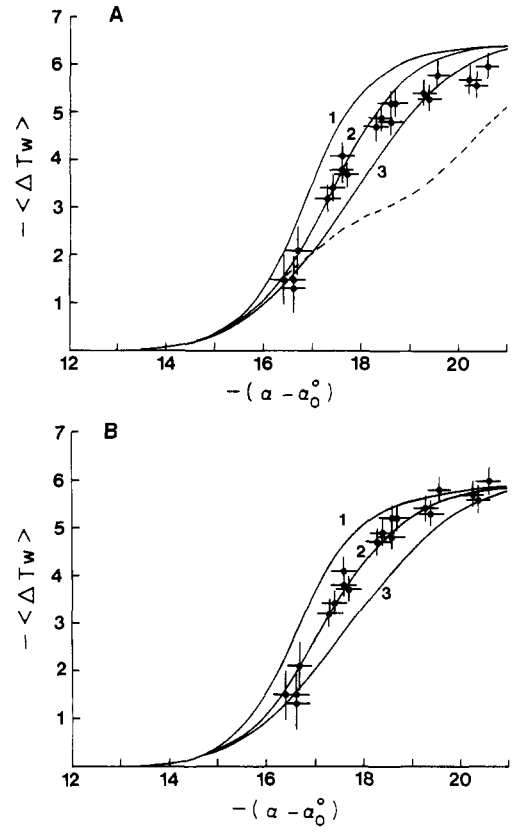

FIGURE 5: Three statistical mechanical models for the supercoiling-induced structural transition in $dG(CG)_6(TA)_4(CA)_6C$. (A) (Model 1) Solid lines depict a situation in which each bp within the sequence can adopt the B state or the Z state and nucleation can occur only once but with equal probability throughout the sequence. Lines 1–3 differ only in the energy required to stabilize an A-T bp in the Z conformation (line 1, 1.1 kcal/mol of bp; line 2, 1.2 kcal/mol of bp; line 3, 1.3 kcal/mol of bp). (Model 2) (Dotted line in A) The $d(TA)_4$ segment is constrained to the B conformation, and the $d(CG)_6$ blocks are treated as independently nucleating units that are coupled energetically only through the free energy of supercoiling, ΔG_τ. (B) Model 3 is identical with model 1 with the exception that the A-T bp are constrained either to the B state or to the unwound state. Similarly, lines 1–3 differ only in the energy required to stabilize an A-T bp in this underwound state (line 1, 0.8 kcal/mol of bp; line 2, 0.9 kcal/mol of bp; line 3, 1.0 kcal/mol of bp). The curves generated in (A) and (B) by these models were compared with the unwinding data accumulated from five two-dimensional gels, which are displayed identically in A and B as a function of $\alpha - \alpha_0^\circ$.

occupancy of each state. A more complete description of the statistical mechanical treatment of competing B–Z transitions will be given elsewhere. Experimental data (manuscript in preparation) are in excellent agreement with the predictions of this model. The transition curve generated by this treatment is shown as the broken line in Figure 5A. The model predicts a biphasic transition, which fits the experimental data poorly, ruling out the possibility that the $d(TA)_4$ segment remains as unperturbed B DNA in response to negative supercoiling.

In the third model, the entire insert undergoes a structural transition, with the base pairs of the $d(CG)_6$ blocks adopting either the B form or the Z form and the base pairs of the $d(TA)_4$ segment adopting either the B form or an unwound state with a helical pitch intermediate between that of B DNA and Z DNA. This model is essentially identical with model 1, with the exception that the a_j unwinding terms for the A-T base pairs have a value of $-(1/10.5)$; that is, we assume no net twist. Shown in Figure 5B are three transition curves, differing only in the energy-related s_j terms for the eight A-T base pairs. The curve generated by setting the free energy of

transition equal to 0.9 kcal/mol of bp appears to be an excellent approximation of the transition occurring in the insert. From this, we conclude that the $d(TA)_4$ segment does undergo a structural transition in response to negative supercoiling, which bridges the two $d(CG)_6$ blocks. However, the structure adopted by this segment appears to be neither right-handed nor left-handed but is rather an underwound structure with a net helical pitch of approximately zero. This analysis also provides two types of energetic information. Although the $d(TA)_4$ segment does not predominate in the Z form from model 1, a minimal estimate of 1.2 kcal/mol of bp can be made for the free energy that would be required to stabilize this segment in the Z form (Figure 5A). Moreover, the free-energy change associated with the actual underwound state of this segment can be estimated from model 3 to be 0.9 kcal/mol of bp.

While the above results support a model in which $d(TA)_4$ has no net twist, the precision of the data cannot rule out models where $d(TA)_4$ has a slight helical twist in either the positive or negative direction. Furthermore, the models employed here assume that the deformation occurring in $d(TA)_4$ is homogeneous, so that the estimated energy values apply to a structural average. We cannot rule out, for example, the possibility that A-T base pairs situated adjacent to the $(CG)_6$ blocks adopt the Z conformation.

Sensitivity of the $d(AT)_4$ Segment to Mung Bean Nuclease. Several underwound DNA conformations have been identified that show sensitivity to the single strand specific nucleases S1 and mung bean nuclease. These conformations include unpaired regions (Kowalski et al., 1976), B–Z junctions [see Kang & Wells (1985) for review], and a supercoiling-induced transition in polypurine–polypyrimidine stretches [Cantor & Efstradiatis (1984) and Pulleyblank et al. (1985) give recent reviews]. In view of the results from the statistical mechanical analysis presented above, it was predicted that the $d(TA)_4$ segment may also show sensitivity to one of these nucleases when situated between two segments of Z DNA.

Shown in Figure 6 are the results obtained when the supercoiled plasmid pZ64 was treated briefly with mung bean nuclease and analyzed on a sequencing gel to determine the location of the cut sites relative to the test sequence. Mung bean nuclease was selected as a probe for this experiment because of its reduced exonucleolytic activity and its ability to function at higher pH values. While the gel does not provide information at nucleotide resolution, it is apparent that cutting in the vicinity of the test insert occurs predominately within the $d(TA)_4$ segment and not within the blocks of $d(CG)_6$. These cut sites are notably absent in pZ64, which is in the relaxed configuration or at a superhelical density estimated to be close to the onset of the transition within the test sequence. The heightened sensitivity of the $d(TA)_4$ segment to mung bean nuclease relative to the flanking $d(CG)_6$ portions of the test sequence provides further evidence that this sequence adopts a conformation that can be classified as neither B DNA nor Z DNA and, therefore, by implication must exist in an intermediate underwound state.

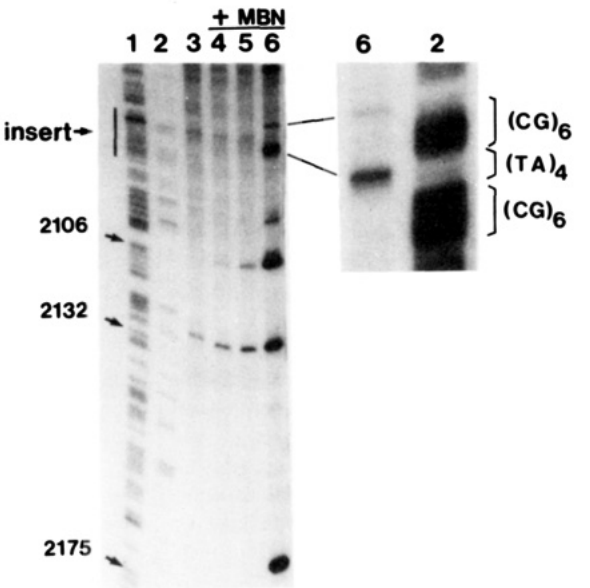

FIGURE 6: Sensitivity of $dG(CG)_6(TA)_4(CG)_6C$ to mung bean nuclease at different superhelix densities. Plasmid pZ64, at three different levels of supercoiling, was treated with mung bean nuclease (MBN) as described in the text. Treated samples were then radioactively labeled by filling in the unique *Tth*III site with [α^{32}P]dCTP by using the Klenow fragment, followed by electrophoresis on 0.8% polyacrylamide gels containing 8 M urea (Maxam & Gilbert, 1980). The *Tth*III site is located at position 2221 on the pBR322 map, 154 bp away from the start of the insert at position 2067. (Lane 1) G+A sequence; (lane 2) G sequence; (lane 3) untreated pZ64; (lanes 4–6) pZ64 at superhelix densities of –0.00, –0.04, and –0.06, respectively.

Discussion

Structure of $d(TA)_4$. In spite of the fact that the $d(TA)_4$ segment of the sequence $dG(CG)_6(TA)_4(CG)_6C$ is alternating purine–pyrimidine residing in phase between two blocks that adopt the Z form readily, it is apparent from the above results that the Z conformation is a difficult structure for $d(TA)_4$ to assume. Evidence that $d(TA)_4$ does indeed undergo a structural alteration in response to negative supercoiling comes primarily from two observations. First, the transition in $dG(CG)_6(TA)_4(CG)_6C$ is cooperative. The $d(CG)_6$ blocks do not flip as individual units, which would be predicted if the intervening sequence remained predominantly in the B form (model 2, Figure 5A). Instead, the flipping of one $d(CG)_6$ block facilitates a transition within the other, presumably via a structural change occurring in the central block of $d(TA)_4$. Second, at levels of supercoiling where $d(TA)_4$ is flanked by Z DNA, this sequence becomes sensitive to mung bean nuclease. Sensitivity of DNA to enzymes such as this has been taken in the past as strong evidence for the non-B-DNA character of the sequence.

Given that the $d(CG)_6$ segments on either side of $d(TA)_4$ adopt the Z form, it is difficult to imagine that any eight base pair structure could exist between these two left-handed DNA segments without being underwound to some degree relative to the B form. Indeed, this point is borne out by the remarkably close fit of the unwinding data, shown in Figure 5, with the statistical mechanical model in which $d(TA)_4$ assumes a structure with a helical pitch intermediate between that of B DNA and Z DNA (model 3, Figure 5B). The degree of helical unwinding observed in the $d(TA)_4$ portion of the insert is compatible with several structures that have been either demonstrated or postulated to exist and to be stabilized by negative supercoiling. These alterations in the helix include (1) cruciforms (Gellert et al., 1983; Lilley, 1980; Panayotatos & Wells, 1981), (2) bulges or unpaired regions of the duplex, (3) paired, side-by-side models [see Saenger (1984) for review], (4) structural perturbations at the boundary between B DNA and Z DNA (B–Z junctions) (see above), and (5) recently proposed structures for polypurine–polypyrimidine stretches (Pulleyblank et al., 1985; Cantor & Efstradiatis, 1984).

Two results presented here argue against the formation of cruciforms or bulges in $d(TA)_4$. Both of these structures contain unpaired bases, which show extreme sensitivity to the single-strand specific nucleases (Kowalski et al., 1976). In contrast, although the $d(TA)_4$ segment in supercoiled plasmids

is significantly sensitive to mung bean nuclease, it is clear from Figure 6 that this region represents a relatively minor target site for this enzyme. In addition, the transition observed here in $d(TA)_4$ occurs with greater facility than can be predicted for cruciform or bulge formation in a sequence of identical length. Greaves et al. (1985) have recently determined that the free energy required for cruciform loop and junction stabilization in $d(AT)_{34}$ is approximately 14 kcal/mol. Since these components are common to all cruciforms, this value is likely to represent a minimum estimate for cruciform formation in $d(TA)_4$. Similarly, it can be calculated from Tinoco's energy determinations for RNA secondary structure (Tinoco et al., 1973) that 16 kcal/mol is required to stabilize an eight base pair bulge. The transition described for $d(TA)_4$ in this work, however, is estimated to require only 7 kcal total (0.9 kcal/mol of bp). We conclude from these findings that the underwound form of $d(TA)_4$ probably maintains a considerable degree of order, derived in part from stacking and base pairing interactions. The relevance of the other duplex alternatives mentioned above to the transition observed here remains to be determined.

Purine–Pyrimidine Alternation and Z-DNA Formation. It has been known for some time that stretches of the alternating sequences $d(CG)_n$ and $d(CA)_n$ form Z DNA readily in negatively supercoiled plasmids. Here, we have shown that eight base pairs of alternating $d(TA)_4$ do not predominate in the Z form even when favorably flanked by strong Z-forming sequences. Increasing the length of the central $d(TA)_4$ segment would further decrease the Z-forming potential of this sequence, since longer stretches of $d(TA)_n$ show a strong tendency for cruciform extrusion. Decreasing the length of $d(TA)_4$ relative to the adjacent $d(CG)_6$ blocks would likely shift the equilibrium in favor of Z DNA in the remaining A-T residues. Wang et al. have observed, for instance, that the DNA oligomers $d(m^5CGTAm^5CG)$ and d(CGCATGCG) both crystallize in the Z conformation (Wang et al., 1984). Regardless of the overall length of $d(TA)_n$, we feel that, in the absence of strong inducement from adjacent sequences, $d(TA)_n$ is unlikely to adopt the Z form under any circumstances.

The poor Z-forming potential of $d(TA)_n$ is pointedly illustrated when considering the minimal energy estimated to flip an A-T base pair into the Z conformation relative to energy values determined previously for other sequences. The free-energy change associated with converting a single base pair from B form to Z form in the sequences $d(CG)_n$ and $d(CA)_n$ has been determined to be 0.33 kcal/mol of bp (Peck & Wang, 1983) and 0.67 kcal/mol of bp (Vologodskii & Frank-Kamenetskii, 1984), respectively. In comparison, the conversion of an A-T bp to Z DNA in the sequence $d(TA)_n$ requires at least 1.2 kcal/mol of bp, approximately twice that for base pairs in $d(CA)_n$ and nearly 4 times that for base pairs in $d(CG)_n$. Recently, it has been determined that the free energy required to stabilize the interaction between two adjacent C or G residues in the Z conformation is also 1.2 kcal/mol of bp (Ellison et al., 1985). From this value, it can be estimated that the Z-forming potential of alternating $d(TA)_n$ is comparable to that of the nonalternating sequence poly(dC)–poly(dG). However, the inability of poly(dC)–poly(dG) to form cruciforms, together with the fact that long stretches of alternating $d(TA)_n$ do form cruciforms, argues that poly(dC)–poly(dG) is probably a better candidate for Z-DNA formation than $d(TA)_n$.

From the above comparisons, it is evident that the energetic advantage afforded to alternating purine–pyrimidine sequences toward Z-DNA formation is lost as the A+T content of the DNA increases. Superimposed upon this trend is the effect of specifically increasing the number of ApT or TpA nearest neighbors contained in a given stretch. The reason that A-T base pairs, either in alternation or out of alternation (Ellison et al., 1985), act to destabilize Z DNA is not obvious at the present time. Energy-minimized models of alternating $d(TA)_n$ and $d(CG)_n$ in the Z form show no steric preference for one sequence over the other (Kollman et al., 1982). The differences between sequences must then arise from differences in solvent interactions. In support of this idea, Wang et al. have shown that in Z-DNA crystals of the hexamer $d(m^5CGTAm^5CG)$ the water molecules are disordered in the vicinity of the A-T base pairs (Wang et al., 1984).

From the studies presented here and elsewhere (Wang et al., 1985; Feigon et al., 1985; Ellison et al., 1985), it is apparent that the rules that govern the sequence dependence of Z-DNA formation are complex. Simple purine–pyrimidine alteration is an inadequate criterion for the selection of sequences of strong Z-forming potential. Moreover, recent studies from this laboratory have suggested that certain types of sequences that do not alternate in purines and pyrimidines may nonetheless show a strong tendency toward the formation of Z DNA (Ellison et al., 1985).

A picture of negatively supercoiled DNA has begun to emerge in which a variety of non-B-DNA structures can exist simultaneously in different sequences. This diversity of structure is strikingly illustrated by considering that the sequence $dG(CG)_6(TA)_4(CG)_6C$ and its flanking sequences, having undergone the supercoiling-induced transition, are composed of as many as seven contiguous structural units, including B DNA, Z DNA, B–Z junctional distortion, and the altered structure of the $d(TA)_4$ segment. All of these structures are contained within 40 bp of DNA. From this viewpoint, it is reasonable to believe that DNA, under extreme conditions of negative superhelicity such as those in form V DNA (Stettler et al., 1979) or in the paranemic complex generated during genetic recombination (Kmiec et al., 1985), will appear as a mosaic of noncanonical structures. The approach taken here will be useful in discerning which structural alternatives prevail in various sequences.

ACKNOWLEDGMENTS

We acknowledge Gary Gryan and Chon Vo for their assistance in the preparation of oligonucleotides.

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Small-Angle Neutron Scattering Study of the Ternary Complex Formed between Bacterial Elongation Factor Tu, Guanosine 5′-Triphosphate, and Valyl-tRNA^{Val}

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Received November 25, 1985; Revised Manuscript Received February 13, 1986

ABSTRACT: The formation of the ternary complex between bacterial elongation factor Tu, GTP, and valyl-tRNA^{Val} has been studied by small-angle neutron scattering. Titrations of the protein with aminoacyl-tRNA solutions in both H_2O and 70% D_2O confirm the expected stoichiometry. The molecular weight obtained for the protein alone is significantly higher than expected and can be explained by postulating a monomer–dimer equilibrium. The titration data are then internally consistent with a dissociation of the dimer on ternary complex formation. The radius of gyration for the ternary complex and the calculation of the separation of the centers of mass of the protein and tRNA components suggest a compact model for the ternary complex.

In bacteria, one of the properties of the soluble polypeptide elongation factor Tu (EF-Tu) is to interact with elongator tRNA to produce the ternary complex EF-Tu·GTP·aminoacyl-tRNA. This complex is then used to promote the enzymatic binding of elongator-tRNA to the ribosome–mRNA complex. A number of chemical and biochemical studies have been described aimed at the determination of the mode of interaction of the two macromolecular components of the complex (Boutorin et al., 1981; Wikman et al., 1982; Antonsson & Leberman, 1984). These would indicate that the protein and nucleic acid moieties have fairly extensive areas of contact, consistent with the high binding constants found

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0006-2960/86/0425-3655$01.50/0