The effect of neighboring AT-rich sequences on the right-handed B to left-handed Z transition was investigated in plasmids. The supercoiled stabilized Z-DNA structure in (CG) tracts 36 and 40 base pairs (bp) in length revealed an unexpected conformational aberration at defined C residues proximal to one end (coil). When the inserts were bilaterally flanked by an 80% AT-rich segment (90 bp on one side and 331 bp on the other), the presence of the perturbed Z-configuration required (CG) stretches longer than 32 bp and bilateral flanking by the AT-rich tracts, since plasmids with the (CG) tracts unilaterally flanked had an orthodox Z-structure. The thermodynamics of the negative supercoil-induced transitions were influenced only slightly by the neighboring AT-rich regions. Hence, the nature of Z-conformations in plasmids is markedly influenced by intrinsic structural features of the (pur-pyr) tract and by seemingly modest changes in the properties of neighboring sequences over a distance of several helical turns.

A number of in vitro investigations on left-handed Z-DNA have been conducted providing diverse information, including the following: kinetics, mechanism, and thermodynamics of formation for polymers and plasmids, types of left-handed conformations, properties of B-Z junctions, effect of sequence on stability, stabilizing factors (solvent, supercoiling, etc.), and capacity of enzymes to utilize as substrates (reviewed in Refs. 1–5). However, little, or no, work has been done on evaluating the effect of neighboring sequences on the properties of left-handed structures.

The in vivo existence and consequences of Z-DNA were demonstrated with the EcoRI methylase, expressed off a cloned gene, which is sensitive to substrate conformational changes (6). The second in vivo assay (7) measured the change in linking number on formation of the Z-helix and revealed the equilibrium between B- and Z-structures. Recent studies (8) showed that the site of cloning of sequences, capable of adopting Z-DNA or triple helix structures, in pBR322-derived vectors had a substantial influence on the frequency of deletions in the inserts. Also, the propensity for a sequence to adopt a Z-helix is a function of its location within a plasmid relative to promoters and the transcriptional state of the cells (9). Both of these results (8, 9) were interpreted in terms of the genetic functions of the cloning sites. If neighboring (flanking) sequences have an influence on the properties of a Z-tract, this may contribute to the observed biological effects. The base composition of flanking sequences affects the kinetics and mechanism of formation of cruciforms (10).

Herein, we have investigated the effect of neighboring AT-rich sequences on the thermodynamics of the formation of Z-DNA structures. Unexpectedly, the bilateral flanking AT-rich sequences cause a structural aberration at a defined locus in the left-handed region.

**MATERIALS AND METHODS**

**Plasmids**—Inserts of varying (CG) lengths were cloned into the BglII site of the vectors pBR1556 and pRW1852. pBR1556 is a pBR322 derivative with an 8-bp BglII linker (GAGATCTG) ligated into the filled-in EcoRI site as described (6). pRW1852 was constructed by ligating an 8-bp BglII linker (GAGATCTG) into the filled-in XbaI site of pColIRAXba (10) (generous gift of Dr. D. M. J. Lilley, University of Dundee, United Kingdom). pColIRAXba is a derivative of pBR322 with a deletion of 622 bp and the replacement of the 375-bp EcoRI-BamHI fragment by 421 bp of AT-rich (80%) sequence from ColE1. The plasmids having inserts in pRW1560 encode resistance to both ampicillin and tetracycline whereas those plasmids having inserts in pRW1852 encode resistance only to ampicillin. The correct sequences and lengths of the inserts for all the plasmids were confirmed by sequencing both of the strands using the primer extension method (9, 11–13).

**Chemical Modifications**—The plasmids were modified with three chemical probes as described, OsO4, (9), bromoacetaldehyde (BAA) (14), and diethylpyrocarbonate (DEPC) (15–17). However, all the modifications were carried out in TBE buffer (0.09 M Tris, 0.09 M boric acid, 2.5 mM EDTA, pH 8.0) instead of the buffers previously used for these probes. Both strands of each of the modified plasmids were then analyzed by primer extension (9, 11–13), except for the DEPC-modified samples which were cleaved by hot piperidine (17) prior to the primer extension analyses. pBR EcoRI clockwise and counterclockwise primers (New England Biolabs) were used to map the inserts in pRW1560. However, in the case of the inserts in pRW1852, synthetic clockwise (from base 36 to 57 of the top strand of the original Coe1 sequence (GenBank)) and counterclockwise (from base 150 to 169 of the bottom strand of the Coe1 sequence) primers were used. These primers were synthesized on an Applied Biosystems 380A oligonucleotide synthesizer using the phosphoramidite method.

**Topoisomer Analyses**—Topoisomeric populations of purified plasmid DNA were prepared using topoisomerase I as described (18). Two-dimensional agarose gel electrophoresis was conducted and analyzed as described (19–21).

**RESULTS**

**Chemical Probing of 40-bp (CG) Tracts—Plasmids** pRW1556 and pRW1856 (Fig. 1), both having the same (CG) length insert, but embedded in different sequence contexts on the two vectors, were probed with OsO4, BAA, and DEPC. These chemical probes were widely used to study non-B DNA

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1 The abbreviations used are: bp, base pair(s); BAA, bromoacetaldehyde; DEPC, diethylpyrocarbonate.
Z-DNA Distortion Caused by Flanking AT-rich Sequences

The table shows the length of the (CG) inserts including the 4 bp of the central EcoRI sites. E, EcoRI site (GAATTC).

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Insert</th>
<th>Vector</th>
<th>Length of CG tract(bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>pRW1556</td>
<td>(CG)46(CG)49</td>
<td>1560</td>
<td>40</td>
</tr>
<tr>
<td>pRW1856</td>
<td>(CG)46(CG)49</td>
<td>1852</td>
<td>40</td>
</tr>
<tr>
<td>pRW1570</td>
<td>(CG)46(CG)49</td>
<td>1560</td>
<td>36</td>
</tr>
<tr>
<td>pRW1870</td>
<td>(CG)46(CG)49</td>
<td>1560</td>
<td>32</td>
</tr>
<tr>
<td>pRW1857</td>
<td>(CG)46(CG)49</td>
<td>1560</td>
<td>32</td>
</tr>
</tbody>
</table>

Fig. 1. Plasmids used in these studies. All plasmids were made by cloning the inserts shown in the table into the BglII site of either the pRW1560 or pRW1852. pRW1560 (6) is a derivative of pBR322. pRW1852 has an AT-rich (80%) ColE1 sequence (I) between the filled-in EcoRI and BamHI sites. The rest of pRW1852 is pBR322. The table shows the length of the (CG) inserts including the 4 bp of the central EcoRI sites. E, EcoRI site (GAATTC).

The results of chemical probing and mapping for pRW1556 and pRW1856, is that in the former, the insert is flanked by the pBR322 sequence but, in the latter, the same insert is flanked by the ColE1 AT-rich (80%) region. This ColE1 region can be divided into two parts, the left side 90-bp colL and the right side 331-bp colR (25). The insert has two symmetric (CG) blocks of 18 bp each which flank an EcoRI site. Since the AT-rich regions contain few convenient restriction sites, we utilized primer extension methods (9, 11-13) to map the chemical modifications.

The results of chemical probing and mapping for pRW1556 and pRW1856 at native supercoil density (7 = -0.050) are shown in Fig. 2. The A and G sequencing lanes of the top strand of pRW1556 are shown in Fig. 2A. Lanes 1 and 2 show the primer extension analyses on pRW1556 treated with bipyridine only (control) or bipyridine plus OsO4. Lane 1 (control) shows no reactivity. The mapping pattern seen in lane 2 for the OsO4-modified bottom strand shows hyper-reactivities at the expected B-Z junctions (15, 26, 27) and at the EcoRI site (9). Similar results were obtained for the BAA-modified DNA (lane 3), as observed earlier (14, 23). However, some nonspecific stop sites were observed (compare to the control (lane 1)). Lane 4 shows the stop sites obtained on DEPC-modified pRW1556. The mapping pattern observed confirms that the insert is in the Z-form (9, 15, 17). The reactivity patterns obtained for the top strand (data not shown) of this plasmid with these three probes are similar to that seen for the bottom strand.

Fig. 2B shows the results for pRW1856, which was treated similarly. The A and G lanes of the sequencing ladder for the top strand are indicated. Lane 1 is the control (unmodified DNA) and shows no reactivity. Lanes 2, 3, and 4 are the mapping patterns for the bottom strand treated with OsO4, BAA, and DEPC, respectively. It can be seen in lanes 2 and 3 that, in addition to the usual hyper-reactive sites observed at the B-Z junctions and the EcoRI site, an additional unexpected hypersensitive band (arrow) is seen at the 5th C on the bottom strand of the first (CG) block (proximal to the colL region) from the 3' direction. This enhanced reactivity is indicative of a distortion or perturbation in the structure at that nucleotide. Also note (lane 4) the enhanced DEPC reactivity of the G residues at this site.

Mapping of the hyper-reactive sites for the top strand of pRW1856 also shows the additional unexpected band within the (CG) block (proximal to colL region) in addition to the expected bands (B-Z and Z-Z junctions). Furthermore, this reactive site on the top strand is shifted by 2 bp to the right of that seen for the bottom strand, that is, at the 7th C from the 5' junction on the (CG) block proximal to the colL region. None of the Cs of the second block (proximal to colR) showed any hyper-reactivity. In summary, these results indicate that, in the case of pRW1856, a distortion of the Z-structure is present only within the (CG) block proximal to colL.

Mapping Patterns for Shorter (CG) Tracts—The potential effect of the total length of the Z-DNA segment was evaluated with a series of shorter inserts containing either 36 or 32 bp. pRW1570 and pRW1870 contain a (CG)12ATT(CG)2 insert in the BglII site of the vectors pRW1560 and pRW1852, respectively (Fig. 1). The total length of this (CG) tract is 36 bp and was shown to adopt a left-handed helix (7). Mapping of the OsO4 reactive sites, at the bp level, of pRW1570 (both top and bottom strands) indicated hyper-reactivity at only the B-Z junctions and the EcoRI site as expected (data not shown). However, the OsO4 reactivity for pRW1870 differed
from the data obtained with pRW1570. In addition to those bands observed for pRW1570, another stop site was detected (as in pRW1856) at the 5th C of the bottom strand from the 3' end on the (CG)$_8$ block (Fig. 3) (proximal to the colL region). For the top strand, this extra reactivity occurred also at the 5th C from the 5' end of the (CG)$_8$ block. These reactivities within the (CG) block were observed when the DNA was modified with either OsO$_4$ or BAA. None of the Cs on the (CG)$_7$ block (proximal to colR sequence) showed any enhanced reactivity. DEPC sites were mapped for both pRW1570 and pRW1870 and the patterns confirmed that the insert was in the Z-form. Hence, it is evident that a conformational distortion occurs at a specific C in the (CG) tract proximal to the colL only, for both the 40- and 36-bp tracts; in the former case, the blocks are symmetric and in the latter are asymmetric in length.

Chemical modification analyses with OsO$_4$ and BAA were carried out on two other plasmids, pRW1557 and pRW1857 (Fig. 1), to determine if the new reactivity was a function only of the total (CG) length. Both these plasmids contain inserts of 32 bp with two symmetric (CG)$_7$ blocks. The plasmids were modified with OsO$_4$ and BAA, and the reactive sites on both strands were mapped by primer extension. Fig. 3 shows the OsO$_4$ reactivity pattern on the bottom strand of pRW1857. The patterns of reactivity for both pRW1557 and pRW1857 on both strands were identical with no unexpected reactivities observed within the (CG) blocks, as found for pRW1856 (Fig. 2B) and pRW1870 (Fig. 3). The only reactivities observed were those indicative of the B-Z and the Z-Z junctions. Similar results were obtained for BAA-modified pRW1857 for both the strands. DEPC modification of both pRW1857 and pRW1570 showed that the inserted formed Z-structures.

From these analyses on chemically modified plasmids containing varying lengths of (CG) tracts, it is clear that the unanticipated perturbation within the (CG) blocks is a function of total (CG) length (requiring an insert length of more than 32 bp) and occurs only in a specific sequence environment.

**Effect of Orientation**—The disappearance of the additional reactivity in pRW1857 prompted this question: was it due to (a) the decrease in (CG) length or (b) some feature of the (CG)$_8$ block proximal to the colL region for pRW1856 and pRW1870, which was not present in pRW1857. To answer this question, another plasmid, pRW1871, was constructed. In this plasmid, the total (CG) length was 36 bp (identical with the insert length in pRW1870). However, the orientation of the asymmetric (CG) blocks was interchanged, i.e., the insert was (CG)$_7$AATT(CG)$_7$ in pRW1871 as opposed to (CG)$_8$ AATT(CG)$_7$ in pRW1870. Thus, in pRW1871, the (CG)$_7$ block neighbors the colL sequence as compared to pRW1870, where the (CG)$_8$ block was proximal to the colL sequence.

OsO$_4$ and BAA modifications and subsequent primer extension analyses were carried out on both strands of pRW1871. Results of the analysis on the bottom strand of OsO$_4$-modified pRW1871 are shown in Fig. 3. A distinct stop site was found at the 5th C of the (CG)$_7$ block (proximal to the colL region) in addition to the other usually reactive sites. This unusual hyper-reactivity was observed also on the 5th C of the top strand. The patterns of reactivity obtained with BAA-modified pRW1871 (top and bottom strands) were identical with that seen for OsO$_4$-modified pRW1871. There was, however, no reactivity seen for any of the Cs within the (CG) block distal to the colL sequence (proximal to colR), i.e., in this case, the (CG)$_8$ block.

Hence, it can be concluded from all the above analyses that the unexpected hyper-reactive band within the (CG) block is dependent on both the total (CG) length and the proximity of the insert relative to the colL region, but not on the specific orientation of the individual (CG) blocks.

**Change of Vector Sequence Background**—To test the effect of unbilaterally flanking AT-rich sequences on the B → Z transition of (CG) blocks, the different length inserts in pRW1556, pRW1570, and pRW1557 were cloned into the BamHI site or the filled-in EcoRI site of pRW1852. In the former set of plasmids, the inserts are flanked only on one side by the AT-rich colL sequence and not when the ColEl sequence was present. These two sets of plasmids were chemically modified with DEPC, BAA, and OsO$_4$, and primer extension analyses were carried out for each of the six plasmids. DEPC modification patterns confirmed that all the inserts in these plasmids were in the Z-conformation. The mapping patterns obtained for the BAA- and OsO$_4$-modified plasmids, however, showed reactive sites only at the B-Z and Z-Z junctions. No other unusual reactive sites within any of the (CG) blocks were detected.

These results, in conjunction with the data presented in Figs. 2 and 3, show that an unusual perturbed structure is formed in (CG) blocks only when flanked bilaterally by the AT-rich ColEl sequence and not when the ColEl sequence unilaterally flanks the inserts.

**Effect of Supercoiling**—Chemical probing by OsO$_4$ was carried out on topoisomeric populations of pRW1871 to determine whether the unusual reactivity within the (CG) block proximal to the colL region was a function of negative super-
helical density. For this purpose, individual topoisomeric populations were prepared (18) and probed with OsO₄ (9). Primer extension analyses of the bottom strand were carried out, and the mapping pattern is shown in Fig. 4. Lanes N and R are the patterns for native supercoiled and relaxed modified plasmids, respectively. The other five lanes show the mapping patterns observed for the modified topoisomeric populations at varying superhelical densities (from $\sigma = -0.01$ to $-0.08$). An increase in negative superhelical density causes enhanced reactivity at the two B-Z junctions, the Z-Z junction, and at the unusually reactive site at the 5th C in the (CG)$_b$ block proximal to the colL region. Therefore, OsO₄ reactivity at all four sites is a function of the presence of Z-DNA.

*Two-dimensional Gel Electrophoresis*—The seven plasmids (Fig. 1) were analyzed by two-dimensional gel electrophoresis (1, 16) to confirm the types of structural transitions involved and to evaluate the energetics. The two-dimensional gel patterns for the sets of plasmids pRW1556/pRW1856 and pRW1570/pRW1870/pRW1871 showed two transitions each. Fig. 5 shows the two-dimensional gel photographs for pRW1570 and pRW1871 (upper panel) and their schematic representation (lower panel). Both these plasmids undergo two transitions each. The relaxation of these transitions corresponded to the Z formation of the individual (CG) blocks. However, the $-\sigma$ values at the midpoint of the first B $\rightarrow$ Z transition were slightly, but reproducibly, less for the inserts in the random sequence environment versus those in the AT-rich ColE1 environment. For pRW1556, the $\sigma$ value was $-0.031$ as compared to pRW1856 where $\sigma = -0.033$. Similarly, for pRW1570, the value was $0.029$ as compared to pRW1870 and pRW1871 where $\sigma = -0.031$. Both the plasmids, pRW1557 and pRW1857, having two symmetric (CG)$_b$ blocks showed only one B $\rightarrow$ Z transition implying the same superhelical density value at which both (CG)$_b$ blocks formed the Z-structure. For pRW1557, the $\sigma$ value was $-0.030$ whereas for pRW1857, $\sigma = -0.032$. (All $\sigma$ values ($\pm 0.005$) were the average of three experimental observations.)

From these results, we deduce that the inserts flanked by AT-rich sequences do form left-handed helices as for those in a random sequence environment. In addition, the energetics of the B $\rightarrow$ Z transitions for the inserts flanked by the pBR322 sequence or by the ColE1 sequence are only slightly different. Interestingly, both the symmetrical (CG)$_b$E(CG)$_b$ and (CG)$_b$E(CG)$_b$ inserts in pRW1856 and pRW1857, respectively, adopted B-structures, Z-structures, and cruciforms above the critical superhelical density. Alternatively, both of these types of inserts, as well as the asymmetrical (CG)$_b$E(CG)$_b$, adopted only Z-helices when cloned in the BglII site of pRW1560; the same behavior was found for this asymmetrical insert in pRW1870 or pRW1871 (with the opposite orientation). Hence, this unprecedented conformational behavior is a function of the presence of the AT-rich tract (Fig. 1) as well as the symmetry of the insert blocks and is under further investigation.

Two-dimensional gel electrophoresis determinations also were performed on plasmids (pRW1846, -1847, -1850, -1876, -1877, and -1890) containing the same inserts unilaterally flanked by either colR or colL. Two transitions were observed for the (CG)$_b$E(CG)$_b$ and (CG)$_b$E(CG)$_b$ blocks as compared to only one transition for the (CG)$_b$E(CG)$_b$, as found for inserts bilaterally flanked by the AT-rich sequences. Also, as described above in bilateral AT-rich environments, the symmetric inserts, i.e. (CG)$_b$E(CG)$_b$ and (CG)$_b$E(CG)$_b$, simulta-
neously adopted B-structures, Z-structures, and cruciforms in these plasmids above the critical superhelical density.

Summary of Modification Patterns—Fig. 6 summarizes schematically the mapping patterns for (CG) inserts of 40 to 32 bp probed with OsO₄ and BAA. These inserts were placed either in a random sequence environment (as for pRW1556, -1557, and -1555), a bilaterally flanked AT-rich environment (pRW1856, -1870, -1871, and -1857), or a unilaterally flanked AT-rich environment with only the colL sequence on one side (pRW1846, -1850, and -1847) or the colR sequence flanking the insert (pRW1876, -1880, and -1877).

The expected reactivities were observed at the 5' and 3' B Z junctions and at the central Z-Z junction (AATT region) in all the plasmids shown, the As and Cs being reactive to BAA and the Ts to OsO₄. However, as shown with arrows in Fig. 6 for pRW1856, additional and unexpected reactivities with both BAA and OsO₄ were observed at the 5th C residue in the bottom strand (from the 3' end and proximal to the colL sequence) and at the 7th C on the top strand (from the 5' end and proximal to the colL sequence). No reactivities were detected on the other (CG) tract (closer to colR).

New chemically reactive sites, indicated by arrows, also were detected for pRW1870 and pRW1871 at the 5th C residue on both of the strands of the (CG)₉ and (CG)₇ tracts, respectively (closer to the colL sequence), with both probes. As expected, no hyper-reactive sites were seen for the (CG) blocks closer to the colR sequence, and the reactivities detected were independent of the symmetry or orientation of the (CG) blocks. In addition, no extra reactive sites were detected for the inserts when unilaterally flanked by AT-rich sequences. In summary, these results demonstrate the formation of a new junction or structural aberration within the (CG) block closer to colL only for inserts bilaterally flanked by the AT-rich ColEl sequence.

DISCUSSION

An unexpected chemical probe reactivity occurs within left-handed (CG) tracts of 36 and 40 bp in length when they are bilaterally flanked by AT-rich regions in supercoiled plasmids. However, the unusual hyper-reactive site was not detected for (CG) tracts of 32 bp in the same sequence context. These results reveal the necessity of the flanking AT-rich regions and a certain length of the (CG) tract. Apparently, sufficient stability is present in the 36- and 40-bp Z-DNA tracts (~3 or more turns of 12.0 bp/turn) to cause the flanking AT-rich sequences to induce the structural aberrations whereas the 32-bp (CG) tract (~2.75 turns of Z-helix) is not stable enough.

The unusual reactivity at certain C residues in the Z-tract proximal to the colL region indicates that a sequence (or structural) feature of the extreme AT-rich (90% AT in a 30-bp segment) colL region is responsible for this effect. It was shown earlier that this part of the colL segment played a key role in the influence of neighboring sequences on the kinetics and mechanism of cruciform extrusion (10, 25).

The requirement for the presence of AT-rich tracts on both sides of the Z-helix, rather than just one side, emphasizes the long range nature (28, 29) of this perturbation. It has been known for some time that AT-rich sequences differ structurally from random sequence DNA (reviewed in Ref. 29). These AT-rich regions have a high degree of structural polymorphism and flexibility and are easily deformed into non-B-DNA structures. Poly(dA-dT)-poly(dA-dT) forms alternating dinucleotide and other types of conformations (30–35). In particular, NMR studies (31, 36) revealed that the backbone conformations between A and T bases in this DNA polymer are different, generating an alternating conformational motif. The large amount of mobility in AT-rich DNA was demonstrated (37, 38). Similar structural variations are known for poly(dA)-poly(dT) (39–41).

Hence, the AT-rich ColEl sequences used herein may be capable of adopting a variety of conformations (29, 39) which are likely to be dynamic in nature. These properties of AT-
rich segments coupled with the torsional stress in the supercoiled plasmids could increase the propensity of DNA breathing (helix opening caused by thermal fluctuations). The resulting conformational or dynamic effects could then be transmitted to the (CG) block where the structural perturbation was detected.

The detailed nature of this conformational distortion is uncertain at present but must involve a helical perturbation in order to cause accessibility to BAA and OsO$_4$, at quite specific loci. BAA attacks adenosine at the N1 and C6 amino group when the base pair is broken (14, 42, 43). OsO$_4$ adds to the C5=C6 double bond of pyrimidines which are accessible to out-of-plane attack. Both probes were used widely to study cruciforms and Z-DNA (15, 23, 27). Whereas these chemical probes are extremely useful for revealing the existence of conformational irregularities with precise molecular resolution (at the base pair level), they do not elucidate the atomic nature of the aberration. We speculate that the (CG) tract neighboring the AT-rich coil sequence simultaneously adopts two different conformations (one from the leftmost B-Z junction to the structural aberration (arrow) and the other in the rest of the left-handed segment) which creates a new junction. Presumably, the reactive 5th (or 7th) cytosine residues are distorted but probably still paired and stacked most of the time. The helix may be bent (or “cracked”) at this location. Previous work (44, 45) showed the existence of left-handed but non-Z types of helices. The next step in elucidating the atomic features of this helical perturbation may require x-ray crystallography or spectroscopic (NMR, etc.) methods, techniques that do not give high resolution information on several kilobase pair plasmids. Hence, these analyses may require the development of systems that mimic negative supercoiling torsion on suitable lovemiyogumolaricitles of a length tractable by x-ray, NMR, etc.

Structural polymorphism of AT-rich regions is of potential biological significance because of their existence in interesting locations in eukaryotic genomes (reviewed in Ref. 46). AT-rich sequences are found in some cases near transcribed genes suggesting their biological role. For example, a long AT-rich region (91%) exists at approximately 450 bp upstream from the Xenopus laevis $\beta$-globin gene (47). Certain intergenic regions of Drosophila melanogaster have a high AT content (81% over 250 bp) (48); however, their exact genomic location is unknown. We demonstrated the influence of flanking AT-rich right-handed sequences on the conformational features of left-handed Z-tracts at specific loci. Furthermore, seemingly small changes (mutations) in a critical effector sequence may have a large effect with respect to stabilizing the structural aberration in the Z-tract. If a specific protein requires this structural aberration for binding, a mutation at a position several helical turns away from the binding site could abolish the site, hence eliminating the resultant biological function.

Thus, these results broaden the possibilities of types of specific interactions of duplex DNA sequences with proteins, drugs, and other ligands as a consequence of flanking sequence effects.

**Acknowledgment**—We thank Timothy A. Fritz for help with some experiments.

**Note Added in Proof**—Two other molecules containing (CG) tracts of 40 and 36 bp (similar to pRW1856 and pRW1870) without the EcoRI site in the middle, embedded in the BgII site of pRW1852, were prepared recently and modified with BAA and OsO$_4$. For the uninterrupted 40 bp (CG) tract, primer extension analyses revealed the unusual reactivity at the same positions as found for pRW1856, i.e. at the seventh C of the top strand and the fifth C of the bottom strand. For the 36-bp continuous (CG) tract, this unusual reactivity was seen at the fifth C on both strands with both probes. Thus, the presence of the AATT sequence does not cause this structural aberration.

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