Characteristics of Z-DNA Helices Formed by Imperfect (Purine-Pyrimidine) Sequences in Plasmids*

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The capacities of three synthetic sequences to adopt left-handed helices were evaluated in recombinant plasmids. The sequences consisted of very short runs of (CG), (n = 2 - 4) interspersed with runs of alternating A\(^+\)T base pairs and/or with regions of non-alternating base pairs. The plasmids were studied by two-dimensional gel electrophoresis to determine the natures of the conformational transitions and their free energies of formation. These results coupled with analyses with chemical (diethyl pyrocarbonate, osmium tetroxide, and bromoacetaldehyde) and enzymatic (S1 nuclease, T\(_7\) gene 3 product, and MHhaI) probes indicated that the entire sequence was adopting a left-handed helix in each case. In one of these sequences, Z-DNA formation necessitated the retention of the anti conformation of one of the guanines in a region of non-alternation. In a sequence which contains out-of-phase regions of alternation, our results indicate the formation of a separate left-handed helix in the central (CG), region, thus forming two Z-Z junctions. In summary, we conclude that only very short regions of alternating CG are necessary to effect the B to Z transition and that this conformational change can be transmitted through non-alternating regions. A set of empirical rules governing the characteristics of the B to Z transition and the types of left-handed helices in supercoiled plasmids was derived from studies on a systematic series of 17 plasmids.

The structural polymorphism of DNA has been well-documented (for reviews, see Refs. 1 and 2). Perhaps the most dramatic of these changes is the conversion of some sequences from a right- to left-handed helix. It has been postulated that this transition may act as a biological trigger (3, 4), either per se or as the result of the associated release of superhelical tension, with the concomitant formation of new supercoil nodes. Accordingly, it is probable that this transition will be highly regulated in vivo, either by a subtle balance of topoisomerase activities or by the action of DNA-binding proteins.

The base sequence and composition of a region affects the conformational transitions and their free energies of formation. These results coupled with analyses with chemical (diethyl pyrocarbonate, osmium tetroxide, and bromoacetaldehyde) and enzymatic (S1 nuclease, T\(_7\) gene 3 product, and MHhaI) probes indicated that the entire sequence was adopting a left-handed helix in each case. In one of these sequences, Z-DNA formation necessitated the retention of the anti conformation of one of the guanines in a region of non-alternation. In a sequence which contains out-of-phase regions of alternation, our results indicate the formation of a separate left-handed helix in the central (CG), region, thus forming two Z-Z junctions. In summary, we conclude that only very short regions of alternating CG are necessary to effect the B to Z transition and that this conformational change can be transmitted through non-alternating regions. A set of empirical rules governing the characteristics of the B to Z transition and the types of left-handed helices in supercoiled plasmids was derived from studies on a systematic series of 17 plasmids.

The abbreviations used are: bp, base pairs; DEPC, diethyl pyrocarbonate.

1. J. A. Blaho, M. J. McLean, J. E. Larson, and R. D. Wells, unpublished data.

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shown in Fig. 1. Both pRW1019 and -1022 resulted from ligation of the same synthetic oligonucleotide into pRW790. The synthesized sequence is that which is found in pRW1022.

Fine Mapping of Diethyl Pyrocarbonate Reactions—These were performed essentially as described (19). Supercoiled DNAs (3 μg) were incubated in 200 μl of 50 mM sodium cacodylate, 1 mM EDTA, pH 8.0, at 37°C in the presence of 10 μl of diethyl pyrocarbonate (Sigma) for 30 min. Excess reagent was then extracted into diethyl ether; the pellet DNA was redissolved in 10 mM Tris-HCl, 0.1 mM EDTA, pH 8.0, and divided into two for digestion and labeling at the EcoRI or HindIII site. A second digestion which generated a 3'-overhang was performed simultaneously. The 5'-overhang was filled in with [α-32P]dATP (Amersham Corp.) in the presence of nonradioactive deoxynucleotide triphosphates with Klenow fragment DNA polymerase (BMB), and the labeled fragments were separated by polyacrylamide gel electrophoresis, eluted from the gel, and precipitated with ethanol several times. The sites of reaction were then determined as described previously (19). For reactions on linear substrates, the plasmids were digested with EcoRI and PstI or with HindIII and SstI; phenol- and ether-extracted; precipitated; redissolved in 50 mM sodium cacodylate, 1 mM EDTA, pH 8.0; and treated with DEPC as described above. The pelleted DNA was then radio-labeled directly as described above.

Other Methods—Fine mapping of osmium tetroxide reaction sites (20), topoisomeric samples of the plasmids (21), and two-dimensional gel electrophoresis (5) were as described. All other materials and methods were as described previously (5, 16, 22-24).

RESULTS

Sequences of the Plasmids

The sequences of the inserts cloned into the BamHI site of pRW790 (5) are shown in Fig. 1. The inserts are the same length (24 bp) in each case, and the base pairs at the vector-insert junctions are identical. We previously reported (5) that the sequence (CG)₆(TA)₄(CG)₄ adopted a left-handed helix in response to negative superhelical stress. The sequences shown in Fig. 1 are all sequence isomeric with this insert (pRW1009 (5)) and were designed to investigate the effect of length and orientation of alternating (CG)₆ regions on the capacity of the entire insert to form left-handed helices. Thus, the insert in pRW1022 has the same total number of C-G and A-T base pairs as the insert in pRW1009 except that these regions are now present as smaller runs interspersed with each other. The insert maintains a perfectly alternating pyrimidine-purine nature throughout its entire length.

The insert in pRW1019 is very similar to that in pRW1022 except that there is an in-phase (with respect to the alternation of pyrimidines and purines) transversion of one G:C base pair so that the longest run of C-G is now 7 bp and there is a region of non-alternation. The insert in pRW1020 is again similar to that in pRW1022 except that the central (GC)₂ region in pRW1022 is converted to (CG)₂ in pRW1020 and thus is out-of-phase with the rest of the potential Z-helix. This region contains two Z-Z junctions (3, 25). It was of interest to determine whether these junctions would be formed, thereby necessitating the formation of three short Z-helices within the 24-bp insert, or whether the influence of the longer flanking blocks would be sufficient to force the central (CG)₂ region into a "reverse Z" configuration. In this case, the pyrimidines would be in the syn configuration and the purines in the anti configuration around the glycosidic bonds, thus maintaining a perfect alternation of these orientations throughout the contiguous Z-helix.

Energetics of Right- to Left-handed Transitions

Topoisomer populations of the plasmids (Fig. 1) were analyzed by two-dimensional agarose gel electrophoresis as reported previously (5). Electrophoresis in the presence of chloroquine in the second dimension enabled the separation of topoisomers which had undergone structural transitions and which thus had a reduced mobility in the first dimension. Measuring the extent of this reduced mobility allows a determination of the nature of the structural transition (5, 22, 26, 27), and determination of the topoisomer required to cause the transition enables a calculation of the free energy of formation of the new conformation.

The conversion of 24 bp of B-DNA with 10.5 bp/twist to Z-DNA with 12.0 bp/twist should cause a relaxation of 4.28 supercoils. The relaxation observed on electrophoresis of topoisomers of the plasmids in Fig. 1 is in agreement with the formation of a left-handed helix throughout the entire insert sequence in each case. Thus, for pRW1019, a relaxation of 4.5 ± 0.1 supercoils is observed; for pRW1020, 4.6 ± 0.1 supercoils are relaxed; and for pRW1022, the relaxation is 4.3 ± 0.1 supercoils. These results are not consistent with the inserts forming either cruciforms or anisomorphic DNA (16) as expected since the sequences are not perfect inverted repeats or direct repeats.

Within this series of homologous plasmid inserts, significant differences are observed in the superhelical density required for the structural transition to occur. This is a consequence of the different free energies of formation for the left-handed helices adopted by these inserts. For each of the plasmids, the free energy required to convert 1 bp of the insert from a right- to a left-handed form (ΔGₑ) was calculated as described previously (5, 28) from the superhelical density required to cause the B to Z transition, and these values are presented in Table I. The previously calculated value (5) for the insert in pRW1009 is included for reference since the sequences in this study are all isomeric.

It can be seen that, relative to ΔGₑ for pRW1009, shortening the lengths of the individual runs of (CG)₆ and inter-sparing runs of (AT), have no significant effect on the free energy of formation of the Z-helix. In contrast, introducing

| Plasmid | ΔGₑ (kcal·mol·bp⁻¹) |
|---------|---------------------|
| pRW1009 | 8.75                |
| pRW1019 | 9.15                |
| pRW1020 | 8.90                |
| pRW1022 | 8.80                |

Fig. 1. Inserts in plasmids used in study. The multiple cloning site is in upper-case letters: a, EcoRI; b, SstI; c, Smal; d, BamHI; e, XbaI; f, HindII; g, PstI; h, HindIII. The positions and sequences of the inserts are in upper-case letters.
an in-phase transversion (pRW1019) increases this value quite significantly, and the introduction of an out-of-phase region (pRW1020) has an even larger effect, relative to the 1022 control, on the Z-forming potential of the entire insert.

The sequences used herein are of identical length and sequence composition, and the same vector is present in all three cases. Hence, these energetic differences must be related to the relative ease with which individual nucleotides adopt the syn conformation (or remain in the anti conformation) in order to accommodate the formation of a Z-helix. Alternatively, the large effect observed for the sequence in pRW1020 may be due to the formation of junction regions between small, discrete Z-helices within the insert.

Rationale for Diethyl Pyrocarbonate and OsO₄ Reactions

DEPC carboxylates purines at N-7 (19, 29), and these modified nucleosides are more susceptible than the parent bases to cleavage by piperidine. In a regular Z-DNA helix, the purine nucleosides have the syn conformation in which N-7 is more accessible to DEPC, and thus these residues show enhanced reactivity toward this probe (19). It has been shown (19, 25) that purines out-of-alternation in a Z-helix do not exhibit hyper-reactivity to DEPC, thus indicating the retention of the anti conformation by these nucleosides, as in the right-handed B-structure.

X-ray crystallography (30) showed that consecutive alternating A-T base pairs stacked differently from G-C base pairs in the Z-form and have an associated change in the base pair twist angles. There are also significant differences in the hydration of the helical groove at the A-T pairs, and it was proposed (30) that this is the reason why A-T base pairs are less stable in the Z-form than are G-C pairs. We have recently reported (6) that sequences containing an alternating (TG)₆ motif exhibit hyper-reactivity toward osmium tetroxide in the Z-form. In these sequences (6), every T and G residue reacted with the probe, indicating significant structural differences between the Z-helices formed by (TG)₆ and by (CG)₆. We proposed that this observed difference in solution is a consequence of the stacking differences observed in the crystal form (30).

Diethyl pyrocarbonate reactions on the plasmids used herein (Fig. 1) gave valuable information on the conformational properties of purine nucleosides in the left-handed helices formed by the inserts. However, these studies gave no information as to the structures of the pyrimidine nucleosides. In order to investigate this question, the plasmids with native superhelical density were reacted with OsO₄. The principal target for this probe is the 5,6-double bond of thymidine residues, where its reaction renders the modified base more susceptible to cleavage with piperidine. Reaction with guanosine residues is also seen in some instances (6), although the nature of the modified species in such cases is not clear. Osmium tetroxide reacts with B-Z junctions (20), cruciforms (6, 34), and other, less well-characterized alternate DNA structures (37). Thus, it is a conformationally sensitive probe for thymidine residues.

To determine the molecular characteristics of the left-handed helices being formed by the inserts in pRW1019–1022, their reactivities toward DEPC and OsO₄ were investigated. The results of these reactions on the plasmids are discussed below, where they are used as the basis for establishing general rules for the formation of left-handed helices in supercoiled molecules.

Rules for Z-DNA Formation

Z-helices May Be Transmitted through Intervening Sequences to Form the Longest Left-handed Helix Possible—The duplex oligonucleotides (CG)₆ and (CG)₈ were shown to form Z-helices when crystallized under certain conditions (31, 32). However, our preliminary results indicate that these sequences do not adopt the Z-form when cloned into plasmids, up to a superhelical density of −0.075. The most significant difference between studies in supercoiled plasmids and studies on oligonucleotides is the presence in plasmids of non-Z sequences flanking the potential Z-helix, thus necessitating the formation of two B-Z junctions. This is the most energetically unfavorable step in the supercoil-induced B to Z transition (23, 24, 33). The failure of the sequence (CG)₆ to adopt the Z-form under superhelical stress suggests that the supercoil relaxation caused by this transition (1.07 superhelical turns) is not sufficient to warrant the formation of two energetically unfavorable junctions. Alternatively, it may be that a 6-bp Z-helix is not stable enough to withstand the torsion generated at the junctions. We previously showed (5) that the sequence (CG)₆ will adopt a left-handed conformation under superhelical stress and that this structure could be transmitted through an intervening sequence to induce a neighboring (CG)₆ region to also adopt the Z-form for the sequence (CG)₆TAC(CG)₆.

Fig. 2 shows the results of DEPC modification on both strands of the insert in pRW1022. In lanes 1 and 3, the modification was performed after digestion of the plasmid with EcoRI and HindIII, respectively, and in lanes 2 and 4, the reaction was performed on the supercoiled plasmid (z = −0.075), with subsequent division of the sample for digestion with EcoRI (lane 2) or HindIII (lane 4) and radiolabeling.

There is little or no enhanced DEPC reactivity of purines outside the potential Z-helix when the reaction is performed on a supercoiled substrate as opposed to a linear substrate. In particular, neither the G nor the A residues on both strands immediately outside the Z-helix (just below the brackets in Fig. 2) are significantly enhanced. In contrast, every purine residue inside the alternating purine-pyrimidine region shows increased reaction with DEPC when the reaction is performed on a supercoiled plasmid, thus indicating that the entire 24-bp insert is forming a Z-helix. At this superhelical density (−0.075), neither (CG)₆ nor (by implication) (GC)₆ alone will adopt a Z-helix, whereas these sequences will adopt the Z-form when separated from each other and from a similar sequence by a few base pairs (Figs. 1 and 2). Furthermore, the free energy of formation of this Z-helix is not significantly greater than that required for Z-helix formation by an isomeric sequence (pBR1098; (CG)₆(TA)₃(CG)₄) in which the two tracts of (CG), flank a central (TA), block.

Thus, the Z-forming potential of (CG)₆ and (GC)₈ is greatly enhanced when they are in proximity to each other and to a slightly longer sequence, (CG),. The sequence (TA), does not form a Z-helix in isolation (34, 35), although this result (Fig. 2) and previous studies (5) show that this sequence motif can be induced to form a left-handed helix when between short Z-forming regions. Since there are clearly sequence and length limitations on the ability of this type of sequence to adopt the Z-form (5, 27), we wish to distinguish intervening sequences which allow transmission of the Z-helix and intervening sequences which prohibit this phenomenon and instead favor the formation of two (or more) separate and independent Z-helices.

Bases Out-of-Alternation May Be Incorporated into a Z-helix: the Preferred Helix Is That Which Maintains an Alternating syn/anti Conformational Motif—Herr (19) showed that a 14-bp region of alternating purine-pyrimidine in pBR322

3 M. J. McLean and R. D. Wells, unpublished data.
4 J. Klysik, W. Zacharias, and R. D. Wells, unpublished data.
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FIG. 2. Fine mapping of DEPC-reactive sites on both strands of pRW1022. In lanes 1 and 2, samples were radiolabeled at the EcoRI site with DNA polymerase, Klenow fragment, and in lanes 3 and 4, labeling was at the HindIII site. In lanes 1 and 3, samples of the plasmid were digested with the appropriate restriction endonucleases prior to treatment with DEPC. In lanes 2 and 4, the DEPC reaction was performed on one sample of the supercoiled plasmid with subsequent division for radiolabeling of the opposite strands. The insert between the BamHI sites is bracketed.

became hyper-reactive toward DEPC at a superhelical density which correlated with that at which other workers (36) suggested Z-DNA formation by this segment. At higher superhelical densities, Herr observed that this enhanced reactivity extended over a 31-bp segment, including regions which are out-of-alternation with the 14-bp Z-helix. Similarly, Johnston and Rich (25) observed that purines up to 19 bp away from a (CG)$_n$ sequence show enhanced reactivity toward this probe at very high (>-0.1) superhelical densities. Prior investigations with other probes (22, 23) demonstrated that the length of Z-helices increased with superhelical densities and that the B-Z junctions moved along the duplex.

Two-dimensional gel electrophoresis of topoisomers of pRW1019 showed that the entire insert was adopting a left-handed helix and that the transition was complete at a superhelical density of -0.055. The insert in this plasmid is identical to that in pRW1022 (Fig. 1) except for an in-phase G to C transversion in the longest (CG)$_n$ block, thus generating a short region that does not maintain the alternating pyrimidine motif. We propose that the increased free energy of formation of the Z-helix for pRW1019 (Table I) is a consequence of the difficulty with which pyrimidine nucleosides adopt the syn conformation. Thus, the second G in the sequence 3'-GGA-5' on the lower strand of this insert should be in the anti conformation to maintain perfect alternation of syn/anti in the insert.

Fig. 3 shows the results of DEPC reaction with both strands of the insert in pRW1019 performed as described above for pRW1022. Again, it can be seen that purines outside the insert are no more reactive toward DEPC when the reaction is performed on the supercoiled plasmid (lanes 2 and 4) compared to the reaction on the linearized plasmid (lanes 1 and 3). In particular, bands due to reaction of the G and A residues immediately outside the potential Z-helix (just out-
side the boxed insert) on either strand are not significantly enhanced.

In contrast, the reaction at the first G residue in the potential Z-helix is enhanced approximately 3-fold over a G residue in the vector when the reaction is performed on a supercoiled substrate (Fig. 3, lanes 2 and 4), whereas the same residues show no significant enhancement in lanes 1 and 3 (linear substrate). This supercoil-dependent enhanced reactivity is evident at every purine in both strands of the 24-bp insert, except at the G residue on the lower strand (asterisk, lanes 1 and 2) which interrupts the alternation of pyrimidines and purines. No supercoil-dependent reaction at this position was observed. The 2 purines on either side of this G residue showed the enhanced reactivity expected of purines in the syn conformation in Z-DNA.

Thus, we conclude that the 24-bp insert in this plasmid (pRW1019) is forming a Z-helix in which every purine nucleoside has the syn conformation, except the second G in the sequence 3'-GGA-5', which retains the anti conformation in order to maintain a perfectly alternating syn/anti relationship throughout the insert. We assume that the complementary C is in the syn conformation.

The longest perfect tract with Z-forming potential in this sequence (pRW1019) is (CG)$_2$C, yet the Z-helix is transmitted through a region of non-alternation and through two short (TA)$_n$ regions to encompass the entire insert. As mentioned above, (CG)$_2$ appears not to form a Z-helix.

The Z-Z Junction Occupies 1 bp—The term Z-Z junction has been used to describe the structural disruption that must occur at the interface between two (or more) Z-helices which are out-of-phase with each other with respect to alternating syn/anti (25). Our previous studies (6) on a molecule containing such a structural feature showed that its presence causes a large increase in the free energy required for the formation of a left-handed helix by the entire insert, relative to an identical (in terms of length and sequence composition) sequence which remained perfectly in-phase. We further showed (6) that the disruption caused by the Z-Z junction extends over only a small distance (a few base pairs at most). The conformational properties of the purines at the Z-Z junction could not be determined in this molecule due to the fact that G residues 5' to thymidines are not reactive toward DEPC (19).

The sequence between the BamHI sites in pRW1020 (Fig. 1) contains two potential Z-Z junctions, that is, the central (CG)$_3$ region is out-of-phase (with regard to an alternating purine-pyrimidine motif) with the potential Z-helices on either side. From two-dimensional gel electrophoresis, the entire 24-bp insert formed a left-handed helix (data not shown) which had a higher free energy of formation than for the isomeric sequences in pRW1019 and 1022 (Table I).

By definition (3), this left-handed helix cannot be a Z-helix, and there must be some conformational preference of the bases around the glycosidic bonds to accommodate left-handed DNA formation. Three of the possibilities are illustrated in Fig. 4. In model 1, every purine nucleoside is shown in the syn conformation (upper-case) and every pyrimidine nucleoside in the anti conformation (lower-case). In this model, the (CG)$_3$TAT and the ATA(CG)$_3$ portions of the molecule are in-phase with each other, and the central (CG)$_3$ forms a mini-Z-helix which remains out-of-phase with the flanking sequences. Each putative Z-Z junction either would occupy no space at all or would extend over 2 bp as shown. In this case, analysis by DEPC would show strong reaction at every purine in the supercoiled molecule.

![Fig. 4. Models for possible nucleoside conformations in pRW1020.](image)

![Fig. 5. Fine mapping of DEPC-reactive sites on both strands of pRW1020.](image)
Model 2 shows a strict alternation of nucleosides in either the syn or anti conformation, regardless of whether the base is a purine or a pyrimidine. Thus, the entire insert remains in-phase. This model proposes that the central (CG)₂ portion of the molecule has the C residues in the syn conformation and the G residues in the anti conformation. If this novel left-handed form were to exist, DEPC analysis would show protection of the two central G residues on each strand, whereas every other purine would show strong reaction. The Z-Z junction in this case would extend over the 4 central base pairs.

In model 3, the central (CG)₂ forms a mini-Z-helix which is in-phase with the flanking sequences, whereas the nearest neighbors (in both cases, an A·T base pair) are in a non-Z conformation with the adenosine shown formally in the anti conformation. Every G residue in the (CG)₂ region is in the syn conformation; and thus, DEPC analysis would show strong reaction at these positions, whereas there would be protection of the A residues on either side if this model structure exists. The Z-Z junction in this case would each occupy 1 bp.

Fig. 5 shows the results of DEPC reaction on both strands of pRW1020 as described above for pRW1022 and pRW1019. As in Figs. 2 and 3, there is no significant enhancement of reaction at purines in the vector when the reaction is performed on the supercoiled molecule, and the G and A residues immediately outside the insert are no more reactive than purines much farther away. In lane 2, it can be seen that every G residue in the 3′-(GC)₄-5′ block exhibits enhanced reactivity to DEPC, as does the first A residue in the sequence ATA. However, the second A residue in this sequence shows protection from reaction with this probe. This is denoted by the asterisk in Fig. 5. The central 3′-(GC)₄-5′ region shows strong reaction at the G residues, and every purine in the remainder of the insert shows enhanced reactivity in the supercoiled form. On the opposite strand, the same situation is apparent, that is, the A residue at the point where the potential Z-helices become out-of-phase (asterisk) is protected from reaction with DEPC, whereas every other purine within the 24-bp left-handed helix is hyper-reactive to this probe in the supercoiled form.

These results suggest that model 3 (Fig. 4) is correct. Thus, the central (CG)₂ region is forming a regular Z-helix which is in-phase with the flanking sequences, whereas its nearest neighbors are in a non-Z conformation. In particular, the adenine bases on opposite strands at these points are not in a high syn conformation about their glycosidic bonds. However, the extent of DEPC modification at these positions (Fig. 5) is not the background level observed for purine nucleosides in the anti conformation (Fig. 3). It may be that there is some contribution from the situation depicted in model 1 (Fig. 4). Models 1 and 3 are not mutually exclusive. However, we have no evidence for the existence of a structure with the conformations illustrated in model 2 (Fig. 4).

Thus, we conclude that in molecules containing Z-Z junctions, the preference is for the separate Z-helices to remain in-phase with each other, leading to a structural disruption at the point where they would otherwise become out-of-phase. This disruption occupies 1 bp and does not prohibit the transmission of left-handedness between short Z-forming regions at the HindIII site.

B, summary of the modification patterns observed for the supercoiled plasmids treated with OsO₄. The length of each line corresponds to the relative intensity of a modification site which was determined by quantitation of a scan of the x-ray film with a Bio-Rad 620 video densitometer. The reproducibility is ±10%.
gions, although there is a high energy cost associated with it (Table I and Refs. 5 and 6).

**Thymidine Residues Embedded in Z-DNA Have Unusual Stacking Properties**—OsO₄ reactions were performed on both strands of the three plasmids (Fig. 1) at native superhelical density. Fig. 6A shows a photograph of the results obtained by reaction of OsO₄ with both strands of pRW1019. Fig. 6B shows the analysis of densitometric analyses of the results from similar treatment of the other plasmids along with those from pRW1019. For each determination, the length of the vertical lines (Fig. 6B) is proportional to the extent of reaction and was normalized relative to bands far removed (~10–20 bp) from the insert sequence. This is necessary to establish hyper-reactivity as opposed to general reaction and to minimize contributions from piperidine-induced cleavage of unmodified bases.

It can be seen that, with one exception, every thymidine in the left-handed helices exhibits hyper-reactivity toward this probe. The exception is the first T residue after the Z-Z junction on the top strand of pRW1020. The reason for this lack of hyper-reactivity is not clear, but it is unlikely to be a consequence of the Z-Z junction per se since a T residue in an almost identical position on the other strand does show hyper-reactivity. The hyper-reactivity of the thymidines in these helices is also unlikely to be due to thermal instability as a consequence of the relatively large amount of AT (25%) in each sequence since similar studies on the sequence (GC)₄AT(GC)₄, contained in a supercoiled plasmid also showed a marked hypersensitivity of the T residues to reaction with OsO₄.

In the Z-helix formed by the insert in pRW1022, osmium tetroxide reacts with every T residue within the insert (Fig. 6B). There is also a significant reaction at a residue on the lower strand which is within the Z-helix itself (Fig. 2), but which also forms part of the BamHI site. There is also a reaction at this position in the other two plasmids (Fig. 6, A and B). No other G residues in the Z-helix of pRW1022 react with this probe. In contrast, the inserts in pRW1019 and -1020 both exhibit a complex pattern of hyper-reactivity at G residues in the Z-form. Presumably the distortions detected by the probe in these sequences are a consequence of the irregularity of the left-handed helices formed by them compared to the regular Z-helix of pRW1022.

All the plasmids show similar patterns of reactivity at the junctions between the right- and left-handed helices. Thus, the junctions at the 5'-end of each strand are more reactive toward OsO₄ than the 3'-junctions (Fig. 6B). Interestingly, the T residues in both BamHI sites of pRW1020 are much less reactive (or nonreactive) than the corresponding sites in the other two plasmids, suggesting that the disruption at the center of this sequence causes a change in the structure of the junction between the right- and left-handed helices.

In summary, it is evident from the results described above that A-T base pairs embedded in Z-DNA are hyper-reactive toward osmium tetroxide. Lilley and co-workers (37) have previously reported that (AT), sequences in linear DNA are hyper-reactive toward OsO₄, although they observed that the sequence (TA), was less reactive than longer sequences. We feel that it is unlikely that the reactivity observed herein is due to a similar structure to that postulated by Lilley and co-workers, but is instead a reflection of the different stacking of A-T base pairs in Z-DNA (30) or the lack of ordering of solvent molecules in the vicinity of these sequences.

**S1 Nuclease, MHiHaI, and Bromoacetaldehyde Reactions**

Studies were performed as described previously (16, 38) to define and characterize further the B to Z transition in these three plasmids. Thus, all the plasmids exhibited supercoil-dependent hypersensitivity to S1 nuclease and to bromoacetaldehyde. From restriction mapping, the reaction sites were near or at the insert in each case. Fine mapping of S1 nuclease and bromoacetaldehyde reactive sites showed strong reactions at the B-Z junctions, as well as at other reactive sites within the left-handed helices themselves (data not shown).

All the plasmids contain multiple recognition sites for HhaI and the corresponding methylase. As shown previously for runs of perfectly alternating (CG), (39, 40), the methylation of these sites in the inserts of the three plasmids (Fig. 1) was inhibited when the sequences were in the left-handed form (data not shown). Furthermore, a preliminary investigation of the rates of methylation of different HhaI sites within the inserts by a fine mapping procedure reveals an all-or-none mechanism of inhibition.

There was no reaction with the T7 gene 3 product at or near the insert in any of the plasmids. Thus, these regions are not forming cruciforms (6). In summary, these results confirm the conclusions described above drawn from the studies with DEPC and OsO₄, namely, the entire insert in each case is forming a left-handed helix, although there are structural peculiarities associated with the presence of A-T base pairs in Z-DNA.

**DISCUSSION**

Herein, we have shown that sequences which do not normally form Z-DNA in isolation can be induced to do so when this will allow transmission of the Z-helix to neighboring regions to form the longest and most stable left-handed helix possible. Whereas Z-DNA formation by these regions requires more energy than that required for an identical length of (CG), (5, 33), the formation of left-handed helices is energetically more favorable than the introduction of B-Z junctions between short Z-forming regions.

Clearly, there are sequence and length preferences for a given region to become left-handed rather than forming B-Z junctions. Thus, we showed previously (22, 23) that a BamHI site and the sequences GTTTG and GACTG could form left-handed helices when flanked by Z-forming regions. Furthermore, in a systematic study (5), we showed that an alternating purine-pyrimidine motif (TATA) is energetically more favorable in the Z-form than a non-alternating (TTTT) sequence.

The same study (5) revealed that up to 6 bp of TA between two short Z-forming regions also form a left-handed helix. Ellison et al. (27) inferred that the central 8 bp of this sequence contained in the insert (CG), (TA), (CG), adopted a structure intermediate between the B- and Z-forms whereas the flanking (CG), regions were fully left-handed. Our preliminary results on the sequence (GC), (AT), (GC), contained in a plasmid indicate that this insert is preferentially extruded as a cruciform rather than forming a Z-helix. Thus, it appears that Z-helices cannot be transmitted through regions longer than 8 bp.

We have also shown that regions out-of-alternation are included in the Z-helix, even though this requires the retention of the anti conformation in purine nucleosides in order to maintain the alternating syn/anti motif in the Z-helix.

It was previously reported (19) that a similar situation is observed in pBR322. Thus, there is 1 guanosine out-of-alternation in an otherwise perfectly alternating 14-bp tract of purine-pyrimidine, and this residue is protected from reaction with DEPC (19). Our results with a systematic series of molecules (Ref. 5 and this work) studied by several techniques...

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5 M. J. McLean, W. Zacharias, and R. D. Wells, unpublished data.
show that quite long regions of non-alternation will be tolerated in a Z-helix, although there is an energetic penalty associated with these transversions which is presumably due to the difficulty with which pyrimidine nucleosides adopt the syn conformation (41).

The systematic nature of our studies enables a calculation of this energy penalty. Table I shows the free energy required to convert 1 bp of each insert from the B- to the Z-form and is thus an average of the contributions for individual base pairs. This value is higher for pRW1019 than for pRW1022, and the only difference between these plasmids is the single G to C transition in the insert of pRW1019. Thus, the difference in total free energy of Z-DNA formation by these two sequences (\(\Delta G_{Z} + 2\Delta G_{B} \)) must equal the extra energy term introduced by the base pair out-of-alternation. From the data presented in Table I, this calculation gives the energy cost of a G to C transversion as 1.2 kcal mol\(^{-1}\). The existence of Z-helices which are more sensitive to slight changes in available free energy would offer more opportunities for the involvement of this unusual DNA structure in biological regulatory processes.

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