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Mutagenesis by Site-Specific Arylamine Adducts in Plasmid DNA: Enhancing Replication of the Adducted Strand Alters Mutation Frequency†

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ABSTRACT: Site specifically modified plasmids were used to determine the mutagenic effects of single arylamine adducts in bacterial cells. A synthetic heptadecamer bearing a single *N*-(guanin-8-yl)-2-aminofluorene (AF) or *N*-(guanin-8-yl)-2-(acetylamino)fluorene (AAF) adduct was used to introduce the adducts into a specific site in plasmid DNA that contained a 17-base single-stranded region complementary to the modified oligonucleotide. Following transformation of bacterial cells with the adduct-bearing DNA, putative mutants were detected by colony hybridization techniques that allowed unbiased detection of all mutations at or near the site of the adduct. The site-specific AF or AAF adducts were also placed into plasmid DNA that contained uracil residues on the strand opposite that bearing the lesions. The presence of uracil in one strand of the DNA decreases the ability of the bacterial replication system to use the uracil-containing strand, thereby favoring the use of the strand bearing the adducts. In a comparison of the results obtained with site specifically modified DNA, either with or without uracil, the presence of the uracil increased the mutation frequencies of the AF adduct by >7-fold to 2.9% and of the AAF adduct by >12-fold to 0.75%. The mutation frequency of the AF adduct was greatly reduced in a *uvrA*⁻ strain while no mutations occurred with the AAF adduct in this strain. The sequence changes resulting from these treatments were dependent on adduct structure and the presence or absence of uracil on the strand opposite the adducts. The AF adduct produced primarily single-base deletions in the absence of uracil but only base substitutions in the uracil-containing constructs. The AAF adduct produced mutations only in the uracil-containing DNA, which included both frame shifts and base substitutions. Mutations produced by both adducts were SOS dependent.

The formation of carcinogen–DNA adducts has been implicated in mutagenesis and carcinogenesis (Singer & Grunberger, 1983). Therefore, an understanding of the molecular mechanisms involved in the formation of mutations is important in determining how chemical carcinogens are involved in tumor formation. Most chemical mutagens produce reactive intermediates that damage DNA at multiple sites and may form more than one type of lesion (Singer & Grunberger, 1983). In addition, the local sequence of the DNA affects the degree to which a given base will react with a chemical as well as the mutational specificity at that site (Miller, 1983; Fuchs, 1984; Warpehoski & Hurley, 1988). The recent use of well-defined site-specific lesions in viral and plasmid genomes has allowed much greater control over both the identity of the adduct and the sequence context in which it resides (Basu & Essigmann, 1988). The mutagenic specificity of the two major adducts produced by aminofluorene derivatives, i.e., the AF[1] and AAF adducts of guanine, has been studied in a number of systems including those using site-specific constructs.

The mutagenic potential of these adducts is thought to be due in part to the distortion that each adduct causes in DNA. The acetylated AAF adduct can induce a conformational change in the DNA molecule by causing the modified guanine to rotate about the glycosidic bond into a syn conformation and insert the AAF moiety bound to guanine into the base-pairing region of the helix (Fuchs & Daune, 1972; Fuchs et al., 1976; Grunberger & Weinstein, 1978). A comparison of mutagenesis studies using aminofluorene derivatives shows that the AAF adduct appears to be primarily a frame-shift mutagen (Fuchs et al., 1981; Koffel-Schwartz et al., 1984). However, one study showed that a site-specific AAF adduct in a plasmid vector gave rise primarily to base-substitution mutations (Moriya et al., 1988), while in another sequence context a single AAF adduct produced only frame shifts (Burnouf et al., 1989). Furthermore, it has been shown that the presence of an AAF adduct in one strand of a double-stranded molecule can cause the loss of that strand such that only the unadducted strand will be used as a template for DNA replication (Koffel-Schwartz et al., 1987). Conformational studies of the nonacetylated AF adduct have shown that it prefers the normal

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[1] Abbreviations: AF, *N*-(guanin-8-yl)-2-aminofluorene; AAF, *N*-(guanin-8-yl)-2-(acetylamino)fluorene; *N*-AcO-TFAAF, *N*-acetoxy-*N*-(trifluoroacetyl)-2-aminofluorene; *N*-AcO-AAF, *N*-acetoxy-*N*-acetyl-2-aminofluorene; AIX medium, LB agar containing 100 μg/mL ampicillin, 70 μg/mL IPTG, and 80 μg/mL X-Gal; bp, base pair; gapped heteroduplex, double-stranded plasmid DNA containing a 17-base gap in the minus strand; HPLC, high-performance liquid chromatography; SDS, sodium dodecyl sulfate, SSC, 150 mM sodium chloride and 15 mM sodium citrate (pH 7.0); TLC, thin-layer chromatography.

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anti configuration without displacement of the guanine (Evans et al., 1980), although it can also assume a syn conformation (Hingerty & Broyde, 1986). AF adducts have been shown to be primarily base-pair mutagens in experiments using randomly modified DNA (Bichara & Fuchs, 1985; Gupta et al., 1988; Carothers et al., 1989; Mah et al., 1989) but have also been shown to produce frame-shift mutations in the Ames test (Beranek et al., 1982) and in a site specifically modified plasmid (Mitchell & Stohrer, 1986).

As a means of probing the effects of these different conformations at a single site in a biologically active DNA molecule, we have constructed plasmid vectors bearing single, site-specific aminofluorene- or 2-(acetylamino)fluorene-substituted guanines. We have also employed a system designed to promote replication of the adducted strand. To accomplish this, plasmids were constructed that contained uracil residues on the strand opposite that bearing the adducts. The uracil residues are converted to abasic sites in vivo, causing a biological inactivation of this strand (Kunkel, 1985; Kunkel et al., 1987) and forcing the cell to used the strand bearing the adducts as a template for DNA replication. Following transformation of bacterial cells with the site specifically modified DNA, mutants were detected by oligonucleotide hybridization techniques that allowed unbiased detection of all mutations produced by the adducts.

Materials and Methods

Plasmid DNA, Bacteriophage, and Bacterial Strains. Plasmid pIBI25, bacteriophage M13K07, *Escherichia coli* NM522 [Δ(lac-proAB), thi, hsdΔ5, supE[F′, proAB, lacIqZΔM15]] (Gough & Murray, 1983), and *E. coli* CJ236 [dut1, ung1, thi1, relA1/pCJ105] (Joyce & Grindley, 1984) were obtained from International Biotechnologies (New Haven, CT). *E. coli* AB1886 [*uvr*A6] (Howard-Flanders et al., 1966) was obtained from Yale University Genetic Stock Center. T_4 polynucleotide kinase, Klenow fragment of DNA polymerase I, and T_4 DNA ligase were obtained from Pharmacia (Piscataway, NJ). T_4 DNA polymerase and Sequenase, T_7 DNA polymerase, were from U.S. Biochemicals (Cleveland, OH). Restriction enzymes *Eco*RI and *Sal*I were from Bethesda Research Laboratories (Gaithersburg, MD); *Spe*I was from Stratagene (La Jolla, CA). Conditions for restriction enzyme digestions were as specified by the suppliers.

Synthesis of [^{3}H]-N-AcO-TFAAF. A solution of 60 mg (0.3 mmol) of [ring-^{3}H]-2-nitrofluorene in 5 mL of freshly distilled tetrahydrofuran was reduced to the hydroxylamine with 18 mg (0.6 mmol) of hydrazine in the presence of 19 mg of 5% palladium/charcoal at 0 °C for 30 min (Westra, 1981). Trifluoroacetic anhydride (60 mg; 1.1 mmol) was added to the reaction mixture, and the reaction was allowed to proceed at room temperature for 80 min. The reaction mixture was then filtered through a bed of Celite and rinsed with ether. The organic phase was washed with H_2O (1 × 5 mL), with saturated $NaHCO_3$ (1 × 5 mL), and again with H_2O (1 × 5 mL) and then extracted with 0.5 N NaOH (2 × 6 mL). The combined NaOH phase was washed once with ether and then acidified to pH 2 with 6 N HCl. The product, [^{3}H]-*N*-hydroxy-*N*-(trifluoroacetyl)-2-aminofluorene (Weeks et al., 1980), was extracted into ether and the ether solution washed with H_2O. The ether solution was treated with 23 mg (0.2 mmol) of acetic anhydride at room temperature for 20 min. The solvent was evaporated under a stream of nitrogen, and the residue was triturated with ether–hexanes to obtain 40 mg (0.1 mmol) of [ring-^{3}H]-*N*-AcO-TFAAF (33% yield, 94% radiochemical purity, 60 mCi/mmol). TLC behavior of this product was identical with that of an authentic nonradioactive sample (R_f = 0.73, benzene/alcohol = 9:1) that was synthesized according to the published procedure (Lee & King, 1981); the product had a melting point of 118–120 °C, and an electron impact mass spectrum that confirmed its identity: (*m/z*, % RA) 335 (M^+, 75), 293 (80), 277 (35), 276 (68), 208 (23), 207 (100), 179 (82).

In addition, reaction of *N*-AcO-TFAAF with dGMP yielded a single product whose TLC behavior and UV and NMR spectra were consistent with those published for *N*-(guanosin-8-yl)-2-aminofluorene (Kriek et al., 1967; King & Phillips, 1969; Kriek & Westra, 1980) (data not shown).

Oligonucleotide Synthesis. The synthetic oligonucleotides used in this study were obtained from Wayne State University Center for Molecular Biology. The crude products were purified by reverse-phase HPLC using an acetonitrile gradient in 0.1 M triethylammonium acetate, pH 7.0. The initial chromatographic separation was carried out with the 5′-(dimethoxytrityl) group attached to the oligomer. Following removal of the dimethoxytrityl group (Atkinson & Smith, 1984), the oligomer was further purified by rechromatography. The product was stored in dried aliquots at −20 °C. The purity of the oligomers at various stages of purification was monitored by end labeling aliquots with [^{32}P]ATP and T_4 polynucleotide kinase. These labeled products were analyzed by polyacrylamide gel electrophoresis (20% acrylamide, 7 M urea) followed by autoradiography. The nucleotide sequence of the synthetic heptadecamer, d(AATTCCACTAGTACCCC), used for site-specific adduct formation was verified by the methods of Maxam and Gilbert (1980).

Reaction of d(AATTCCACTAGTACCCC) with N-AcO-TFAAF and N-AcO-AAF. For the initial chemical analysis of the oligonucleotide adducts, tritiated substrates were used. For the biological studies, unlabeled compounds were used. Reaction conditions and subsequent purifications were the same in both cases. The purified heptadecamer was dissolved in 2 mM sodium citrate (pH 7.0) and mixed with a 5-fold molar excess of [^{3}H]-*N*-AcO-TFAAF or [^{3}H]-*N*-Aco-AAF that had been freshly dissolved in 95% ethanol. The final ethanol concentration was 20%. Following incubation at 37 °C for 60 min, the mixtures were extracted with diethyl ether (3 × 1 mL). The reaction products were purified by reverse-phase HPLC on a Hamilton PRP-1 column using a 20-min linear gradient of 10–25% acetonitrile in 0.1 M triethylammonium acetate (pH 7.0) at a flow rate of 1.0 mL/min. Tritium in the eluant was detected with a Flo-I radioactivity flow detector (Radiomatic Instruments, Tampa, FL). Only the material corresponding to the upper 50% of the major UV-absorbing peaks was used for further analysis and for subsequent mutagenicity studies. Aliquots of the peaks corresponding to the modified heptadecamers were dried under vacuum and incubated with anhydrous trifluoroacetic acid at 70 °C for 1 h (Tang & Lieberman, 1983). This material was dried under vacuum and redissolved in methanol for analysis by reverse-phase HPLC. The samples were also analyzed by 5′-end labeling and polyacrylamide gel electrophoresis as indicated above.

As a measure of the stability of the AF adduct, a portion of tritiated, AF-modified heptadecamer was incubated in 0.1 N NaOH at 37 °C for 60 min. The pH was adjusted to approximately 7.0, and aliquots were subjected to trifluoroacetic acid hydrolysis and HPLC as described above or end labeled with [^{32}P]ATP and analyzed by polyacrylamide gel electrophoresis. In order to verify the position of the site-specific AF adduct, aliquots of the purified AF-modified heptadecamer were treated with 1 M piperidine as previously

described (Johnson et al., 1986). This treatment has been shown to cause strand breaks specifically at the site of an AF adduct (Bichara & Fuchs, 1985; Johnson et al., 1986). The product of this treatment was compared to an unmodified heptadecamer that had been subjected to Maxam–Gilbert sequencing reactions specific for cleavage at guanines. These samples were analyzed by polyacrylamide gel electrophoresis and autoradiography. ^{32}P-Labeled aliquots of the AAF-modified heptadecamer were treated with T_4 DNA polymerase under conditions in which the enzyme functions as a 3′ → 5′ exonuclease followed by polyacrylamide gel electrophoresis and autoradiography. The presence of an AAF adduct has been shown to completely block the exonuclease activity of the enzyme one base 3′ to the site of the lesion (Fuchs, 1984). The product of this reaction was compared to an oligonucleotide (dT) size ladder (data not shown).

DNA Preparation. The plasmid pIBI25 is derived from the pEMBL plasmids (Dente et al., 1983) and contains both the pBR322 and M13 origins of replication. Coinfection with an M13 derivative, M13K07, allows the positive strand of the plasmid to be packaged and extruded into the supernatant. Single-stranded DNA was generated by superinfecting plasmid-bearing cultures of *E. coli* NM522 with the helper phage M13K07 (Messing, 1988). The single-stranded DNA was isolated by a modified phenol extraction procedure (Messing, 1983). Large-scale preparation of double-stranded plasmid DNA was achieved by a modification of the Triton X-100 method (Clewell & Helsinki, 1972); purification was by centrifugation in cesium chlorite–ethidium bromide density gradients. Small-scale preparations of double-stranded plasmid DNA for sequencing were prepared as described (Holmes & Quigley, 1981) followed by RNase treatment and poly(ethylene glycol) precipitation (Hattori & Sakaki, 1986).

Construction of PMC17 DNA. pIBI25 was digested with *Eco*RI and *Sal*I, and the 33-bp *Eco*RI–*Sal*I fragment was removed by ultrafiltration using a Centricon 30 filter apparatus (Amicon; MW 30 000 cutoff). A double-stranded duplex was prepared from the unmodified heptadecamer and a second 17-base oligonucleotide that allowed formation of cohesive *Eco*RI and *Sal*I ends (Figure 1). These oligonucleotides had been phosphorylated at their 5′ ends with ATP and T_4 polynucleotide kinase and the excess ATP removed by chromatography on a Nensorb 20 column (NEN/Du Pont). HPLC analysis of aliquots of kinase reactions used throughout this study showed that the efficiency of these reactions was at least 95%. Following Nensorb 20 purification, the oligonucleotides were annealed, and the resulting duplex, d(pAATTCCACTAGTACCCC)·d(pTCGAGGGGTACTAGTGG), was ligated into the large *Eco*RI–*Sal*I fragment of pIBI25. An aliquot of the ligation mixture was used to transform *E. coli* NM522, and the presence of the insert was confirmed by the appearance of light blue colonies on AIX medium as opposed to the dark blue phenotype of the wild-type pIBI25 DNA. Further proof of the insert was determined by the recombinant DNA's sensitivity to digestion by *Spe*I and insensitivity to digestion by *Sal*I and by DNA sequencing of the positive strand (Sanger et al., 1977). This recombinant plasmid containing the 17-base insert was termed pMC17.

In order to prepare plasmid DNA containing uracil residues, an aliquot of pMC17 DNA was used to transform *E. coli* CJ236 cells. This strain is deficient in dUTPase and uracil-*N*-glycosylase activities, allowing uracil residues to be inserted into DNA (Kunkel, 1985). It is estimated that transformants arising in this strain contain approximately 5% of their thymine residues substituted by uracil (Sagher & Strauss, 1983).

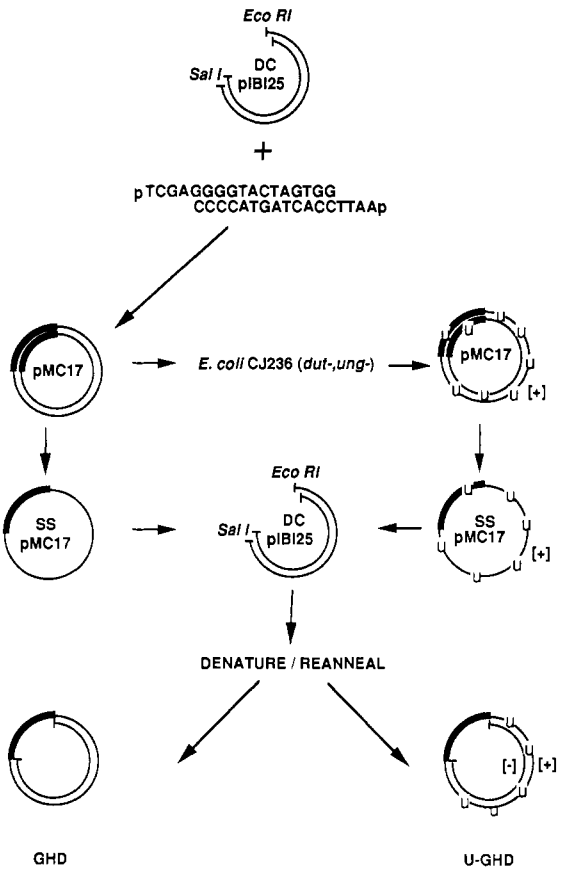

FIGURE 1: Schematic of DNA constructs. pMC17 was constructed as described under Materials and Methods. To construct gapped heteroduplex molecules, the large *Eco*RI–*Sal*I fragment of pIBI25 (DC pIBI25) was mixed with single-stranded pMC17 (+/−uracil) and denatured by dialysis against 95% formamide. The DNA was allowed to renature by dialysis against decreasing concentrations of formamide.

Preparation of Gapped Heteroduplex DNA. The method of construction of gapped heteroduplex DNA is shown in Figure 1. A 10-fold molar excess of single-stranded pMC17 DNA (2871 bases) was mixed with the large *Eco*RI–*Sal*I fragment of pIBI25 (2854 bp) and allowed to denature/renature by dialysis against decreasing concentrations of deionized formamide at room temperature (Lundquist & Olivera, 1982). This was followed by dialysis against 0.18 M KPO_4 buffer (pH 7.0) and chromatography on hydroxylapatite to remove excess single-stranded DNA. The majority of single-stranded DNA eluted in the 0.18 M KPO_4 while the gapped heteroduplex and the remaining double-digested pIBI25 eluted in 0.3 M KPO_4 buffer. The concentration of gapped heteroduplex DNA at each step was estimated by comparison to standards following electrophoresis through 0.8% agarose gels containing 0.5 μg/mL ethidium bromide.

In order to prepare DNA that contained uracils in the strand opposite the adducts, the same dialysis procedure was performed with uracil-containing pMC17 (Figure 1). The hydroxylapatite purification step was omitted since introduction of the uracil-containing single-stranded DNA into *E. coli* did not produce any ampicillin-resistant clones (data not shown).

Ligation of Heptadecamers into Gapped Heteroduplex DNA. In a typical reaction, 0.5 μg (100 pmol) of kinased heptadecamer (with or without the AF/AAF adduct) was mixed with 2 μg (1 pmol) of gapped heteroduplex DNA in the presence of 50 mM Tris (pH 7.6), 10 mM $MgCl_2$, 1 mM ATP, 1 mM DTT, and 10 Weiss units of T_4 DNA ligase in a volume of 50 μL. This mixture was incubated at 25 °C for 2 h. Following the addition of another 10 units of ligase, the

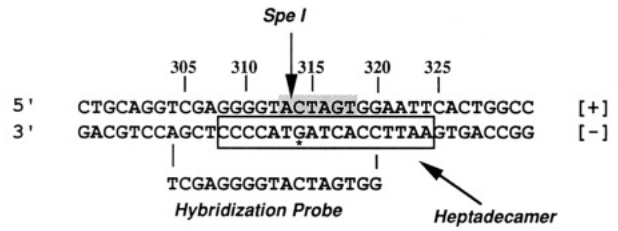

FIGURE 2: Sequence surrounding the site-specific adducts. The site-specific adduct site is designated with an asterisk; the *Spe*I recognition sequence (ACTAGT) is shaded.

mixture was incubated for another 2 h at 20 °C and then at 37 °C for 1 h.

In order to ensure that the correct sequence was generated, aliquots of the ligation mixtures were incubated with restriction enzymes, *Eco*RI, *Sal*I, or *Spe*I, and electrohoresed through 0.8% agarose gels. Analysis of the *Spe*I digestions also allowed a determination of the presence of the AF or AAF adduct since the presence of these adducts inhibits the enzyme. The sequence resulting from the ligations is shown in Figure 2.

Transformation. The site specifically modified plasmids were used to transform *E. coli* NM522 and AB1886 with or without the prior induction of SOS functions. The UV dose for SOS induction was 30–32 J/m^2 for NM522 and 4–6 J/m^2 for AB1886 (Koffel-Schwartz et al., 1984; Bichara & Fuchs, 1985), which gave a 10–20% survival in both strains after irradiation. Following irradiation, the cells were shaken at 37 °C for 20 min in the dark. SOS-induced and noninduced cells were made competent by $CaCl_2$ treatment (Cohen et al., 1972). All transformation procedures were carried out within 2 h of UV irradiation. Transformants were selected for ampicillin resistance on AIX medium.

For some experiments, in order to enrich the mutant population, aliquots of cells were removed after the heat-shock procedure and grown in LB broth for 2 h at 37 °C. Ampicillin was added to the media to a final concentration of 100 μg/mL, and the cells were grown overnight at 37 °C. Small-scale preparations of plasmid DNA from these cultures were obtained, and the DNA was incubated with *Spe*I for 60 min at 37 °C. Various dilutions of this DNA were used to transform *E. coli* NM522 cells. No SOS induction was used for this second transformation.

Detection of Mutants. Colonies resulting from transformations were randomly selected and toothpicked in a grid onto AIX plates followed by incubation at 37 °C overnight. The colonies were transferred to Amersham Hybond-N filters, and the DNA was fixed to the filters by irradiation at 300 nm on a Fotodyne Model 3000 transilluminator for 5 min. The filters were incubated for 2 h at room temperature with a solution of 6× SSC, 5× Denhard's solution, 0.5% SDS, and 2 × 10^6 cpm of a ^{32}P-labeled oligonucleotide that was complementary to the sequence containing the modified guanine of the plasmid. The filters were washed twice in 2× SSC at room temperature and autoradiographed. The filters were then washed with 2× SSC for 10 min at 48 °C and autoradiographed again. Colonies that showed a signal at room temperature but not at 48 °C were subjected to DNA sequence analysis (Sanger et al., 1977). A control colony was included on each filter that was the result of a transformation with DNA that contained a G → T transversion at the site of modification. Loss of signal by this colony with the above methods demonstrated that base substitutions as well as frame shifts could be detected.

RESULTS

Preparation of AF- and AAF-Modified Heptadecamers. HPLC analysis of the products of the reaction between the synthetic heptadecamer and [^{3}H]-*N*-AcO-TFAAF or [^{3}H]-*N*-AcO-AAF showed single UV-absorbing peaks in addition to that representing the unmodified heptadecamer (Figure 3). These were the only UV-absorbing peaks associated with radioactivity, and their UV spectra were consistent with AF- and AAF-modified oligonucleotides (Johnson et al., 1986, 1987). The early-eluting radioactive peaks presumably contain solvolysis products not extractable into ether. The lack of a detectable UV signal would rule out any nucleotide being present at these positions of the chromatographs. On the basis of the oligonucleotide employed, the reaction yield following HPLC purification was approximately 15% for the *N*-AcO-TFAAF reaction and 25% for the *N*-AcO-AAF reaction. Hydrolysis of a portion of the modified oligomers with anhydrous trifluoroacetic acid followed by HPLC analysis resulted in products whose only radioactive and UV-absorbing peaks coeluted with the trifluoroacetic acid hydrolysis products of authentic standards of dGMP–AF or dGMP–AAF (data not shown).

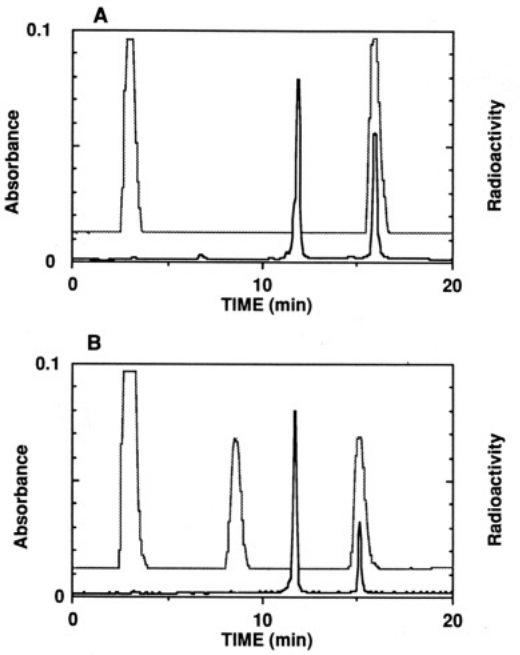

FIGURE 3: HPLC purification of the synthetic heptadecamer following reaction with [^{3}H]-*N*-AcO-TFAAF and [^{3}H]-*N*-AcO-AAF. Conditions are as described under Materials and Methods. Solid lines represent the absorbance at 260 nm. Dashed lines represent tritium. (Panel A) Reaction with [^{3}H]-*N*-AcO-TFAAF; (panel B) reaction with [^{3}H]-*N*-AcO-AAF.

Polyacrylamide gel electrophoresis and autoradiography of ^{32}P-labeled samples of the modified and unmodified heptadecamers showed the presence of a single band in each case, indicating the purity of these preparations (Figure 4). The modified heptadecamers migrated more slowly through the gel than the unmodified sample. Treatment of a ^{32}P-labeled sample of *N*-AcO-TFAAF-modified heptadecamer with 1 M piperidine at 90 °C for 1 h showed the presence of an oligonucleotide that comigrated with a species produced by subjecting an unmodified heptadecamer to Maxam–Gilbert reactions specific for cleavage at guanine residues (Figure 4). Treatment of a ^{32}P-labeled sample of *N*-AcO-AAF-modified heptadecamer with T_4 DNA polymerase under conditions that promote the 3′ → 5′ exonuclease activity of the enzyme showed a single band that migrated to a position between 12 and 13 bases, corresponding to a 12-mer bearing an a adduct (Figure 4). This is the expected product of this digestion since the presence of an AAF adduct has been shown to completely

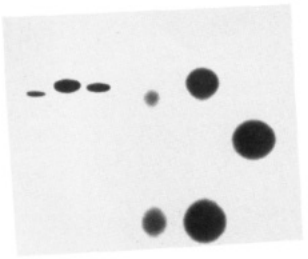

FIGURE 4: Analysis of ^{32}P-labeled samples of the synthetic heptadecamer after HPLC purification. Samples were 5′-end labeled with ^{32}P by T_4 polynucleotide kinase, electrophoresed through a 20% denaturing polyacrylamide gel, and visualized by autoradiography: (lane A) HPLC-purified heptadecamer; (lane B) AF-modified heptadecamer following HPLC purification; (lane C) AAF-modified heptadecamer following HPLC purification; (lane D) Maxam–Gilbert G-reaction cleavage product of the unmodified heptadecamer; (lane E) AF-modified heptadecamer treated with 1 M piperidine; (lane F) AAF-modified heptadecamer following treatment with T_4 DNA polymerase.

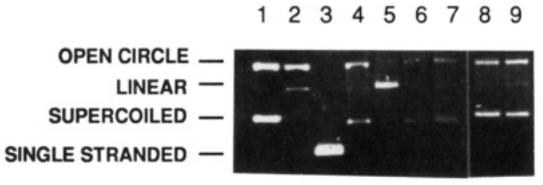

FIGURE 5: Agarose gel electrophoresis of samples used in construction of site specifically modified vectors. The 0.8% gel contained 0.5 mg/mL ethidium bromide; (lane 1) pIBI25 DNA; (lane 2) gapped heteroduplex DNA; (lane 3) single-stranded pMC17 DNA; (lane 4) ligation product using the unmodified heptadecamer; (lane 5) *Spe*I digestion of unmodified ligation product; (land 6) ligation product using the AF-modified heptadecamer; (lane 7) *Spe*I digestion of AF-modified ligation product; (lane 8) ligation product using the AAF-modified heptadecamer; (lane 9) *Spe*I digestion of AAF-modified ligation product.

block the exonuclease activity of T_4 DNA polymerase one base 3′ to the site of the lesion (Fuchs, 1984). In addition, a sample of the AF-modified heptadecamer showed no evidence of an imidazole ring-opened product (Tang & Lieberman, 1983) following treatment with 0.1 M NaOH at 37 °C for 1 h when analyzed by trifluoroacetic acid hydrolysis and HPLC or by polyacrylamide gel electrophoresis (data not shown).

Preparation of the Gapped Heteroduplex. The agarose gel in Figure 5 shows the purified preparation of the gapped heteroduplex in lane 2. Following the formamide dialysis treatment and hydroxylapatite chromatography, the sample contains unhybridized double-stranded linear DNA and a species that electrophoreses to a position corresponding to nicked, open-circular DNA, which is the gapped heteroduplex DNA. It was estimated that approximately 80% of the double-digested pIBI25 DNA reanealed with the single-stranded DNA to form the heteroduplex. Analogous results were obtained with uracil-containing DNA.

Ligation of Heptadecamers into Gapped Heteroduplex DNA. Initial characterization of the ligation products was achieved by agarose gel electrophoresis following incubation with various restriction enzymes. As shown in Figure 5, in the presence of ethidium bromide, a band appears following ligation of the unmodified or modified heptadecamers that corresponds to supercoiled DNA (lanes 4, 6, and 8). A comparison of the DNA in lanes 7 and 9 with that in lane 5 shows that only the ligation products employing the unmodified heptadecamer are cleaved by *Spe*I. The presence of AF or AAF adducts inhibits the enzyme as has been seen with these and other bulky adducts (Naser et al., 1988).

Table I: Effect of Uracil Residues on the Transformation Efficiency of *E. coli* NM522 Cells

	transformation efficiency (cfu/ng of DNA)		
	no adduct	AF adduct	AAF adduct
−uracil	238	192	188
+uracil	6	5	8
percent	3	3	4

Futher analysis showed that while the gapped heteroduplex itself is not cleaved by *Eco*RI, the ligation products were all digested by this enzyme but not by *Sal*I (data not shown). To assess the biological activity of the ligation products, aliquots of the ligation mixtures were used to transform *E. coli* NM522 cells. The presence of the adducts did not appreciably lower the transformation efficiency of the DNA compared to that of the unmodified plasmid (Table I).

Introduction of plasmid DNA into an *E. coli ung*$^-$ *dut*$^-$ strain causes uracil residues to be incorporated into the plasmid genome due to increased intracellular levels of dUTP and the lack of a functional uracil-*N*-glycosylase to remove the uracils once incorporated (Kunkel, 1985; Kunkel et al., 1987). Introduction of the uracil-containing DNA into wild-type (*ung*$^+$) *E. coli* cells will cause removal of the uracil residues by the host cell's uracil-*N*-glycosylase, resulting in the formation of abasic sites (Lindahl, 1982). The presence of these abasic sites is thought to inhibit DNA synthesis by blocking DNA polymerases (Schaaper & Loeb, 1981; Kunkel, 1984) and also causes strand breaks due to the activities of cellular AP endonucleases (Lindahl, 1982). When incorporated into one strand of a double-stranded molecule, as in the present study, the uracil-containing strand is biologically inactivated, causing the non-uracil strand to be the primary template for DNA synthesis. That only one strand is being used as a template is reflected in the low transformation efficiency of this DNA as shown in Table I. The uracil-containing DNA was approximately 30-fold less efficient in transforming *E. coli* NM522 than was DNA that did not contain uracil. The presence of the single AF or AAF adducts did not appreciably alter the transformation efficiency relative to that of unmodified DNA.

As a measure of the strand bias induced by the presence of the uracil residues in only one strand of these molecules, a 17-base oligomer whose sequence was identical with that bearing the adducts except that the single guanine was replaced by a thymine was ligated into the uracil-containing gapped heteroduplex, resulting in a C/T mismatch within the *Spe*I restriction site. An aliquot of this ligation product was used to transform *E. coli* NM522 cells, which have a functional uracil-*N*-glycosylase gene. This should yield DNA that is resistant to *Spe*I if the strand containing the G → T change is used for a template during DNA replication, and *Spe*I-sensitive DNA if the uracil-containing strand is used for a template. Following the transformation, DNA from 20 ampicillin-resistant clones was isolated and incubated with *Spe*I. Out of 20 samples, 15 were resistant to the enzyme (75%), indicating that in these samples the non-uracil strand was used primarily as a template for DNA replication. In a control experiment, the oligomer containing the G → T change was ligated into a gapped heteroduplex that did not contain uracil in either strand, and the above experiment was repeated. In this case only 2 clones out of 12 (17%) were resistant to digestion by *Spe*I.

Mutagenesis Studies. The mutation frequencies induced by the adducts are shown in Tables II and III. These include

Table II: Mutagenesis Induced by Site-Specific Adducts in *E. coli* NM522 Cells

adduct	−uracil		+uracil	
	−SOS	+SOS	−SOS	+SOS
none	<0.22 (0/476)[a]	<0.23 (0/431)	ND	<0.17 (0/576)
AF	0.17 (1/576)	0.39 (8/2070)	0.17 (1/576)	2.9 (13/454)
AAF	<0.17 (0/576)	<0.06 (0/1728)	<0.17 (0/576)	0.75 (5/667)

[a] Single-base mutations (%) excluding ligation sites.

Table III: Mutagenesis Induced by Site-Specific Adducts in *E. coli* AB1886 (*uvrA*⁻) Cells with Uracil-Containing DNA

adduct	−SOS	+SOS
none	ND	<0.22 (0/462)[a]
AF	<0.17 (0/576)[b]	0.35 (2/576)[c]
AAF	<0.17 (0/576)	<0.17 (0/576)

[a] Single-base mutations (%) excluding ligation sites. [b] Mutation frequencies do not include a single large deletion and two recombinational mutations as described under Results. [c] The mutations induced were a single G → T and a single G → A base substitution targeted to the modified base.

single-base substitutions and/or deletions at bases near the adduct site and at the adduct site, but not at either ligation point nor in the sequence of the gapped heteroduplex itself. The complete mutation spectra produced by these adducts is shown in Figure 6.

In non-uracil DNA only the AF adduct produced mutations at or near the site of the adduct, and with one exception these mutations were SOS dependent. In uracil-containing DNA both adducts were mutagenic, and again the mutations were SOS dependent. In addition, in the presence of uracil on the opposite strand of the plasmid, the mutation frequency for both adducts increased, but no effect was seen without SOS induction. In SOS-induced *E. coli* NM522 cells, the AF adduct yielded a 17-fold higher mutation frequency than background in the uracil constructs and a 7-fold increase in mutagenesis compared to non-uracil DNA. The AAF adduct, which produced no mutations in normal DNA, had a mutation frequency 4-fold higher than background in SOS-induced cells utilizing the uracil-containing DNA and a 12-fold increase in mutagenesis compared to non-uracil DNA.

When present in non-uracil DNA, the AF adduct caused primarily single-base deletions with the majority of these being targeted to the modified base. A single G → T transversion was also observed at this site. Mutations also occurred at bases that were near but not at the AF adduct site. These mutations included two T deletions two bases 5′ to the adduct and one C deletion three bases 5′ to the adduct that occurred in SOS-induced cells. In contrast, in molecules with uracil in the opposite strand, the AF adduct produced only base-substitution mutations targeted to the modified base with the majority (11/13) being G → T transversions. The AAF adduct, on the other hand, produced no mutations in non-uracil DNA, but in uracil-containing DNA, both frame shifts and base substitutions were detected at bases near to, but not at, the site of the lesion, as well as a single G → T transversion at the adduct site. One A → T transversion was observed one base 5′ to the adduct while two −1 deletions were observed three bases 5′ to the adduct. An 18-base deletion was also observed with the AAF adduct that ranged from 6 bases 5′ to the adduct to 11 bases 3′ to the adduct. A larger AAF-induced deletion was detected by altered mobility on an agarose gel, but this sample was not sequenced.

In order to increase the likelihood of detecting mutants within the *Spe*I site in non-uracil plasmids, DNA was isolated from transformations of SOS-induced cells, incubated with *Spe*I, and used for a second transformation. This treatment

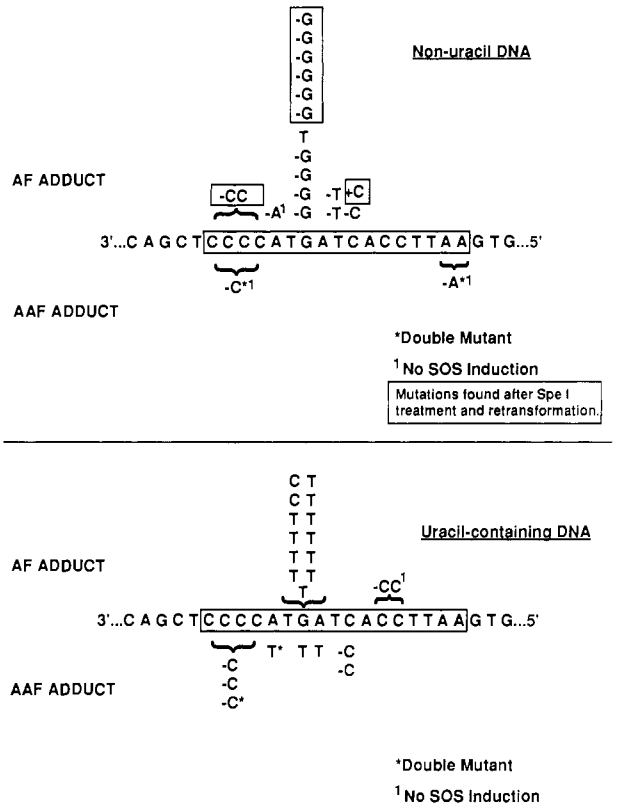

FIGURE 6: Mutational spectra induced by the site-specific adducts. The mutations are shown for the non-uracil DNA in the top panel and for the uracil-containing DNA in the bottom panel. The heptadecamer sequence is enclosed by the solid box. The mutations observed after treatment with *Spe*I are also shown in boxes. All mutations were SOS dependent except where noted.

should linearize any DNA without sequence changes in the immediate region of the adduct since the adducted guanine is within the *Spe*I site. While the single-enzyme treatment did not digest all the DNA without sequence changes within the *Spe*I site, the *Spe*I-treated DNA had a transformation efficiency of less than 2% of that of miniprep DNA that had not been subjected to any enzyme treatment. From each group (i.e., control and AF- and AAF-modified DNA), 384 independent colonies were screened for mutations. Only the AF-modified DNA gave rise to mutations, with the majority of these being single-base deletions at the site of the adduct and one C insertion three bases 5′ to the adduct (Figure 7). One untargeted CC deletion mutation was observed at the *Sal*I ligation site. No mutations were seen with either the unmodified or the AAF-modified DNA with this protocol. While this treatment served to confirm the appearance of G deletions in non-uracil DNA containing an AF adduct, there was no clonal selection for these mutants, and hence, they may not represent independent events.

To examine the effect of bacterial host cells on mutational specificity, *E. coli* AB1886 (*uvrA*⁻) cells were also used for transformations with uracil-containing DNA. In the *uvrA*⁻ cells, the AF adduct produced two base substitutions at the site of the modified guanine, a G → A transition and a G →

T transversion. These mutations occurred in SOS-induced cells, giving rise to a mutation frequency of 0.35%. In cells without SOS induction, two recombinational mutations occurred. These two mutants had 14 bases of the plasmid from position 304 to position 317, which included the adduct site, replaced by a 12-base sequence that exhibited <60% homology with any other sequence of the plasmid. The fact that both mutants had exactly the same sequence suggests that they did not occur independently. A single large deletion was also detected in cells treated with AF-modified DNA but was not sequenced. No mutations were detected in the *uvrA*⁻ cells treated with the AAF-modified DNA, with or without SOS induction. The mutation frequencies given in Table III for the various treatments do not include the large frame shifts and rearrangements that were detected.

Discussion

In a previous report we examined the effect of single, site-specific AF and AAF adducts in a phage vector using an in situ hybridization procedure that identified, without bias, both targeted mutations and those occurring in the immediate vicinity of the modified base (Gupta et al., 1989). Here we extend these studies by monitoring the effect of site specifically placed AF or AAF adducts within a different sequence context of a plasmid vector. For some experiments, the plasmid was constructed so that it contained uracil residues in the strand opposite the adducts. The conversion of these uracils to abasic sites in vivo allowed us to study the effect of forcing replication of an adducted strand on mutagenesis in bacterial cells.

It is apparent from the data in Tables II and III that the mutations observed in this study were dependent on the induction of host cell SOS function as has been seen previously with these adducts (Koffel-Schwartz et al., 1984; Bichara & Fuchs, 1985; Gupta et al., 1988). In a comparison of the results obtained with SOS-induced *E. coli* NM522 cells, it was found that in non-uracil DNA only the AF adduct produced mutations. In contrast, the AAF adduct produced no mutations in the non-uracil construct. These data are consistent with a bias toward replication of the nonadducted strand during plasmid replication due to blockage of DNA polymerases by bulky adducts (Koffel-Schwartz et al., 1987). Furthermore, the ability of the AF adduct to induce mutations given this strand bias is in agreement with studies demonstrating the ability of DNA polymerases to bypass AF adducts with greater efficiency than AAF adducts (Moore et al., 1982; O'Conner & Stohrer, 1985; Michaels et al., 1987).

As a means of counteracting the replication bias induced by the presence of bulky adducts in one strand of the plasmid, we have used constructs that utilize a modification of the site-directed mutagenesis protocol developed by Kunkel et al. (1985). By use of this methodology, the adducts were inserted into plasmids bearing uracil residues on the strand opposite the adducts. The uracil is degraded in vivo, causing the adduct-bearing strand to be the primary template used for DNA replication. The use of the uracil-containing plasmids produced a marked increase in mutagenicity for both AF and AAF adducts in SOS-induced cells. These data suggest that biasing replication of an adduct-bearing DNA strand in SOS-induced cells may lead to more efficient mutagenesis by polymerase-blocking lesions.

In contrast, when uracil-containing plasmids bearing the single adducts were used to transform a *uvrA*-deficient strain of *E. coli* (AB1886), the mutagenic effects were comparable to that observed in wild-type (NM522) cells in the absence of uracil. Previous studies with these adducts in randomly modified plasmid DNA showed that mutation frequencies did not increase in the same *uvrA*⁻ strain nor was prior induction of SOS functions required (Koffel-Schwartz et al., 1984). In experiments using the analogous C-8-aminobiphenyl adduct, mutagenesis in *uvrA*⁻ or *uvrC*⁻ strains was not increased over that observed in wild-type cells (Lasko et al., 1988). Chambers et al. (1988) showed that the percentage of transition mutations produced by a site-specific O^6-methylguanine adduct base paired with cytosine decreased markedly in the AB1886 strain relative to a wild-type strain (AB1157). The O^6-methylguanine adduct has recently been shown to be a substrate for Uvr endonuclease excision in vitro (Voigt et al., 1989). Thus, the differences in repair synthesis under SOS-induced conditions may play a role in the mutations observed in the present study. However, given that the NM522 and AB1886 strains are not isogenic, other cellular factors may be involved.

The differences in the type of mutations observed in the presence and absence of uracil should be noted. In non-uracil DNA, the AF adduct produced primarily frame-shift mutations although one G → T transversion was also observed. In the uracil-containing constructs, the AF adduct produced only base-substitution mutations in both repair-deficient and wild-type strains. Previous studies have shown that G → T transversions are the predominant type of mutation produced by AF adducts in randomly modified DNA in both bacterial and mammalian systems (Bichara & Fuchs, 1985; Gupta et al., 1988; Carothers et al., 1989; Mah et al., 1989). In addition, NMR studies have demonstrated that AF-modified guanine can base pair with adenine, implying a possible mechanism for this type of mutation (Norman et al., 1989). However, the AF adduct has also been shown to cause deletion mutations (Gupta et al., 1988; Ross et al., 1988), and one study has shown that AF adducts can produce both deletions and base substitutions at the same guanine base (Bichara & Fuchs, 1985).

The shift to base-substitution mutations in the uracil constructs may be due to cellular factors that cause mutational pathways for these adducts to be modified in the presence of DNA damage on the strand opposite the adduct. For example, the kinetics of adduct bypass may be altered in such a way that adduct-induced alterations in DNA conformation that contribute to the formation of frame shifts (such as slippage of the template strand) are less likely to occur. One could also postulate that the presence of uracil residues might induce a glycosylase activity such that the AF lesions are converted to abasic sites. It has been shown that adenine is the nucleotide most often incorporated opposite an abasic site (Loeb & Preston, 1986), and this would explain the G → T transversions observed in this study. Arguing against this is the fact that no effect was induced by the presence of uracil without prior induction of SOS functions. Moreover, there is no evidence that AF adducts are converted to abasic sites in vivo.

The AAF adduct also produced base substitutions in the uracil-containing constructs, but only one of these occurred at the adducted base, and frame-shift mutations were detected as well. AAF adducts have been shown to produce primarily frame-shift mutations in randomly modified DNA (Koffel-Schwartz et al., 1984), and some sequences have been shown to be particularly prone to AAF-induced deletions (Burnouf et al., 1989). However, in other sequence contexts, base substitutions make up most if not all of the mutations observed with site-specific AAF adducts (Moriya et al., 1988; Gupta et al., 1989). The observation of mutations at bases near to but not at the site of the adducts used in the present study suggests that these adducts, which are known to cause significant distortion in DNA molecules, may perturb the rep-

lication machinery at sites somewhat removed from the damage, as has been observed in other studies (Schaaper & Glickman, 1982; Kuchino et al., 1987; Gupta et al., 1989).

While no impurities could be detected in the adduct preparations (Figure 4), this does not preclude the possibility that an undetected contaminant is responsible for some of the observed mutations. However, any contaminant would have to be extremely mutagenic since it would be present at exceedingly low concentrations relative to the major adduct.

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Physical Studies of DNA Premelting Equilibria in Duplexes with and without Homo dA·dT Tracts: Correlations with DNA Bending†

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ABSTRACT: We have employed a variety of physical methods to study the equilibrium melting and temperature-dependent conformational dynamics of dA·dT tracts in fractionated synthetic DNA polymers and in well-defined fragments of kinetoplast DNA (kDNA). Using circular dichroism (CD), we have detected a temperature-dependent, "premelting" event in poly(dA)·poly(dT) which exhibits a midpoint near 37 °C. Significantly, we also detect this CD "premelting" behavior in a fragment of kDNA. By contrast, we do not observe this "premelting" behavior in the temperature-dependent CD spectra of poly[d(AT)]·poly[d(AT)], poly(dG)·poly(dC), poly[d(GC)]·poly[d(GC)], or calf thymus DNA. Thus, poly(dA)·poly(dT) and kDNA exhibit a common CD-detected "premelting" event which is absent in the other duplex systems studied in this work. Furthermore, we find that the anomalous electrophoretic retardation of the kDNA fragments we have investigated disappears at temperatures above approximately 37 °C. We also observe that the rotational dynamics of poly(dA)·poly(dT) and kDNA as assessed by singlet depletion anisotropy decay (SDAD) and electric birefringence decay (EBD) also display a discontinuity near 37 °C, which is not observed for the other duplex systems studied. Thus, in the aggregate, our static and dynamic measurements suggest that the homo dA·dT sequence element [common to both poly(dA)·poly(dT) and kDNA] is capable of a temperature-dependent equilibrium between at least two helical states in a temperature range well below that required to induce global melting of the host duplex. We suggest that this "preglobal" melting event may correspond to the thermally induced "disruption" of "bent" DNA.

Crystal structures of proteins, nucleic acids, and their complexes have provided valuable structural frameworks for developing an understanding of the interactions that control gene regulation. However, by its very nature, crystallography emphasizes the static or, at best, the time-averaged structure of a macromolecule. As a result, models for site-specific binding events often have a "lock and key" quality that tries to explain recognition in terms of rigid partners. One example of such thinking is the proposal that certain sequences of DNA are "bent" (Nelson et al., 1987; Coll et al., 1987; Alexeev et al., 1987). To be specific, the slow electrodiffusion constant in polyacrylamide gels of helices with periodic arrays of homo dA·dT segments [e.g., kinetoplast DNA (kDNA)] has been interpreted as the effect of a static curvature of the helix (Diekmann & Wang, 1985; Woo & Crothers, 1984). In addition, certain aspects of gene regulation may involve the binding-induced physical distortion of the DNA helix away from a linear "B" form (Frederick et al., 1984; Liu-Johnson et al., 1986; Hogan et al., 1987). Consequently, certain sequences of DNA may be statically bent while other sequences inherently may be more deformable (elastic). Identifying and distinguishing between these two classes of DNA "bending" should be of importance in our understanding of ligand-DNA interactions as well as in our understanding of the influence of sequence on DNA structure.

In this paper we examine several physical aspects of the unusual properties of the homopolymer poly(dA)·poly(dT), since it is believed that this particular sequence motif is closely related to many aspects of "bent" DNA and the abnormalities exhibited by kDNA (Srinivasan et al., 1987; Maroun & Olson, 1988). We note the similarities in several "premelting" physical properties of poly(dA)·poly(dT) and kDNA and contrast these properties with those of other synthetic and natural DNA duplexes which do not contain homo dA·dT tracts (also called "A tracts"). The "premelting" properties we report are to be differentiated from those associated with the large body of previous studies on DNA premelting [Doty et al., 1959; Ts'o & Helmkamp, 1961; Fresco, 1961; Freund

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