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Differential Sequence Dynamics of Homopolymeric and Alternating AT Tracts in a Small Plasmid DNA†

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Received April 23, 1990; Revised Manuscript Received October 4, 1990

ABSTRACT: The location of OsO_4 bispyridine hyper- and hyporeactivity in a small deletion derivative of plasmid ColE1 (PTC12, 1727 bp) has been determined for approximately 70% of the molecule. Thymine bases in homopolymeric $(dA)_n \cdot (dT)_n$ tracts ($n \geq 4$) were always found to be resistant toward OsO_4 modification. DNA supercoiling did not destabilize these tracts. The extent of OsO_4 bispyridine reactivity of homopolymeric $(dA)_n \cdot (dT)_n$ tracts, where $n = 3$, was found to be dependent on the rate of base unpairing of the sequence immediately 5′ and 3′ to the tract. Repressed OsO_4 reactivity of thymine bases in $(dA)_3 \cdot (dT)_3$ tracts was observed if immediately both 5′ and 3′ to the tract were stable DNA sequences composed of GC base pairs and/or a homopolymeric $(dA)_n \cdot (dT)_n$ tract ($n \geq 4$). Homopolymeric tracts of $n = 3$ not having adjacent sequences with repressed unpairing rates did not show reduced levels of OsO_4 bispyridine reactivity. Alternating $d(TA)_n$ tracts ($n \geq 2$) were found to exhibit hyperreactivity with OsO_4. The extent of this hyperreactivity was dependent on the length of the tract and superhelical torsional stress. The distribution and frequency of homopolymeric $(dA)_n \cdot (dT)_n$ ($n \geq 4$) tracts in *Escherichia coli* promoter sequences were examined, and the possible implications of these tracts on promoter function are discussed.

Patterns of local nucleotide sequence within promoters and regulatory sequences may be important determinants of biological activity, and we are interested in evaluating the structural dynamics of different sequence motifs that are frequently represented in *Escherichia coli* promoters. Homopolymeric and alternating AT sequences fall into this category (Hawley & McClure, 1983; Pivec et al., 1985; Galas et al., 1985; Harley & Reynolds, 1987; Travers, 1989). Hexamer sequences partly composed of alternating AT sequences are a key feature of the −10 region of prokaryotic promoters, and these sequences are involved in unpairing events leading to RNA polymerase–promoter open complex formation (Hawley & McClure, 1983; Pivec et al., 1985; Galas et al., 1985; Harley & Reynolds, 1987). Homopolymeric dA·dT sequences are present among prokaryotic promoters, particularly 5′ to the −35 hexamer recognition sequence (Galas et al., 1985; Deuschle et al., 1986; Plaskon & Wartell, 1987); however, these sequences are also found at a lower frequency in the spacer region between the −35 and the −10 recognition sequences of the promoter and within the −10 region of a small number of *E. coli* promoters (Hawley & McClure, 1983; Harley & Reynolds, 1987).

Homopolymeric and alternating AT sequences differ dramatically in structure and dynamic properties. The characteristics of homopolymeric $(dA)_n \cdot (dT)_n$ sequences are as follows: (1) short, phased runs of $(dA)_n \cdot (dT)_n$ tracts (where $n \geq 4$) result in DNA bending (Wu & Crothers, 1984; Koo et al., 1986); (2) there is a resistance toward nucleosome reconstitution (Kunkel & Martinson, 1981) and DNase I cleavage (Drew & Travers, 1984); (3) hydroxyl radical cleavage of the adenine strand (Burkhoff & Tullius, 1987) progressively decreases in a 5′ to 3′ direction; (4) there is a reduced binding affinity for the intercalative drugs ethidium bromide (Bresloff & Crothers, 1981), propidium (Jones et al., 1986), and daunomycin (Chaires, 1983) compared to most other DNA sequences including alternating AT sequences; (5)

† Studies supported by National Institutes of Health Grant CA17077 awarded to J.L., principal investigator.
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0006-2960/91/0430-2664$02.50/0

there is a resistance to environmental conditions (humidity, salt concentration) that induce other sequences to undergo a B to A form DNA transition (Pilet et al., 1975); (6) thermal melting studies of self-complementary AT-containing oligonucleotide dodecamers found that the melting temperature of the homopolymeric oligo duplex $d(A_6T_6)$ was approximately 12 °C higher than that of the duplex with an alternating $d(AT)_6$ sequence (Wilson et al., 1987).

Crystallography of a B-type dodecamer containing a central $dA_6 \cdot dT_6$ tract has revealed a high degree of propeller twist for the base pairs in the homopolymeric sequence that maximizes purine–purine stacking interactions (Alexeev et al., 1987; Nelson et al., 1987; Coll et al., 1987). The propeller twist appears to be involved in narrowing the minor groove to 9.1 Å compared to 12 Å for standard B-DNA. The propellor twist also generates a system of bifurcated hydrogen bonds in which the carbonyl oxygen of thymine receives both a hydrogen bond from the N6-H of the complementary adenine and a diagonal hydrogen bond, across the major groove, from the N6-H of the adenine stacked above the complementary adenine. A zig-zag system of hydrogen bonds down the major groove is generated from this pattern of bifurcated hydrogen bonds (Nelson et al., 1987). A structural model for homopolymeric $(dA)_n \cdot (dT)_n$ sequences has been developed (Chuprina, 1987; Poltev et al., 1988), in which the minor groove contains a spine of hydration with water molecules hydrogen bonded to the N3 atoms of adenines and O2 atoms of thymines of adjacent base pairs, thereby bridging bases of the opposite strands of the double helix and narrowing the minor groove. The spine of hydration model for homopolymeric $(dA)_n \cdot (dT)_n$ sequences is the energetically optimal structure by energy minimization calculations (Lipanov & Chuprina, 1987). The minimum size of the homopolymeric AT tract necessary for formation of the proposed spine of hydration is four base pairs (Chuprina, 1987). Crystallographic studies did not detect the proposed spine of hydration (Nelson & Klug, 1988), but very recent experimental evidence for extensive hydration comes from volume and heat changes that occur when netropsin displaces water upon binding to poly(dA)·poly(dT) (Marky & Kupke, 1989).

The dynamics of base pair transitions for alternating AT and homopolymeric $(dA)_n \cdot (dT)_n$ tracts has been measured by using imino proton magnetic resonance (Patel et al., 1985a,b; Leroy et al., 1988). Thymidine imino proton exchange for the TATAAT Pribnow box and TATA sequences, centrally located in self-complementary dodecamers, have exchange times 3–5 times faster than those observed for an AATT segment (Patel et al., 1985a,b). Leroy et al. (1988) found that the rate of opening of the central AT base pair in the oligonucleotide duplexes d(CGCAAAGCG) and d(CGCAAAAAGCG) differs substantially, with the central AT base pair (bp)[1] in the poly(A) tract with five adenine bases exchanging 20 times slower than the central AT base pair in the duplex with only three consecutive adenine bases.

Nucleotide resolution detection of unpairing transitions using imino proton magnetic resonance requires the use of small oligonucleotide duplexes. However, an understanding of the helix destabilizing influences of superhelical torsional stress on different sequence motifs is also of considerable importance since overwhelming evidence has been presented showing that DNA supercoiling plays a crucial role in DNA metabolic processes for prokaryotes (Gellert, 1981; Wang, 1985; Pruss & Drlica, 1989). In eukaryotes, a variety of indirect observations suggest that DNA supercoiling is also important in gene expression (Wang, 1985; Weintraub, 1985; North, 1985). Consequently, an experimental strategy for exploring base unpairing in large DNA molecules is of considerable importance. To address this question we have made use of osmium tetraoxide (OsO_4) and diethyl pyrocarbonate (DEPC) modification to probe for unpairing transitions in supercoiled and linearized DNA under different salt conditions in a small deletion derivative of ColE1 DNA (PTC12, 1727 bp), with the focus of our attention on homopolymeric and alternating AT sequences.

Osmium tetraoxide bispyridine reacts across the C5–C6 double bond of pyrimidine bases [thymine ≫ cytosine] to form an osmate ester. Lukasova et al. (1984) found that the reaction kinetics were much faster for denatured DNA vs native DNA. Limited modification (~1%) of duplex DNA showed no detectable changes in CD spectra or T_m, and a differential pulse polarographic peak characteristic for single-strand DNA was absent (Lukasova et al., 1984). The crystal structure of an OsO_4 bispyridine adduct of thymine has been solved (Kistenmacher, 1976; Neidle & Stuart, 1976). Molecular modeling using the above monomeric crystal structure data demonstrated that an OsO_4 bispyridine adduct can not exist in intact B form DNA due to steric hindrance (Lukasova et al., 1984; Furlong et al., 1989). The above results suggested that OsO_4 bispyridine modification can only occur when a thymine residue is sufficiently unstacked and the C5–C6 double bond of the base is accessible to the reagent without steric clashes with other neighboring groups. On the basis of the above results, OsO_4 bispyridine modification has been used successfully as a probe for thymine bases in regions of DNA known to be unpaired: (1) cruciform loops (Lilley & Palecek, 1984); (2) B–Z junction (Palecek et al., 1987); (3) the center and 3′ junction of the pyrimidine strand in intramolecular triplexes (Wells et al., 1988; Htun & Dahlberg, 1988; Johnston, 1988); (4) the detection of single mismatch sites (Cotton et al., 1988; Cotton & Campbell, 1989). The latter study established that matched bases near mismatches are often reactive (Cotton & Campbell, 1989). This laboratory has found that a thymine base opposite a single nucleotide gap in a 115 base pair duplex represents a highly preferential target site for OsO_4 bispyridine modification (Chan et al., 1989). Reactivity was limited to the exposed thymine base with a slight amount of reactivity occurring at thymine bases located within two to three base pairs of the gap. Reactivity of thymine bases adjacent to the gap probably resulted from increased unwinding at the gap ends and could be suppressed by reduction of the temperature during OsO_4 modification. The highly preferential reactivity of only thymine bases at or near the gap site in the 115 base pair duplex demonstrates that osmium tetraoxide did not perturb the helix structure of the DNA fragment and acts as a passive probe capable of detecting fully unstacked thymine bases and thymine bases with an increased propensity toward unstacking. Furlong et al. (1989) have examined the temperature dependence of localized hyperreactivity of an AT-rich DNA sequence in supercoiled DNA by using OsO_4 bispyridine and bromoacetaldehyde (BAA) modification. They found that a sharp transition toward increased BAA modification for the target sequence occurred between 25 and 30 °C, whereas OsO_4 showed a gradual, approximately linear increase in reactivity as the temperature increased from 0 to 15 °C. The temperature dependences of the respective BAA and OsO_4 bispyridine reactions suggested that OsO_4 is capable of detecting transient

[1] Abbreviations: DEPC, diethyl pyrocarbonate; BAA, bromoacetaldehyde; bp, base pair.

unstacking events, while BAA requires infrequent larger scale helix opening (Furlong et al., 1989). In summary, all of the studies cited above support the conclusion that OsO_4/pyridine modification occurs at accessible thymines located in either stable single-strand regions or in structurally perturbed regions. Furthermore, transiently unpaired T residues are accessible to modification. No evidence has been obtained that OsO_4/pyridine acts as a denaturant, i.e., promoting unpairing by the destabilization of the secondary structure of DNA.

Diethyl pyrocarbonate carbethoxylates the N7 atom of purine bases (Leonard et al., 1971) and has been shown to react preferentially with purine bases in the loop of cruciforms (Furlong & Lilley, 1986) and throughout a Z-DNA duplex. The syn conformation of purines in Z-DNA results in greater exposure of the N7 atom to DEPC attack (Johnston & Rich, 1985). Despite the fact that DEPC has been shown to exhibit some specificity toward unpaired purine bases, its reaction with purines throughout a Z-DNA duplex demonstrates that enhanced DEPC reactivity can occur in the absence of base unpairing.

The location of OsO_4 bispyridine hyper- and hyporeactivity has been determined for approximately 70% of PTC12 DNA. The relative extent of modification at specific sites in the DNA reflect the rate of base unpairing, and differential sequence dynamics were readily discernable by examination of OsO_4 reactivity. In particular, homopolymeric $(dA)_n \cdot (dT)_n$ tracts greater than or equal to four base pairs in length were found to always be resistant toward OsO_4 bispyridine modification under both superhelical torsional stress and topological relaxed states. However, the adenines in these tracts were reactive to DEPC. Alternating $d(TA)_n$ ($n \geq 2$) sequences routinely exhibited hyperreactivity with OsO_4.

Materials and Methods

Bacterial Cultures and Plasmid Purification. *E. coli* K12, c2180 was obtained from Peter Chan, and supercoiled plasmid DNA was isolated as described earlier (Hale et al., 1983). Plasmid PTC12 was constructed in this laboratory by Peter Chan and consists of the ColE1 *Taq*I E and B fragments, which contain the origin of replication and the colicin immunity gene (Chan et al., 1985). Supercoiled PTC12 DNA was isolated by centrifugation in a CsCl/ethidium bromide density gradient, and its purity was confirmed by agarose gel electrophoresis. Topological isomers of PTC12 DNA were generated by wheat germ DNA topoisomerase I (prepared by David Wood in this laboratory) as described earlier (Singleton & Wells, 1982). The superhelical density of native PTC12 DNA was measured by direct band counting after two-dimensional gel electrophoresis (Wang et al., 1982) using PTC12 DNA topological isomers for reference.

Osmium Tetraoxide Reactions. All reactions with OsO_4 (Aldrich) were at 37 °C for the times indicated in the figure legends. Restriction enzyme linearized PTC12 DNA was dephosphorylated with calf intestinal alkaline phosphatase (CIP) and purified by phenol extraction, ether extraction, and ethanol precipitation prior to reaction with OsO_4. Reaction solutions contained either 100 or 50 μg/mL supercoiled or linearized PTC12 DNA, 20 mM Tris-HCl, pH 7.4, 1 mM EDTA, 100 or 50 mM NaCl, 2% pyridine, and 2 mM OsO_4 (final reaction conditions). Reactions were terminated by precipitation with ethanol and sodium acetate, pH 5.4. Precipitants were resuspended in 0.3 M sodium acetate, pH 5.4, reprecipitated with ethanol, washed extensively with 70% ethanol, and brought to dryness.

Diethyl Pyrocarbonate Reactions. Supercoiled PTC12 DNA (50 μg/mL final) was reacted with DEPC at 37 °C for the times indicated in the legend to Figure 4. Reactions were at either pH 4.7, or pH 7.2. The low pH DNA solution contained 50 μg/mL supercoiled PTC12 DNA, 20 mM Tris-HCl, pH 7.6, 100 mM NaCl, and 1 mM EDTA in a 1.4-mL final volume. The reaction was initiated by addition of 35 μL of DEPC (Aldrich, 97%) and vigorous mixing. The final pH of this DEPC reaction solution was 4.7. Alternatively, 35 μL of DEPC was added to a 1.4-mL solution containing 50 μg/mL supercoiled PTC12 DNA, 50 mM sodium cacodylate, pH 7.2, 50 mM NaCl, and 1 mM EDTA. The final pH of this solution was 7.2. The reactions were terminated and the samples purified as described in the OsO_4 section above.

Restriction Digestions, End-Labeling, and Fragment Purification. Following OsO_4 or DEPC reaction of supercoiled PTC12 DNA, purified DNA samples were digested with either *Eco*RV, *Hae*2, *Nru*I, or *Bst*YI to completion (see Figure 1 for mapping scheme). All of these enzymes cut only once in PTC12, creating a linear molecule of 1727 bp. Restriction buffers employed for each enzyme digestion were as suggested by the manufacturer. DNA samples cleaved with either *Nru*I, *Eco*RV, or *Hae*2 were then dephosphorylated with calf intestinal alkaline phosphatase and purified by phenol extraction, ether extraction, and ethanol precipitation. The linearized and dephosphorylated fragments were then 5′-end-labeled by using [γ-^{32}P]ATP (ICN, crude, >7000 Ci/mmol, 160 mCi/mL) and T4 polynucleotide kinase (USB). Unincorporated label was removed by passage through two consecutive Sephadex G-50 spin columns that were equilibrated with ddH_2O. *Bst*YI-digested DNA samples (10 μg) were 3′-end-labeled in *Bst*YI restriction buffer containing [α-^{32}P]dATP (Amersham, 10 mCi/mL, 6000 Ci/mmol; 1.67 μM final), dGTP (39.2 μM final), and 2.5 units of DNA polymerase I (Klenow fragment; USB) for 30 min at room temperature. The 3′-end-labeling was terminated by phenol extraction and passage through two consecutive Sephadex G-50 spin columns as described earlier. End-labeled DNA samples were then further digested with a second restriction enzyme that cuts singly in PTC12 (see Figure 1A), creating two fragments of differing lengths with either the top or bottom strand uniquely labeled. Fragments were separated by electrophoresis on a 1% agarose gel (IBI) in a Tris–acetate–EDTA buffer. The fragment bands were located by staining with ethidium bromide and were excised. The gel slices were placed in a IBI UEA electroeluter, and the DNA electroeluted into a 3 M sodium acetate salt bridge as described by the manufacturer. Following electroelution, the salt bridge was removed from each well and the fragments were precipitated with ethanol.

Formic Acid Reactions, Piperidine Cleavage, and Electrophoresis. Unmodified PTC12 DNA was also restricted, 5′- or 3′-end-labeled, and purified as described above. Formic acid (A + G) reactions and piperidine cleavage were performed as described previously (Chan et al., 1989). After piperidine cleavage, samples were brought to dryness in a speed vac. The pellet was then resuspended and dried 8–9 times, each resuspension being in 50 μL of 20% ethanol. The final DNA fragment pellet was resuspended in 20 μL of a 95% deionized formamide, 0.01% (w/v) xylene cyanol, 0.01% bromophenol blue solution, heated at 94 °C for 4 min, and then placed on ice. Approximately 4 μL of each sample was loaded into each well of a 6% polyacrylamide (30:1 acrylamide/bisacrylamide), 7 M urea sequencing gel (0.4 mm × 40 cm × 80 cm) (Bio-Rad Sequi-Gen nucleic acid sequencing cell) and resolving at 3100 V (about 32 mA). Following electrophoresis, the gel was transferred to Whatmann 3MM chromatography paper,

covered with saran wrap, and exposed against an X-ray film.

Results

Plasmid PTC12 and Mapping Scheme. Plasmid PTC12 (1727 bp) is a deletion derivative of the parent plasmid ColE1 (6646 bp). PTC12 was formed by ligation of ColE1 *Taq*I B and E fragments and therefore contains nucleotide sequences 1–1322 and 6242–6646 of ColE1 DNA (Chan et al., 1985). The first nucleotide position in PTC12 is at the single *Eco*RI site following the designation for the ColE1 DNA sequence (Chan et al., 1985). The average negative superhelical density of PTC12 DNA was found to be −0.054, as described under Materials and Methods. The restriction digestion protocols employed in fine-mapping adduct sites is illustrated in Figure 1A. In each of the four protocols shown, the DNA is digested with a single restriction enzyme that cuts once in PTC12 and is then either 5′- or 3′-end-labeled. The location of restriction sites that were end-labeled in each protocol is designated with solid boxes. Digestion with a second restriction enzyme in each case generates two fragments of differing lengths with only one strand of the fragment labeled. Only the *Bst*YI–*Eco*RV protocol involved 3′-end-labeling; all others were 5′-end-labeled (Figure 1A).

Fine Mapping of OsO_4 Bispyridine Adducts in Supercoiled and Linearized PTC12 DNA. If chemical modification can occur only in the unpaired state, base pairs must transiently open in order for reaction to occur. Under these circumstances the observed rate of reaction of a chemical probe is equal to the probability or fraction of open conformations times the rate of reaction of the unpaired state with the probe (von Hippel & Wong, 1971; Utiyama & Doty, 1971). The fractional unpairing for a specific site in a population of identical DNA molecules is essentially the equilibrium constant for unpairing at that site. Helix destabilization caused by DNA superhelical torsional stress will increase the magnitude of the fractional unpairing or the conformational equilibrium constant for different sites in supercoiled DNA and the rate of chemical modification will be enhanced relative to linear DNA. Consequently, in order to obtain comparable band intensities for supercoiled and linearized DNA, the latter must be reacted for longer time periods as described below. The extent of OsO_4/pyridine reactivity for the indicated regions of PTC12 DNA under supercoiled (reacted for 3 h at 37 °C) and linearized (reacted for 17.5 h at 37 °C) conditions is shown in Figure 2, lanes b and c and lanes e and f, for the 5′[^{32}P]-*Hae*2–*Eco*RV B and 5′[^{32}P]*Nru*I–*Hae*2 A restriction fragments, respectively. Appropriate unmodified restriction fragments were treated with formic acid and run adjacent to OsO_4 modified samples to provide a purine sequence reference (lanes a and d). At positions 1157 and 369 in PTC12 DNA there are homopolymeric $(dA)_n$·$(dT)_n$ tracts with $n = 5$ and $n = 4$, respectively (lanes a–c and d–f). For both the supercoiled (lanes b and e) and linearized (lanes c and f) DNA samples, very little OsO_4 reactivity occurred in these homopolymeric AT tracts compared to thymine bases (see positions 1155 and 381) in alternating or mixed sequence tracts. The precise nucleotide sequences shown in the fine-mapping experiments are given in Figure 1B (sequences 1–3). A resistance to unpairing or long base pair life times is a characteristic of every homopolymeric $(dA)_n$·$(dT)_n$ tract with $n \geq 4$ (see below).

Also shown in Figure 2 (lanes b and c) is the relative sensitivity of homopolymeric $(dA)_n$·$(dT)_n$ tracts (where $n = 3$) toward OsO_4 modification. Three consecutive thymine bases at positions 1130–1132 and 1113–1115 show considerable differences in OsO_4 bispyridine reactivity in both the modified supercoiled (lane b) and linearized (lane c) samples. The three

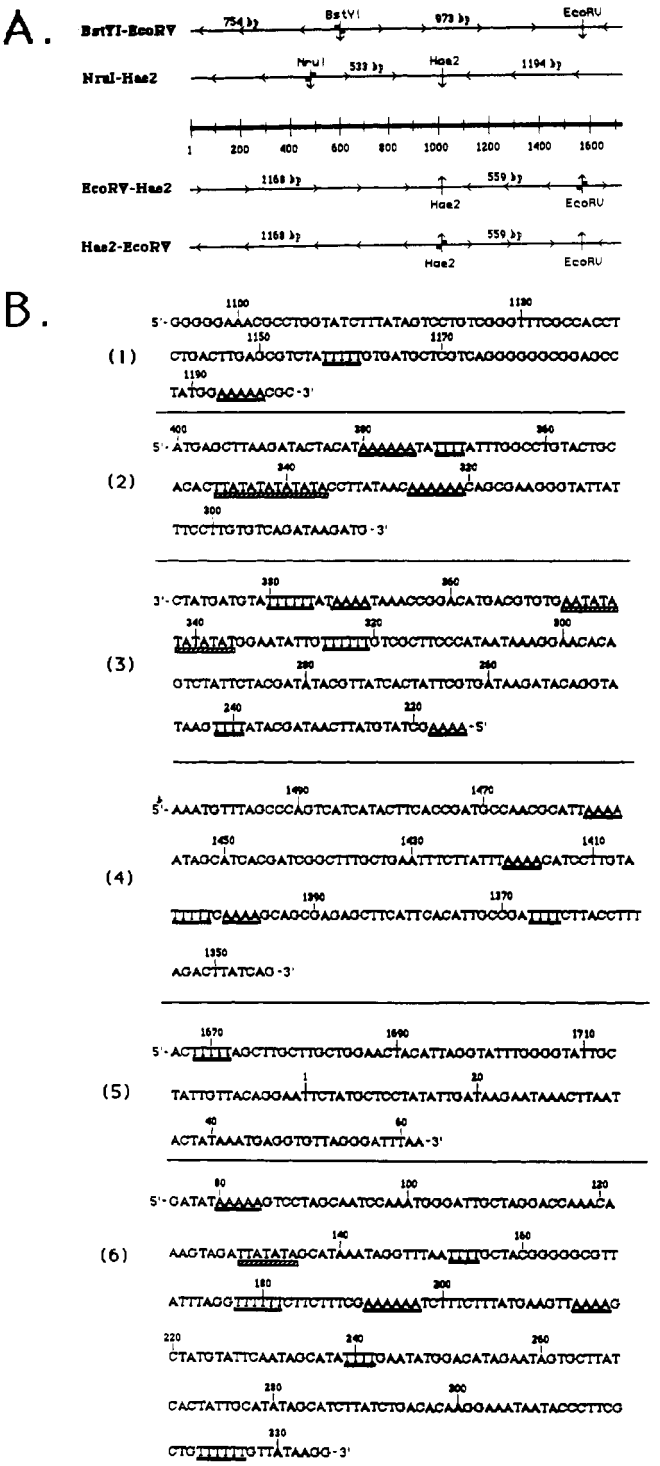

FIGURE 1: (A) Linear map of PTC12 (1727 bp) and restriction digestion protocols for mapping adduct sites. The location of the single recognition sites of *Bst*YI, *Eco*RV, *Nru*I, and *Hae*2 restriction endonulceases are indicated. Each thin line represents a double digestion protocol used in mapping adducts sites. PTC12 DNA was converted to a linear molecule by the first cleavage and either 5′- or 3′-end-labeled. Respective linear molecules labeled at each end were cleaved into two fragments (larger fragment designated A and smaller fragment designated B in text) to generate a single labeled fragment for mapping of chemical adduct sites. For Figures 2–4, restriction fragments will be designated in the order of cleavage, e.g., *Hae*2–*Eco*RV B (bottom thin line). Solid boxes indicate either the 5′-[^{32}P]-end-labeled positions for the *Nru*I, *Eco*RV, and *Hae*2 digests or the 3′[^{32}P]-end-labeled position for the *Bst*YI restriction digest (see Materials and Methods). The direction of the sequence ladder is shown by arrows. (B) PTC12 sequence regions of OsO_4 or DEPC reactivity mapping that are presented in this paper. The locations of homopolymeric (solid line) or alternating (hatched line) AT regions and 5′ and 3′ ends are indicated.

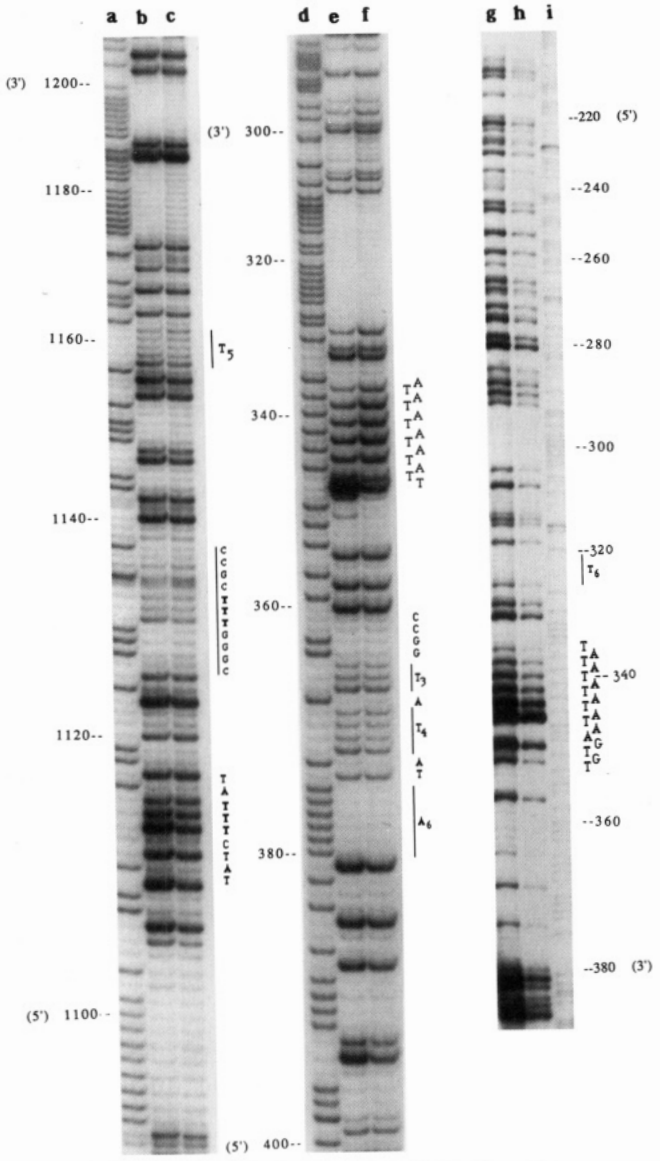

FIGURE 2: Nucleotide resolution mapping of OsO_4 bispyridine thymine adducts for PTC12 DNA modified in the supercoiled and linear states. The supercoiled and linear forms of PTC12 DNA (100 μg/mL) were reacted with OsO_4/pyridine for 3 and 17.5 h, respectively, at 37 °C in a pH 7.4 buffer containing 100 mM NaCl (see text). End-labeled fragments of modified DNA were obtained by the restriction digestion protocols presented in Figure 1. These fragments were then treated with piperidine plus heat to induce cleavage at modified thymines followed by the separation of cleavage products on a sequencing gel. Reactivity patterns for OsO_4/pyridine modification under supercoiling and linear relaxed conditions are shown in adjacent lanes b and c and lanes e and f for the 5'[^{32}P]-*Hae*2–*Eco*RV B and 5'[^{32}P]-*Nru*I–*Hae*2 A restriction fragments, respectively. Appropriate unmodified restriction fragments were treated with formic acid and run adjacent to OsO_4-modified samples to provide a purine sequence references (lanes a and d). Supercoiled PTC12 DNA (50 μg/mL) was also reacted with OsO_4/pyridine in a buffer (pH 7.4) containing either 50 mM NaCl (lane g) or 100 mM NaCl (lane h) for 1 h at 37 °C, and reactivity differences were compared for the 3'[^{32}P]-*Bst*YI–*Eco*RV B fragment. Lane i is the formic acid generated purine sequence lane as described above.

consecutive thymine bases at positions 1130–1132 showed very little OsO_4 reactivity, whereas considerable modification occurred at the thymine bases located at positions 1113–1115. Differences in OsO_4 reactivity for these two $(dA)_3$·$(dT)_3$ tracts appear to result from the sequences immediately 5' and 3' to the tracts. In particular, the four G-C base pairs on both sides of the $(dA)_3$·$(dT)_3$ tract (at positions 1130–1132) provide very stable flanking sequences (Gotoh & Takashira, 1981a,b), thereby reducing thymine reactivity in this tract. The extent of OsO_4 reactivity is also reduced in another $(dA)_n$·$(dT)_n$ tract ($n = 3$) at positions 367–365 (Figure 2, lanes e and f), in which there are very stable flanking sequences: four G-C base pairs 3' and a $(dA)_4$·$(dT)_4$ tract 5' to the $(dA)_3$·$(dT)_3$ tract. The 20 base pair region extending from 380–360 shows a very low extent of OsO_4 modification although the AT content is 80% in this short segment of the molecule.

Thymine bases in the alternating $d(TA)_6$ tract (347–336) show a hyperreactivity with OsO_4/pyridine in both the supercoiled (lane e) and the *Nru*I-linearized (lane f) molecule. The OsO_4 reactivity is uniformly distributed throughout the alternating TA tract in the linearized molecule, whereas in the supercoiled molecule the most intense band corresponds with the thymine base at 348 (see Figure 1B (sequence 2) and Figure 2, lane e). The hyperreactivity of the alternating TA tract in supercoiled PTC12 DNA has also resulted in some OsO_4 modification of cytosine bases at positions 349 and 351 (Figure 2, lane e). In order to further examine this alternating TA tract, supercoiled PTC12 DNA was reacted with OsO_4/pyridine for 1 h at 37 °C in a buffer containing either 50 mM NaCl (Figure 2, lane g) or 100 mM NaCl (Figure 2, lane h); the DNA was purified, digested with *Bst*YI, and 3'-end-labeled (see Materials and Methods and Figure 1A). This labeling protocol allows us to examine the extent of OsO_4 reactivity on the opposite strand of that shown in lanes e and f. Again, the alternating TA tract was hyperreactive with OsO_4. Hyperreactivity in this region of the molecule extends from about 352 to 338. The alternating $d(TA)_6$ tract shows a gradient of band intensity. This gradient would be expected if there were multiple reacted sites (see Discussion). Although bands in the 50 mM NaCl reaction (lane g) appear more intense than those in the 100 mM NaCl reaction (lane h), the patterns of reactivity appear identical. The intensity difference of bands could largely be explained as a result of more radioactive label (2.87 times more) loaded into lane g as opposed to lane h. Even though the alternating $d(TA)_6$ tract (348–336) is hyperreactive with OsO_4, reactivity of thymine bases in the homopolymeric $(dA)_n$·$(dT)_n$ ($n = 5$ and 6) tracts located nearby at 326–321 and 380–375 show little OsO_4 reactivity [Figure 2, lanes g and h; Figure 1B (sequence 3)]. The alternating $d(TA)_2$ tract located at position 280 also shows some hyperreactivity with OsO_4 bispyridine (Figure 2, lanes g and h).

The patterns of OsO_4 bispyridine reactivity in another region of PTC12 are shown in Figure 3. Lanes a, c, and f provide the appropriate formic acid, A + G sequencing reference for the OsO_4-modified samples. Supercoiled (lanes b, d, and g) or EcoRV-linearized (lane e) PTC12 DNA was reacted with OsO_4/pyridine as described in Figure 3. Osmium tetraoxide reactivity of thymine bases in four homopolymeric $(dA)_n$·$(dT)_n$ tracts ($n = 4$, 5, and 6) is shown. The nucleotide sequence and location of alternating and homopolymeric AT tracts corresponding to the results presented in Figure 3 are given in Figure 1B (sequences 4–6). Homopolymeric $(dA)_n$·$(dT)_n$ tracts ($n \geq 4$) found at positions 1405 ($n = 5$), 1367 ($n = 4$), 152 ($n = 4$), and 177 ($n = 6$) all showed a reduced amount of OsO_4 reactivity (Figure 3, lanes d, e, and g). The short alternating $d(TA)_3$ region located at 130–135 showed some hyperreactivity with OsO_4 (lane g).

Figures 2 and 3 showed that the patterns of OsO_4 bispyridine reactivity in PTC12 in the supercoiled and linearized conformations are quite similar. The amount of total OsO_4 reactivity in a restriction fragment can be estimated by the amount of full length strands remaining after piperidine/heat

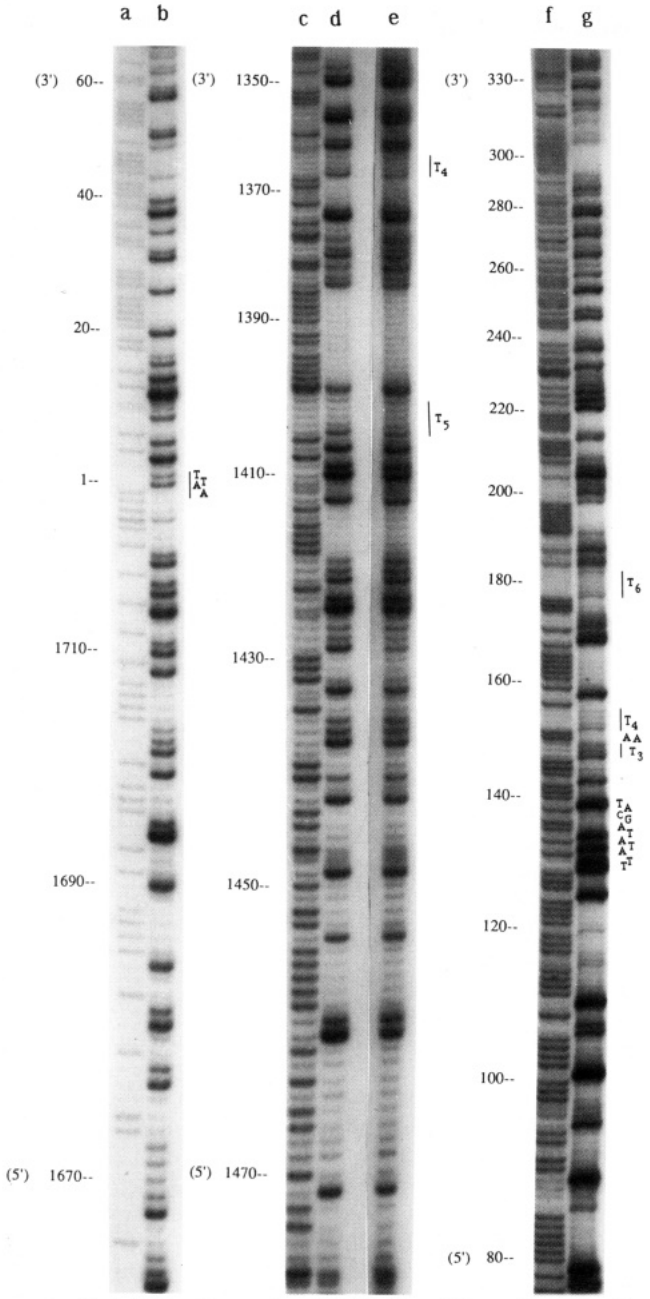

FIGURE 3: Additional mapping of OsO_4 bispyridine adduct sites for modified supercoiled or linearized PTC12 DNA. Supercoiled and *EcoRV*-linearized PTC12 forms (100 μg/mL) were reacted with OsO_4/pyridine for 6 and 17 h, respectively, at 37 °C in a reaction buffer (pH 7.4) containing 100 mM NaCl (see Materials and Methods). Lanes b and g show the location and relative intensities of OsO_4/pyridine adduct sites for the 5′[^{32}P]-*Eco*RV–*Hae*2 A fragment isolated from modified supercoiled DNA. Lanes a and f represent the respective purine reference sequences generated by formic acid treatment. Lanes c, d, and e show respectively the purine sequence lane and the location of OsO_4/pyridine adducts for modification under supercoiling conditions and under linear relaxed conditions for the 5′[^{32}P]-*Eco*RV–*Hae*2 B fragment.

induced cleavage of adduct sites. As indicated in the legends to Figures 2 and 3, linearized PTC12 DNA samples were respectively reacted with OsO_4 for nearly 6 and 3 times longer than the supercoiled DNA samples. We observed slightly more full length strands in the linearized vs the supercoiled sample (data not shown), indicating that the reactivity levels were close but not precisely identical. As we have observed with other single-strand specific chemicals, the torsional stress of negative supercoiling results in much greater rates of base unpairing and chemical modification than would occur in the nontorsionally strained linearized molecule under identical buffer conditions. As indicated, the average negative superhelical density of PTC12 DNA was −0.054. This superhelical torsional stress did not result in substantial destabilization of the homopolymeric $(dA)_n$·$(dT)_n$ ($n \geq 4$) tracts, as indicated by the extent of OsO_4 bispyridine reactivity.

Diethyl Pyrocarbonate Fine Mapping. We have also investigated alternating and homopolymeric AT regions in PTC12 DNA by DEPC probing (Figure 4). Unlike OsO_4 experiments, homopolymeric $(dA)_n$·$(dT)_n$ tracts ($n \geq 4$) do not appear to be resistant toward DEPC modification at either pH 4.7 (lanes b–e) or pH 7.2 (lanes g–i). The DEPC reactivity of adenine bases in $(dA)_5$·$(dT)_5$ and $(dA)_6$·$(dT)_6$ tracts at positions 1459 (lanes b–e) and 380 (lanes g–i) shows a gradient of DEPC reactivity with maximal reactivity being one nucleotide before the 3′ end of the tract. The adenines in the alternating $d(TA)_6$ tract (346–336) do not show greater reactivity than the adenines in the homopolymeric $(dA)_n$·$(dT)_n$ tracts ($n \geq 4$) located nearby. We believe that DEPC has a greater rate of reaction with the structurally altered homopolymeric AT DNA relative to the reactivity of transient unpaired adenine residues.

Distribution of Homopolymeric $(dA)_n$·$(dT)_n$ Tracts ($n \geq 4$) in E. coli Promoters. The number of occurrences and location of homopolymeric AT tracts were examined for 172 *E. coli* promoter sequences listed by Pivec et al. (1985) and Hawley and McClure (1983). Promoter sequences (−47 to +11) were analyzed at each nucleotide position for an adenine or thymine base involved in a homopolymeric $(dA)_n$·$(dT)_n$ tract ($n \geq 4$). The total number of occurrences at each position is shown in Figure 5. Of the 172 promoters examined, 107 (62.2%) contain at least one homopolymeric AT tract. Of the promoters containing a tract, they have as a mean 1.41 homopolymeric AT tracts per promoter ($\sigma = 0.626$) and 4.59 base pairs per tract ($\sigma = 0.84$). Two noteworthy regions that are relatively devoid of homopolymeric AT tracts (see Figure 5) extend from the −39 to −34 and the −9 to −1 regions of promoters.

Discussion

We have shown that OsO_4 bispyridine modification of DNA provides a sensitive probe for analysis of discrete conformational changes in a DNA molecule. Homopolymeric $(dA)_n$·$(dT)_n$ tracts ($n \geq 4$) were found to contain a unique structure characterized by a reduced rate of base unpairing, whereas alternating $d(TA)_n$ ($n \geq 1$) sequences were found to unpair readily. Further destabilization of alternating AT tracts occurred under superhelical torsional stress whereas homopolymeric $(dA)_n$·$(dT)_n$ tracts ($n \geq 4$) retained duplex stability.

The reduced level of OsO_4 reactivity for homopolymeric $(dA)_n$·$(dT)_n$ tracts ($n \geq 4$) in PTC12 DNA is in complete accord with the special structural features of this sequence motif outlined in the introduction. Once a homopolymeric AT tract reaches four base pairs in length it adopts this unique structure that is also characterized by long base pair lifetimes by imino proton exchange studies on synthetic oligonucleotide duplexes (Leroy et al., 1988). In contrast, the homopolymeric AT tracts of three base pairs or less in length do not possess the uniquely stable DNA conformation discussed above since the extent of base unpairing in these tracts is directly dependent on the rate of unpairing of adjacent sequences (Figure 2). The tendency of adjacent sequences to influence the rate of unpairing of an internal sequence is consistent with the earlier reported process of telestability (Burd et al., 1975a,b). Homopolymeric $(dA)_n$·$(dT)_n$ tracts ($n \geq 4$) show repressed rates of base unpairing regardless of the sequence of adjacent

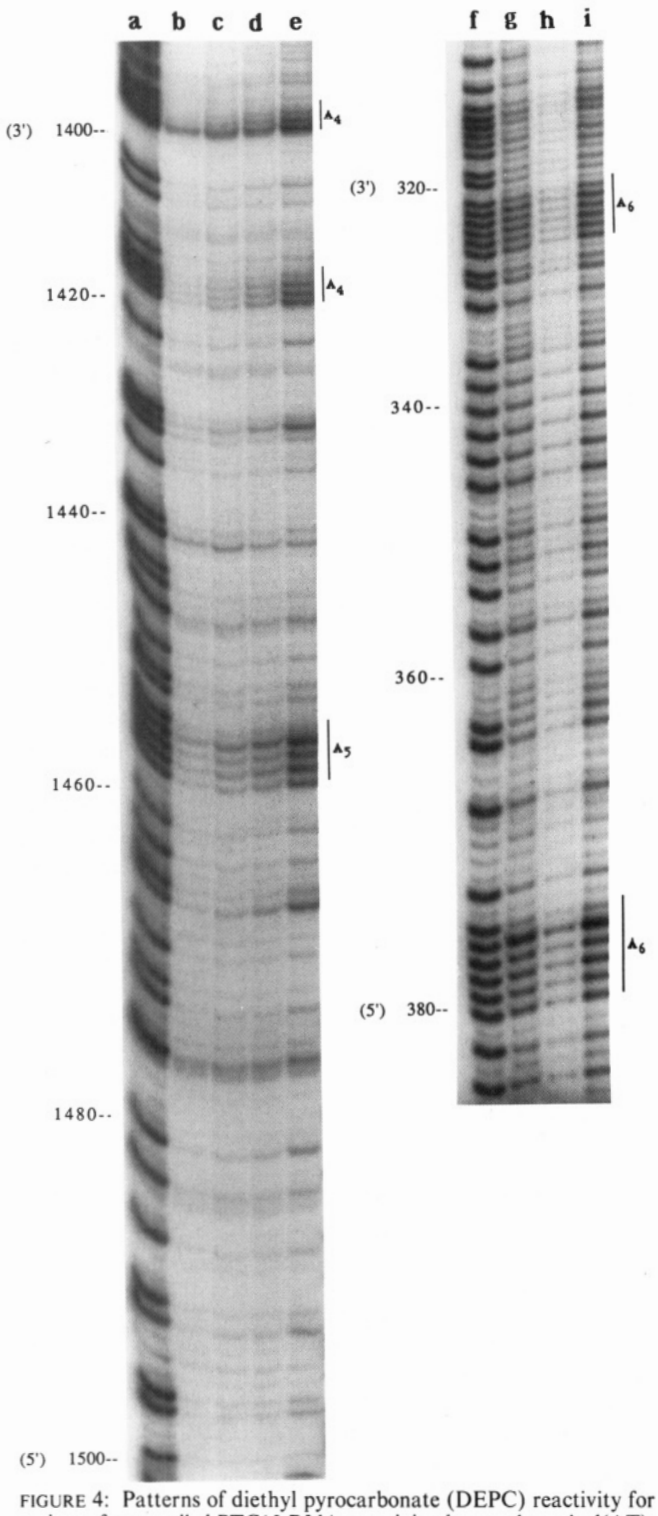

FIGURE 4: Patterns of diethyl pyrocarbonate (DEPC) reactivity for regions of supercoiled PTC12 DNA containing homopolymeric $d(AT)_n$ tracts. Left panel: the position and amount of DEPC reactivity for the 5′[^{32}P]-*Eco*RV–*Hae*2 B fragment isolated from supercoiled PTC12 DNA modified with DEPC at pH 4.7; lanes b–e represent 37 °C reactions for 5 min, 10 min, 20 min, and 1 h, respectively. Lane a is the purine sequence reference lane generated by formic acid. Right panel: the position and amount of DEPC reactivity for the 5′-[^{32}P]-*Nru*I–*Hae*2 A fragment isolated from supercoiled PTC12 DNA modified with DEPC at pH 7.2; lanes g–i represent 37 °C reactions for 30 min, 1 h, and 1.5 h, respectively. Lane f is the purine sequence reference lane generated by formic acid.

base pairs. For example, under the ionic conditions explored in this study, the stability of a homopolymeric $(dA)_5$·$(dT)_5$ tract is maintained even when a highly unstable alternating $d(TA)_6$ tract is located nearby (Figure 2).

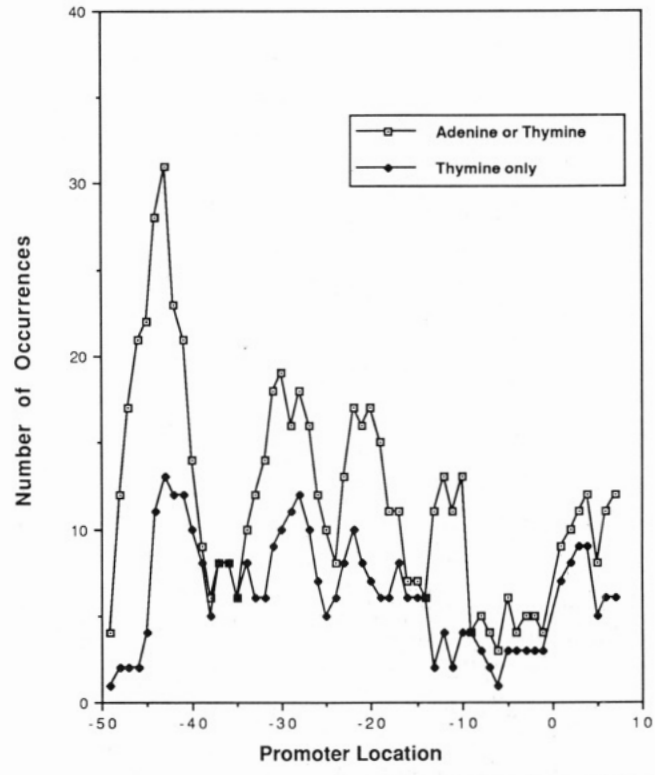

FIGURE 5: Distribution of homopolymeric $(dA)_n$·$(dT)_n$ tracts (where $n \geq 4$) in 172 *E. coli* promoter sequences. Promoter nucleotide sequences were from Pivec et al. (1985) and included positions −49 to +11. At each promoter nucleotide position, the number of occurrences of adenine or thymine bases involved in a homopolymeric $(dA)_n$·$(dT)_n$ tract ($n \geq 4$) is shown. Position 0 in the promoter does not truly exist in the nucleotide sequence (only −1 and +1); however, this location was mathematically necessary for programs used in plotting.

To further examine the homopolymeric $(dA)_n$·$(dT)_n$ tracts ($n \geq 4$), we also investigated the pattern of diethyl pyrocarbonate reactivity for these regions. Contrary to results observed with OsO_4, homopolymeric $(dA)_n$·$(dT)_n$ tracts ($n \geq 4$) were not resistant toward DEPC modification and in some cases showed a slight hyperreactivity. In some tracts a gradient of increasing DEPC reactivity of adenine bases was observed in the 5′ to 3′ direction with maximal reactivity being one nucleotide before the 3′ end of the tract (Figure 4). Despite the fact that DEPC has been shown to exhibit some specificity toward unpaired purine bases, its reaction with purines throughout a Z-DNA duplex demonstrates that enhanced DEPC reactivity can occur in the absence of base unpairing. Very recently Bernues et al. (1990) studied the capacity of homopolymeric tracts cloned into supercoiled DNA to form triplex structures in the presence of zinc ions. For the case of a $d(T/A)_{60}$ insert, triplex formation did not occur; however, the polyadenine tract was hyperreactive to DEPC. This result is in accord with our observations. It appears that the conformational properties of homopolymeric AT tracts also provide greater exposure of the N7 atom of adenine toward DEPC attack.

Alternating AT sequences have the normal B-DNA geometry and are not resistant toward nucleosome reconstitution and DNase I cleavage. Long alternating $d(AT)_n$ tracts, where n is 16 or more, have been shown (Greaves et al., 1985; Lilley & McClellan, 1987) to be structurally bimorphic. In linear DNA fragments, these tracts unpair at a very high rate as detected by single-strand endonucleases and chemical modification (Lilley & McClellan, 1987). In supercoiled DNA, these AT tracts have been shown (Lilley & McClellan, 1987)

to be in a two-state conformational equilibrium between the rapidly unpairing conformation found in linear DNA and a cruciform. The alternating $d(TA)_6$ tract centered at about 342 in PTC12 was hypereactive with OsO_4 (Figure 2, lanes e, g, and h). The most intense bands in the tract were located at 348 (lane e) and 346 (lanes g and h). Samples modified in the superhelical conformation showed a nonlinear gradient of decreasing band intensity, whereas adducts were evenly distributed throughout the same TA tract for DNA molecules modified in the linearized conformation. If there are multiple adducts in a tract, we can only detect the adduct site closest to the labeled end of the fragment upon piperidine cleavage; the other cleavage events will generate unlabeled fragments. Combinatorial mathematics can be used to estimate band intensity ratios resulting from multiple adducts in the $d(TA)_6$ tract. Each T position of the tract is assigned a letter for purposes of identification as follows: ^{32}P-5′-$T_aT_bAT_cAT_dA$-$T_eAT_fAT_gA$-3′ (Figure 2, lane e), and we calculate the number of combinations at each of the a–g sites in the tract for 2, 3, and 4 adducts, respectively. From the combinatorial analysis, molecules with 2 adducts per tract will have a T_a/T_c band intensity ratio of 1.5. For 3 and 4 adducts per tract, the T_a/T_c band intensity ratio will increase to 2.5 and 5, respectively. Visual examination of band intensities indicates that the intensity of the T_a band appears to be approximately 3-fold greater than the T_c band (Figure 2, lane e). A comparable estimate can be made for the T_a/T_c (3′ to 5′) intensity ratio for the complementary strand of the alternating tract (Figure 2, lanes g and h). Consequently, we estimate that the $d(TA)_6$ tract has approximately three adducts. These combinatorial distributions will be true only if reactivity is random and all thymine bases in a tract are equally accessible for reaction. A comparison of the experimental results to combinatorial distributions represents an oversimplification, since the results must reflect a weighted distribution of molecules with different numbers of adducts in the alternating AT tract. The gradient of band intensity for this situation would be dependent on the fraction of molecules with 1, 2, ..., n adducts in the tract. Consequently, we believe that a weighted distribution of supercoiled molecules with different numbers of adducts per tract would more adequately account for the gradient of band intensity seen in the alternating AT tract of Figure 2. While the latter cannot be obtained from the data, we can conclude from the considerations raised above that under superhelical torsional stress alternating AT tracts undergo sufficient destabilization to cooperatively unwind and that multiple modification events can occur in the tract.

In contrast to the results for superhelical DNA, we observed an even distribution of OsO_4 reactivity for the $d(TA)_6$ tract in the linearized sample, suggesting that the tract contains only one adduct and single unpairing events occur to expose thymines for OsO_4 modification. Each base pair in the tract has an equal life time. As indicated earlier, the TpA doublet has a relatively fast proton exchange rate (Patel et al., 1985a,b). For small complementary oligonucleotides, imino proton exchange occurs by a single base pair opening event (Gueron et al., 1987). Our results for linearized DNA are consistent with the NMR observations cited above; single unpairing events exposing thymines for modification appear to be responsible for the cleavage patterns. Short alternating $d(TA)_n$ (n = 2 and 3) regions in PTC12, containing TpA steps, showed some preferential OsO_4 reactivity of thymine bases. The TpA dinucleotide step has been shown by Drew et al. (1985) to be especially sensitive toward supercoiling induced base unstacking.

Cruciform formation in superhelical DNA will reduce the superhelical density, thereby lowering the free energy of the molecule. The mechanistic pathways for forming cruciforms and other stable transconformations are of considerable interest. Lilley's laboratory has investigated the influence of flanking sequences on the kinetics of cruciform extrusion in supercoiled ColE1 DNA derivatives (Sullivan & Lilley, 1986; Sullivan et al., 1988; Lilley, 1988). At 0 mM NaCl (buffered by 10 mM Tris-HCl at pH 7.5) AT-rich sequences of ColE1 flanking an inverted repeat sequence are responsible for inducing cruciform extrusion (13-bp stem and 4-base loop cruciform) following a mechanistic pathway that proceeds via a relatively large denatured region. This mechanistic pathway for cruciform kinetics was called C-type kinetics, and the inducing flanking sequences were designated C-type inducing sequences. Deletion analysis of the flanking sequences led to the discovery that a short 30-bp sequence, GGGATTTAATTATTCTTTATTGATATAAAAA, was sufficient to confer C-type kinetics (Sullivan et al., 1988). It can be noted that this core inducing sequence contains five TpA steps, two TTT tracts, three TT doublets, and one homopolymeric A_5 tract which is directly next to the extruded cruciform. The 30-bp sequence is dominated by sequence motifs that are very susceptible to unpairing. Under very low ionic strength conditions, the homopolymeric A_5 tract must be disrupted to form a denaturation bubble that would include the cruciform sequence. C-Type cruciform extrusion kinetics are suppressed by increased ionic strength, which stabilizes the core inducing sequence, preventing the formation of a denaturation bubble (Sullivan & Lilley, 1986; Sullivan et al., 1988; Lilley, 1988).

In order to consider the possible biological consequences of DNA structural motifs, we have examined 172 known *E. coli* promoter sequences (Hawley & McClure, 1983; Pivec et al., 1985) and have determined the distribution and number of occurrences of adenine or thymine bases involved in a homopolymeric $(dA)_n$·$(dT)_n$ tract ($n \geq 4$) (see Figure 5). These tracts were found on average to be located at positions −43, −29.5, −20.5, and −11. The region with the greatest incidence of homopolymeric AT tracts was at position −43, whereas the −39 to −34 and −9 to −1 sequences were particularly devoid of these tracts. The −35 and −10 regions of promoters contain hexamer sequences involved in *E. coli* RNA polymerase–promoter interactions, and one might expect that these regions would not contain or have a reduced frequency of homopolymeric $(dA)_n$·$(dT)_n$ ($n \geq 4$) tracts. The distribution of the trinucleotide sequences ApApT/ApTpT and ApApA/TpTpT in *E. coli* promoter sequences were determined by Travers (1988) and also found to occur on average at −9, −20, −30, and −42. Travers (1988, 1990) has suggested that if promoter DNA is wrapped around RNA polymerase the periodic modulation of these trinucleotide sequences in 10-bp intervals may facilitate this process.

As indicated earlier, short, phased runs of $(dA)_n$·$(dT)_n$ tracts ($n \geq 4$) result in DNA bending (Wu & Crothers, 1984; Koo et al., 1986). Helix deflection at the junctions between a homopolymeric $(dA)_n$·$(dT)_n$ tract and normal B form DNA appears to be the most likely structural basis for stable DNA bending (Wu & Crothers, 1984; Koo & Crothers, 1986). Evidence has accumulated that phased $(dA)_n$·$(dT)_n$ tracts ($n \geq 4$) located upstream of the −35 region of promoters can activate some promoters or synthetic promoter constructs (McAllister & Achberger, 1989; Bracco et al., 1989; Collis et al., 1989). McAllister and Achberger (1989) propose that the curved DNA deflects the helix backbone toward the

promoter bound RNA polymerase, allowing for upstream DNA interactions with the enzyme which promote open complex formation. A similar explanation appears reasonable for the observations that upstream DNA sequences containing properly positioned 10-bp phased homopolymeric $(dA)_n$·$(dT)_n$ tracts can replace CRP transcriptional activation of a *gal* promoter construct in which the −35 region was deleted (Bracco et al., 1989). Theoretical conformational analysis of bending with different ordinary B form terminal structures and homopolymeric tracts has shown that different types of bending can result from the alterations of junction base pairs in the B structure (Chuprina & Abagyan, 1988). If different types of bending can result from junction alterations then there could be variations in helix deflection affecting the positioning of RNA polymerase. Homopolymeric AT tracts located at other positions of relative high incidence in promoters may play a role in the orientation of RNA polymerase on the DNA molecule that could affect binding and open complex formation. Homopolymeric AT tracts, as mentioned previously, are also very hydrated, and their location in promoters could influence binding stability by the favorable entropy change resulting from water displacement from the DNA upon protein binding (Record, 1988). It appears that the effects of homopolymeric AT tracts on promoter function could be complex and clearly remain to be determined.

Although techniques such as NMR and X-ray crystallography have provided a large body of information on the structure of DNA, these techniques can be limited with respect to the size of the DNA molecule that can be studied, and therefore short synthetic oligonucleoties have to be employed. The use of chemical probes does not have such size constraints and has previously shown utility in the resolution of the secondary structure of both rRNA and tRNA (Mougel et al., 1987; Moazed et al., 1986; Theobald et al., 1988). The use of various chemical probes that require differing base and DNA structural properties for reaction should be of great utility in the examination of discrete conformational changes in a DNA molecule. Chemical probes have proven of great utility for the analysis of protein–DNA interactions (Tullius, 1989). Considerable progress has been made in the detection of unpaired bases in RNA polymerase–promoter open complexes using dimethyl sulfate methylation of unpaired cytosines (Kirkegaard et al., 1983), potassium permanganate oxidation of unpaired thymines (Sasse-Dwight & Gralla, 1989), and DEPC modification of adenines (Buckle & Buc, 1989). Chloroacetaldehyde modification of unpaired adenines and cytosines can also be used to fine map single-strand regions in promoter open complexes (Chan et al., manuscript in preparation). By utilizing a variety of chemical probes that detect major and minor groove interactions and unpaired sites, we envision that considerable structural information can be obtained for a variety of protein–DNA interacting systems.

Acknowledgments

We thank Dr. Peter T. Chan for supplying PTC12.

Registry No. Poly[(dA)·(dI)], 25464-54-4; poly[d(TA)], 31834-53-4; adenine, 73-24-5; thymine, 65-71-4.

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