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Diethyl pyrocarbonate: A chemical probe for secondary structure in negatively supercoiled DNA

(chemical modification/alternating purines and pyrimidines/pBR322/Z-DNA)

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ABSTRACT **Purine residues located within regions of DNA that have the potential to form left-handed Z-helical structures are modified preferentially by diethyl pyrocarbonate; this hyperreactivity is dependent on the degree of negative superhelicity of the circular DNA molecules. As negative superhelical density increases, guanosines in a 32-base-pair alternating G-C sequence and adenosines (but not guanosines) in a 64-base-pair alternating A-C/G-T sequence become 5- to 10-fold more reactive to diethyl pyrocarbonate. The negative superhelical densities at which enhanced reactivity occurs are similar to those reported for the point at which left-handed helices form within plasmids carrying these DNA sequences. Probing of negatively supercoiled pBR322 with diethyl pyrocarbonate reveals a hyperreactive region 31 base pairs in length of which only 9 base pairs are a perfect alternating purine and pyrimidine sequence; the reactivity of purines within this sequence indicates that purines in the *anti* conformation, or guanosines in the *syn* conformation with neighboring 3′ thymidines, are not hyperreactive in the Z-DNA form.**

DNA is a flexible molecule that can exist in a variety of conformations. One of the parameters influencing the equilibrium between these conformations is the stress created by negative supercoils; increasing the negative supercoil density within closed circular DNA molecules has been shown to stabilize the formation of single-stranded DNA, cruciform structures, and the double-stranded Z-DNA form (1). Of these structures, Z-DNA is the best characterized, because of the molecular detail afforded by high-resolution x-ray diffraction studies (reviewed in ref. 2).

Z-DNA is a left-handed double helix with a dinucleotide repeating unit. In the dinucleotide repeat, the bases along each strand alternate between the *syn* and *anti* conformations; these two conformations are differentiated by a 180° rotation of the base around the glycosyl bond. Both pyrimidines and purines are equally stable in the *anti* conformation (the structure found in the right-handed helix of B-DNA); in the *syn* conformation, however, pyrimidines are less stable than purines (3, 4). The relative instability of pyrimidines in the *syn* conformation offers an explanation for the fact that certain stretches of alternating purines and pyrimidines, e.g., repeating G-C or A-C/G-T stretches, are particularly favored for Z-DNA formation when under negative superhelical stress (5–9). The crystal structure of a nonalternating purine and pyrimidine oligonucleotide that is in the Z-form (10) implies that certain of these sequences can also be induced to form Z-DNA under sufficient negative superhelical stress.

Two methods that have been used to identify Z-DNA in closed circular molecules formed as a result of negative supercoiling stress are (*i*) analysis of electrophoretic mobility, which can change with the linking number of the DNA, and (*ii*) binding of antibodies directed against the Z-form. The first method detects the loss of supercoils and, therefore, decreasing linking number caused by the transition from a right-handed B-helix to the left-handed Z-form. With the second method, anti-Z-DNA antibodies are bound to negatively supercoiled molecules, and binding sites are identified either by electron microscopy or by the retention of antibody-bound DNAs to nitrocellulose filters (7). An advantage of the electrophoretic analyses is that formation of Z-DNA cannot be affected by interactions with antibodies. Alternatively, the antibody studies have the advantages that sites of Z-DNA formation can be identified at higher negative superhelical densities and the Z-DNA sites can be more readily mapped. Neither of the methods, however, maps Z-DNA formation at the nucleotide level.

A method that does identify structure at the nucleotide level in both DNA and RNA is chemical modification followed by strand scission at the modified sites (11, 12). This strategy relies on conformation-dependent reactivity of bases to particular reagents. Diethyl pyrocarbonate, which carbethoxylates purines at the N-7 atom (13, 14), is one such reagent. In RNA, purines in double-stranded but not single-stranded regions are refractory to diethyl pyrocarbonate modification (12). In double-stranded DNA, however, purines are accessible to this reagent (15). One interpretation of these results is that diethyl pyrocarbonate is less reactive to purines in an RNA A-helix than to purines in a DNA B-helix. Such a possible conformational sensitivity suggested that diethyl pyrocarbonate might serve to distinguish different helical structures in DNA, in particular the B- and Z-forms. I therefore compared the relative diethyl pyrocarbonate reactivity of purines within three sequences favored for Z-DNA formation under negative superhelical stress: (*i*) an alternating G-C sequence, (*ii*) an alternating A-C/G-T sequence, and (*iii*) a region of pBR322 containing a stretch of nine alternating purines and pyrimidines (5–9, 16, 17). I report here that, indeed, certain purines within all three of these sequences become hyperreactive to diethyl pyrocarbonate upon negative supercoiling.

MATERIALS AND METHODS

Plasmid DNAs. The three plasmid DNAs used, pLP32, pAN064, and pBR322, were prepared by alkaline NaDodSO$_4$ extraction (18) and CsCl equilibrium density gradient centrifugation. DNAs with different linking numbers were prepared by treatment with calf thymus topoisomerase I (Bethesda Research Laboratories) in the presence of ethidium bromide, as described (6, 7), and the average linking number of each DNA preparation was determined by the band counting method (6, 19). The superhelical density of each sample was determined by comparison to DNAs treated

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Abbreviation: bp, base pair(s).

with topoisomerase I under the conditions of diethyl pyrocarbonate modification. Linear DNAs were generated by digestion with *Pvu* II for pLP32 and pANO64 and *Eco*RV for pBR322.

Diethyl Pyrocarbonate Modification. Linear and supercoiled plasmid DNAs were modified by diethyl pyrocarbonate essentially as described (15). DNAs (1 μg) were suspended in 200 μl of 50 mM sodium cacodylate, pH 7.1/1 mM EDTA. The tubes were placed on ice and 3 μl of diethyl pyrocarbonate was added to each sample. The samples were incubated at 20°C for a total of 15 min. Because diethyl pyrocarbonate is relatively insoluble in water, the samples were thoroughly mixed at the beginning and halfway through the 15-min incubation. The reactions were terminated by the addition of 50 μl of 1.5 M sodium acetate containing tRNA at 100 μg/ml, followed by two sequential rapid ethanol precipitations, as described (11, 20). The samples were then suspended and stored in 50 μl of 10 mM Tris chloride, pH 8.0/0.1 mM EDTA. Electrophoresis of the modified samples showed that little nicking of the closed circular molecules had occurred during the chemical modification.

End-Labeling, Strand Cleavage, and Gel Electrophoresis. After digestion with the appropriate restriction enzyme, modified DNAs were labeled at either the 5′ end with polynucleotide kinase or the 3′ end with the large fragment of DNA polymerase I. End-labeled fragments were recleaved with a second restriction enzyme and purified by electrophoresis through 5% native polyacrylamide gels. Fragments were eluted by diffusion overnight at room temperature in 0.5 M ammonium acetate/0.05% $NaDodSO_4$/1 mM EDTA with 10 μg of tRNA carrier. After ethanol precipitation, the strands of the purified fragments were cleaved at the sites of diethyl pyrocarbonate modification by treatment with 25 μl of 1 M piperidine in sealed capillary tubes at 90°C for 5 min (3′ end-labeled fragments) or for 30 min (5′ end-labeled fragments). The samples were lyophilized twice and suspended in 100 μl of H_2O. The Cerenkov radioactivity of each sample was measured, and the same number of Cerenkov counts of each sample was transferred to new tubes and lyophilized. The samples were suspended in 3 μl of 99% formamide/10 mM EDTA containing dyes and denatured at 90°C for 1 min, and the modified sites were identified by electrophoresis through polyacrylamide gels in 8.3 M urea/100 mM Tris borate, pH 8.3/2 mM EDTA. Chemical degradation sequencing reactions were as described (20). The relative reactivities of particular sites were measured by scanning exposures of the gels with a Hoeffer GS300 densitometer.

RESULTS

Substrates to Study the Negative-Supercoil-Dependent Reactivity to Diethyl Pyrocarbonate of Alternating Purine and Pyrimidine Sequences. Three test plasmids, pLP32, pANO64, and pBR322, were used to compare the negative-supercoil-dependent reactivity of purines to diethyl pyrocarbonate. pLP32 contains a 32-base-pair (bp) alternating G-C sequence inserted at the *Bam*HI site of pBR322 (6) that forms Z-DNA at relatively low negative superhelical densities (6, 7). pANO64 contains a 250-bp segment of the mouse immunoglobulin κ light chain gene between the *Eco*RI and *Cla* I sites of pBR322 (8); within this segment lies a near-perfect 64-bp sequence of alternating A-C/G-T (one of the cytosine residues is replaced by a thymidine). This segment binds anti-Z-DNA antibodies under conditions of sufficient negative supercoil stress (8), and a similarly sized stretch of alternating A-C/G-T residues has been shown to form a left-handed helix by electrophoretic analyses (9). Although pBR322 does not contain long stretches of alternating purines and pyrimidines, it does contain a 14-bp stretch of alternating purines and pyrimidines near the unique *Ava* I site with only one nucleotide out of alternation; this region has been identified as forming a negative-supercoil-dependent Z-DNA segment by antibody binding (7, 16, 17).

Plasmid DNAs of different negative superhelical densities were produced as described in *Materials and Methods*. The reactivities of specific bases to diethyl pyrocarbonate were measured by modifying the DNAs with diethyl pyrocarbonate (15), isolating 3′ or 5′ singly end-labeled fragments containing the regions of interest, cleaving the DNA at the sites of modification with piperidine (11), and electrophoresing the cleavage products through denaturing polyacrylamide gels.

Reactivity of Guanosines Within an Alternating G-C Stretch to Diethyl Pyrocarbonate. The negative-supercoil-dependent diethyl pyrocarbonate reactivity of the 32-bp alternating G-C stretch of pLP32 is shown in Fig. 1 (lanes 3–7). The reactivity of the G-C stretch (identified by brackets in the figure) within linear (lane 3) and closed circular pLP32 DNAs with average superhelical densities of −0.014 (lane 4) and −0.029 (lane 5) is similar to the reactivity of purines within the sequences flanking the G-C stretch. However, at higher superhelical densities [−0.045 (lane 6) and −0.062 (lane 7)], the reactivity of guanine residues within the G-C stretch increases dramatically as shown by the 5- to 10-fold increase in band intensity (compare lanes 5 and 6). This transition in diethyl pyrocarbonate reactivity correlates with formation of a left-handed helix within the alternating G-C stretch (see *Discussion*).

To demonstrate that the observed diethyl pyrocarbonate hyperreactivity of purines was due to modification under the defined reaction conditions, two identical samples of supercoiled pLP32 were treated with diethyl pyrocarbonate, but only one of the samples (Fig. 1, lane 2) was incubated for 15 min before the reactions were terminated. The pattern of bands for the "unreacted" sample (lane 1) shows that some cleavage at purines has occurred; however, hyperreactivity of the alternating G-C stretch (lane 2) depends on the 15-min incubation with diethyl pyrocarbonate.

Adenosines, but Not Guanosines, Are Hyperreactive Within an Alternating A-C/G-T Sequence. The supercoil-dependent reactivity of purines within a repetitive $(A\text{-}C/G\text{-}T)_{32}$ sequence is shown in Fig. 2. Linearized plasmid (lanes 1) and closed circular (CC; lanes 2–5) pANO64 DNAs of increasingly negative superhelicity were modified with diethyl pyrocarbonate and digested with *Eco*RI; one-half of each sample was then 5′ end-labeled to examine the alternating G-T strand (Fig. 2*a*), while the other half was 3′ end-labeled to observe the reactivity of the A-C stretch (Fig. 2*b*).

The adenosine residues within the $(A\text{-}C)_{32}$ stretch in the less negatively supercoiled pANO64 DNAs (Fig. 2*b*, lanes 1 and 2) exhibit reactivities similar to those of purines within the flanking sequences. At superhelical densities of −0.064 and greater, however, there is a dramatic increase in adenosine reactivity within the $(A\text{-}C)_{32}$ domain (Fig. 2*b*, lanes 3–5). The relative increase in adenosine reactivity is similar to that of guanosines in the alternating G-C stretch of pLP32.

Fig. 2*a* shows the modification pattern of guanosines within the stretch of alternating G-T residues opposite the $(A\text{-}C)_{32}$ strand. Unlike the adenosines, the guanosine residues do not display such a dramatic supercoil-dependent increase in reactivity, even though the patterns on the left and right sides of the gel are the result of the same modification reactions. (The slight increase in reactivity of the guanosines in lane 3 of Fig. 2*a* may indicate an intermediate conformation in which guanosines are more accessible.) Since the guanosines flanked by cytidines in pLP32 do become hyperreactive at elevated superhelical densities, the non-hyperreactivity of the guanosines in pANO64 probably reflects an effect of the neighboring thymidine residues. Adenosines do not appear to be as strongly protected by neighboring

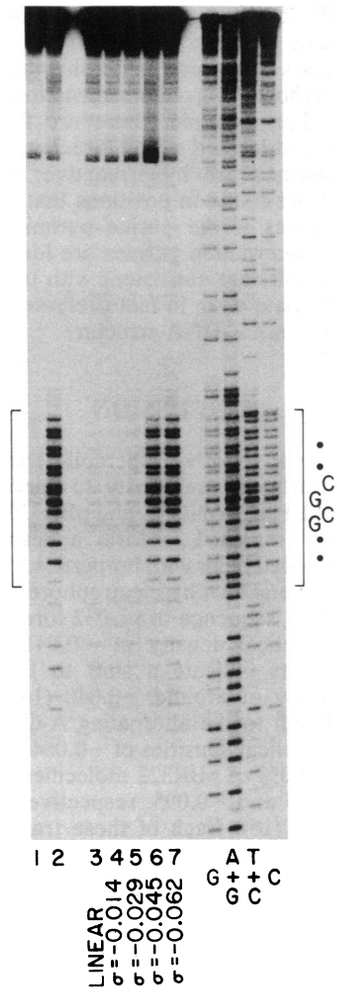

FIG. 1. Diethyl pyrocarbonate modification of pLP32 shows a negative-supercoil-dependent hyperreactivity of guanosines within a 32-bp alternating G-C sequence. Linear and closed circular pLP32 DNAs were modified with diethyl pyrocarbonate; the modified DNAs were digested with *Taq* I, 3′ end-labeled, and then digested with *Sph* I; and the 259-bp *Taq* I–*Sph* I restriction fragment containing the 32-bp sequence of alternating G-C was purified. The piperidine cleavage products were separated by electrophoresis through a 10% polyacrylamide gel. Lanes: 1 and 2, pLP32 DNA at the native superhelical density was treated with diethyl pyrocarbonate, not allowing (lane 1) or allowing (lane 2) a 15-min incubation after diethyl pyrocarbonate addition; 3, linear pLP32 DNA; 4–7, closed circular pLP32 DNAs of increasing negative supercoil densities were modified with diethyl pyrocarbonate. Supercoil densities (σ) of each closed circular DNA sample are given below each lane. The chemical degradation sequence patterns shown at the right locate the reactive purines within the sequence of pLP32. The irregular spacing of bands in the G-C stretch is due to "compression" resulting from the self-complementary G-C sequence.

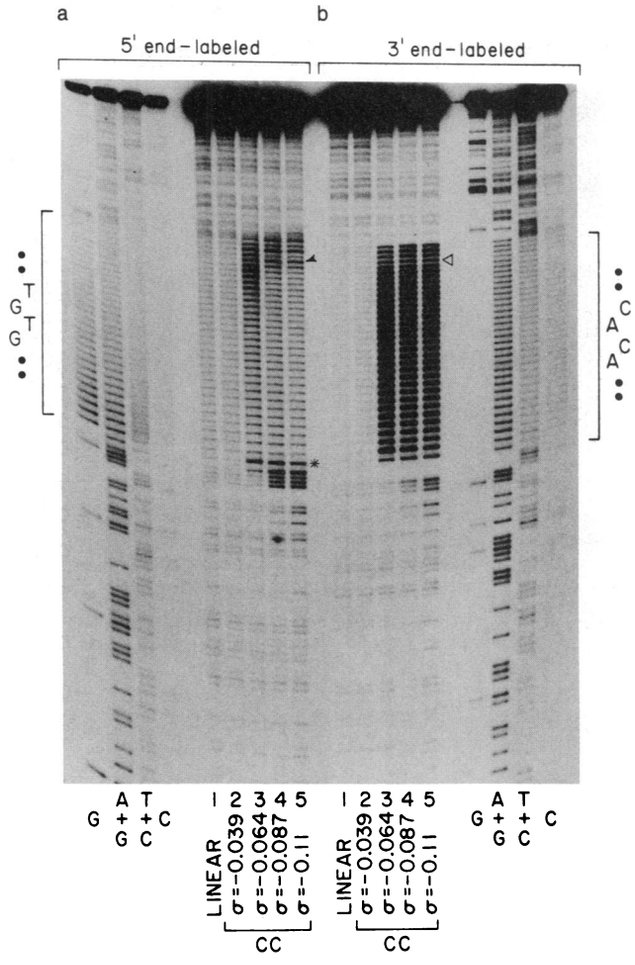

FIG. 2. Adenosines, but not guanosines, exhibit negative-supercoil-dependent hyperreactivity to diethyl pyrocarbonate in a 64-bp segment of alternating A-C/G-T. Linear (lanes 1) and closed circular (lanes 2–5) pANO64 DNAs were modified with diethyl pyrocarbonate. Modified DNAs were cleaved with *Eco*RI and either 5′ (*a*) or 3′ (*b*) end-labeled. Then, end-labeled fragments were digested with *Hin*dIII, the small *Eco*RI–*Hin*dIII fragment was purified, and the strands were cleaved with piperidine. The strand-cleavage products were electrophoresed through a 6% polyacrylamide gel. The chemical degradation sequencing patterns to identify the reactive purines are shown on the left and the right. Supercoil densities (σ) of the closed circular (CC) DNAs are shown below each lane. *, ◀, and ◁, Hyperreactive adenosine residues.

thymidines from diethyl pyrocarbonate attack as guanosines are: two adenosines, one within the alternating G-T stretch (◀) and one surrounded by thymidines at the 5′ end of the G-T stretch (*), exhibit hyperreactivity with increasingly negative superhelicity, and adenosines flanking a thymidine in the A-C strand (◁ and the band just below) are hyperreactive. The hyperreactivity of purines outside the A-C/G-T stretch in the more negatively supercoiled samples (lanes 4 and 5) suggests that altered secondary structures also exist outside the A-C/G-T sequence.

Negative Superhelical-Dependent Hyperreactivity of Purines Within a 31-bp Segment of pBR322. The region of pBR322 containing the nearly perfect 14-bp alternating purine and pyrimidine sequence was chosen to examine the reactivity of purines within a sequence lacking long stretches of alternating purines and pyrimidines. The diethyl pyrocarbonate modification pattern of this region in differentially supercoiled pBR322 DNA is shown in Fig. 3. The two sets of lanes (1–5) represent a single set of DNA modifications in which one portion (*a*) was labeled at the 5′ end and the other portion (*b*) was labeled at the 3′ end of the unique pBR322 *Ava* I site. As with pLP32 and pANO64, the less supercoiled samples of pBR322 (lanes 1 and 2) generate a pattern in which all of the purines within this region of pBR322 are modified similarly by diethyl pyrocarbonate. At an average negative superhelical density of −0.067 (lanes 3), the purines within the region containing the 14-bp sequence begin to exhibit hyperreactivity in both the 5′ and 3′ end-labeled strands (compare the purine bands in lanes 2 and 3 across from the stippled bar); as the negative superhelical stress increases to −0.091 and −0.12 (lanes 4 and 5), the hyperreactivity of purines within this region becomes more pronounced. At these higher superhelical densities, a well-defined block of hyperreactiv-

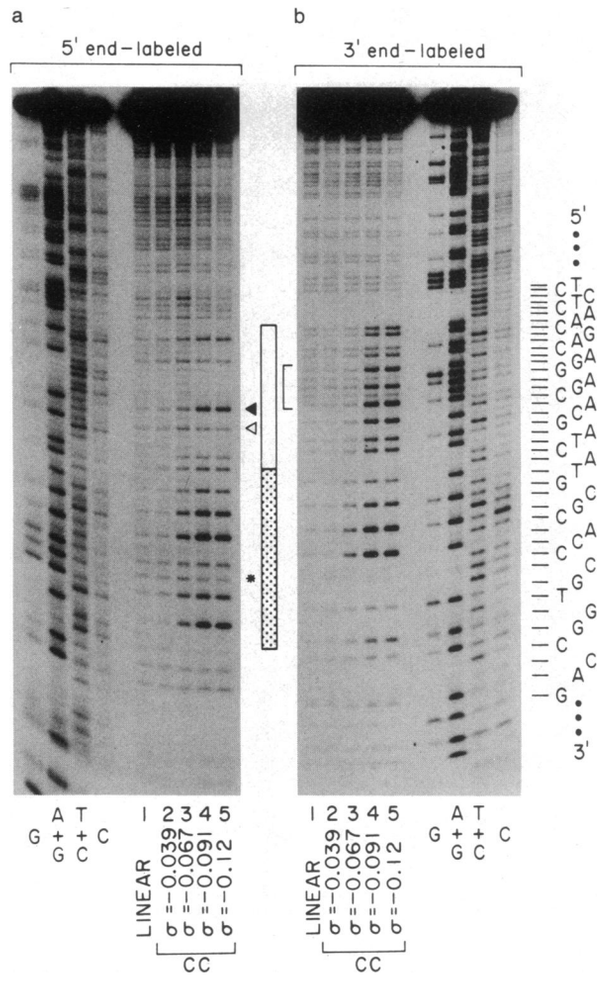

FIG. 3. Purines within a 31-bp region of pBR322, containing a short stretch of alternating purines and pyrimidines, are increasingly reactive to diethyl pyrocarbonate on negative supercoiling. Linear (lanes 1) and negatively supercoiled (CC; lanes 2–5) pBR322 DNAs were modified with diethyl pyrocarbonate. The modified DNAs were digested with *Ava* I, 5′ (*a*) or 3′ (*b*) end-labeled, and redigested with *Hin*fI. The piperidine cleavage products were separated by electrophoresis through a denaturing 10% polyacrylamide gel. The sequencing patterns to identify the purines are shown on the left and on the right, with the 3′ end-labeled sequence of this region of pBR322 shown to the far right. The negative supercoil densities (σ) are shown below each lane. The bar outlines the 31-bp region of diethyl pyrocarbonate hyperreactivity; the stippled portion corresponds to the 14-bp region of alternating purines and pyrimidines in which one nucleotide (*) is out of alternation. ◁ and ◀, Guanosine residues lying, respectively, 5′ and 3′ to a thymidine residue. The bracket outlines five purine residues in a row that exhibit an alternating pattern of hyperreactivities.

ity extends for 31 bp (outlined by the entire bar in Fig. 3), with the 14-bp sequence of imperfect purine and pyrimidine alternation located to one side.

The sequence of the pBR322-hyperreactive region is shown boxed in Fig. 4. Twenty-three of the 31 purines within this region of pBR322 are hyperreactive at the higher superhelical densities (circled bases). The 8 non-hyperreactive purines fall into two classes. First, four guanosines that are followed by thymidines at the 3′ position (noted by black bars in the figure) are not hyperreactive. This is best observed in the two guanines in the sequence G-T-G, identified by triangles in the 5′ end-labeled patterns of Fig. 3; the guanosine 3′ to the thymidine residue is hyperreactive (▲) whereas the 5′ guanosine is not hyperreactive (△). These results suggest that the lack of increased reactivity of guanosines in the alternating G-T stretch of pANO64 is due to the thymidines that lie 3′ of the guanosines.

The second class of purines exhibiting no increased reactivity consists of those lying out of alternation with respect to the 9-bp core. For example, there are five consecutive purines in the 3′ end-labeled strand (bracket, Fig. 3) but only three of the purines are hyperreactive; the non-hyperreactive purines are found in positions that would be occupied by pyrimidines if the purine–pyrimidine core were extended (out of alternation purines are identified by Xs in Fig. 4). These results are consistent with those expected if diethyl pyrocarbonate were in fact preferentially modifying alternate residues in a Z-DNA structure.

DISCUSSION

I have described the negative-supercoil-dependent patterns of diethyl pyrocarbonate reactivity to specific regions of three plasmids: pLP32, pANO64, and pBR322; each of these regions has been reported to form a negative-supercoil-dependent left-handed helix with properties of Z-DNA. Peck and Wang (21) have shown by electrophoretic analyses that the alternating G-C sequence of pLP32 forms a left-handed helix at a superhelical density of −0.041, and antibody binding experiments indicate a shift to the Z-form at a superhelical density of around −0.040 (16). Anti-Z-DNA antibodies bind well to the alternating A-C/G-T stretch of pANO64 at superhelical densities of −0.064 (8), and approximately 70% and 90% of pBR322 molecules at superhelical densities of −0.065 and −0.095, respectively, bind to anti-Z-DNA antibodies (16). Each of these transition points is similar to the superhelical densities at which purines in these sequences become hyperreactive to diethyl pyrocarbonate (Figs. 1–3).

Although the various measurements of Z-DNA formation have not been carried out under the conditions of diethyl pyrocarbonate modification, the close correlations suggest that the supercoil-dependent diethyl pyrocarbonate hyperreactivity of purines within these regions reflects the formation of left-handed Z-DNA. One sequence for which a correlation between anti-Z-DNA antibody binding and diethyl pyrocarbonate hyperreactivity is not readily apparent is the early promoter/enhancer region of the simian virus 40 genome. Anti-Z-DNA antibodies have been shown to bind to this region (22; A. Nordheim, personal communication) but I have been unable to identify hyperreactive purines on either strand of this region in negatively supercoiled simian virus 40 DNA (unpublished results).

The site of diethyl pyrocarbonate attack that renders purines susceptible to piperidine cleavage is probably the N-7 atom; carbethoxylation occurs readily at this site and destabilizes the imidazole ring (13, 14), thereby creating a piperidine-sensitive site. Comparison of the B- and Z-structures indicates that the N-7 atom is more accessible in the *syn* conformation of Z-DNA (23). The structure of Z-DNA is therefore consistent with purines in the *syn* conformation showing enhanced reactivity to diethyl pyrocarbonate. The characteristics of the diethyl pyrocarbonate modification patterns described suggest two rules for the accessibility of purines in the Z-form: the non-hyper-reactivity of *syn* guanosines 5′ of thymidine residues and non-hyper-reactivity of purines in the Z-DNA *anti* conformation. These characteristic modification patterns should prove useful in distinguishing Z-DNA structures from other supercoil-induced structures that might also show diethyl pyrocarbonate hyperreactivity.

The effect of 3′ flanking thymidines on the reactivity of guanosines may result from protection by the thymidine C-5 methyl group; this methyl group lies near the N-7 atom of *syn*

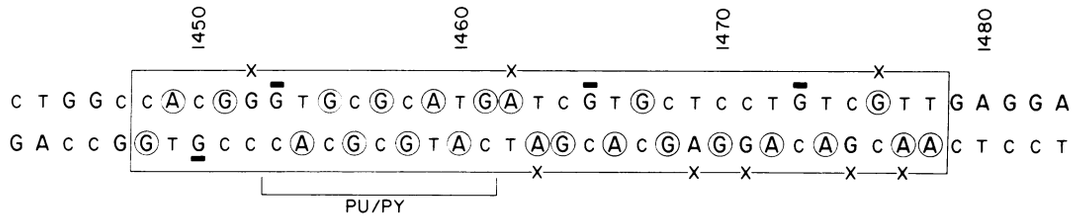

FIG. 4. Nucleotide sequence of pBR322 between nucleotides 1443 and 1483 showing the sites of hyperreactivity to diethyl pyrocarbonate. The unique *Ava* I site lies to the left of the sequence shown. The upper strand summarizes the 5′ end-labeled pattern in Fig. 3; the lower strand represents the 3′ end-labeled pattern. The large open box delineates the limits of hyperreactivity. X, Purines that are out of alternation with respect to the 9-bp core of alternating purines and pyrimidines (labeled PU/PY). The four non-hyperreactive guanosines that are adjacent to 3′ thymidine residues are identified by black bars. Negative-supercoil-dependent hyperreactive purines showing a more than 2-fold increase on the upper strand or a more than 3-fold increase on the lower strand are circled.

guanosines in the Z-form (24). The protection may be less pronounced for adenosines because A·T base pairs are stacked differently than G·C base pairs in Z-DNA (24). Protection of out-of-alternation purines probably reflects purines lying in the *anti* conformation of Z-DNA, because the N-7 atom of purines in the *anti* conformation is not as accessible to the solvent as the N-7 atom of purines in the *syn* configuration (10). There are four out-of-alternation purines within the 31-bp hyperreactive segment of pBR322 that are not protected from diethyl pyrocarbonate modification (Fig. 4). These hyperreactivities may reflect different secondary structures at these bases.

Other chemical reagents whose actions are sensitive to the conformational changes induced by negative supercoiling should also prove useful in characterizing Z-DNA formation. Johnston and Rich (25) have identified four such reagents, two of which, hydroxylamine and osmium tetroxide, modify residues at potential junctions between the Z- and B-forms.

The patterns of diethyl pyrocarbonate reactivity to pBR322 suggest a well-defined 31-bp segment of altered secondary structure. If the hyperreactivity to diethyl pyrocarbonate within this region reflects the formation of Z-DNA then the results presented here indicate that (*i*) nonalternating purine and pyrimidine sequences can form the major portion of a Z-DNA structure in naturally occurring sequences and (*ii*) if there is a requirement for a short (in this case 9-bp) core of alternating purines and pyrimidines for Z-DNA formation, that core need not be centrally located. These conclusions suggest that, *in vivo*, a Z-DNA stabilizing factor could bind to a specific sequence, for example alternating purines and pyrimidines, and asymmetrically affect the secondary structure of neighboring sequences by inducing Z-DNA formation in the flanking region.

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