Sequence-Specific Alkylation and Cleavage of DNA Mediated by Purine Motif Triple Helix Formation[†]

Kathryn B. Grant and Peter B. Dervan*

The Beckman Institute, California Institute of Technology, Pasadena, California 91125

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ABSTRACT: An *N*-bromoacetyl electrophile attached to the 5′-phosphate group of a purine-rich oligonucleotide affords sequence-specific alkylation of duplex DNA (at 37 °C, pH 7.4) through the formation of a specific purine·purine·pyrimidine triple-helical complex. In a 645 bp restriction fragment containing three consecutive guanine bases adjacent to the 3′-end of an oligonucleotide binding site, the yield of single-strand cleavage after piperidine treatment is 80% at the guanine base directly adjacent to the binding site and 88% overall. In an 837 bp restriction fragment containing two adjacent inverted repeats of the third strand binding site and a single 3′-guanine base, yields of single-strand cleavage are 87% on each strand at the 3′-guanine base. Double-strand cleavage was obtained in 61% yield at a single site in a 6.6 kbp plasmid containing the 837 bp fragment. Extension of triple helix mediated DNA alkylation from the pyrimidine to purine motif formally extends the number of sites in duplex DNA that can be cleaved in a sequence-specific and nucleotide-specific manner in good yields.

Oligodeoxyribonucleotide-directed triple helix formation is a general method for the sequence-specific recognition of double-helical DNA (Moser & Dervan, 1987; Le Doan et al., 1987). Oligodeoxyribonucleotides bind to a broad range of sequences in duplex DNA to form stable triple-helical complexes which are sensitive to single-base mismatches (Singleton & Dervan, 1992; Beal & Dervan, 1992; Best & Dervan, 1995; Greenberg & Dervan, 1995). Two classes of triplex DNA are known to exist. In the pyrimidine motif, pyrimidine oligodeoxyribonucleotides bind in a parallel orientation to purine tracts of the underlying duplex by forming specific Hoogsteen hydrogen bonds in the major groove (Moser & Dervan, 1987; Praseuth et al., 1988; Rajagopal & Feigon, 1989; de los Santos et al., 1989; Radhakrishnan et al., 1991a). In this motif, thymine (T) recognizes adenine in an adenine-thymine base pair (AT) to form a T·AT triplet, and protonated cytosine (C^+) in the third strand forms a C^+·GC triplet. In the purine motif, G·GC, A·AT, and T·AT triplets result from the formation of reverse Hoogsteen hydrogen bonds in the major groove of DNA between an antiparallel purine-rich third strand and the purine strand of the duplex (Cooney et al., 1988; Beal & Dervan, 1991; Pilch et al., 1991; Durland et al., 1991; Radhakrishnan et al., 1991b, 1993; Radhakrishnan & Patel, 1993; Svinarchuk et al., 1995).

Triple helix formation has been utilized to afford enzyme-mediated, site-specific cleavage of duplex DNA. An oligonucleotide attached to a staphylococcal nuclease adduct produces double-strand cleavage of plasmid DNA in 75% yield (Pei et al., 1990). Cleavage occurs at multiple positions adjacent to the oligonucleotide binding site, likely due to conformational flexibility of the nuclease. A second ap-

proach, termed Achilles heel cleavage, involves transient protection of an oligonucleotide binding site on duplex DNA by triple helix formation. Methylation of the protected duplex, followed by deprotection, and then treatment with a restriction endonuclease afford cleavage of the duplex DNA at the triplex binding site. The Achilles heel method has been used to effect the single-site, double-strand cleavage of yeast chromosome III (340 kbp) in 94% yield (Strobel & Dervan, 1991).

For a strictly chemical approach, triple helix forming oligonucleotides have been tethered to DNA cleaving moieties. Mechanisms of cleavage include (i) oxidation of the deoxyribose ring, (ii) covalent photo-cross-linking, and (iii) electrophilic alkylation of the bases. Fe-EDTA[1] attached to pyrimidine-rich oligonucleotides produces double-strand cleavage of duplex DNA by an oxidative mechanism (Moser & Dervan, 1987; Strobel et al., 1988; Strobel & Dervan, 1990; Beal & Dervan, 1991). Although cleavage is typically inefficient (6−25%), the Fe-EDTA moiety generates a nonspecific diffusible oxidant that produces multiple cleavage sites which provide information with regard to issues of ligand orientation and groove location (Dervan, 1986, 1991). Under UV irradiation, 3-azidoproflavine (Le Doan et al., 1987) and *p*-azidophenacyl (Praseuth et al., 1988) modified oligonucleotides react with duplex DNA to form photo-cross-linked species which can be cleaved under alkaline conditions. Cross-linking occurs at bases adjacent to the oligonucleotide binding site to produce single-strand cleavage of the DNA duplex in low yield.

The most efficient and selective chemical method for site-specific cleavage of duplex DNA entails the use of *nondif-*

[1] Abbreviations: bp, base pair(s); EDTA, ethylenediaminetetraacetic acid; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; HPLC, high-performance liquid chromatography; MALDI TOF, matrix-assisted laser desorption ionization time-of-flight; NMR, nuclear magnetic resonance; TE, Tris-HCl/EDTA; TFA, trifluoroacetyl; THF, tetrahydrofuran; TLC, thin-layer chromatography; UV, ultraviolet; FAB-MS, fast atom bombardment mass spectrometry.

fusible alkylating electrophiles. When attached to oligonucleotides, these electrophiles are nucleotide-specific, targeting the N-7 position of guanine bases located at discrete sites adjacent to the triple-helical complex. Oligonucleotides equipped with *N*-bromoacetyl (Povsic & Dervan, 1990; Povsic et al., 1992) and ethano-5-methyldeoxycytidine (Shaw et al., 1991) alkylate duplex DNA on one strand in very high yields (>95%). Quantitative cleavage of the duplex DNA is effected by treatment with base (e.g., piperidine) at high temperatures (90−95 °C).

An *N*-bromoacetyl electrophile attached to the 5-position of a thymine at the 5′-end of a pyrimidine oligonucleotide binds in the major groove of duplex DNA and alkylates a guanine two base pairs to the 5′-side of the oligonucleotide binding site (Povsic & Dervan, 1990; Povsic et al., 1992). The oligonucleotide is covalently attached to the target sequence by the reaction of the electrophilic bromo-substituted carbon atom of *N*-bromoacetyl with the N-7 position of the guanine. Piperidine treatment of alkylated DNA product results in depurination and strand cleavage. By reacting the *N*-bromoacetyloligonucleotide with adjacent inverted repeats of the oligonucleotide binding site, double-strand cleavage was obtained at a single site on a 6.7 kbp linearized plasmid in 85% yield and on a 340 kbp yeast chromosome in 85−90% yield (Povsic et al., 1992). Advantages conferred by the use of the *N*-bromoacetyl functionality are the nondiffusible and nucleotide-specific nature of the electrophile, high reactivity toward DNA and low reactivity toward aqueous buffer components under physiologically relevant conditions (37 °C, pH 7.4), and the production of 5′- and 3′-phosphate termini (Baker & Dervan, 1989) which may be ligated to complementary DNA restriction fragments (Povsic et al., 1992).

Methods of triple helix mediated DNA cleavage employing *N*-bromoacetyl and other nondiffusible DNA cleaving moieties have so far been limited to pyrimidine motif recognition. Application of *N*-bromoacetyl methodology to the purine motif would greatly extend the number of unique sites in double-helical DNA that can be cleaved in a nucleotide-specific and sequence-specific manner. In the pyrimidine motif, $5'\text{-(pyrimidine)}_m\text{(purine)}_n\text{-}3'$-type DNA sequences correspond to potential sites for double-strand cleavage (Figure 1A). Double-strand cleavage in the purine motif would afford recognition sequences of the $5'\text{-(purine)}_m\text{(pyrimidine)}_n\text{-}3'$ type, enlarging the sequence repertoire substantially (Figure 1B).

Here we report that an *N*-bromoacetyl electrophile attached to the 5′-phosphate group of a thymine at the 5′-end of a purine-rich oligonucleotide alkylates guanine bases adjacent to the 3′-side of an oligonucleotide binding site in a purine motif triple-helical complex. Control experiments conducted with a 645 bp restriction fragment containing three contiguous guanine bases to the 3′-side of the triple helix binding site revealed that the duplex was cleaved on one strand and that the major site of cleavage was at a single guanine base immediately adjacent to the triplex site. Through the design of a construct containing adjacent inverted repeats of the oligonucleotide binding site and a single 3′-guanine base, single-strand cleavage was obtained in high yield at the 3′-guanine base on each strand of an 837 bp restriction fragment. Using this approach, double-strand cleavage was achieved at a single site in a 6.6 kbp plasmid in good yield.
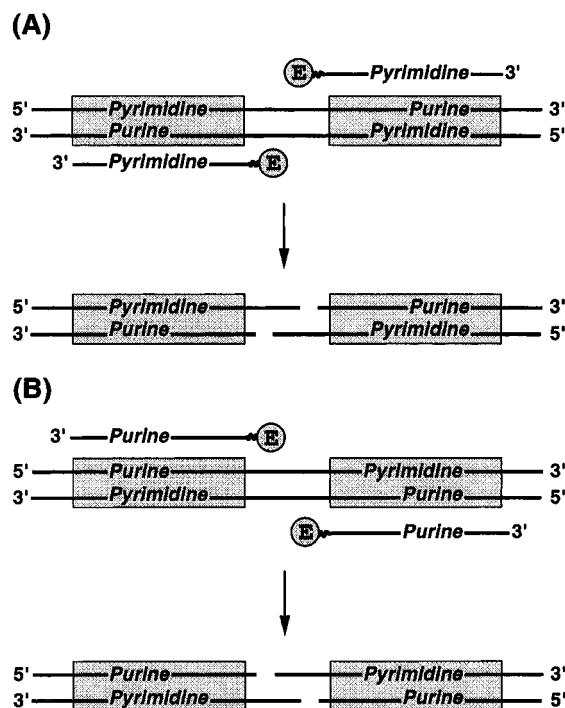
(A)

(B)

FIGURE 1: Double-strand alkylation of duplex DNA mediated by triple helix formation. Piperidine treatment results in cleavage of both DNA strands. (A) Pyrimidine-rich (*N*-bromoacetyl)oligonucleotides binding to adjacent inverted purine tracts on duplex DNA in a *pyrimidine motif* triple-helical complex target $5'\text{-(pyrimidine)}_m\text{-(purine)}_n\text{-}3'$-type sequences on the sense strand. (B) Purine-rich (*N*-bromoacetyl)oligonucleotides binding to adjacent inverted purine tracts on duplex DNA in a *purine motif* triple-helical complex target sequences of the $5'\text{-(purine)}_m\text{(pyrimidine)}_n\text{-}3'$ type.

MATERIALS AND METHODS

Materials and General Methods. NMR spectra were recorded on a General Electric QE 300 spectrometer operating at 300 MHz. Chemical shifts were reported using the isotopic impurity peak of the NMR solvent as an internal standard. A Hewlett Packard 8452A diode array spectrophotometer was used for UV−visible spectra. High-resolution FAB mass spectra were recorded by the Mass Spectrometry Facility at the California Institute of Technology with a VG ProSpec mass spectrometer. MALDI TOF mass spectra were recorded on a Vestec Voyager RP research-grade instrument by the Protein/Peptide Micro Analytical Facility at the Beckman Institute (California Institute of Technology). Reagents used were of the highest available purity and were obtained from Aldrich Chemical Co. except for β-(cyanoethyl)-*N*,*N*-(diisopropylchloro)phosphoramidite which was obtained from Sigma Chemical Co. Absolute ethanol was purchased from Quantum Chemical, and anhydrous THF stored over molecular sieves was purchased from Fluka. Analytical thin-layer chromatography was performed using 5 cm × 7.5 cm 200 μm silica-coated aluminum sheets with a UV fluorescent indicator (E. Merck). Mesh 230−400 silica gel 60 (E. M. Science) was used for flash chromatography. All enzymes were used according to the manufacturers' instructions in the supplied buffers. Restriction endonucleases were obtained from New England Biolabs or from Boehringer Mannheim. Radioactive nucleotides were obtained from Amersham Life Science.

N-(Trifluoroacetyl)-5-amino-1-pentanol **2**. Commercially available 5-amino-1-pentanol, **1** (1.05 mL, 9.69 mmol),

was dissolved in absolute ethanol (11.85 mL). Ethyl trifluoroacetate (3.46 mL, 29.1 mmol), dissolved in 1.21 mL of ethanol, was added by dropwise addition. The reaction was refluxed under argon for 3 h. The product mixture was concentrated *in vacuo*, redissolved in 4% MeOH in CH_2Cl_2, and loaded onto a flash column. The *N*-TFA-aminopentanol **2** was eluted with 6% MeOH in CH_2Cl_2 and was concentrated *in vacuo* to a clear, colorless oil (1.50 g, 77.9% from **1**): TLC 6% MeOH, CH_2Cl_2, ruddy-brown spot (ninhydrin), $R_f = 0.26$; ^{1}H NMR (CDCl$_3$) δ 6.58 (bs, 1 H), 3.64−3.69 (t, 2 H, $J = 6.2$ Hz), 3.35−3.41 (m, 2 H), 1.56−1.68 (m, 4 H), 1.42−1.49 (m, 2 H); ^{13}C NMR (CDCl$_3$) δ 157.4 (q, $J = 36.4$ Hz), 115.9 (q, $J = 287.5$ Hz), 62.3, 39.8, 31.7, 28.4, 22.8; high-resolution FAB-MS calculated for C_7, F_3, H_{13}, N_1, O_2 [MH]$^+$ 200.0898, found 200.0892.

N-TFA-(Aminopentyl)phosphoramidite 3. Phosphoramidite **3** was prepared from *N*-(trifluoroacetyl)-5-amino-1-pentanol **2** (250.0 mg, 1.26 mmol) according to a procedure reported for *N*-(trifluoroacetyl)-6-aminohexyl-β-(cyanoethyl)-*N,N*-diisopropylphosphoramidite (Sinha & Striepeke, 1991). Flash column chromatography (25% EtOAc/5% Et$_3$N in hexane) afforded a clear, colorless oil (319.2 mg, 63.7% from **2**): TLC 25% EtOAc/5% Et$_3$N in hexane, bright yellow spot (ninhydrin), $R_f = 0.38$; ^{1}H NMR (CDCl$_3$) δ 6.79 (bs, 1 H), 3.70−3.88 (m, 2 H), 3.52−3.69 (m, 4 H), 3.31−3.38 (m, 2 H), 2.61−2.65 (t, 2 H, $J = 6.2$ Hz), 1.56−1.65 (m, 4 H), 1.38−1.48 (m, 2 H), 1.15−1.17 (d, 12 H, $J = 6.6$ Hz); high-resolution FAB-MS calculated for C_{16}, F_3, H_{30}, N_3, O_3, P_1 [MH]$^+$ 400.1977, found 400.1990.

Oligodeoxyribonucleotide Amine 5. The oligodeoxyribonucleotide 5′-d(TGGGGTGGGGTGGGT)-3′ was synthesized by standard automated solid-support β-(cyanoethyl)-phosphoramidite chemistry using an Applied Biosystem Model 394 DNA synthesizer. *N*-TFA-(Aminopentyl)phosphoramidite **3** was directly incorporated onto the 5′-terminus by automated synthesis. The TFA protecting group was removed during the ammonium hydroxide deprotection step (30% aqueous ammonium hydroxide, 45 °C, 24 h) to produce oligodeoxyribonucleotide amine **5**. The coupling efficiency of the *N*-TFA-(aminopentyl)phosphoramidite was estimated to be ∼95% by UV-shadowing of crude oligodeoxyribonucleotide **5** resolved on an analytical 20% denaturing polyacrylamide gel (19:1 monomer/bis). Oligodeoxyribonucleotide amine **5** was then purified by two rounds of preparative polyacrylamide gel electrophoresis. DNA was eluted from an excised gel band by the crush and soak method (100 mM NaCl in TE, pH 7.5, 37 °C, 14 h), filtered through a 0.45-μm Centrex filter (Schleicher & Schuell), and desalted on a Sep-Pak Plus C18 cartridge (Waters). The recovery of purified oligodeoxyribonucleotide **5** was determined by UV−visible spectrophotometry. The DNA was lyophilized and stored at −20 °C: UV(H$_2$O) $\lambda_{max} = 254$ nm, $\epsilon_{260} = 145\,900$ M^{-1} cm^{-1}; MALDI TOF-MS calculated for [M-H]$^-$ 4940.2, found 4940.6.

(N-Bromoacetyl)oligonucleotide 6. Oligodeoxyribonucleotide amine **5** (10 nmol in 10 μL of 200 mM sodium tetraborate buffer, pH 8.5) was treated with 250 mM *N*-hydroxysuccinimidyl bromoacetate in DMF (10 μL) at room temperature for 5 min. The (*N*-bromoacetyl)oligonucleotide product was precipitated with 0.3 M sodium acetate, pH 5.2, in 3 volumes of EtOH, washed with 70% EtOH, and then dissolved in water. The solution was desalted on a NAP 5 column (Pharmacia Biotech). The (*N*-

bromoacetyl)oligonucleotide (eluted from the column in 1 mL of deionized, distilled water) was quantitated by UV−visible spectrophotometry and then stored in solution at −78 °C. Analysis by analytical reverse-phase HPLC (Rainin Microsorb-MV, 5 μm C18, 4.6 mm × 250 mm column) using a gradient of 0−50% CH$_3$CN (1.25%/min) in 100 mM TEAA, pH 6.5, indicated clean conversion of starting amine **5** to product **6** (∼89%).

Plasmid Construction. The plasmid pPB19AG was prepared previously (Beal & Dervan, 1991). Plasmid pUCLEU2DS6 was prepared by ligating a duplex containing the sequence 5′-d(AGGGAGGGGGAGGGGAGAATTCTC-CCCTCCCCTCCCT)-3′ into *Xho*I-digested pUCLEU2 (Povsic et al., 1992). DNA was purified using a Quiagen plasmid mega kit. The sequence of the inserted duplex was confirmed by dideoxy sequencing using a Sequenase Version 2.0 DNA kit (United States Biochemical). Other biochemical manipulations including the transformation of competent *E. coli* cells (Stratagene, XL-1 blue) were performed according to established laboratory protocol (Sambrook et al., 1989).

Alkylation and Cleavage of Duplex DNA at Nucleotide Resolution. *Hin*dIII-linearized pPB19AG was 3′-end-labeled using Sequenase Version 2.0 (United States Biochemical) and [α-^{32}P]ddATP. Unincorporated nucleotides were removed with a G-50 Sephadex spun column (Boehringer Mannheim). The DNA was then digested with *Ssp*I to produce restriction fragments 645 and 2054 bp in length which were resolved on a 1% agarose gel. The 645 bp fragment containing the target site was excised, and DNA was eluted by spinning the gel slice through siliconized glass wool. DNA was precipitated from the eluate with 0.3 M sodium acetate, pH 5.2, in 3 volumes of EtOH, washed with 70% EtOH, dissolved in deionized, distilled water, and then desalted on a NICK column (Pharmacia Biotech). The purified DNA was diluted to a concentration of 1200 cpm/μL with deionized, distilled water and was stored at −78 °C until use.

Plasmid pUCLEU2DS6 was digested with *Nar*I to produce 5466 and 1134 bp fragments. An aliquot of the DNA was labeled using a DNA 5′-end-labeling kit (Boehringer Mannheim) and [γ-^{32}P]ATP according to the manufacturer's instructions. A second aliquot was labeled at the 3′-end with Sequenase Version 2.0 and [α-^{32}P]dCTP. For both the 3′- and 5′-end-labeling reactions, unincorporated nucleotides were removed with a G-50 Sephadex spun column. Labeled DNA was digested with *Pst*I to produce restriction fragments 3073, 2393, 837, and 297 bp in size. The digest was resolved on a 1% agarose gel, and the 837 bp fragment containing the target sequence was excised from the gel and purified as described above.

Typical reaction conditions for alkylation experiments were 1 μM (*N*-bromoacetyl)oligonucleotide **6**, 20 mM HEPES, pH 7.4, 0.8 mM Co(NH$_3$)$_6$Cl$_3$, and 9600 cpm of DNA in a total volume of 20 μL. Reactions were incubated at 37 °C for 24 h, precipitated with 40 μg of glycogen and 0.3 M sodium acetate, pH 5.2, in 2.5 volumes of EtOH, and washed with 70% EtOH. The DNA was dissolved in 100 μL of 1.0% piperidine, heated at 90 °C for 30 min, and then lyophilized to dryness. Cleavage products were separated on denaturing polyacrylamide gels (8.0% for pPB19AG and 6% for pUCLEU2DS6). To determine cleavage yields, the gels were scanned with a Molecular Dynamics 400S PhosphorImager. The storage-phospor autoradiograms were

quantitated using Image Quant v. 3.22 software (Molecular Dynamics). Chemical DNA sequencing reactions were performed as described (Iverson & Dervan, 1987; Williamson & Celander, 1990).

Double-Strand Alkylation and Cleavage of Plasmid DNA. *Sal*I-linearized pUCLEU2DS6 was 3′-end-labeled with Sequenase Version 2.0 and [α-^{32}P]TTP. Unincorporated nucleotides were removed with a G-50 Sephadex spun column. The eluate was extracted 3 times with phenol/chloroform/isoamyl alcohol (25:24:1) and once with chloroform/isoamyl alcohol (24:1). DNA was precipitated in 0.3 M sodium acetate, pH 5.2, in 3 volumes of EtOH, washed with 70% EtOH, and then desalted on a NICK column. The DNA was diluted to a final concentration of 1200 cpm/μL with deionized, distilled water.

Alkylation reactions were 1 μM (*N*-bromoacetyl)oligonucleotide **6**, 20 mM HEPES, pH 7.4, 0.8 mM Co(NH$_3$)$_6$-Cl$_3$, and 12 000 cpm of DNA in a total volume of 20 μL. After incubation at 37 °C for 24 h, the DNA was precipitated with 40 μg of glycogen and 0.3 M sodium acetate, pH 5.2, in 2.5 volumes of EtOH, washed with 70% EtOH, and dissolved in 50 μL of 0.25% piperidine, 100 mM NaCl, 10 mM Tris-HCl pH 8, and 10 mM EDTA. The reactions were topped with light mineral oil and then heated at 65 °C for 12 h. Cleavage products were resolved on a 0.8% agarose gel. Yields were determined by Molecular Dynamics PhosphorImager technology as described above. A molecular weight marker comprised of restriction fragments 6634, 4634, 3500, 3134, and 2000 bp in length was obtained by partial digestion of the labeled DNA with *Nar*I.

Kinetics. Alkylation reactions were performed as described above. Individual reactions were quenched by EtOH precipitation after 2, 3, 4, 6, 8, 10, 22, and 46 h. Piperidine treatment, electrophoresis, and gel quantitation were performed as described. A plot of ln ([DNA]$_{intact}$/[DNA]$_{total}$) as a function of time was linear over two half-lives, indicating first-order kinetics. Rate constants and half-lives were then derived from linear regression curve fits ($k = -$slope, $t_{1/2} = 0.693/k$).

RESULTS AND DISCUSSION

Synthesis of (N-Bromoacetyl)oligonucleotide **6**. The design of (*N*-bromoacetyl)oligonucleotide **6** was based on model building studies of a purine motif triple-helical complex. These studies suggested that alkylation of a guanine base adjacent to the 3′-side of the triple helix binding site could be achieved by using an aminopentyl linker to tether the *N*-bromoacetyl moiety to the 5′-phosphate group of the oligonucleotide. The scheme used to synthesize (*N*-bromoacetyl)oligonucleotide **6** is outlined in Figure 2. (*N*-Trifluoroacetamido-β-(cyanoethyl)-*N,N*-diisopropylphosphoramidite **3** was synthesized in two steps from known starting material. Treatment of 5-amino-1-pentanol **1** with ethyl trifluoroacetate afforded the corresponding *N*-trifluoroacetamido alcohol **2** which was reacted with β-(cyanoethyl)-*N,N*-(diisopropylchloro)phosphoramidite to give phosphoramidite **3**. The *N*-trifluoroacetamido-modified oligodeoxyribonucleotide **4** was synthesized by standard automated solid-support β-(cyanoethyl)phosphoramidite chemistry with phosphoramidite **3** directly incorporated onto the 5′-terminus during the synthesis. The base-labile TFA protecting group was removed during the ammonium hydroxide deprotection step
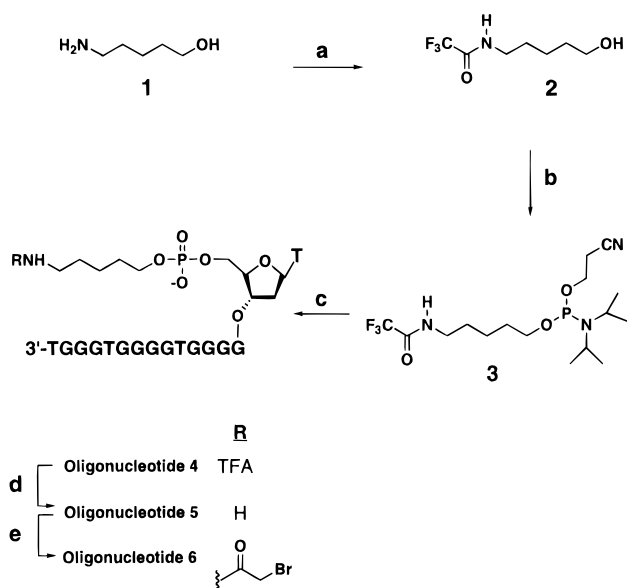

FIGURE 2: Synthesis of (*N*-bromoacetyl)oligonucleotide **6**: (a) CF$_3$-CO$_2$C$_2$H$_5$, EtOH, reflux, 78%; (b) (i) diisopropylethylamine, THF, 0 °C; (ii) β-(cyanoethyl)-*N,N*-(diisopropylchloro)phosphoramidite, room temperature, 64%; (c) standard automated DNA synthesis; (d) NH$_4$OH/H$_2$O, 45 °C; (e) *N*-hydroxysuccinimidyl bromoacetate, DMF/100 mM sodium tetraborate buffer, pH 8.5, room temperature.

to produce oligodeoxyribonucleotide amine **5**. The coupling efficiency of the *N*-TFA-aminophosphoramidite was estimated to be ~95% by analytical polyacrylamide gel electrophoresis. The amino-modified oligonucleotide was then reacted with *N*-hydroxysuccinimidyl bromoacetate to produce (*N*-bromoacetyl)oligonucleotide **6**.

Single-Strand Alkylation and Cleavage of Duplex DNA. Preliminary studies were conducted using plasmid pPB19AG (Beal & Dervan, 1991) which contains the well-characterized purine motif triple helix binding site 5′-d(AGGGAGGG-GAGGGGA)-3′ (Greenberg & Dervan, 1995) as well as three guanine bases in the duplex directly adjacent to the 3′ end of the site. These initial experiments would indicate the efficiency and specificity of single-stand alkylation of duplex DNA by (*N*-bromoacetyl)oligonucleotide **6**. Plasmid pPB19AG was linearized with *Hin*dIII, 3′-end-labeled with [α-^{32}P]ddATP, and then cut with *Ssp*I to produce a 645 bp restriction fragment containing the sequence of interest. The radiolabeled DNA (9600 cpm) was treated with (*N*-bromoacetyl)oligonucleotide **6** at 1 μM concentration [20 mM HEPES, pH 7.4, 0.8 mM Co(NH$_3$)$_6$Cl$_3$]. The alkylation reactions were incubated at 37 °C for 24 h. Treatment with 1.0% piperidine (90 °C, 30 min) effected single-strand cleavage of the alkylated duplex DNA. Cleavage efficiencies were determined by storage-phosphor autoradiography of reaction products separated on an 8.0% denaturing polyacrylamide gel. The analysis revealed that the 645 bp restriction fragment was cleaved in 80 ± 2% yield (averaged over two trials) at the guanine base immediately adjacent to the triplex site. Minor cleavage was observed at the guanines one and two bases removed (7% and 1% yield, respectively). Alkylation experiments conducted with 5′-end-labeled DNA demonstrated that no modification occurred on the opposite strand of the duplex.

Time course experiments were conducted in order to determine rate constants for alkylation of the 645 bp *Hin*dIII/*Ssp*I pPB19AG restriction fragment by (*N*-bromoacetyl)-
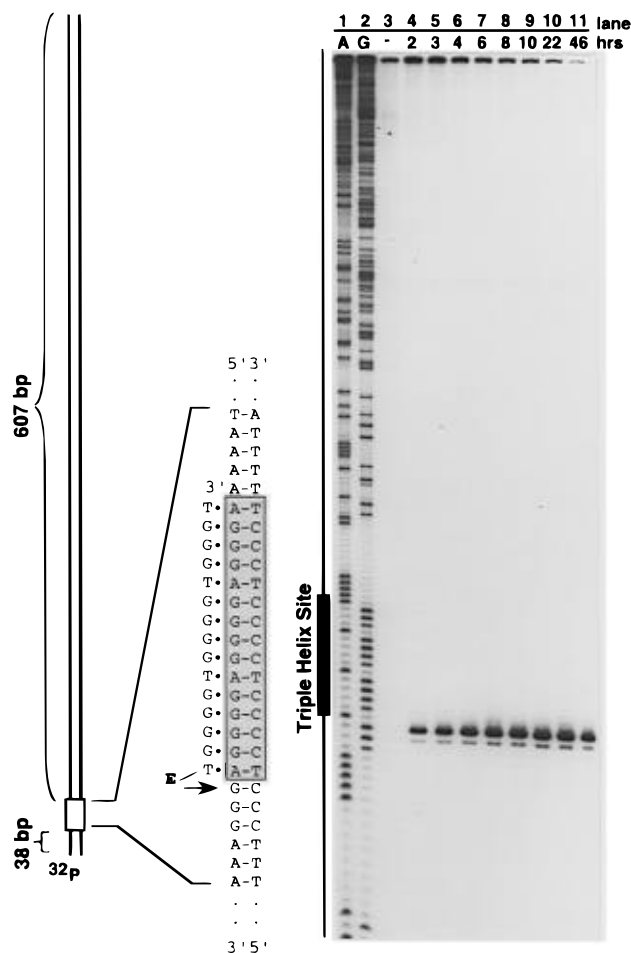
FIGURE 3: (Left) 645 bp *Hin*dIII/*Ssp*I restriction fragment derived from pPB19AG (Beal & Dervan, 1991) showing the location and sequence of the triple-helical complex. The major position of alkylation is indicated with an arrow. (Right) Autoradiogram of an 8.0% denaturing polyacrylamide gel of a time course experiment involving the reaction of (*N*-bromoacetyl)oligonucleotide **6** with the ^{32}P 3′-end-labeled 645 bp fragment. Lane 1: A-specific chemical sequencing reaction (Iverson & Dervan, 1987). Lane 2: G-specific chemical sequencing reaction (Williamson & Celander, 1990). Lane 3: 645 bp restriction fragment incubated in the absence of (*N*-bromoacetyl)oligonucleotide **6** and treated with piperidine. Lanes 4−11: same restriction fragment incubated in the presence of (*N*-bromoacetyl)oligonucleotide **6** for 2, 3, 4, 6, 8, 10, 22, and 46 h and then treated with piperidine.

Table 1: First-Order Rate Constants (k) and Half-Lives ($t_{1/2}$) for Single-Strand Alkylation Reactions at 37 °C for the 645 bp Fragment, pPB19AG[a]

site[b]	k (s^{-1})[c]	$t_{1/2}$ (h)[c]
G1	3.5 ($\pm$0.1) $\times$ 10^{-5}	5.5 ($\pm$0.2)
G2	1.7 ($\pm$0.1) $\times$ 10^{-6}	115.8 ($\pm$3.6)
G3	1.1 ($\pm$0.2) $\times$ 10^{-7}	1744.0 ($\pm$258.0)

[a] Data are averaged over 3 trials. [b] Site of alkylation. G1 is the guanine base adjacent to the 3′-end of the triplex binding site; G2 and G3 are one and two bases removed, respectively. [c] Errors are given as standard deviation.
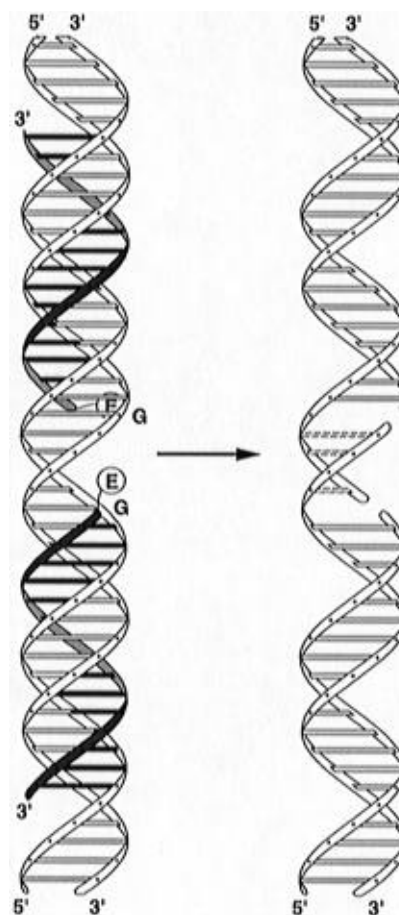
FIGURE 4: Double-strand cleavage of duplex DNA mediated by a purine motif triple-helical complex. Identical (*N*-bromoacetyl)-oligonucleotides binding to adjacent inverted purine tracts on the duplex alkylate one guanine base to the 3′-side of each binding site. Depurination and strand cleavage are effected by warming in the presence of piperidine.

oligonucleotide **6**. Alkylation reactions performed in parallel were incubated at 37 °C. After 2, 3, 4, 6, 8, 10, 22, and 46 h time intervals, individual reactions were quenched by EtOH precipitation. Following piperidine treatment, reaction products were resolved on a denaturing polyacrylamide gel (Figure 3). Values for [DNA]$_{intact}$ and [DNA]$_{total}$ were derived from analyses of the storage-phosphor autoradiograms. First-order kinetics were indicated by linear plots of ln ([DNA]$_{intact}$/[DNA]$_{total}$) vs time. Rate constants and half-lives are summarized in Table 1. Within the purine motif triple-helical complex, the half-life for alkylation is 5.5 h at the guanine immediately adjacent to the 3′-end of the triplex binding site. Rates of alkylation at the guanines one and two bases removed are 20 and 333 times slower than that of the major site of reaction. For comparison, the half-life reported for site-specific alkylation by an (*N*-bromoacetyl)oligonucleotide in a pyrimidine motif triple-helical complex is 6.2 h at the major site of alkylation (Povsic & Dervan, 1990).

Double-Strand Cleavage of Duplex DNA at Nucleotide Resolution. Incorporation of two adjacent inverted triplex binding sites into duplex DNA would afford cleavage of both DNA strands. The specificity of the single-strand cleavage experiments indicated that an optimal design would entail placement of a single guanine base immediately adjacent to the 3′-side of each triplex binding site (Figure 4). Based on these considerations, pUCLEU2DS6 was prepared by ligating a duplex containing the sequence 5′-d(AGGGAGGGGAGGG-GAGAATTCTCCCCTCCCCTCCCT)-3′ into *Xho*I-digested pUCLEU2 (Povsic et al., 1992). Restriction fragments from a *Nar*I digest of pUCLEU2DS6 were 5′-end-labeled with [γ-^{32}P]ATP or 3′-end-labeled with [α-^{32}P]dCTP. The labeled DNA was digested with *Pst*I to produce restriction fragments 837 bp in size containing the inverted repeats. DNA labeled

FIGURE 5: (Left) 837 bp restriction fragment obtained by digesting pUCLEU2DS6 with *Nar*I and *Pst*I. The location and sequence of the adjacent triple-helical complexes and the positions of alkylation are indicated. (Right) Autoradiogram of a 6% denaturing polyacrylamide gel revealing cleavage products at nucleotide resolution obtained from the reaction of (*N*-bromoacetyl)oligonucleotide **6** with the 837 bp restriction fragment. Lanes 1−4 contain 3′-end-labeled DNA. Lanes 5−8 contain 5′-end-labeled DNA. Lanes 1 and 8: A-specific chemical sequencing reactions (Iverson & Dervan, 1987). Lanes 2 and 7: G-specific chemical sequencing reactions (Williamson & Celander, 1990). Lanes 3 and 6: 837 bp restriction fragment incubated in the absence of (*N*-bromoacetyl)oligonucleotide **6** and treated with piperidine. Lanes 4 and 5: 837 bp restriction fragment incubated in the presence of (*N*-bromoacetyl)-oligonucleotide **6** and treated with piperidine.

Table 2: First-Order Rate Constants (*k*) and Half-Lives (*t*$_{1/2}$) for Double-Strand Alkylation Reactions at 37 °C for the 837 bp Fragment, pUCLEU2DS6[a]

label[b]	site[c]	k (s^{-1})[d]	$t_{1/2}$ (h)[d]
3′	G1	7.7 (±0.1) × 10^{-5}	2.5 (±0.1)
5′	G1	6.9 (±0.4) × 10^{-5}	2.8 (±0.2)

[a] Data are averaged over 4 trials. [b] Position of ^{32}P end-label. [c] Site of alkylation. G1 is the guanine base adjacent to the 3′-end of the triplex binding site. [d] Errors are given as standard deviation.

FIGURE 6: (Left) *Sal*I-linearized plasmid pUCLEU2DS6 (6.6 kbp) with the location and sequence of the adjacent triple-helical complexes. The positions of alkylation are indicated with arrows. (Right) Autoradiogram of a 0.8% nondenaturing agarose gel showing cleavage products from the reaction of (*N*-bromoacetyl)-oligonucleotide **6** with *Sal*I-linearized pUCLEU2DS6 labeled with ^{32}P at both 3′-ends. Lane 1: molecular weight marker consisting of *Sal*I-linearized pUCLEU2DS6 partially digested with *Nar*I. Lane 2: linearized DNA incubated in the absence of (*N*-bromoacetyl)-oligonucleotide **6** and treated with piperidine. Lane 3: linearized DNA incubated in the presence of (*N*-bromoacetyl)oligonucleotide **6** and treated with piperidine.

on either strand (9600 cpm) was reacted with (*N*-bromoacetyl)oligonucleotide **6** at 1 μM concentration [20 mM HEPES, pH 7.4, 0.8 mM Co(NH$_3$)$_6$Cl$_3$] at 37 °C for 24 h. The alkylated DNA was treated with 1.0% piperidine at 90 °C for 30 min and then resolved on a 6.0% denaturing polyacrylamide gel (Figure 5). Analysis of the storage-phosphor autoradiogram confirmed that cleavage occurred at a single guanine base on each strand at the position immediately adjacent to the 3′-end of the triplex binding site. Cleavage efficiencies calculated over four trials were 87 ± 4% and 87 ± 1% for the 3′-end-labeled strand and the 5′-end-labeled strand, respectively. DNA controls incubated in the absence of (*N*-bromoacetyl)oligonucleotide **6** were not cleaved. The high efficiency of alkylation associated with the triplex sites suggests that triple helix formation by the guanine-rich (*N*-bromoacetyl)oligonucleotide was not significantly inhibited by self-alkylation or by the formation of guanine quartet structures (Henderson et al., 1987; William-

son et al., 1989). In addition, the location of the cleavage shows that the (*N*-bromoacetyl)oligonucleotide was aligned in the expected antiparallel orientation with respect to the purine strand of the duplex, consistent with the formation of a purine motif triple-helical complex.

In order to determine rate constants for alkylation of the 837 bp *Nar*I/*Pst*I pUCLEU2DS6 restriction fragment, individual alkylation reactions were quenched by EtOH precipitation after 2, 3, 4, 6, 8, 10, 22, and 46 h time intervals.

Kinetic data were derived from linear plots of ln ([DNA]$_{intact}$/[DNA]$_{total}$) vs time (Table 2). Half-lives of alkylation at the guanine base adjacent to the 3′-side of each triplex binding site are 2.5 and 2.8 h for the 3′- and 5′-end-labeled strands, respectively. These values are somewhat faster than the half-life at the major site of alkylation in the 645 bp *Hin*dIII/*Ssp*I pPB19AG restriction fragment used for single-strand cleavage experiments (5.5 h, Table 1). The difference may be due in part to sequence-dependent and/or alkylation-dependent variations in local secondary structure and to the presence of two additional flanking guanine bases on the pPB19AG fragment which compete for alkylation.

Double-Strand Alkylation and Cleavage of Plasmid DNA. To determine the yield of double-strand cleavage, plasmid pUCLEU2DS6 was linearized with *Sal*I and 3′-end-labeled with [α-^{32}P]dTTP. (*N*-Bromoacetyl)oligonucleotide **6** (1 μM) was reacted with the radiolabeled DNA (12 000 cpm) at 37 °C for 24 h [20 mM HEPES, pH 7.4, 0.8 mM Co-(NH$_3$)$_6$Cl$_3$]. The alkylated plasmid was cleaved under nondenaturing conditions by treatment with 0.25% piperidine at 65 °C for 12 h. Reaction products were resolved on a 0.8% nondenaturing agarose gel (Figure 6). A storage-phosphor autoradiogram of the gel showed that the 6.6 kbp linearized plasmid was cleaved at one site, producing discrete fragments 2.2 and 4.4 kbp in size. The average yield of double-strand cleavage calculated over seven trials was 61 ± 7%. In the controls, no cleavage was observed in the absence of (*N*-bromoacetyl)oligonucleotide **6**.

Conclusion. In this study, a purine-rich oligonucleotide was tethered to a *N*-bromoacetyl electrophile to afford sequence-specific and nucleotide-specific alkylation of duplex DNA through the formation of a purine motif triple helix. Using this approach, the single-site, double-strand cleavage of a 6.6 kbp plasmid containing two adjacent inverted triplex binding sites was obtained in 61% yield. Until now, methods of triple helix mediated DNA cleavage employing a nondiffusible DNA cleaving moiety have been limited to pyrimidine motif recognition. By extending this methodology to the purine motif, we have increased the sequence repertoire in duplex DNA that can be targeted for double-strand cleavage. The site-specific alkylation by an (*N*-bromoacetyl)oligonucleotide in the antiparallel purine motif increases the versatility of the triple helix approach for the manipulation of megabase DNA by strictly chemical methods.

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