Kinetic Analysis of Sequence-Specific Alkylation of DNA by Pyrimidine Oligodeoxyribonucleotide-Directed Triple-Helix Formation

Matthew J. Taylor and Peter B. Dervan*

Arnold and Mabel Beckman Laboratories of Chemical Synthesis, Division of Chemistry and Chemical Engineering, California Institute of Technology, Pasadena, California 91125. Received March 12, 1997[⊗]

Attachment of a nondiffusible bromoacetyl electrophile to the 5-position of a thymine at the 5′-end of a pyrimidine oligodeoxyribonucleotide affords sequence-specific alkylation of a guanine base in duplex DNA two base pairs to the 5′-side of a local triple-helical complex. Products resulting from reaction of 5′-^{E}TTTT^{Me}CTTTT^{Me}C^{Me}CTTT^{Me}CTTTT-3′ at 37 °C with a 29 base pair target duplex are determined by a gel mobility analysis to be oligonucleotides terminating in 5′- and 3′-phosphate functional groups, consistent with a mechanism involving alkylation, glycosidic bond cleavage, and base-promoted strand cleavage. The guanine-(linker)-oligonucleotide conjugate formed upon triple-helix-mediated alkylation at the N^7 position of a guanine base in a 60 base pair duplex was identified by enzymatic phosphodiester hydrolysis of the alkylation products followed by reversed phase HPLC analysis. To determine the rate enhancement achieved by oligonucleotide-directed alkylation of duplex DNA, a comparison of rates of alkylation at N^7 of guanine in double-stranded DNA by the *N*-bromoacetyloligonucleotide and 2-bromoacetamide was performed by a polyacrylamide gel assay. The reaction within the triple-helical complex on a restriction fragment was determined at 200 nM *N*-bromoacetyloligonucleotide to have a first-order rate constant k_1 of (2.7 ± 0.5) × 10^{-5} s^{-1} ($t_{1/2}$ = 7.2 h). The reaction of 2-bromoacetamide with a 39 base pair duplex of sequence corresponding to the restriction fragment targeted by triple-helix formation was determined to have a second-order rate constant k_2 of (3.6 ± 0.3) × 10^{-5} M^{-1} s^{-1}. A comparison of the first-order and second-order rate constants for the unimolecular and bimolecular alkylation reactions provides an effective molarity of 0.8 M for bromoacetyl within the triple-helical complex.

INTRODUCTION

The design of sequence-specific DNA-cleaving molecules requires the integration of recognition and cleavage in a single molecule. Three approaches to the bifunctional design of these molecules include attachment of functional groups to DNA-binding molecules that can achieve oxidation of the deoxyribose backbone (*1*), electrophilic modification of the bases (*2*), or hydrolysis of the phosphodiester bond. Of these classes, DNA-binding molecules that perform electrophilic modification of the bases can provide additional specificity due to the different nucleophilicity of positions on the bases in DNA (e.g. N^7 of guanine or N^3 of adenine).

Oligonucleotide-directed triple-helix formation is one of the most powerful methods for the sequence-specific recognition of single sites within megabase pair double-helical DNA (*3*, *4*). Pyrimidine oligodeoxyribonucleotides bind purine tracts in the major groove of DNA parallel to the purine Watson−Crick strand, through formation of specific Hoogsteen hydrogen bonds to the purine Watson−Crick bases (Figure 1). Specificity is derived from thymine (T) recognition of adenine−thymine (AT) base pairs (TAT triplet) and N^3-protonated cytosine (C+) recognition of guanine−cytosine (GC) base pairs (C+GC triplet).

Efforts have been successful in the use of oligonucleotides for directing electrophiles to react at the N^7-position of guanine in the major groove via triple-helix formation (*5*). Early examples utilizing 5′-aromatic chloroethylamine-modified pyrimidine oligonucleotides demonstrated alkylation at adjacent guanine bases with modest yield (*5a*,*b*). Ethano-5-methyl-2′-deoxycytidine residues at the 3′-end of oligonucleotides (*5d*,*e*) and oligonucle-

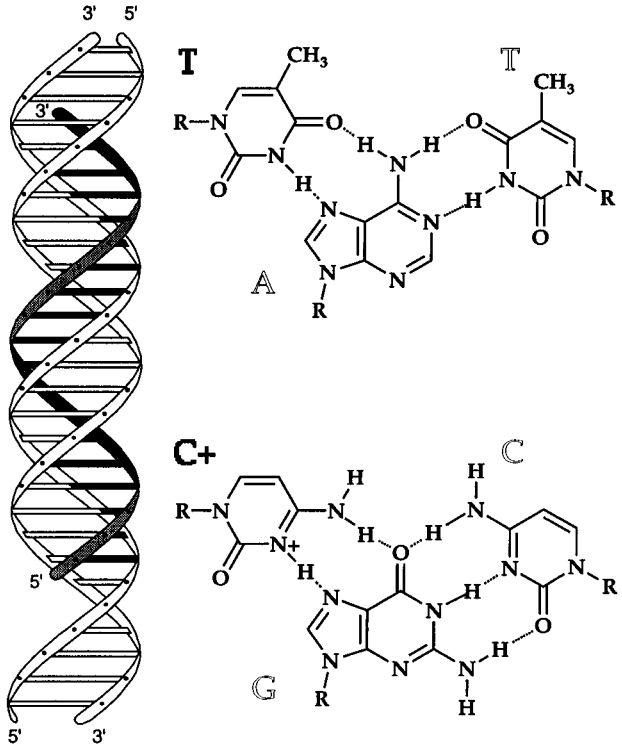

Figure 1. Model representing a pyrimidine-rich oligonucleotide bound in the major groove of double-stranded DNA and base triplets formed upon binding.

* Author to whom correspondence should be addressed [telephone (818) 395-6002; fax (818) 683-8753; e-mail dervan@cco.caltech.edu].

[⊗] Abstract published in *Advance ACS Abstracts,* April 15, 1997.

S1043-1802(97)00035-9 CCC: $14.00

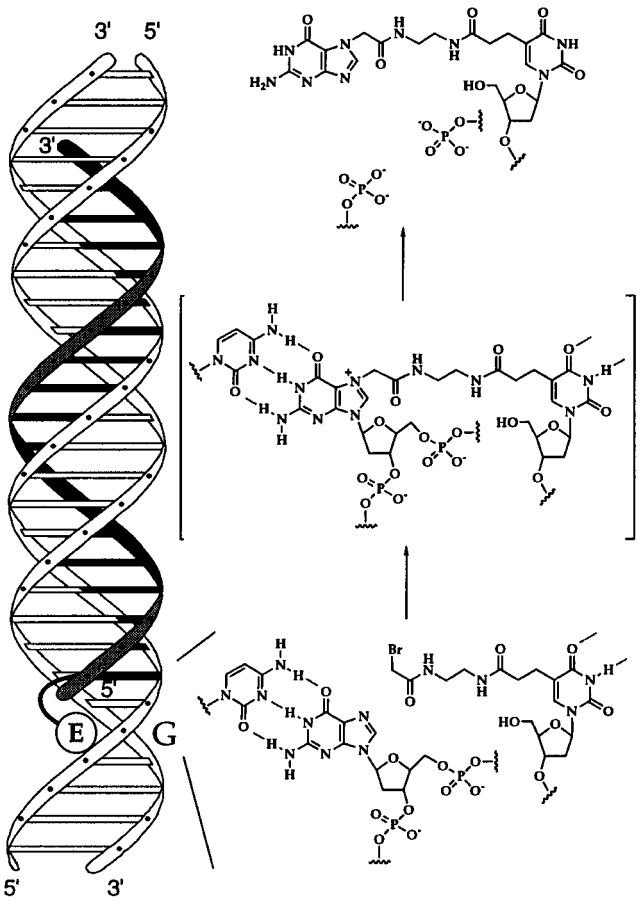

Figure 2. Bromoacetyl electrophile is localized in the major groove by triple-helix formation proximal to a GC base pair (G) in the Watson−Crick duplex target site. Alkylation at N7 of guanine followed by depurination results in cleavage of the deoxyribose backbone.

otides equipped with *N*-bromoacetyl at the 5′-end react with one strand of a duplex DNA target site in very high yield (*5c*). Pyrimidine oligodeoxyribonucleotides bound in the major groove and equipped with a bromoacetyl moiety at the 5′-end position the electrophile proximal to a guanine 2 base pairs to the 5′-side of the target sequence. Reaction of the electrophilic carbon of *N*-bromoacetyl with N^7 of the guanine base adjacent to the local triple helix results in covalent attachment of the oligonucleotide to the target sequence. Upon warming in the presence of base, depurination at the position of alkylation occurs and cleavage of one strand of the DNA backbone is observed (*5c*) (Figure 2).

More recently, alkylation of both strands of double-helical DNA has been demonstrated (*6*). Pyrimidine oligonucleotides modified at both the 5′-end (for same-strand alkylation) and 3′-end (for opposite-strand alkylation) with aromatic chloroethylamines cross-link short double-helical DNA at guanine bases separated by 22−26 base pairs in 80% yield (*6b*). *N*-Bromoacetyloligonucleotides bind adjacent inverted purine tracts on double-helical DNA by triple-helix formation and alkylate single guanine positions on opposite strands (*6a*). Double-strand cleavage at a single site within yeast chromosome III occurs in 85−90% yield, demonstrating the utility of this nonenzymatic approach for atom-specific reaction and cleavage of megabase pair double-helical DNA. Further design of these systems will undoubtedly benefit from an understanding in quantitative terms of what has been gained in reaction rate and specificity upon covalent attachment of electrophiles to oligonucleotides for alkylation of DNA.

We report here a product and kinetic analysis of pyrimidine oligonucleotide-directed bromoacetyl alkylation of double-stranded DNA. Products of N^7 alkylation of guanine in the target duplex DNA and the pyrimidine-rich Hoogsteen strand of the bound triple-helical complex are characterized. Rate constants for the alkylation reaction at N^7 of guanine within the triple-helical complex and the reaction of 2-bromoacetamide with N^7 of guanine within duplex DNA are determined, allowing for an estimate of the effective molarity of bromoacetyl obtained upon attachment of the electrophile to the Hoogsteen strand of a local triple-helical complex.

EXPERIMENTAL PROCEDURES

^{1}H NMR and ^{13}C NMR spectra were recorded at 300 MHz on a General Electric QE 300 Spectrometer. Chemical shifts were recorded in parts per million using the proteo NMR solvent as a reference. Biochemical manipulations were carried out according to standard procedures (*7*) unless otherwise noted. Adenine-specific (*8*) and guanine-specific (*7*) sequencing reactions were performed as previously described. Radioactive nucleotides were purchased from Amersham and ICN. T4 polynucleotide kinase, calf alkaline phosphatase, Klenow fragment, and glycogen were obtained from Boehringer Mannheim. *Hin*dIII was obtained from New England Biolabs. *Ssp*I was obtained from Gibco/BRL. Terminal transferase was obtained from USB. Dimethyl sulfate, piperidine, and 2-bromoacetamide were obtained from Aldrich. Purity of 2-bromoacetamide was verified by ^{1}H NMR. Acrylamide was purchased from Bio-Rad. Cobalt hexammine trichloride was obtained from Fluka. Snake venom phosphodiesterase (type VIII) was purchased from Sigma. NAP-5, NAP-25, and NICK columns were purchased from Pharmacia. All reagents were used without further purification unless otherwise noted. High-resolution FAB mass spectra were obtained at the California Institute of Technology Chemistry/Biology Mass Spectrometry Facility. Matrix-assisted laser desorption/ionization time of flight mass spectra were obtained at the Protein and Peptide Microanalytical Facility at the California Institute of Technology.

High-pressure liquid chromatography (HPLC) was performed on a Hewlett-Packard 1090 liquid chromatograph with a HPLC 3D Chemstation. Analytical reversed phase HPLC was performed on a Vydac 201HS reversed phase HPLC column. Oligonucleotide purification was accomplished on a Pharmacia LKB FPLC using a ProRPC HR 16/10 reversed phase column (160 mm × 10 mm). Ion retardation resin (AG 11A8) and cation exchange cellulose (Cellex-CM) were purchased from Bio-Rad. Reversed phase resin (LiChroprep RP-18) was purchased from EM Separations.

Synthesis and Purification of Oligonucleotides. Oligonucleotides were synthesized on an Applied Biosystems Model 380B DNA synthesizer using standard phosphoramidite chemistry (*9*) and deprotected with concentrated NH_4OH at 55 °C for 24 h. Following lyophilization, purification of 5′-OH-oligonucleotide was achieved using denaturing polyacrylamide gel electrophoresis. Purification of the 5′-O-dimethoxytrityloligonucleotide was achieved using reversed phase FPLC (solvent A, 100 mM ammonium acetate, pH 7.0; solvent B, 40% acetonitrile in solvent A). Following lyophilization of appropriate fractions and removal of the dimethoxytrityl protecting group (80% acetic acid for 20 min followed by lyophilization), purification of the deprotected oligonucleotide was achieved by reversed phase FPLC. Following purification, oligonucleotides were desalted on a Sep-Pak car-

tridge (Waters) and quantitated by UV−vis spectroscopy (extinction coefficients in M^{-1} cm^{-1}: dT = 8 700, dG = 11 500, dA = 15 400, dC = 7 400).

Synthesis and Analysis of *N*-Bromoacetyloligonucleotides. FMOC-protected aminonucleoside (*5c*) was incorporated at the 5′-end of the oligonucleotide 5′-TTT^{Me}-CTTTT^{Me}C^{Me}CTTT^{Me}CTTTT-3′ as the 5′-O-DMT-3′-phosphoramidite with a modification of standard coupling protocols. The last coupling step in the oligonucleotide synthesis was performed with a 0.15 M solution of the β-cyanoethyl phosphoramidite in tetrahydrofuran (distilled from calcium hydride) on a 1 μmol scale. Following deprotection at 55 °C for 15 h, the 5′-O-DMT-oligonucleotide was purified by reversed phase FPLC, deprotected, and further purified by reversed phase FPLC. Acylation of the resulting oligonucleotide−amine was then performed. Ten nanomoles of oligonucleotide was dissolved in 10 μL of 200 mM borate buffer, pH 8.9. Ten microliters of a 30 mM solution of freshly prepared *N*-hydroxysuccinimidyl bromoacetate in *N*,*N*-dimethylformamide (stored over molecular sieves) was added, the resulting solution mixed well, and, after 30 s, diluted to 0.5 mL with water and passed through an NAP-5 column (Sephadex G-25, equilibrated with water). Following elution with 1.0 mL of water, the *N*-bromoacetyloligonucleotide was quantitated by UV−vis spectroscopy (extinction coefficient of 153 000 M^{-1} cm^{-1} determined from the sum of dT = 8 700, dT^E = 8 700, and 5-methyl-dC = 5 700) and stored at −20 °C. The reaction was judged quantitative by HPLC analysis of enzyme degradation products. To the oligonucleotide were added 18 μL of water, 2.5 μL of 10× dephosphorylation buffer (500 mM Tris-HCl, 1 mM EDTA, pH 8.5), 2.5 μL of 2 M $MgCl_2$, and 2 μL of snake venom phosphodiesterase (2 units/0.7 mL of enzyme). Following incubation at 37 °C for 2 h, samples were filtered (0.2 μm Nylon-66 membrane, Rainin) and 23.5 μL was injected. HPLC conditions: Vydac C_{18} 201HS reversed phase column preequilibrated to 50 °C; solvent A, 100 mM ammonium acetate, pH 6.0; solvent B, acetonitrile; gradient, 0−10 min at 0% B, 10−40 min to 5% B, 40−60 min at 5% B, 60−70 min to 10% B, 70−85 min at 10% B, 85−90 min to 0% B. Retention times and relative peak areas: 5-methyl-2′-deoxycytidine 5′-monophosphate, 10.3 min, 2.6; thymidine 5′-monophosphate, 15.0 min, 14.6; hydroxy-(linker)-thymidine, 35.2 min, 0.2; bromo-(linker)-thymidine, 52.6 min, 0.8. Mass spectrum of *N*-bromoacetyloligonucleotide (MALDI-TOF): calcd 5934.9, found 5935.7.

5′-^{32}P-End Labeling of the 29 Base Pair Duplex. Fifty picomoles of single-stranded oligonucleotide 5′-GCTAGTAAAAGAAAAGGAAAGAAAAGTCG-3′ was labeled with ^{32}P at the 5′-end by treatment with 45 units of T4-polynucleotide kinase and [γ-^{32}P]dATPγP for 45 min at 37 °C. Following phenol/chloroform extraction, ethanol precipitation, and hybridization with 100 pmol of complementary single-stranded oligonucleotide, the 5′-^{32}P-end-labeled duplex was subjected to 14% nondenaturing polyacrylamide gel electrophoresis, visualized by autoradiography, excised from the gel, and eluted with 200 mM NaCl/1 mM EDTA at 37 °C overnight. The DNA was filtered (0.45 μm), ethanol precipitated, passed through a NICK column (Sephadex G-50, equilbrated with water), and stored at −20 °C.

3′-^{32}P-End Labeling of 29 Base Pair Duplex. Fifty picomoles of single-stranded oligonucleotide 5′-GCTAGTAAAAGAAAAGGAAAGAAAAGTCG-3′ was labeled with [α-^{32}P]ddAαP at the 3′-end by treatment with 85 units of terminal deoxynucleotidyl transferase and [α-^{32}P]-ddATPαP for 45 min at 37 °C. Unincorporated nucleotide was removed by passage through a NICK column (Sephadex G-50, equilibrated with water), then butanol was extracted, and the nucleotide was lyophilzed to dryness. Radiolabeled single-stranded oligonucleotide was then subjected to 20% denaturing polyacrylamide gel electrophoresis, visualized by autoradiography, excised from the gel, and eluted with 200 mM NaCl/1 mM EDTA at 37 °C overnight. The DNA was filtered (0.45 μm) and ethanol precipitated. Following hybridization with 100 pmol of complementary single strand, the 3′-^{32}P-end-labeled duplex was purified by 14% nondenaturing polyacrylamide gel electrophoresis, visualized by autoradiography, excised from the gel, and eluted with 200 mM NaCl/1 mM EDTA at 37 °C overnight. The DNA was filtered (0.45 μm), ethanol precipitated, passed through a NICK column (Sephadex G-50, equilibrated with water), and stored at −20 °C.

Enzymatic Characterization of DNA Termini. End-labeled 29 base pair duplex (100 000 cpm) with and without *N*-bromoacetyloligonucleotide (2.5 μM) was incubated in 20 mM HEPES, pH 6.8, with 1 mM $Co(NH_3)_6Cl_3$ in a volume of 20 μL at 4 °C for 2 h and then at 37 °C for 24 h. Two microliters of glycogen in water (20 mg/mL), 2 μL of 3 M sodium acetate, pH 5, and 60 μL of absolute ethanol were then added. Following chilling at −70 °C for 20 min and centrifugation at 14 000 rpm for 40 min, the supernatant was discarded. Seventy percent ethanol/water was added, and following centrifugation at 14 000 rpm for 5 min, the supernatant was discarded. Fifty microliters of water was added and, after vigorous mixing, the reaction was dried *in vacuo*. To each tube was added 100 μL of 10% piperidine. Following incubation at 90 °C for 30 min, reactions were dried *in vacuo*. Samples were then lyophilized twice from 50 μL of water.

For the 3′-end analysis, a portion (10 000 cpm) of 5′-^{32}P-end-labeled product as well as products of dimethyl sulfate cleavage were dissolved in separate reactions in dephosphorylation buffer (20 mM Tris-HCl, pH 6.6, 20 mM $MgCl_2$, 10 mM β-mercaptoethanol), and 10 units of T4 polynucleotide kinase was added. After 1 h at 37 °C, reactions were ethanol precipitated. For the 5′-end analysis, a portion (20 000 cpm) of the 3′-[α-^{32}P]ddA-end-labeled product as well as products of dimethyl sulfate cleavage were dissolved in separate reactions in calf alkaline phosphatase (CAP) buffer, and 2 units of CAP was added. After 45 min at 37 °C, reactions were ethanol precipitated. Reaction products, as well as control and G-reactions, were dissolved in formamide loading buffer and subjected to gel electrophoresis on 1:20 cross-linked 20% denaturing polyacrylamide gels. Following electrophoresis, gels were visualized by autoradiography.

***N*-Hydroxysuccinimidyl 2-Bromoacetate (*10*).** To a solution of *N*-hydroxysuccinimide (0.6 g, 5.4 mmol) and 2-bromoacetic acid (0.75 g, 5.4 mmol) in dioxane (30 mL) was added *N*,*N*-dicyclohexylcarbodiimide (1.3 g, 6.5 mmol). The resulting white suspension was stirred for 2 h and then filtered into 100 mL of petroleum ether. The resulting white precipitate was collected by filtration, washed with petroleum ether, and dried *in vacuo* to provide 0.5 g (38%) of *N*-hydroxysuccinimidyl 2-bromoacetate as a white, crystalline solid: ^{1}H NMR (DMSO-d_6) δ 2.82 (s, 4H), 4.62 (s, 2H); ^{13}C NMR (DMSO-d_6) δ 23.1, 26.0, 164.7, 170.3; IR (KBr) 3060 (vw), 2990 (vw), 2943 (vw), 1812 (m), 1781 (m), 1736 (s), 1356 (m), 1211 (m), 1074 (m) cm^{-1}; HRMS calcd for $C_6H_6NO_4Br$ 234.9479, found 234.9469.

Amino-(Linker)-Thymidine (T-NH$_2$). To a flask containing 237 mg (0.75 mmol) of thymidine methyl ester **1** (*11*) was added 5 mL of ethylenediamine (freshly distilled from sodium hydroxide). The clear solution was stirred at 50 °C for 7 h and then at room temperature

for 16 h. The ethylenediamine was removed *in vacuo* to give a clear oil. Evaporation with methanol/toluene to remove trace ethylenediamine yielded a clear oil, and upon repeated evaporation with acetonitrile, 247 mg (96%) of **T-NH$_2$** was obtained as a white, hygroscopic, fluffy solid: ^{1}H NMR (CD_3OH) δ 2.08−2.15 (m, 2H), 2.29−2.33 (m, 2H), 2.47−2.52 (m, 2H), 2.56−2.61 (m, 2H), 3.11 (dd, J = 6.4 Hz, J = 2.3 Hz, 2H), 3.66 (dd, J = 21 Hz, J = 4 Hz, 2H), 3.79 (q, J = 3.4 Hz, 1H), 4.28 (m, 1H), 6.18 (t, J = 6.7 Hz, 1H), 7.70 (s, 1H); ^{13}C NMR (CD_3OH) δ 24.3, 35.8, 41.2, 41.9, 42.8, 62.9, 72.1, 86.3, 88.8, 114.1, 138.8, 152.5, 166.1, 175.3; IR (KBr) 3362 (s), 3261 (m), 2937 (w), 2855 (w), 1702 (s), 1637 (s), 1560 (m), 1282 (m), 1104 (m), 926 (w) cm^{-1}; HRMS calcd for $C_{14}H_{22}N_4O_6$ 342.1534, found 342.1561.

Bromo-(Linker)-Thymidine (T-Br). To a solution of **T-NH$_2$** (19 mg, 55 μmol) in water (0.2 mL) was added *N*-hydroxysuccinimidyl 2-bromoacetate (25 mg, 106 μmol). The solution was vigorously mixed for 5 min and then loaded onto a 2 g Sep-Pak cartridge equilibrated with water. The column was washed first with water and then with 50% acetonitrile/water. Lyophilization afforded 6 mg (24%) **of T-Br** as a fluffy, white solid: ^{1}H NMR (D_2O) δ 2.29−2.38 (m, 2H), 2.40−2.45 (t, J = 7.1 Hz, 2H), 2.56−2.61 (t, J = 5.9 Hz, 2H), 3.30 (s, 4H), 3.71−3.81 (m, 2H), 3.86 (s, 2H), 3.87−3.89 (m, 1H), 3.98−4.02 (m, 1H), 4.41−4.46 (m, 1H), 6.23−6.28 (t, J= 6.7 Hz, 1H), 7.65 (s, 1H); ^{13}C NMR (D_2O) δ 23.0, 28.1, 34.4, 38.4, 38.8, 39.3, 61.2, 70.5, 85.2, 86.6, 113.3, 138.3, 151.6, 165.6, 170.2, 175.5; IR (KBr) 3507 (m), 3365 (s), 3049 (m), 2931 (m), 2802 (w), 1686 (s), 1655 (s), 1625 (m), 1560 (m), 1424 (m), 1048 (m) cm^{-1}; HRMS calcd for $C_{16}H_{24}N_4O_7Br$ 463.0823, found 463.0821.

Hydroxy-(Linker)-Thymidine (T-OH). To a solution of *N*-hydroxysuccinimide (1.5 g, 13.2 mmol) and glycolic acid (1 g, 13.2 mmol) in dioxane (16 mL) was added *N*,*N*-dicyclohexylcarbodiimide (3.3 g, 15.8 mmol). The resulting white suspension was stirred for 2 h and then filtered into 100 mL of petroleum ether. The resulting white precipitate was collected by filtration, washed with petroleum ether, and dried *in vacuo* to provide 1.2 g of a white solid, used without further purification. To a solution of **T-NH$_2$** (44 mg, 129 μmol) in water (0.44 mL) was added the NHS-ester of glycolic acid (34 mg). The solution was vigorously mixed for 5 min and then stirred for 4 h. The reaction mixture was dried *in vacuo* to a yellow oil. The oil was dissolved in 50% aqueous acetonitrile (300 μL) and then adsorbed onto silica gel. Column chromatography (0−7% water/acetonitrile), followed by adsorption and elution from reversed phase resin (LiChroprep RP-18) afforded 7 mg (14%) of **T-OH** as a white solid: ^{1}H NMR (DMSO-d_6) δ 2.03−2.07 (m, 2H), 2.20−2.26 (t, J = 7.4 Hz, 2H), 2.38−2.43 (t, J = 7.4 Hz, 2H), 3.08−3.14 (m, 4H), 3.53−3.58 (m, 2H), 3.73−3.75 (m, 1H), 3.77 (s, 2H), 4.21−4.23 (m, 1H), 5.01−5.05 (m, 1H), 5.22−5.24 (d, J = 4 Hz, 1H), 6.12−6.17 (t, J = 6.9 Hz, 1H), 7.63 (s, 1H), 7.81−7.84 (m, 1H), 7.85−7.90 (m, 1H), 11.28 (s, 1H); ^{13}C NMR (D_2O) δ 23.0, 34.4, 38.4, 38.6, 38.8, 60.9, 61.2, 70.5, 85.2, 86.6, 113.3, 138.3, 151.5, 165.5, 175.2, 175.5; IR (KBr) 3448 (s), 3413 (s), 3072 (m), 2931 (m), 1690 (s), 1655 (s), 1561 (m), 1543 (m), 1273 (m), 1096 (m), 1032 (w) cm^{-1}; HRMS calcd for $C_{16}H_{24}N_4O_8$ 400.1588, found 400.1607.

***N*^{7}-Carboxymethylguanine (*12*).** To a flask containing guanosine (1.8 g, 6.6 mmol), iodoacetic acid (4.8 g, 26 mmol), and lithium hydroxide (0.31 g, 12.8 mmol) was added 12 mL of water. The resulting suspension was stirred at reflux for 70 min, during which time a clear, dark red solution was formed. The solution was cooled, and solid sodium thiosulfate was added until the red solution turned to a light pink color. The solution was then adjusted to pH 5.5 with LiOH(s). After storage overnight at 4 °C, the pink precipitate was collected by filtration. Following recrystallization from 5% aqueous acetic acid 350 mg (26%) of N^7-carboxymethylguanine was obtained as a pale pink solid: ^{1}H NMR (NaOD/D_2O) δ 4.86 (s, 1H), 7.71 (s, 1H); ^{13}C NMR (NaOD/D_2O/DMSO-d_6) δ 40.0 (DMSO-d_6), 50.8, 111.6, 144.0, 160.0, 162.2, 166.5, 176.5; IR (KBr) 3537 (s), 3316 (s), 3124 (s), 2694 (s), 1659 (s), 1502 (w), 1474 (m), 1232 (m), 1103 (w), 897 (w) cm^{-1}; HRMS calcd for $C_7H_7N_5O_3$ 209.0548, found 209.0545.

Guanine-(Linker)-Thymidine (T-G). To 1.5 mL of 50% aqueous pyridine, pH 8.5, was added N^7-carboxymethylguanine (20 mg, 96 μmol), 4-nitrophenol (133 mg, 960 μmol), and thymidine−amine **T-NH$_2$** (36 mg, 105 μmol). 1-[3-(Dimethylamino)propyl]-3-ethylcarbodiimide hydrochloride (EDCI) (total 576 μmol) was then added to the resulting yellow solution in portions of $^1/_5$ equivalent. The solution was lyophilized, dissolved in 2 mL of 10 mM NH_4OAc, pH 4.5, and extracted with an equal amount of chloroform. Removal of residual pyridine was achieved by passage of the aqueous solution through cation exchange cellulose (CM-Sephadex). The reaction was then subjected to purification by reversed phase chromatography (LiChroprep C_{18} resin), affording a further purified mixture of adduct **T-G** and *p*-nitrophenol, which was then lyophilized, dissolved in water, and passed through ion retardation resin (Bio-Rad AG 11A8) to remove *p*-nitrophenol. Lyophilization afforded the authentic standard **T-G** (2 mg) as a fluffy, white solid: ^{1}H NMR (DMSO-d_6) δ 2.04−2.08 (m, 2H), 2.21−2.26 (t, J = 7.4 Hz, 2H), 2.38−2.43 (t, J = 7.5 Hz, 2H), 3.31 (s, 4H), 3.53−3.57 (m, 2H), 3.73−3.77 (m, 1H), 4.21−4.23 (m, 1H), 4.85 (s, 2H), 5.01−5.04 (t, J = 5.1 Hz, 1H), 5.23−5.24 (d, J = 4.2 Hz, 1H), 6.10 (s, 2H), 6.13−6.17 (t, J = 6.9 Hz, 1H), 7.65 (s, 1H), 7.79−7.81 (s, 1H), 7.83 (s, 1H), 8.17 (s, 1H), 10.72 (s, 1H), 11.31 (s, 1H); ^{13}C NMR (DMSO-d_6) δ 23.0, 34.5, 38.5, 38.9, 39.8, 48.6, 61.7, 70.7, 84.3, 87.6, 109.0, 113.1, 137.6, 144.5, 150.8, 153.2, 155.2, 160.1, 163.8, 167.4, 172.0; IR (KBr) 3358 (s), 2924 (m), 2848 (w), 1686 (s), 1560 (m),1384 (m), 1273 (w), 1108 (w) cm^{-1}; HRMS calcd for $C_{21}H_{27}N_9O_8$ 533.1977, found 533.1989.

HPLC Analysis of Products of Oligonucleotide-Directed Alkylation. Twenty nanomoles of single-stranded oligonucleotide 5′-TCGGTACCCGGGGATCT-AAAGTAAAAGAAAAGGAAAGAAAAAGCTTT-CTTCTTCCCTATC-3′ (ϵ = 6.0 × 10^5 M^{-1} cm^{-1}) and complementary oligonucleotide (ϵ = 5.7 × 10^5 M^{-1} cm^{-1}) were hybridized as follows: Oligonucleotides were combined in 400 μL of water and passed through a NAP-5 column (Sephadex G-25, equilibrated with water). To the 1.0 mL of eluent in a 1.7 mL microfuge tube was added 100 μL of 200 mM HEPES, pH 7.0. The solution was heated to 90 °C for 9 min and then cooled to room temperature. After 15 h, the resulting solution was then combined in a 15 mL Falcon tube with 4.5 mL of water, 570 μL of 200 mM HEPES, pH 7.0, and a stirbar. After the solution was cooled to 4 °C with stirring, 20 nmol of *N*-bromoacetyloligonucleotide in 1 mL of water was added, followed by 170 μL of 8 mM cobalt hexammine trichloride (to give a final concentration of 2.7 μM oligonucleotides). This solution was stirred at 4 °C for 1.5 h and then placed in an oil bath equilibrated to 37 °C. The cloudy solution was stirred, and after 10 min, the first aliquot (1.1 mL, 3 nmol) was removed for workup. The sample was pipetted into a 1.7 mL microfuge tube with 100 μL of 2 M sodium chloride (170 mM NaCl final concentration). The resulting clear

solution was heated to 90 °C for 15 min and then cooled to room temperature. The solution was diluted to 2.5 mL and passed through a NAP-25 column (Sephadex G-25, equilibrated with water), the 3.5 mL eluent was collected in two 2 mL microfuge tubes, and the solutions were dried *in vacuo*. Product was then transferred to a 0.7 mL microfuge tube with 100 μL of water, followed by washes of three 100 μL aliquots of water to ensure complete transfer of material. Samples were then dried *in vacuo* and stored at −20 °C. The above procedure was repeated at time points of 3, 7, 10.5, 19.2, 29, and 69.5 h (relative to the time of the first sample withdrawn from the reaction mixture).

Just prior to HPLC analysis, to each tube were added 18 μL of water, 2.5 μL of 10× dephosphorylation buffer (500 mM Tris-HCl/1 mM EDTA, pH 8.5), 2.5 μL of 2 M $MgCl_2$, and 2 μL of snake venom phosphodiesterase (2 units/0.7 mL of enzyme). Following incubation at 37 °C for 2 h, samples were filtered (0.2 μm Nylon-66 membrane, Rainin) and 23.5 μL was injected. A blank run with 23.5 μL of water injected between each run was performed to prevent contamination of HPLC chromatograms from previous runs. HPLC conditions: Vydac C_{18} 201HS reversed phase column preequilibrated to 50 °C; solvent A, 100 mM ammonium acetate, pH 6.0; solvent B, acetonitrile; gradient, 0−10 min at 0% B, 10−40 min to 5% B, 40−60 min at 5% B, 60−70 min to 10% B, 70−85 min at 10% B, 85−90 min to 0% B. Retention times: 2′-deoxycytidine 5′-monophosphate, 7.7 min; 5-methyl-2′-deoxycytidine 5′-monophosphate, 10.7 min; thymidine 5′-monophosphate, 14.5 min; 2′-deoxyadenosine 5′-monophosphate, 26.6 min; hydroxy-(linker)-thymidine nucleoside, 38.4 min; guanine-(linker)-thymidine nucleoside, 47.8 min; bromo-(linker)-thymidine nucleoside, 55.2 min; undigested oligodeoxyribonucleotides, 70−80 min; authentic standard **T-G** injected under identical solution conditions, 48.5 min; hydroxy-(linker)-thymidine, 39.0 min; bromo-(linker)-thymidine, 55.5 min.

Cloning of Plasmid pUCINET. The plasmid pUCINET was constructed by ligation of the duplex formed between oligonucleotides of sequence 5′-GATCATACACATACTAATACTAGTAAAAGAAAAGGAAAGAAAA-3′ and 5′-TCGATTTTCTTTCCTTTTCTTTTACTAGTATTAGTATGTGTAT-3′ with pUC19 previously digested with *Bam*HI and *Sal*I. Ligation products were used to transform Epicurian Coli XL 1 Blue competent cells. Colonies were selected for α-complementation on Luria−Bertani medium agar plates containing 50 μg/mL ampicillin and XGAL and IPTG solution. Large scale plasmid purification was performed using Qiagen purification kits according to the manufacturer's protocol. Plasmid DNA concentration was determined from absorbance at 260 nm using the relation 1 OD unit = 50 μg/mL duplex DNA. The sequence of the inserted region was verified by dideoxy sequencing of the resulting recombinant plasmid.

Kinetic Measurements: *N*-Bromoacetyloligonucleotide Alkylation of the 666 Base Pair Restriction Fragment. Plasmid pUCINET was linearized with the restriction enzyme *Hin*dIII. Eight micrograms of DNA was labeled at the 3′-end using Klenow to incorporate [α-^{32}P]dATPαP and [α-^{32}P]TTPαP. Following a chase fill-in with dATP, TTP, dCTP, and dGTP the reaction was extracted with phenol/chloroform, and the 3′-^{32}P-end-labeled DNA ethanol precipitated. The DNA was digested with *Ssp*I, producing fragments 2 and 0.7 kbp in size. Following ethanol precipitation, separation by 5% polyacrylamide gel electrophoresis, and visualization of the gel by autoradiography, the 666 base pair fragment containing the target site was excised from the gel and eluted with 200 mM NaCl/1 mM EDTA at 37 °C overnight. The radiolabeled restriction fragment was filtered (0.45 μm), ethanol precipitated, and passed through a NICK column (Sephadex G-50, equilibrated with water); $^1/_{10}$ volume of 200 mM Bis-Tris acetate, pH 7.0, was added, and the mixture was stored at −20 °C.

A 2× stock solution was made containing 3′-^{32}P-end-labeled *Hin*dIII/*Ssp*I pUCINET restriction fragment (4 000 cpm/μL), 40 mM Bis-Tris acetate, pH 7.0, and 1.6 mM cobalt hexammine trichloride. The stock solutions were then aliquoted into microfuge tubes, and to each was added an equivalent volume of 2× aqueous *N*-bromoacetyloligonucleotide at 2, 0.4, and 0.2 μM concentrations. The solutions were incubated overnight at 8 °C, and then each was split into seven tubes and put at 37 °C. After 30 min, the first tube (arbitrarily designated time equals zero) was quenched by the addition of 2 μL of glycogen (20 mg/mL), 2 μL of 3 M sodium acetate, and 60 μL of ethanol. Samples were stored at −80 °C until completion of the experiment. Subsequent time points were treated as described. The samples were then centrifuged at 14 000 rpm for 30 min, the supernatants discarded, and reactions dried *in vacuo*. One hundred microliters of 10% aqueous piperidine was added, and reactions were heated to 90 °C for 30 min. Reactions were lyophilized, then lyophilized twice from 50 μL of water, and dissolved in formamide loading buffer. Separation of products was performed by 1:20 cross-linked 8% denaturing polyacrylamide gel electrophoresis (5 000 cpm/lane). Dried polyacrylamide gels were analyzed by storage phosphor autoradiogram.

Band densities were determined by quantitative analysis of storage phosphor autoradiograms using a PhosphorImager (Model 400S) and Image Quant (Molecular Dynamics) software. Integrated volumes were determined for individual bands, and numerical values were transferred to Microsoft Excel. Bands were assigned as depicted in Figure 7 to determine values for $[D]_i$ and $[D]_t$, and $\ln([D]_i/[D]_t)$ was plotted versus time using the program KaleidaGraph to determine from the slope the value for k_1. Kinetics were first-order in target DNA concentration over 2 half-lives and provided rate constants of $(2.4 \pm 0.3) \times 10^{-5}$, $(2.7 \pm 0.5) \times 10^{-5}$, and $(1.6 \pm 0.4) \times 10^{-5}$ s^{-1} for 1, 0.2, and 0.1 μM concentrations of *N*-bromoacetyloligonucleotide, respectively.

Kinetic Measurements: 2-Bromoacetamide Alkylation of the 39 Base Pair Duplex. Fifty picomoles of single-stranded oligonucleotide 5′-ATACACATACTAATACTAGTAAAAGAAAAGGAAAGAAAA-3′ was labeled with ^{32}P at the 5′-end by treatment with 45 units of T4 polynucleotide kinase and [γ-^{32}P]dATPγP for 45 min at 37 °C. Following ethanol precipitation the DNA was resuspended in formamide loading buffer and purified by 20% denaturing polyacrylamide gel electrophoresis. Following elution, ethanol precipitation, and hybridization with excess complementary single-stranded oligonucleotide, the 5′-^{32}P-end-labeled duplex was then subjected to 12% nondenaturing polyacrylamide gel electrophoresis, visualized by autoradiography, excised from the gel, and eluted with 200 mM NaCl/1 mM EDTA at 37 °C overnight. The DNA was filtered (0.45 μm), ethanol precipitated, and passed through a NICK column (Sephadex G-50, equilibrated with water); $^1/_{10}$ volume of 200 mM Bis-Tris acetate, pH 7.0, was added, and the mixture was stored at −20 °C.

A 2× stock solution was made containing 5′-^{32}P-end-labeled 39 base pair duplex (8 000 cpm/μL), 40 mM Bis-Tris acetate, pH 7.0, and 1.6 mM cobalt hexammine trichloride. The stock solutions were then aliquoted into microfuge tubes, and to each was added an equivalent volume of 2× aqueous 2-bromoacetamide (made fresh,

volumetrically) at 20, 40, 60, 100, 120, 140, 160, and 180 mM concentrations. Reactions were each then aliquoted to six microfuge tubes and incubated at 37 °C for 25 min. The first tube (arbitrarily designated time equals zero) was quenched by brief exposure to liquid nitrogen, followed by the addition of 2 μL of glycogen (20 mg/mL), 2 μL of 3 M sodium acetate, and 60 μL of ethanol. Samples were stored at −80 °C until completion of the experiment, and subsequent time points were treated as described. The samples were then centrifuged at 14 000 rpm for 30 min, the supernatants discarded, and reactions dried *in vacuo.* One hundred microliters of 10% aqueous piperidine was added, and reactions were heated to 90 °C for 30 min. Reactions were lyophilized, then lyophilized twice from 50 μL of water, and dissolved in formamide loading buffer. Separation of products was performed by 1:20 cross-linked 15% denaturing polyacrylamide gel electrophoresis (10 000 cpm/lane). Dried polyacrylamide gels were analyzed by storage phosphor autoradiography.

Band densities were determined by quantitative analysis of storage phosphor autoradiography as described above. Bands were assigned as depicted in Figure 9 to determine values for $[D]_i$ and $[D]_t$, and $[D]_i/[D]_t$ was plotted versus time using the program KaleidaGraph to determine from the slope values for k_{obs} for two or three rate determinations at each concentration of 2-bromoacetamide. An analysis of the data indicated that disappearance of fully intact DNA at the highest concentration of 2-bromoacetamide (90 mM) over 3 half-lives followed pseudo-first-order kinetics. In subsequent determinations of observed rate constants, initial rates were used providing values of $(3.4 \pm 0.2) \times 10^{-7}$, $(6.4 \pm 0.8) \times 10^{-7}$, $(9.6 \pm 0.9) \times 10^{-7}$, $(1.7 \pm 0.2) \times 10^{-6}$, $(2.2 \pm 0.1) \times 10^{-6}$, $(2.4 \pm 0.01) \times 10^{-6}$, $(2.4 \pm 0.1) \times 10^{-6}$, and $(3.4 \pm 0.1) \times 10^{-6}$ s^{-1} for 10, 20, 30, 50, 60, 70, 80, and 90 mM 2-bromoacetamide concentrations, respectively. Average k_{obs} values were then plotted against concentration of 2-bromoacetamide to determine, from the slope of the line, a rate constant k_2 of $(3.6 \pm 0.3) \times 10^{-5}$ M^{-1} s^{-1}.

RESULTS AND DISCUSSION

Products of Reaction at Target Duplex. The production of phosphate termini is consistent with a mechanism involving base-promoted DNA cleavage at the abasic site within the target duplex resulting from alkylation at the N^7 position of guanine and depurination. The 29 base pair duplex of sequence composition 5′-GCTAGTAAAAGAAAAGGAAAGAAAAGTCG-3′ possessing a GC base pair 2 base pairs to the 5′-side of the homopurine tract was synthesized and 5′- or 3′-^{32}P-end labeled for 3′- and 5′-terminal analysis, respectively. Radiolabeled duplexes were reacted with *N*-bromoacetyloligonucleotide of sequence composition 5′-^{E}TTTT^{Me}-CTTTT^{Me}C^{Me}CTTT^{Me}CTTTT-3′ at 2.5 μM concentration (20 mM HEPES, pH 6.8, 1 mM cobalt hexammine trichloride) for 24 h at 37 °C, and following piperidine treatment (90 °C, 30 min), the reactions were analyzed by high-resolution polyacrylamide gel electrophoresis. Treatment of the radiolabeled duplex with dimethyl sulfate followed by piperidine affords authentic standards of the expected phosphate-terminal oligonucleotides (*13*). Alkylation by the *N*-bromoacetyloligonucleotide occurs at the targeted guanine base proximal to the local triple helix, and oligonucleotide products comigrate by gel electrophoresis with products of dimethyl sulfate-treated DNA (Figure 3).

For further evidence of terminal phosphate functionality at the site of strand cleavage, oligonucleotide products were treated with the appropriate enzyme to achieve

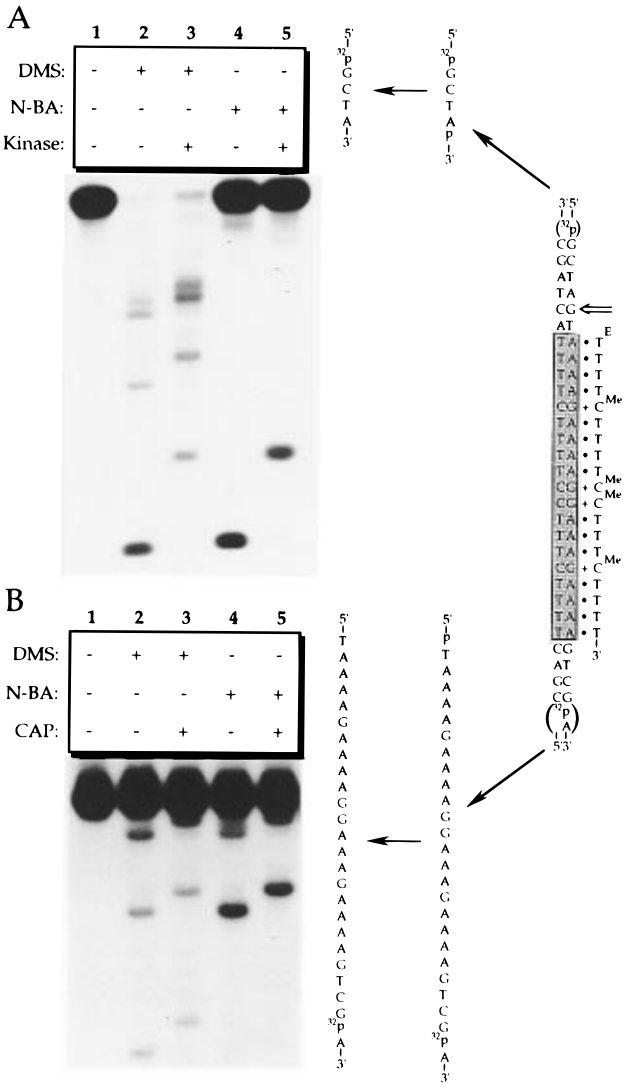

Figure 3. Autoradiograms of 20% denaturing polyacrylamide gels used for characterization of the DNA termini resulting from oligonucleotide-directed alkylation and base-promoted strand cleavage at the targeted guanine base: (A) 3′-end analysis using a 5′-end-labeled 29 base pair target duplex and T4 polynucleotide kinase; (B) 5′-end analysis using a 3′-end-labeled 29 base pair target duplex and calf alkaline phosphatase. (Lane 1) Controls, 24 h with no *N*-bromoacetyloligonucleotide; (lane 2) duplex treated with dimethyl sulfate; (lane 3) enzymatically treated dimethyl sulfate products; (lane 4) duplex treated with 2.5 μM *N*-bromoacetyloligonucleotide for 24 h; (lane 5) enzymatically treated products of oligonucleotide-directed alkylation. T^E is bromoacetyl attached to the 5-position of thymine, and ^{Me}C is 5-methyl-2′-deoxycytidine, previously shown to facilitate binding in oligonucleotide-directed triple-helix formation.

hydrolysis of the 5′- and 3′-phosphate groups. The 5′-end-labeled oligonucleotide fragment possessing the putative 3′-phosphate produced in the alkylation reaction was subjected to treatment with T4 polynucleotide kinase, an enzyme that can catalyze the hydrolysis of 3′-phosphate groups (*14*). Following this treatment, a retardation of gel mobility is observed (Figure 3A). The observed shift in gel mobility is identical to that observed for the products of dimethyl sulfate alkylation of DNA subjected to kinase treatment. The analysis was repeated for the 3′-^{32}P-end-labeled product possessing the putative 5′-phosphate using calf alkaline phosphatase, an enzyme that can catalyze the hydrolysis of 5′-phosphate groups. Again, a product of slower mobility is produced, and the observed shift in gel mobility for the phosphatase-treated products of dimethyl sulfate

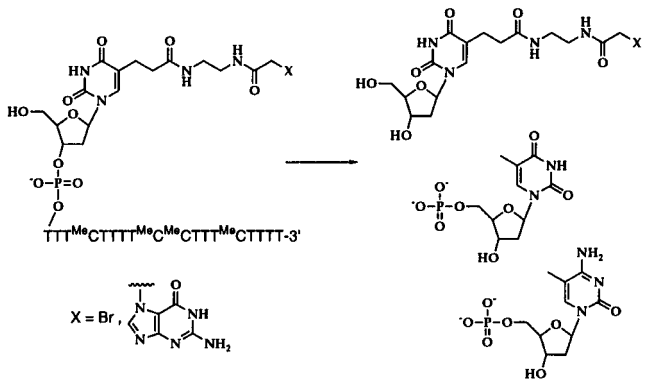

Figure 4. Enzymatic phosphodiester hydrolysis of the modified pyrimidine-rich Hoogsteen strand by the 3′-exonuclease snake venom phosphodiesterase produces thymidine nucleosides with linker arm modifications at the 5-position, thymidine 5′-monophosphate, and 5-methyl-2′-deoxycytidine 5′-monophosphate. These products can then be separated and identified by reversed-phase HPLC.

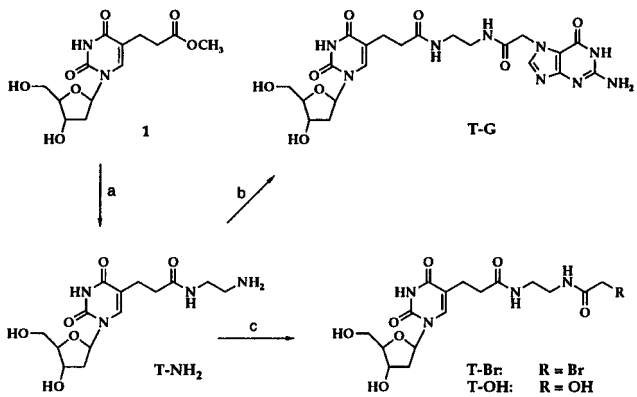

Figure 5. Scheme for the synthesis of authentic standards for HPLC analysis of products of oligonucleotide-directed alkylation of duplex DNA: (a) ethylenediamine (neat), 23 h; (b) N^7-carboxymethylguanine, 4-nitrophenol, pyridine/water, EDCI; (c) *N*-hydroxysuccinimidyl 2-bromoacetate, water (for T-Br) or *N*-hydroxysuccinimidyl 2-hydroxyacetate, water (for T-OH).

alkylation of DNA is equivalent (Figure 3B). These data are consistent with the formation of phosphate termini at the site of strand cleavage.

Identification of the Guanine-(Linker)-Oligonucleotide Conjugate Formed in the Alkylation Reaction. Alkylation at the N^7 atom of guanine within the Watson−Crick duplex is achieved by the bromoacetyl electrophile appended to the 5′-end of the Hoogsteen strand bound in the local triple-helical complex. Identification of the guanine-(linker)-oligonucleotide conjugate formed after depurination would provide direct evidence for covalent bond formation. To address this issue, reversed phase HPLC analysis can be performed on the reaction products enzymatically digested by a 3′-exonuclease. Products of this hydrolysis reaction are nucleotides (resulting from internal and 3′-terminal residues) and nucleosides (resulting from 5′-terminal residues) (Figure 4) (*15*). Nucleosides resulting from modifications of the linker arm at the 5-position of the 5′-terminal thymidine in the pyrimidine-rich Hoogsteen strand can then be separated by standard reversed phase chromatographic techniques from a potentially large background of nucleotides.

N-Bromoacetyloligonucleotide (20 nmol, 2.7 μM) of sequence composition 5′-^{E}TTTT^{Me}CTTTT^{Me}C^{Me}CTTT^{Me}-CTTTT-3′ was bound to the 60 base pair duplex (20 nmol, 2.7 μM) of sequence composition 5′-TCGGTAC-CCGGGGATCTAAAGTAAAAGAAAAGGAAA-GAAAAAGCTTTCTTCTTCCCTATC-3′ and a time course performed (20 mM HEPES, pH 7.0, 0.2 mM cobalt hexammine trichloride, 37 °C). Three nanomole aliquots were removed over time and heated to 90 °C to depurinate N^7-alkylated guanine bases (*16*). Cleavage products were then digested with snake venom phosphodiesterase and subjected to HPLC analysis. For comparison, standards of products expected from enzymatic digestion of unreacted *N*-bromoacetyloligonucleotide, as well as products of N^7-alkylation of guanine and hydrolysis of starting material, were synthesized from the thymidine methyl ester **1** (*11*) (Figure 5).

The reversed phase HPLC analysis of the reaction products is shown in Figure 6. The authentic guanine-(linker)-thymidine standard (**T-G**) expected from the alkylation reaction elutes at 48.5 min under these HPLC conditions (Figure 6a). Featured in the time course (Figure 6b−d) is the presence of a compound with a retention time of 47.8 min (**T-G**, Figure 6). The relative amount of this product increases during the course of the reaction and comigrates upon coinjection with the authentic standard **T-G** (data not shown). The amount of 5-methyl-2′-deoxycytidine 5′-monophosphate (retention

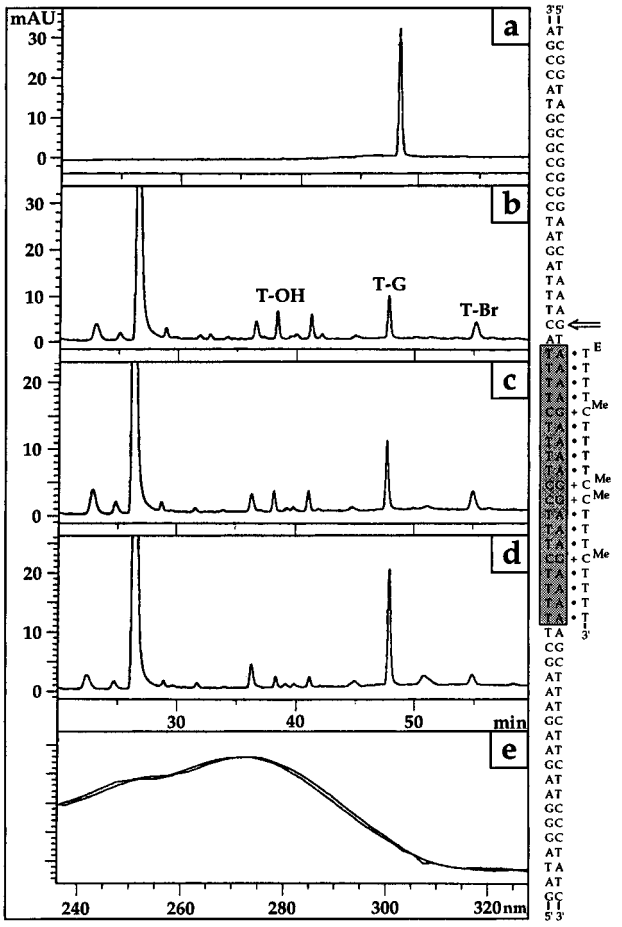

Figure 6. HPLC analysis of the enzymatically digested products of oligonucleotide-directed alkylation of double-stranded DNA. Peak heights for the time course are normalized according to peak area ratio with 5-methyl-2′-deoxycytidine 5′-monophosphate, and chromatograms were monitored at 260 nm: (a) HPLC trace of authentic guanine-(linker)-thymidine standard **T-G**; (b−d) HPLC traces of enzyme digests of oligonucleotides (3 nmol) from an alkylation reaction at times 3, 10.5, and 29 h; (e) UV spectrum of the peak at 47.8 min in (d) superimposed and normalized with the UV spectrum of authentic standard **T-G** in (a). Labels for peak assignments are as follows: **T-OH** for hydroxy-(linker)-thymidine, **T-G** for guanine-(linker)-thymidine, and **T-Br** for bromo-(linker)-thymidine nucleoside.

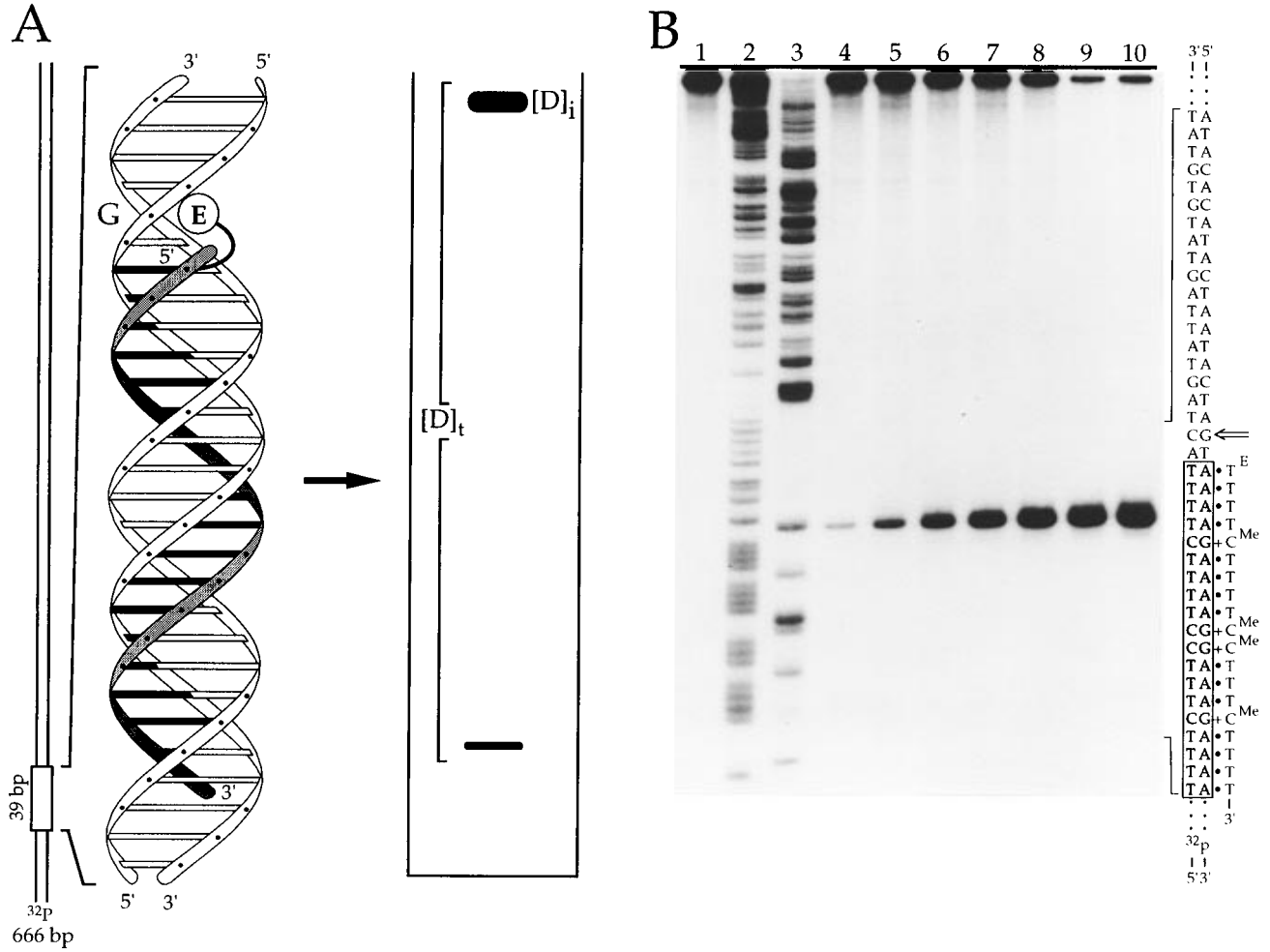

Figure 7. (A) Localization of bromoacetyl (E) by triple-helix formation results in alkylation at a single guanine base (G) in duplex DNA. Following base treatment and polyacrylamide gel electrophoresis, oligonucleotides resolved are those containing intact guanine base (band $[D]_i$) and the oligonucleotide resulting from alkylation at the guanine base. (B) Autoradiogram of an 8% denaturing polyacrylamide gel used to analyze the products of oligonucleotide-directed alkylation and base-promoted strand cleavage within the 3′-^{32}P-end-labeled 666 base pair restriction fragment: (lane 1) control lane, 24 h with no *N*-bromoacetyloligonucleotide; (lane 2) A-specific sequencing reaction; (lane 3) G-specific sequencing reaction; (lanes 4−10) reaction of 200 nM *N*-bromoacetyloligonucleotide with duplex DNA at 37 °C at times 0, 1, 3, 5, 8.8, 12.7, and 27 h.

time = 11 min) remains constant over time and provides an internal standard for relative peak area comparison. Upon superimposition and normalization of the UV spectra of peak **T-G** and the authentic standard, they are shown to be identical (Figure 6e). These data indicate that the covalent bond formation occurs at the N^7 position of guanine proximal to the triple-helical complex. Two additional features of the time course are the presence of peaks consistent with retention times of the starting material (55.2 min, **T-Br**, Figure 6) and the nucleoside formed by hydrolysis (38.4 min, **T-OH**, Figure 6). The relative amounts of **T-Br** and **T-OH** decrease over time, consistent with conversion in the alkylation reaction of *N*-bromoacetyloligonucleotide to guanine-(linker)-oligonucleotide conjugate. As the amount of **T-OH** follows the disappearance of **T-Br**, the presence of the former product likely results from hydrolysis during the enzymatic digest (2 h, pH 8.5) of starting material remaining from the alkylation reaction. This reaction is also seen to occur in control digests of the *N*-bromoacetyloligonucleotide.

Rates of Oligonucleotide-Directed and 2-Bromoacetamide Alkylation of Double-Stranded DNA. It has been demonstrated that localization of bromoacetyl by triple-helix formation on duplex DNA achieves alkylation at one guanine base. To determine the rate enhancement obtained upon tethering of the bromoacetyl moiety to the triple-helical complex, the rate of the intramolecular reaction by the *N*-bromoacetyloligonucleotide at a single guanine base in duplex DNA is compared to the rate of intermolecular alkylation by 2-bromoacetamide at the same guanine base.

The *N*-bromoacetyloligonucleotide of sequence composition 5′-^{E}TTTT^{Me}CTTTT^{Me}C^{Me}CTTT^{Me}CTTTT-3′ is capable of site-specific alkylation of a guanine base 2 base pairs to the 5′-side of the homopurine target site within the 666 base pair fragment of double-stranded DNA derived from plasmid pUCINET (Figure 7). A plot of $\ln([D]_i/[D]_t)$ versus time is linear (Figure 8), indicating that the reaction is first-order in DNA target concentration, and provides a rate constant k_1 of $(2.7 \pm 0.5) \times 10^{-5}$ s^{-1} ($t_{1/2} = 7.2$ h) for the alkylation reaction within the bound complex (*17*). Under these conditions (20 mM Bis-Tris acetate, pH 7.0, 0.8 mM cobalt hexammine trichloride, 37 °C) the rate is maximal down to 200 nM concentration of *N*-bromoacetyloligonucleotide.

For determination of the rate constant of 2-bromoacetamide alkylation at the N^7 position of guanine within duplex DNA, an alkylation reaction on double-stranded DNA (D) by 2-bromoacetamide (E) to form product (P) can be described by

$$D + E \xrightarrow{k_2} P$$

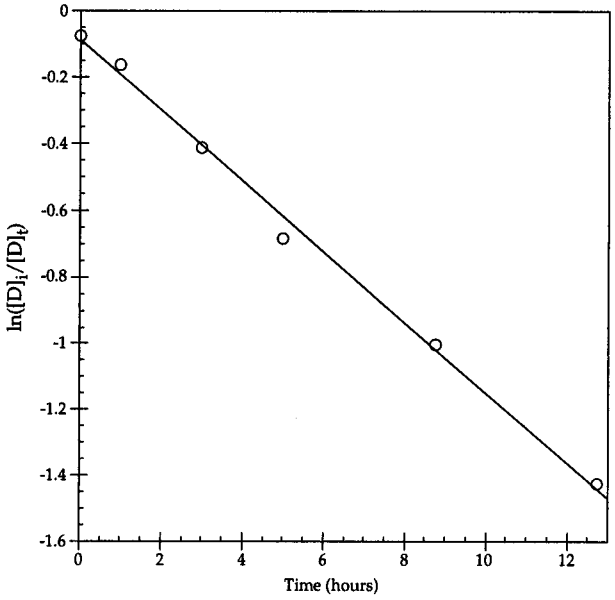

Figure 8. Determination of the rate constant k_1 for oligonucleotide-directed alkylation at the N^7 position of guanine within duplex DNA. Shown is a plot of $\ln([D]_i/[D]_t)$ versus time at 200 nM *N*-bromoacetyloligonucleotide concentration, which provides from an average of four determinations from the slope of the straight line a rate constant k_1 of $(2.7 \pm 0.5) \times 10^{-5}$ s^{-1}.

where k_2 represents the second-order rate constant for alkylation at the N^7 position of one guanine base G in the duplex. The rate of disappearance of duplex containing intact guanine base G is then described by the second-order rate expression $-d[D]/dt = k_2[E][D]$. Formally, the rate of disappearance of fully intact duplex DNA is a sum of the rates of reaction at all guanine bases in the duplex. What is desired in this analysis, however, is the rate of alkylation at guanine base G. To determine this rate, the products of alkylation on a duplex can be resolved on a polyacrylamide gel into two experimentally distinguishable catagories—those containing intact guanine base G (bands $[D]_i$, Figure 9A) and those resulting from alkylation at guanine base G. For this purpose, a 39 base pair duplex of sequence composition identical to the sequence in plasmid pUCINET was synthesized.

Duplex DNA was 5′-^{32}P-end labeled and incubated with 2-bromoacetamide (20 mM Bis-Tris acetate, pH 7.0, 0.8 mM cobalt hexammine trichloride, 37 °C). Reactions were analyzed over time, and cleavage occurs at all guanine bases in the radiolabeled strand of the duplex (Figure 9). Plots of $[D]_i/[D]_t$ versus time under initial rate conditions (*18*) provide observed rate constants (k_{obs}) for the alkylation reaction at various concentrations of 2-bromoacetamide (data not shown). The slope from a plot of observed rate constant versus concentration of 2-bromoacetamide then provides a rate constant k_2 of $(3.6 \pm 0.3) \times 10^{-5}$ M^{-1} s^{-1} for the alkylation reaction of 2-bromoacetamide at N^7 of guanine within duplex DNA (Figure 10).

Included in the observed rate enhancement is the effect of local triple-helix formation on the reactivity of the targeted guanine base to nucleophilic substitution. To determine the magnitude of this effect, an oligonucleotide of identical sequence composition to the *N*-bromoacetyloligonucleotide but lacking the linker arm and electro-

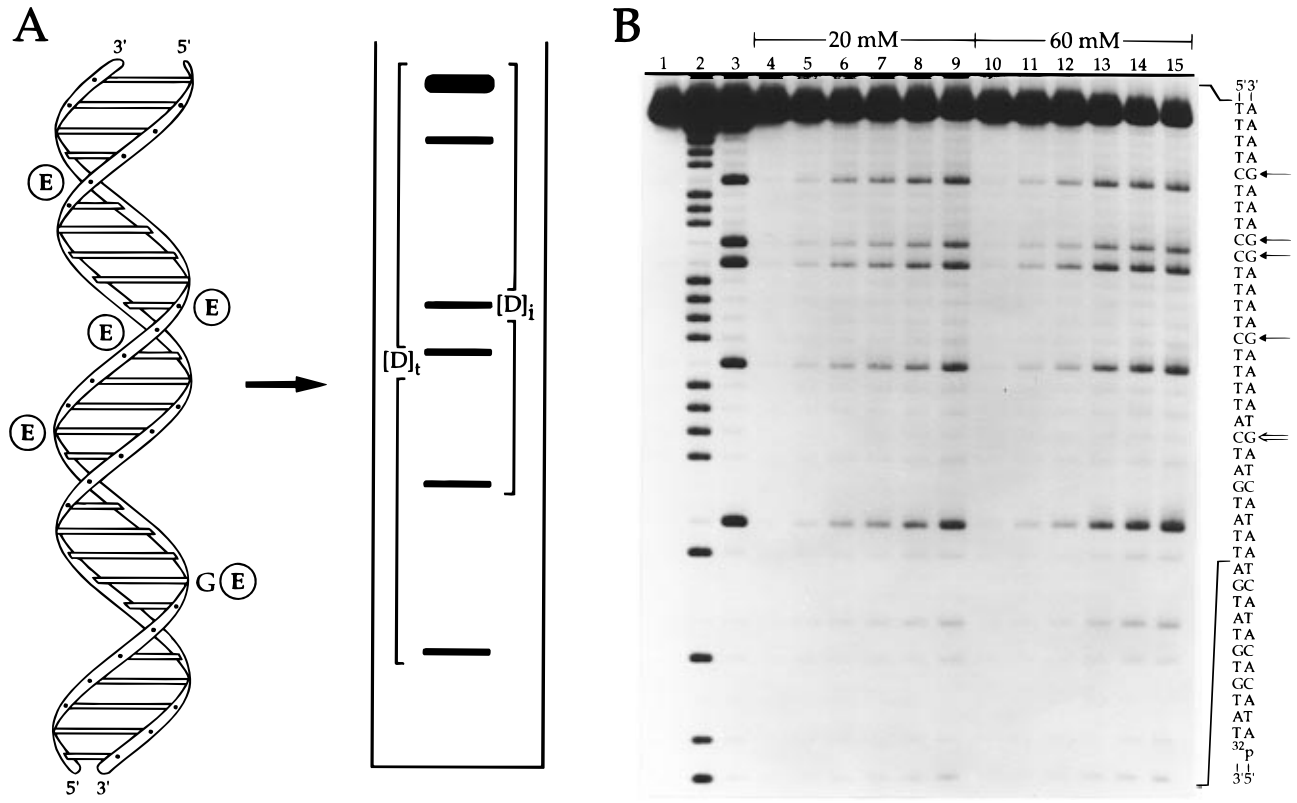

Figure 9. (A) Reaction of free 2-bromoacetamide (E) in solution with duplex DNA results in alkylation of all guanine bases in the radiolabeled strand of the duplex. Base treatment and polyacrylamide gel electrophoresis then resolve a set of oligonucleotides containing the intact guanine base (bands $[D]_i$) and the oligonucleotide resulting from alkylation at the guanine base. (B) Autoradiogram of a 15% denaturing polyacrylamide gel used to analyze the products of intermolecular 2-bromoacetamide alkylation at the N^7 position of guanine and base-promoted strand cleavage within the 5′-^{32}P-end-labeled duplex: (lane 1) control lane, 24 h with no 2-bromoacetamide; (lane 2) A-specific sequencing reaction; (lane 3) G-specific sequencing reaction; (lanes 4−9) reaction of 20 mM 2-bromoacetamide with duplex DNA at times 0, 2.1, 5.8, 9.0, 12.0, and 24.0 h; (lanes 10−15) reaction of 60 mM 2-bromoacetamide with duplex DNA at times 0, 0.9, 2.1, 5.0, 8.0, and 12.0 h.

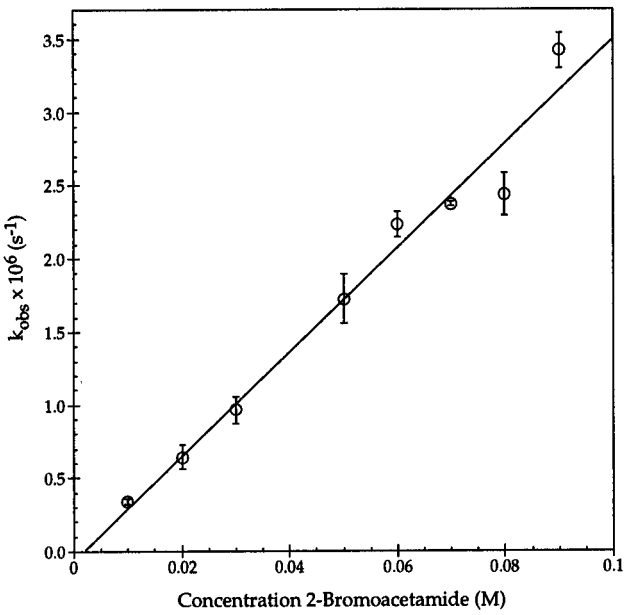

Figure 10. Determination of the rate constant k_2 for the intermolecular alkylation reaction of 2-bromoacetamide at the N^7 position of guanine within duplex DNA. Shown is a plot of k_{obs} versus concentration of 2-bromoacetamide, which provides from the slope a rate constant k_2 of $(3.6 \pm 0.3) \times 10^{-5}$ M^{-1} s^{-1} for the alkylation reaction. Error bars represent standard deviation about the mean for two or three rate determinations at each concentration of 2-bromoacetamide.

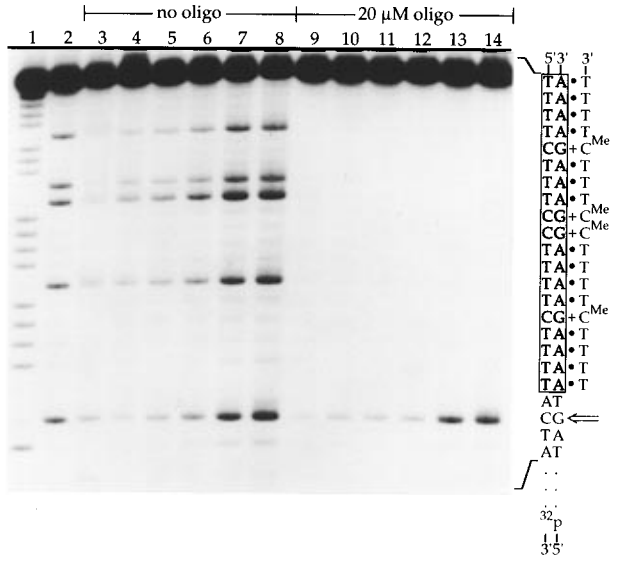

Figure 11. Autoradiogram of a 15% denaturing polyacrylamide gel used to determine the effect of local triple helix formation on the rate of alkylation at N^7 of guanine in duplex DNA by 2-bromoacetamide: (lane 1) A-specific sequencing reaction; (lane 2) G-specific sequencing reaction; (lanes 3−8) reaction of 80 mM 2-bromoacetamide with duplex DNA at times 0, 0.5, 1.0, 2.0, 4.9, and 7.9 h; (lanes 9−14) reaction of 80 mM 2-bromoacetamide with triplex DNA at times 0, 0.5, 1.0, 2.0, 4.9, and 7.9 h.

philic functionality was synthesized and 2-bromoacetamide alkylation reactions were performed in the presence (20 μM) and absence of the Hoogsteen strand of the local triple-helical complex (Figure 11). Binding of the Hoogsteen strand in the major groove protects the N^7 position of guanine from alkylation, resulting in a footprint at the binding site on the duplex. The data then provides observed rate constants at 80 mM 2-bromoacetamide concentration of $(2.4 \pm 0.1) \times 10^{-6}$ and $(1.2 \pm 0.1) \times 10^{-6}$ s^{-1} for alkylation of the targeted guanine base in double-helical and triple-helical DNA, respectively. This indicates an approximate 2-fold decrease in the rate of nucleophilic substitution upon local triple-helix formation.

The effective molarity of bromoacetyl at one position in the duplex upon localization of the alkylation reaction by oligonucleotide-directed triple-helix formation can be derived from the ratio of rate constants for the unimolecular and bimolecular reactions. This provides an effective local concentration of bromoacetyl at the targeted G-C base pair of 0.8 M in the bound complex. Though encouraging with regard to convenient reaction times in this system ($t_{1/2} = 7.2$ h at 200 nM concentration of oligonucleotide), this value does not nearly approach the effective molarities obtained by enzymes (*19*), revealing current limitations in the design of bifunctional molecules for recognition and reaction on DNA.

ACKNOWLEDGMENT

We are grateful for generous support from the National Institutes of Health (GM-35724).

LITERATURE CITED

(1) (a) Hertzberg, R. P., and Dervan, P. B. (1982) Cleavage of Double-Helical DNA by (Methidiumpropyl-EDTA)iron(II). *J. Am. Chem. Soc. 104*, 313. (b) Hertzberg, R. P., and Dervan, P. B. (1984) Cleavage of DNA with Methidiumpropyl-EDTA-Iron(II): Reaction Conditions and Product Analyses. *Biochemistry 23*, 3934. (c) Schultz, P. G., Taylor, J. S., and Dervan, P. B. (1982) Design and Synthesis of a Sequence-Specific DNA Cleaving Molecule. (Distamycin-EDTA)iron(II). *J. Am. Chem. Soc. 104*, 6861. (d) Mack, D. P., Iverson, B. L., and Dervan, P. B. (1988) Design and Chemical Synthesis of a Sequence-Specific DNA-Cleaving Protein. *J. Am. Chem. Soc. 110*, 7572. (e) Mack, D. P., and Dervan, P. B. (1990) Nickel-Mediated Sequence-Specific Oxidative Cleavage of DNA by a Designed Metalloprotein. *J. Am. Chem. Soc. 112*, 4604. (f) Bruice, T. W., Wise, J. G., Rosser, D. S. E., and Sigman, D. S. (1991) Conversion of Lambda Phage Cro into an Operator-Specific Nuclease. *J. Am. Chem. Soc. 113*, 5446.

(2) (a) Baker, B. F., and Dervan, P. B. (1985) Sequence-Specific Cleavage of Double-Helical DNA. *N*-Bromoacetyldistamycin. *J. Am. Chem. Soc. 107*, 8266. (b) Baker, B. F., and Dervan, P. B. (1989) Sequence-Specific Cleavage of DNA by *N*-Bromoacetyldistamycin—Product and Kinetic Analysis. *J. Am. Chem. Soc. 111*, 2700. (c) Boger, D. L., and Mesini, P. (1995) DNA Alkylation Properties of CC-1065 and Duocarmycin Analogs Incorporating the 2,3,10,10a-Tetrahydrocyclopropa-[*d*]benzo[*f*]quinol-5-one Alkylation Subunit—Identification of Subtle Structural Features that Contribute to the Regioselectivity of the Adenine N3 Alkylation Reaction. *J. Am. Chem. Soc. 117*, 11647.

(3) (a) Moser, H. E., and Dervan, P. B. (1987) Sequence-Specific Cleavage of Double Helical DNA by Triple Helix Formation. *Science 238*, 645. (b) Povsic, T. J., and Dervan, P. B. (1989) Triple Helix Formation by Oligonucleotides on DNA Extended to the Physiological pH Range. *J. Am. Chem. Soc. 111*, 3059. (c) Strobel, S. A., and Dervan, P. B. (1991) Single-Site Enzymatic Cleavage of Yeast Genomic DNA Mediated by Triple Helix Formation. *Nature 350*, 172.

(4) (a) LeDoan, T., Perrouault, L., Praseuth, D., Habhoub, N., Decout, J.-L., Thuong, N. T., Lhomme, J., and Helene, C. (1987) Sequence-Specific Recognition, Photocrosslinking, and Cleavage of the DNA Double Helix by an Oligo-[α]-Thymidylate Covalently Linked to an Azidoproflavine Derivative. *Nucleic Acids Res. 15*, 7749. (b) Perrouault, L., Asseline, U., Rivalle, C., Thoung, N. T., Bisagni, E., Giovannangeli, C., Le Doan, T., and Helene, C. (1990) Sequence-Specific Artificial Photoinduced Endonucleases Based on Triple Helix-Forming Oligonucleotides. *Nature 344*, 358. (c) Thuong, N. T., and Helene, C. (1993) Sequence-Specific Recognition and Modification of Double-Helical DNA by Oligonucleotides. *Angew. Chem., Int. Ed. Engl. 32*, 666.

(5) (a) Fedorova, O. S., Knorre, D. G., Podust, L. M., and Zarytova, V. F. (1988) Complementary Addressed Modifica-

tion of Double-Stranded DNA within a Ternary Complex. *FEBS Lett. 228*, 273. (b) Vlassov, V. V., Gaidamakov, S. A., Zarytova, V. F., Knorre, D. G., Levina, A. S., Nikonova, A. A., Podust, L. M., and Fedorova, O. S. (1988) Sequence-Specific Chemical Modification of Double-Stranded DNA with Alkylating Oligodeoxyribonucleotide Derivatives. *Gene 72*, 313. (c) Povsic, T. J., and Dervan, P. B. (1990) Sequence-Specific Alkylation of Double-Helical DNA by Oligonucleotide-Directed Triple-Helix Formation. *J. Am. Chem. Soc. 112*, 9428. (d) Shaw, J-P., Milligan, J. F., Krawczyk, S. H., and Matteucci, M. (1991) Specific, High Efficiency, Triple-Helix-Mediated Cross-Linking to Duplex DNA. *J. Am. Chem. Soc. 113*, 7765. (e) Young, S. L., Krawczyk, S. H., Matteucci, M. D., and Toole, J. J. (1991) Triple Helix Formation Inhibits Transcription Elongation *In Vitro. Proc. Natl. Acad. Sci. U.S.A. 88*, 10023.

(6) (a) Povsic, T. J., Strobel, S. A., and Dervan, P. B. (1992) Sequence-Specific Double-Strand Alkylation and Cleavage of DNA Mediated by Triple-Helix Formation. *J. Am. Chem. Soc. 114*, 5934. (b) Kutyavin, I. V., Gamper, H. B., Gall, A. A., and Meyer, R. B. (1993) Efficient, Specific Interstrand Cross-Linking of Double-Stranded DNA by a Chlorambucil-Modified, Triplex-Forming Oligonucleotide. *J. Am. Chem. Soc. 115*, 9303.

(7) Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) *Molecular Cloning*, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

(8) Iverson, B. L., and Dervan, P. B. (1993) Adenine-Specific DNA Chemical Sequencing Reaction. *Methods Enzymol. 218*, 222.

(9) Gait, M. J. (1984) *Oligonucleotide Synthesis: A Practical Approach*; IRL Press, Oxford, U.K., and references cited therein.

(10) (a) de Groot, N., Lapidot, Y., Panet, A., and Wolman, Y. (1966) The Synthesis of N-Acetylphenylalanyl-sRNA. *Biochem. Biophys. Res. Commun. 25*, 17. (b) Pelligrini, M., Oen, H., and Cantor, C. R. (1972) Covalent Attachment of a Peptidyl-Transfer RNA Analog to the 50S Subunit of *Escherichia coli* Ribosomes. *Proc. Natl. Acad. Sci. U.S.A. 69*, 837.

(11) Dreyer, G. B., and Dervan, P. B. (1985) Sequence-Specific Cleavage of Single-Stranded DNA: Oligodeoxynucleotide-EDTA·Fe(II). *Proc. Natl. Acad. Sci. U.S.A. 82*, 968.

(12) Zurlo, J., Curphey, T. J., Hiley, R., and Longnecker, D. S. (1982) Identification of 7-Carboxymethylguanine in DNA from Pancreatic Acinar Cells Exposed to Azaserine. *Cancer Res. 42*, 1286.

(13) Maxam, A. M., and Gilbert, W. (1980) Sequencing End-Labeled DNA with Base-Specific Chemical Cleavages. *Methods Enzymol. 65*, 499.

(14) Cameron, V., and Uhlenbeck, O. C. (1977) 3′-Phosphatase Activity in T4 Polynucleotide Kinase. *Biochemistry 16*, 5120.

(15) Razzell, W. E., and Khorana, H. G. (1959) Studies on Polynucleotides: IV. Enzyme Degradation. The Stepwise Action of Venom Phosphodiesterase on Deoxyribo-Oligonucleotides. *J. Biol. Chem. 234*, 2114.

(16) Hemminki, K., Peltonen, K., and Vodicka, P. (1989) Depurination from DNA of 7-(2-Aminoethyl)-Guanine and Ring-Opened 7-Methylguanines. *Chem.-Biol. Interact. 70*, 289.

(17) Bevington, P. R., and Robinson, D. K. (1990) *Data Reduction and Error Analysis for the Physical Sciences*, 2nd ed., McGraw-Hill, New York.

(18) Frost, A. A., and Pearson, R. G. (1961) *Kinetics and Mechanism*, 2nd ed., pp 40−46, Wiley, New York.

(19) Page, M. I., and Jencks, W. P. (1971) Entropic Contributions to Rate Accelerations in Enzymic and Intramolecular Reactions and the Chelate Effect. *Proc. Natl. Acad. Sci. U.S.A. 68*, 1678.

BC970035X