avoids a *discrete* carbanion intermediate and allows the rates of the reactions to be explained. Our proposal provides a possible explanation for the observed stereochemical courses of enzyme-catalyzed β-elimination reactions that is based on the pK_as of the α-protons of the carbon acid substrates.

Acknowledgment. We thank Professors Richard L. Schowen, W. W. Cleland, John W. Kozarich, and Debra Dunaway-Mariano for valuable discussions. This research was supported by GM-34572 and GM-40570 from the National Institutes of Health (to J.A.G.) and by the National Science Foundation (to P.G.G.).

Sequence-Specific Double-Strand Alkylation and Cleavage of DNA Mediated by Triple-Helix Formation

Thomas J. Povsic, Scott A. Strobel, and Peter B. Dervan*

Contribution from the Arnold and Mabel Beckman Laboratories of Chemical Synthesis, California Institute of Technology, Pasadena, California 91125. *Received February 12, 1992*

Abstract: Attachment of the nondiffusible electrophile *N*-bromoacetyl to the 5-position of a thymine at the 5'-end of a pyrimidine oligodeoxyribonucleotide affords sequence specific alkylation of a guanine two base pairs to the 5'-side of a local triple-helix complex in >96% yield. *N*-Bromoacetyloligodeoxyribonucleotides bind adjacent inverted purine tracts on double-helical DNA by triple-helix formation and alkylate single guanine positions on opposite strands at 37 °C (pH 7.4). After depurination, double-strand cleavage at a single site within plasmid DNA (4 kp in size) occurs in greater than 85% yield. The resulting DNA fragments from site-specific alkylation and cleavage can be ligated with DNA fragments generated by restriction endonuclease digestion. This nonenzymatic approach which couples sequence-specific recognition with sequence-dependent cleavage affords double-strand site-specific cleavage in megabase size DNA. A yeast chromosome, 340 000 base pairs in size, was cleaved at a single site in 85-90% yield.

Reliable models for the sequence-specific recognition of double-helical DNA by low molecular weight peptides,[1,2] small protein-DNA binding domains,[3,4] and oligonucleotide triple-helix motifs[5-8] now exist. This is due, in part, to a combination of footprinting and affinity cleaving methods[1] for determining sequence specificities, groove locations, and binding orientations of ligands on DNA and the direct structural characterization of some of these complexes by nuclear magnetic resonance spectroscopy[8] and X-ray diffraction analyses.[9] The design of sequence-specific DNA *cleaving* molecules requires the integration of two separate functions, recognition and cleavage in a single molecule. One approach is to combine DNA binding molecules with reactive functionalities capable of oxidation of the deoxyribose,[10,11] electrophilic modification of the bases,[12,13] or hydrolysis of the phosphodiester backbone.[14]

Two criteria for successful bifunctional design are (i) the incorporation of a specific reaction at a designated atom within the bound ligand-DNA complex and (ii) cleavage yields that are quantitative under physiologically relevant conditions. In order to approach quantitative reactions on DNA, the cleaving functionality must be sufficiently reactive to allow modification on DNA at reasonable rates at 37 °C, be inert to aqueous media and buffer components, and not suffer unimolecular decomposition in competition with the desired reaction on DNA. In order to position reactive moieties within angstroms of a single atom

(1) Dervan, P. B. *Science* **1986**, *232*, 464 and references cited therein.
(2) (a) Schultz, P. G.; Taylor, J. S.; Dervan, P. B. *J. Am. Chem. Soc.* **1982**, *104*, 6861. (b) Taylor, J. S.; Schultz, P. G.; Dervan, P. B. *Tetrahedron* **1984**, *40*, 457. Youngquist, R. S.; Dervan, P. B. *J. Am. Chem. Soc.* **1985**, *107*, 5528. Griffin, J. H.; Dervan, P. B. *J. Am. Chem. Soc.* **1986**, *108*, 5008. (c) Wade, W. S.; Dervan, P. B. *J. Am. Chem. Soc.* **1987**, *109*, 1574. (d) Griffin, J. H.; Dervan, P. B. *J. Am. Chem. Soc.* **1987**, *109*, 6840. (e) Youngquist, R. S.; Dervan, P. B. *J. Am. Chem. Soc.* **1987**, *109*, 7564.
(3) (a) Sluka, J. P.; Bruist, M.; Horvath, S. J.; Simon, M. I.; Dervan, P. B. *Science* **1987**, *238*, 1129. (b) Sluka, J. P.; Griffin, J. H.; Mack, J. P.; Dervan, P. B. *J. Am. Chem. Soc.* **1990**, *112*, 6369. (c) Oakley, M. G.; Dervan, P. B. *Science* **1990**, *248*, 847. (d) Sluka, J. P.; Horvath, S. J.; Glasgow, A. C.; Simon, M. I.; Dervan, P. B. *Biochemistry* **1990**, *29*, 6551. (e) Mack, D. P.; Sluka, J. P.; Shin, J. A.; Griffin, J. H.; Simon, M. I.; Dervan, P. B. *Biochemistry* **1990**, *29*, 6561. (f) Graham, K. S.; Dervan, P. B. *J. Biol. Chem.* **1990**, *265*, 16534.
(4) (a) Steitz, T. A. *Q. Rev. Biophys.* **1990**, *23*, 205-280. (b) Harrison, S. C.; Aggarwal, A. K. *Annu. Rev. Biochem.* **1990**, *59*, 933-969.
(5) (a) Moser, H. E.; Dervan, P. B. *Science* **1987**, *238*, 645. (b) Strobel, S. A.; Moser, H. E.; Dervan, P. B. *J. Am. Chem. Soc.* **1988**, *110*, 7927. (c) Povsic, T. J.; Dervan, P. B. *J. Am. Chem. Soc.* **1989**, *111*, 3059. (d) Griffin, L. C.; Dervan, P. B. *Science* **1989**, *245*, 967. (e) Strobel, S. A.; Dervan, P. B. *J. Am. Chem. Soc.* **1989**, *111*, 7286. (f) Maher, L. J.; Wold, B. J.; Dervan, P. B. *Science* **1989**, *245*, 725. (g) Horne, D. A.; Dervan, P. B. *J. Am. Chem. Soc.* **1990**, *112*, 2435. (h) Maher, L. J.; Dervan, P. B.; Wold, B. J. *Biochemistry* **1990**, *29*, 8820. (i) Plum, G. E.; Park, Y. W.; Singleton, S.; Dervan, P. B.; Breslauer, K. T. *Proc. Natl. Acad. Sci. U.S.A.* **1990**, *87*, 9436. (j) Strobel, S. A.; Dervan, P. B. *Science* **1990**, *249*, 9428. (k) Strobel, S. A.; Dervan, P. B. *Nature* **1991**, *350*, 172. (l) Distefano, M. D.; Shin, J. A.; Dervan, P. B. *J. Am. Chem. Soc.* **1991**, *113*, 5901. (m) Horne, D. A.; Dervan, P. B. *Nucleic Acids Res.* **1991**, *19*, 4963. (n) Strobel, S. A.; Doucette-Stamm, L. A.; Riba, L.; Housman, D. E.; Dervan, P. B. *Science* **1991**, *254*, 1639. (o) Maher, L. J.; Dervan, P. B.; Wold, B. *Biochemistry* **1992**, *31*, 70.
(6) (a) Le Doan, T.; Perrouault, L.; Praseuth, D.; Habhoub, N.; Decout, J. L.; Thuong, N. T.; Lhomme, J.; Helene, C. *Nucleic Acids Res.* **1987**, *15*, 7749. (b) Praseuth, D.; Perrousult, L.; Le Doan, T.; Chassignol, M.; Thuong, N. T.; Lhomme, J.; Helene, C. *Proc. Natl. Acad. Sci. U.S.A.* **1988**, *85*, 1349. (c) Francois, J.-C.; Saison-Behmoaras, T.; Chassignol, M.; Thuong, N. T.; Helene, C. *J. Biol. Chem.* **1989**, *264*, 5891. (d) Lyamichev, V. I.; Mirkin, S. M.; Frank-Kamenetskii, M. D.; Cantor, C. R. *Nucleic Acids Res.* **1988**, *16*, 2165. (e) Francois, J. C.; Saison-Behmoaras, T.; Thuong, N. T.; Helene, C. *Proc. Natl. Acad. Sci. U.S.A.* **1989**, *86*, 9198. (f) Perrouault, L.; Asseline, U.; Rivalle, C.; Thuong, N. T.; Bisagni, E.; Giovannangeli, C.; Le Doan, T.; Helene, C. *Nature* **1990**, *344*, 358. (g) Takasugi, M.; Guendouz, A.; Chassignol, M.; Decout, J. L.; Lhomme, J.; Thuong, N. T.; Helene, C. *Proc. Natl. Acad. Sci. U.S.A.* **1991**, *88*, 5602. (h) Mergny, J. L.; Sun, J. S.; Rougee, M.; Montenay-Garestier, T.; Barcelo, F.; Chomilier, J.; Helene, C. *Biochemistry* **1991**, *30*, 9791.
(7) (a) Cooney, M.; Czernuszewicz, G.; Postel, E. H.; Flint, S. J.; Hogan, M. E. *Science* **1988**, *241*, 456. (b) Beal, P. A.; Dervan, P. B. *Science* **1991**, *251*, 1360.
(8) For example see: (a) Rajagopal, P.; Feigon, J. *Nature* **1989**, *239*, 637. (b) de los Santos, C.; Rosen, M.; Patel, D. *Biochemistry* **1989**, *28*, 7282. (c) Radhakrishnan, I.; de los Santos, C.; Patel, D. J. *J. Mol. Biol.* **1991**, *221*, 1408. (d) Radhakrishnan, I.; de los Santos, C.; Live, D.; Patel, D. J. *Biochemistry* **1991**, *30*, 9022.
(9) (a) Wolberger, C.; Vershon, A. K.; Liu, B.; Johnson, A. D.; Pabo, C. O. *Cell* **1991**, *67*, 517. (b) Kissinger, C. R.; Liu, B.; Martin-Blanco, E.; Kornberg, T. B.; Pabo, C. O. *Cell* **1991**, *67*, 517. (c) Jordan, S. R.; Pabo, C. O. *Science* **1988**, *242*, 893-899. (d) Aggarwal, A. K.; Rodgers, D. W.; Drottar, M.; Ptashne, M.; Harrison, S. C. *Science* **1988**, *242*, 899.
(10) (a) Hertzberg, R. P.; Dervan, P. B. *J. Am. Chem. Soc.* **1982**, *104*, 313. (b) Hertzberg, R. P.; Dervan, P. B. *Biochemistry* **1984**, *23*, 3934.
(11) (a) Mack, D. P.; Iverson, B. L.; Dervan, P. B. *J. Am. Chem. Soc.* **1988**, *110*, 7572. (b) Mack, D. P.; Dervan, P. B. *J. Am. Chem. Soc.* **1990**, *112*, 4604.
(12) (a) Baker, B. F.; Dervan, P. B. *J. Am. Chem. Soc.* **1985**, *107*, 8266. (b) Dervan, P. B.; Baker, B. F. *Ann. N.Y. Acad. Sci.* **1986**, *472*, 51. (c) Baker, B. F.; Dervan, P. B. *J. Am. Chem. Soc.* **1989**, *111*, 2700.
(13) Boger, D. L.; Munk, S. A.; Zarrinmayeh, H. *J. Am. Chem. Soc.* **1991**, *113*, 3980.
(14) Basile, L. A.; Raphael, A. L.; Barton, J. K. *J. Am. Chem. Soc.* **1987**, *109*, 7550.

0002-7863/92/1514-5934$03.00/0

proximal to a DNA binding site, knowledge of the groove location and relative base pair position within the ligand–DNA complexes is pivotal. This information can be provided by use of the affinity cleaving technique which relies on the generation of a *nonspecific diffusible* oxidant (hydroxyl radical) at a discrete location within a bound ligand–DNA complex by the attachment of EDTA·Fe to a DNA binding molecule.[1] Replacement of a *diffusible* nonspecific DNA cleaving moiety with a *nondiffusible* moiety[11-14] would provide the necessary specificity to target single nucleotide positions within large double-helical DNA. Nondiffusible DNA cleaving moieties that react at the DNA backbone (sugar oxidation, phosphodiester hydrolysis) would be expected to be less sequence specific than modification of the bases with electrophiles. An early example of sequence-dependent recognition coupled with sequence-dependent cleavage is the reaction of *N*-bromoacetyldistamycin with DNA.[12] *N*-Bromoacetyldistamycin binds DNA in the minor groove at five base pair A,T rich sites and alkylates single adenine positions at N3 within those sites at different rates at 37 °C.[12] An example of sequence-dependent recognition coupled with sequence-independent cleavage is the reaction of Ni(II)·GGH(Hin 139–190) with DNA.[11] The Ni(II)·GGH segment oxidizes a single deoxyribose position in the adjacent minor groove of the DNA binding site for the helix–turn–helix protein, Hin 139–190.

Triple-Helix Motif for Sequence-Specific DNA Recognition. 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.[5,6] Specificity is derived from thymine (T) recognition of adenine·thymine (AT) base pairs (TAT triplet) and N3-protonated cytosine (C+) recognition of guanine·cytosine (GC) base pairs (C + GC triplet). The identification of other natural base triplets,[5d,7] alternate strand triple-helix formation,[5g] and the design of nonnatural base triplets[15] have demonstrated the generalizability of triple-helix formation for the recognition of mixed sequences containing all four base pairs.

Oligonucleotides conjugated to EDTA·Fe can specifically bind double-helical DNA by triple-helix formation and produce double-strand cleavage at single sites in DNA.[5a] However, the chemical yields for double-strand oxidative cleavage by oligonucleotides equipped with EDTA·Fe are typically low.[5a] In the case of plasmid (4 kb)[5a] and λ phage (48.5 kb)[5b] DNA, the yields for site-specific double-strand cleavage are 15 and 25%, respectively. Such yields are adequate for addressing questions related to molecular recognition, including binding specificities, site size, and orientation.[5a] In the case of megabase DNA, the yields decline further.[5j] If the full potential of oligonucleotide-directed recognition of double-helical DNA is to be realized for site-specific cleavage of DNA of high complexity, the design of oligonucleotides equipped with moieties capable of *quantitative modification* of DNA is an area for further development.

Enzymatic Cleavage Mediated by Triple-Helix Formation. There appears to be two separate approaches which have in common coupling *enzymatic* cleavage with oligonucleotide-directed recognition of DNA. One is to attach DNA cleaving enzymes to oligonucleotides. An oligonucleotide–staphylococcal nuclease adduct cleaves plasmid DNA in 75% yield.[16] Although the nuclease is, in a formal sense, nondiffusible, multiple cleavage sites at the binding site are observed, likely due to conformational flexibility of the attached enzyme.[16] The other approach is termed Achilles heel cleavage and involves transient site-specific protection from enzymatic methylation mediated by triple-helix formation, followed by triple-helix disruption and cleavage by a restriction enzyme.[5f,k,n] Enzymatic cleavage mediated by this approach has been shown to cleave a single site in a yeast chromosome (340 kp in size) in 95% yield[5k] and human chromosome 4 (200 megabase pairs in size) in 80–90% yield.[5n] It remains a challenge

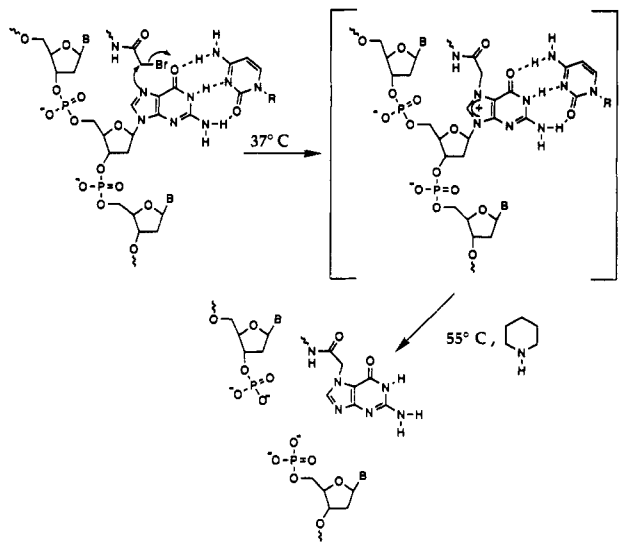


Figure 1. The *N*-bromoacetyl electrophile is localized in the major groove by triple-helix formation proximal to a GC base pair (G) in the Watson–Crick duplex target site. Alkylation at N7 of G followed by depurination results in backbone cleavage to afford 5′ and 3′ phosphate termini.[17,19]

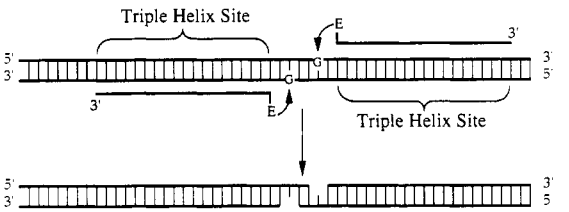


Figure 2. Oligodeoxyribonucleotides equipped with an electrophile (E) at the 5′-end bind to adjacent inverted binding sites on double-helical DNA by triple-helix formation and alkylate at single guanine positions on opposite strands. After depurination, double-strand cleavage produces sequence-specific overhangs suitable for ligation.

for chemists to match the enzymatic yields for DNA cleavage (75–95%) with rational mechanism-based chemical approaches for quantitative modification of DNA within a triple-helix complex.

Sequence-Specific Alkylation of Double-Helical DNA by Triple-Helix Formation. Recently, oligonucleotides equipped with electrophiles have shown promise for the sequence-specific alkylation and, after depurination, cleavage of double-helical DNA on one strand.[17,18] Oligonucleotides equipped with aromatic chloroethylamines at the 5′-end afforded cleavage of one strand of double-helical DNA in modest yield.[18ab] However, oligonucleotides equipped with *N*-bromoacetyl[17] or ethano-5-methyldeoxycytidine[18c] react with one strand of a duplex target site in very high yield. Pyrimidine oligodeoxyribonucleotides bound in the major groove and equipped with an *N*-bromoacetyl moiety at the 5′-end position the electrophile proximal to a guanine two base pairs to the 5′-side of the target sequence. Reaction of the electrophilic carbon of *N*-bromoacetyl with N7 of guanine 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. The DNA termini are 5′ and 3′ phosphates (Figure 1).[19]

Despite the high yields, oligonucleotide-directed sequence-specific alkylation of single GC base pair positions in the major groove of DNA is limited to cleavage of one strand. The question

(15) (a) Koh, J. S.; Dervan, P. B. *J. Am. Chem. Soc.* **1992**, *114*, 1470. (b) Kiessling, L. L.; Griffin, L. C.; Dervan, P. B. *Biochemistry* **1992**, *31*, 2829.
(16) Pei, D.; Corey, D. R.; Schultz, P. G. *Proc. Natl. Acad. Sci. U.S.A.* **1990**, 9858.
(17) Povsic, T. J.; Dervan, P. B. *J. Am. Chem. Soc.* **1990**, *112*, 9428.
(18) (a) Fedorova, O. S.; Knorre, D. G.; Podust, L. M.; Zarytova, V. F. *FEBS Lett* **1988**, *228*, 273. (b) Vlassov, V. V.; Gaidamakov, S. A.; Zarytova, V. F.; Knorre, D. G.; Levina, A. S.; Nekona, A. A.; Podust, L. M.; Fedorova, O. A. *Gene* **1988**, *72*, 313. (c) Shaw, J. P.; Milligan, J. F.; Krawczyk, S. H.; Matteucci, M. *J. Am. Chem. Soc.* **1991**, *113*, 7765.
(19) Povsic, T. J. Ph.D. Thesis, California Institute of Technology, 1992.

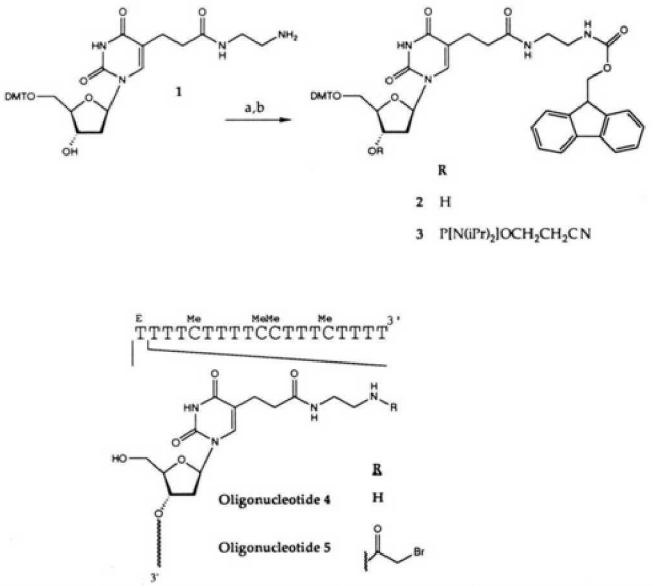


Figure 3. Scheme for construction of Fmoc-protected thymidine 2-cyanoethyl *N,N*-diisopropylphosphoramidite **3** used in the synthesis of oligonucleotide–amine **4**. Modification of oligonucleotide amine **4** with *N*-hydroxysuccinimidyl bromoacetate afforded *N*-bromoacetyloligonucleotide **5**. T^E is a thymidine with an *N*-bromoacetyl attached by a short linker to the 5-position of the pyrimidine.

arises whether high-yield site-specific reactions could be extended to *both strands* of a double-helical DNA target site. For example, two adjacent triple-helix sites with two *N*-bromoacetyloligonucleotides bound on opposite Watson–Crick strands would afford double-strand alkylation and subsequent cleavage after depurination. This could result in DNA fragments with sequence-specific overhangs and phosphate termini suitable for ligation by restriction enzymes. If the overall alkylation yields are sufficiently high for both strands of DNA, this nonenzymatic approach to recognition by triple-helix formation and base-specific modification by electrophiles might allow single-site double-strand cleavage of megabase DNA (Figure 2).

We report here that *N*-bromoacetyloligonucleotides at μM concentrations bind two adjacent inverted purine tracts on double-helical DNA, each containing a guanine residue two base pairs from its 5′-terminus and double-strand cleave DNA at a single site in high yield (Figure 2). From analyses of cleavage patterns on high-resolution polyacrylamide gels, the cleavage on each strand is at a single nucleotide position. Analysis of a 6.7-kb plasmid revealed that double-strand cleavage is achieved at a single site in 85% yield. Additionally, treatment with base generates DNA termini that can be ligated to complementary DNA fragments produced by restriction enzyme digestion. With regard to chromosomal DNA of high complexity, the nonenzymatic reaction is sufficiently efficient and specific that it produces double-strand cleavage at a single site in high yield within a yeast chromosme 340 000 bp in size.

Results and Discussion

Synthesis of *N*-Bromoacetyloligodeoxyribonucleotide (5). The synthesis of 5′-*O*-DMT-3′-phosphoramidite **3** proceeds in two steps from the known aminodeoxyribonucleoside **1**[20] (Figure 3). Protection of the primary amine with 9-fluorenylmethyl chloroformate followed by reaction of the 3′-hydroxyl with β-cyanoethyl-*N,N*-diisopropylchlorophosphoramidite[21] affords a thymidine derivative suitable for use in standard automated oligodeoxyribonucleotide synthesis. Incorporation of the modified base at the 5′-end of an oligonucleotide was achieved in high yield. Deprotection of the primary amine occurs quantitatively under

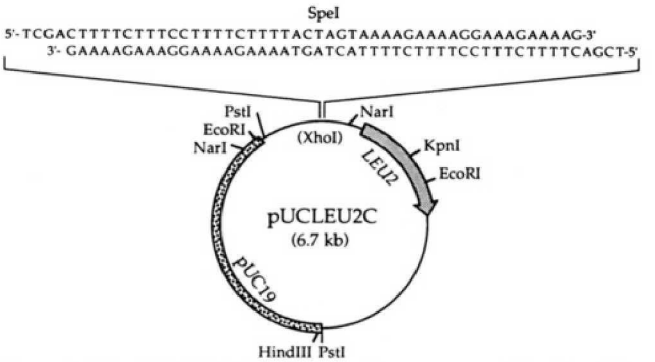


Figure 4. Plasmid pUCLEU2C showing the sequence and location of the triple-helix target sites and the location of the restriction enzyme sites used in this study.

standard deprotection conditions (concentrated NH_4OH, 55 °C, 20 h), as analyzed by reverse-phase and anion-exchange HPLC and polyacrylamide electrophoresis. Oligodeoxyribonucleotide amine **4** was allowed to react with the *N*-hydroxysuccinimide ester of bromoacetic acid to afford *N*-bromoacetyloligodeoxyribonucleotide (**5**) which was purified by anion exchange HPLC.[17,19]

Construction of the Target Sequence. Adjacent inverted binding sites for triple-helix formation would afford alkylation and cleavage of both Watson–Crick DNA strands. Consideration of the cleavage patterns produced by triplex-mediated alkylation indicates that a target sequence of the type 5′-(pyrimidine)$_m$NC(N)$_n$GN-(purine)$_m$-3′ which contains two inverted binding sites for triple-helix formation on adjacent strands may be suitable for double-strand cleavage. This places cleavage positions on both strands of the inverted binding sites separated by *n* base pairs. The target site was designed with a single guanine two base pairs to the 5′-side of each triplex binding site, the position most efficiently alkylated by the bound *N*-bromoacetyloligonucleotide **5**.[17] A single G, flanked by A, T, or C, promotes cleavage specific to a single nucleotide position on each strand. Upon treatment with base, overhangs of size $n + 1$ are produced, reminiscent of the type generated by some restriction enzymes (when $n = 2$). The double-strand cleavage could result in DNA termini compatible for ligation with those produced via cleavage with a restriction endonuclease.

Based on the above considerations, the sequence 5′-CTTTTCTTTCCTTTTCTTTTACTAGTAAAAGAAAAG-GAAAGAAAAG-3′ was cloned into the pUCLEU2 plasmid to generate pUCLEU2C (Figure 4). This sequence contains binding sites on each strand for the *N*-bromoacetyloligonucleotide **5** of sequence composition 5′-TTTT^{Me}CTTTT^{Me}C^{Me}CTTT^{Me}CT-TTT-3′ previously shown to efficiently modify the purine strand.[17] After modification of the targeted guanine by the *N*-bromoacetyloligonucleotide within the triple-helical complex, depurination and strand scission would occur, resulting in a 3′ overhang (CTA-3′) compatible with one end (TAG-3′) generated by SfiI cleavage at the site (5′-GGCCTCTA/GGGCC-3′). By cloning the site into pUCLEU2 plasmid containing the *LEU2* gene,[22] a gene essential for biosynthesis of leucine, it is possible to specifically introduce this target site into yeast chromosome III. The PstI fragment from pUCLEU2 containing both the *LEU2* gene and the target sequence was homologously recombined into chromosome III of a *leu2* deficient yeast strain (SEY6210). Recombinants were selected by growth on minimal media lacking leucine. The target site would be positioned on chromosome III such that cleavage should produce 230- and 110-kb products.[5k] This facilitates the study of the specificity of double-strand cleavage and yields afforded by *N*-bromoacetyloligonucleotides (i) on DNA restriction fragments at single base pair resolution by denaturing polyacrylamide gel electrophoresis (ii) on plasmid DNA 6.7 kb in size and (iii) DNA of megabase pair complexity as analyzed

(20) Dreyer, G. B.; Dervan, P. B. *Proc. Natl. Acad. Sci. U.S.A.* **1985**, *82*, 968.

(21) Gait, M. J., Ed. *Oligonucleotide Synthesis: a Practical Approach*; IRL Press: Oxford, 1984; and references cited therein.

(22) Broach, J. R. et al. *Gene* **1979**, *8*, 121. Andrea-dis, A.; Hsu, Y. P.; Kohlhaw, G. B.; Schimmel, P. *Cell* **1982**, *31*, 319. Fischhoff, D. A.; Waterson, R. H.; Olson, M. V. *Gene* **1984**, *27*, 239.

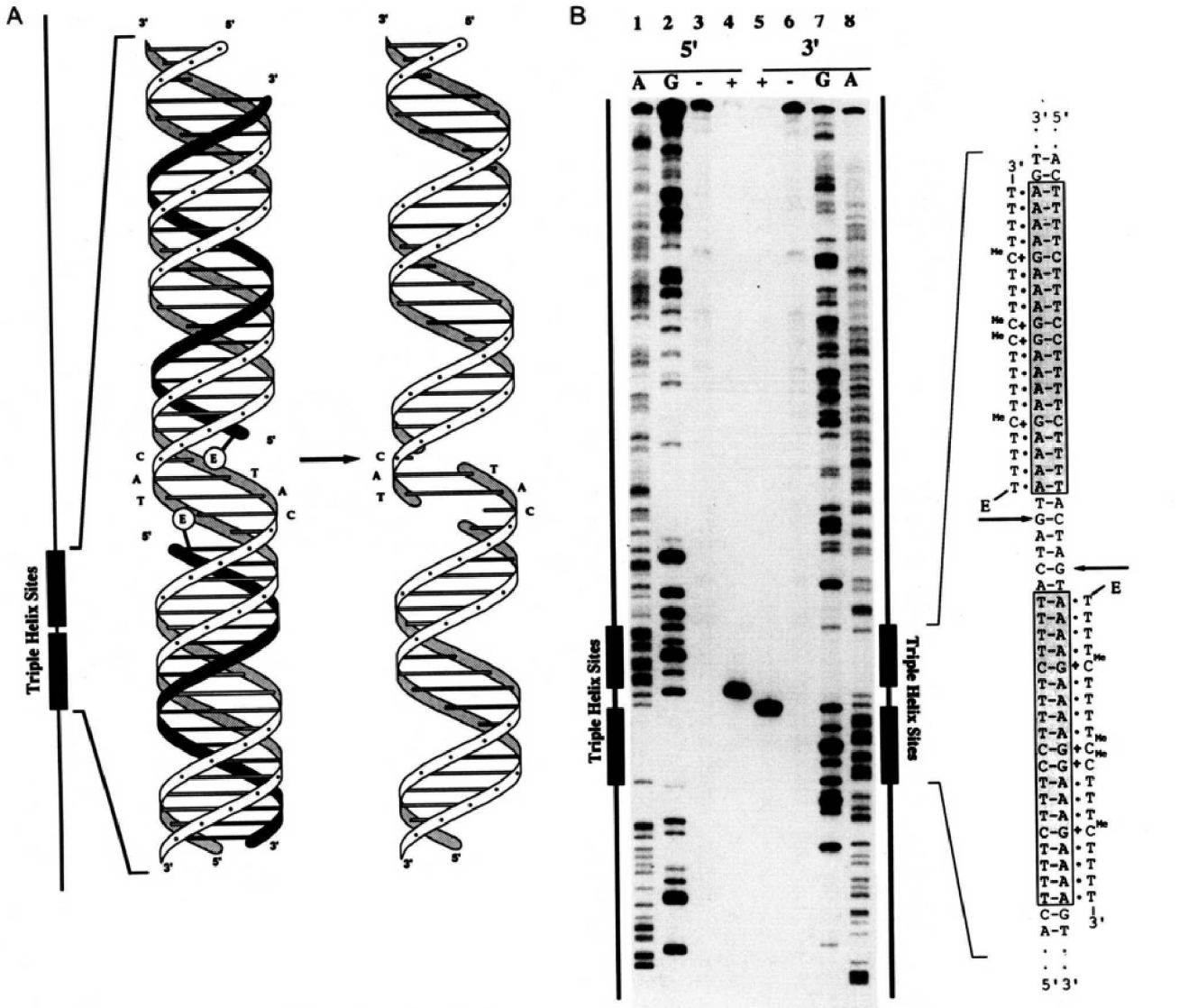


Figure 5. (A) Ribbon model of *N*-bromoacetyloligonucleotide-directed double-strand cleavage of DNA by triple-helix formation at inverted adjacent binding sites. Cleavage occurs at a single nucleotide position on each Watson–Crick strand within each triple-helix complex. (B) Left: Autoradiogram of a high-resolution denaturing polyacrylamide gel revealing cleavage products from reaction of *N*-bromoacetyloligonucleotide **5** with a 0.9-kbp restriction fragment from plasmid pUCLEU2C labeled at the 3′- and 5′-end with ^{32}P. Lanes 1–4 are 5′-end-labeled DNA. Lanes 5–8 contain 3′-end-labeled DNA. Lanes 1 and 8 are A-specific chemical sequencing reactions for the 5′- and 3′-end-labeled fragments.[29] Lanes 2 and 7 are G-specific chemical sequencing reactions for the 5′- and 3′-end-labeled fragments.[30] Lanes 3 and 6 are controls that contain DNA incubated for 36 h in the absence of *N*-bromoacetyloligonucleotide **5** followed by treatment with piperidine. Lanes 4 and 5 contain DNA incubated for 36 h with *N*-bromoacetyloligonucleotide **5** followed by treatment with piperidine. Right: Sequence of the oligonucleotide and DNA target site. The major sites of modification are indicated by arrows.

by pulsed field gel electrophoresis.

High-Resolution Analysis of Opposite-Strand-Cleavage Specificity. A 974-bp NarI/EcoRI restriction fragment from pUCLEU2C containing the inverted triple-helix sites was radiolabeled with ^{32}P approximately 210 bp from the target sites (Figure 5A).[23] DNA labeled on either strand (3′ or 5′) was allowed to react with *N*-bromoacetyloligonucleotide **5** at 1 μM concentration at 37 °C for 36 h (0.8 mM $Co(NH_3)_6^{3+}$, 20 mM HEPES, pH 7.4).[17,24] After treatment with 0.1 M piperidine, (90 °C, 30 min), analysis of the high-resolution polyacrylamide gel used to separate the cleavage products reveals that modification occurred at a single deoxyguanosine located two bases to the 5′-side of each oligonucleotide binding site (Figure 5B). Quantitative analyses of storage-phosphor autoradiograms reveals that the cleavage yields were >96% on each strand.

A plot of ln $[DNA]_{intact}/[DNA]_{total}$ vs time (pseudo-first-order conditions) is linear, indicating that the reaction between oligonucleotide **5** and double-helical DNA is first order in DNA target concentration with first-order rate constants that correspond to a half-life for alkylation within the triple-helical complex of 5.5 and 6.5 h for the 3′- and 5′-end-labeled strands, respectively.[19] The measured rates at adjacent occupied inverted sites are not significantly different from that reported for reaction of the *N*-bromoacetyloligonucleotide **5** when only a single binding site is present.[17]

Double-Strand Cleavage of Plasmid DNA. To determine the yield of double-strand cleavage, *N*-bromoacetyloligonucleotide **5** at 1 μM concentration (0.8 mM $Co(NH_3)_6^{3+}$, 20 mM HEPES, pH 7.4) was allowed to react for 36 h at 37 °C with a linear plasmid DNA, 6.7 kbp in size. Cleavage at the adjacent triple-helix target sites should produce DNA fragments 3.3 and 3.4 kilobase pairs in size which could be analyzed by nondenaturing gel electrophoresis. Treatment of the alkylated plasmid with 0.1% piperidine at 55 °C for 12 h in a buffer containing 10 mM EDTA,

(23) Samblook, J.; Fritsch, E. F.; Maniatis, T. *Molecular Cloning*, 2nd ed.; Cold Spring Harbor Laboratory: Cold Spring Harbor, NY, 1989.

(24) It is known that triple-helix formation is facilitated by the addition of certain cations.[5,6] However, the presence of spermine and Tris buffers reduced the cleavage reaction yields, likely due to reaction with the bromoacetyl moiety. Therefore, the polycation $Co(NH_3)_6^{3+}$ and HEPES buffer were substituted.[17] In control experiments, we observed no significant reaction between $Co(NH_3)_6^{3+}$ and the *N*-bromoacetyloligonucleotide (at 37 °C).

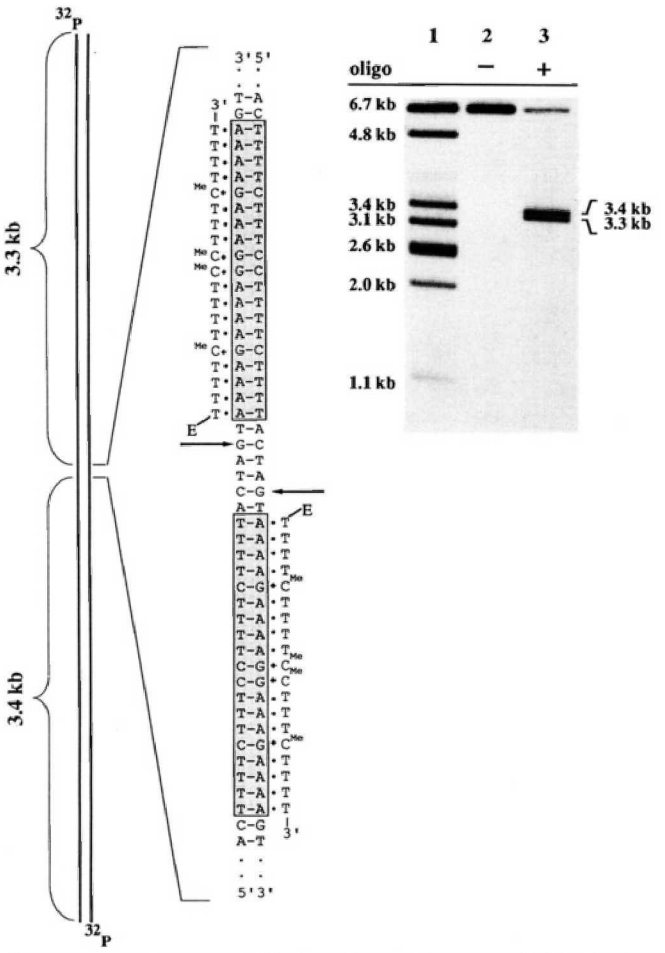


Figure 6. Left: Diagram of linearized plasmid pUCLEU2C indicating location and sequence of oligonucleotide binding sites. The sites of alkylation and subsequent DNA cleavage are indicated. Right: Autoradiogram of a 1.0% agarose gel of cleavage products from the reaction of oligonucleotide **5** with HindIII plasmid pUCLEU2C (6.7 kbp in length) with ^{32}P at the 3′-ends. Lane 1: DNA size markers obtained by digestion with restriction endonucleases. Sizes are indicated to the left of the band. Lane 2: DNA incubated in the absence of oligonucleotide **5** and treated with piperidine. Lane 3: DNA incubated with oligonucleotide **5** and treated with piperidine.

10 mM Tris·HCl, pH 8.0, and 100 mM NaCl resulted in two DNA fragments (Figure 6). Radiolabeled DNA was used to determine the efficiency of double-helical cleavage. The ^{32}P end-labeled products resulting from double-strand cleavage were separated by agarose gel electrophoresis. Integration of storage-phosphor autoradiogram signals indicated double-strand-cleavage efficiencies of 85%. In a control, no cleavage was observed in the absence of *N*-bromoacetyloligonucleotide **5**.

Enzymatic Ligation of DNA Products Resulting from Double-Strand Cleavage by *N*-Bromoacetyloligonucleotides. Cleavage of pUCLEU2C with *N*-bromoacetyloligonucleotide **5** produced ends with the 3′ overhanging sequence 5′-CTA-3′ (Figure 7). End product analysis following piperidine workup indicated that the 3′- and recessed 5′-termini of both ends contain phosphate groups.[19] Ligation reactions between restriction enzyme digested products involve esterification of the 5′ phosphate with the 3′-hydroxyl of an adjacent nucleotide. Digestion of the plasmid pUCSfiI with the enzyme SfiI produced a DNA product with the 3′ overhanging sequence 5′-TAG-3′, which should be complementary to the end of the DNA product resulting from reaction with *N*-bromoacetyloligonucleotide **5**. Because of the 3′-end of the SfiI product contains a free hydroxyl group, it could be ligated to the 5′ phosphate of the *N*-bromoacetyloligonucleotide treated DNA. The oligonucleotide **5** derived cleavage products of pUCLEU2C (EcoRI cut) were ligated into the SfiI/EcoRI fragment of pUCSfiI by T4 DNA ligase. The colonies were screened by α-complementation and five of each of the resultant plasmids were sequenced.[23] All products were consistent with simple ligation of each of the *N*-bromoacetyloligonucleotide/EcoRI cut fragments into the StiI/EcoRI sites of pUCSfiI. Guanine was not detected at the ligation site consistent with depurination following alkylation. Thus, this synthetic cleavage agent produced DNA cleavage products that can be ligated into restriction enzyme digested DNA (Figure 7).

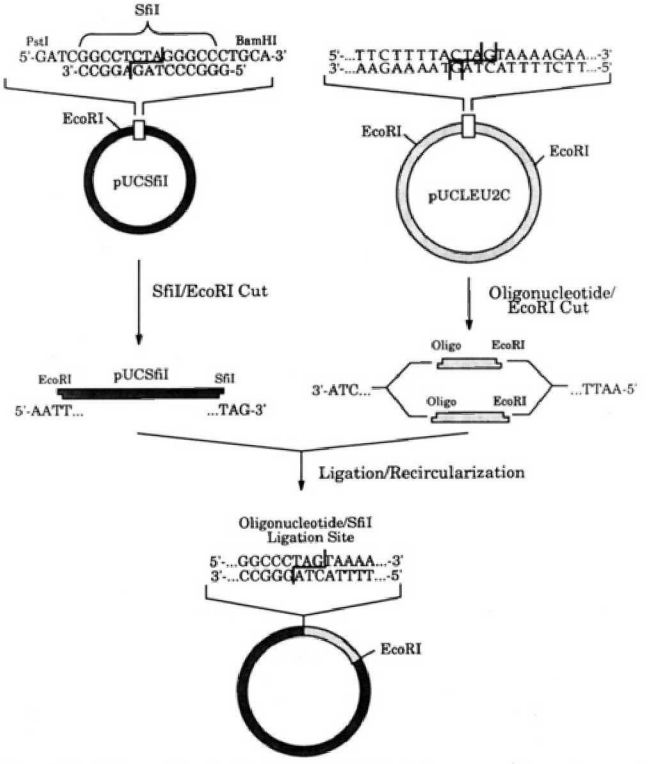


Figure 7. Scheme for the ligation of DNA cleavage products obtained by reaction of oligonucleotide **5** with plasmid pUCLEU2C into the complementary SfiI site of plasmid pUCSfiI. Incubation of plasmid pUCLEU2C with *N*-bromoacetyloligonucleotide **5** followed by piperidine treatment results in double-strand cleavage products with a 3′ overhang (CTA-3′). Digestion with the restriction endonuclease EcoR I produces two fragments containing one 5′-AATT and one CTA-3′ overhang. Both of these products are complementary with the SfiI/EcoRI digestion product of pUCSfiI (5′-AATT and TAG-3′ overhangs). After circularization and transformation of bacterial cells with the new plasmid, DNA was isolated and sequenced to verify the production of ligatable ends.

Double-Strand Cleavage of a Yeast Chromosome at a Single Site. Oligonucleotides equipped with the nonspecific DNA cleaving moiety EDTA·Fe(II) are capable of binding to and cleaving DNA at a specific site within a yeast chromosome but in low yield. Moreover, unless conditions are carefully chosen, oligonucleotides–EDTA·Fe bind and cleave at partially homologous secondary sites. Coupling a base-specific cleaving moiety at the 5′-end of an oligonucleotide capable of binding by triple-helix formation may result in a molecule that binds at secondary sites but does *not react* in the absence of a guanine residue two base pairs to the 5′-side of the local triplex. Further double-strand cleavage would occur only if triple-helix sites are present on *both* strands of the DNA within a reasonable number (<20–30) of base pairs for double-strand modification, cleavage, and denaturation to occur.

Chromosomal DNA from the appropriate yeast strain, SE-Y6210C, was prepared in low melting point (LMP) agarose to prevent mechanical shearing of the megabase DNA. The DNA was allowed to react with the *N*-bromoacetyloligonucleotide **5** using two cleavage cycles. This was accomplished by allowing the chromosomal DNA to react at 37 °C with oligonucleotide **5** as described above (1.0 mM $Co(NH_3)_6^{3+}$, 20 mM Hepes, pH 7.4, 10 mM EDTA) followed by washes in a low-salt, high-pH buffer to disrupt the triple helix and to remove unreacted oligo-

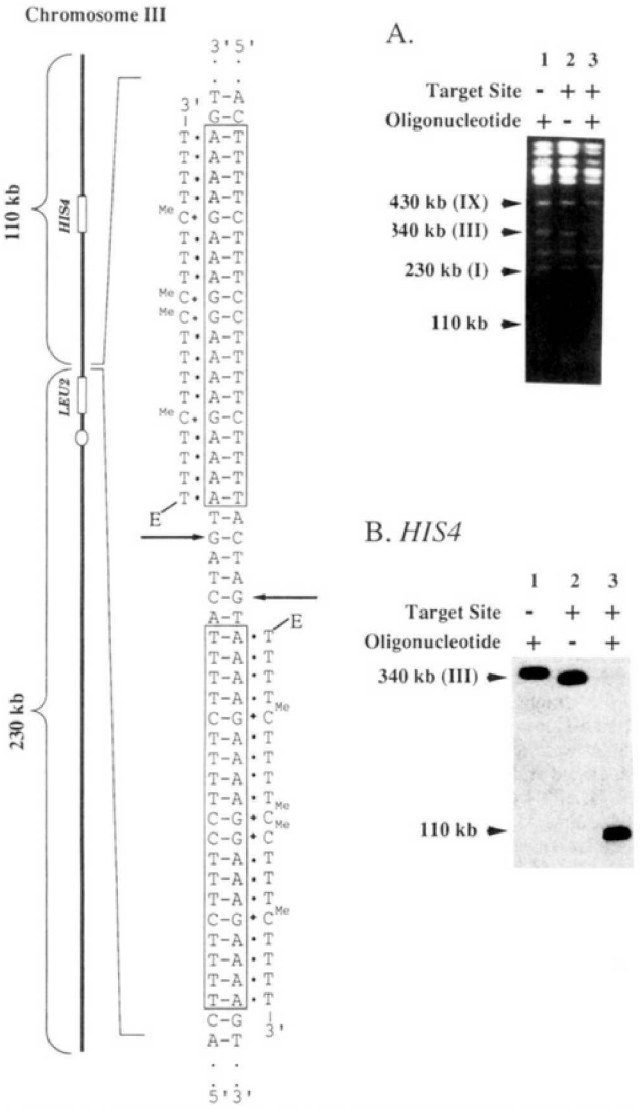


Figure 8. Cleavage of yeast chromosome III by *N*-bromoacetyloligonucleotide **5**. Left: Diagram of yeast chromosome III indicating location and sequence of oligonucleotide binding sites and the *HIS*4 locus used for cleavage analysis. The binding site sequence and sites of alkylation for subsequent site-specific DNA cleavage are indicated. Right: DNA from yeast strains SEY6210 (no target site) and SEY6210C (+ target site) allowed to react with *N*-bromoacetyloligonucleotide **5**. Lane 1: Control with DNA from yeast strain SEY6210 lacking triple-helical target site incubated with *N*-bromoacetyloligonucleotide **5** followed by treatment with piperidine. Lane 2: Control with DNA from yeast strain SEY6210C incubated in the absence of *N*-bromoacetyloligonucleotide **5** followed by treatment with piperidine. Lane 3: DNA from yeast strain SEY6210C incubated with *N*-bromoacetyloligonucleotide **5**, followed by treatment with piperidine. (A) Separation of yeast chromosomes by pulsed field gel electrophoresis in agarose and stained with ethidium bromide. (B) Autoradiogram of a DNA blot hybridization of gel in part A with a 250-bp HIS 4 fragment radiolabeled with ^{32}P by random-primer extension. Locations of the intact chromosome II (340 kbp) and the 110-kbp cleavage fragment are indicated. Yield of site-specific cleavage is 90%.

nucleotide from the solution. The DNA was reequilibrated in triple-helix alkylation buffer and a second aliquot of *N*-bromoacetyloligonucleotide **5** added. The DNA was then treated with 0.1% piperidine at 55 °C for 12 h to effect double-strand cleavage. The cleavage products were separated by pulsed field gel electrophoresis and visualized by ethidium bromide staining and hybridization with the chromosome III specific marker, *HIS4*.[25]

Reaction of total yeast chromosomal DNA with *N*-bromoacetyloligodeoxyribonucleotide (**5**) resulted in the cleavage of chromosome III to produce 230- and 110-kb products (Figure 8).[26] No other cleavage products could be detected by ethidium bromide staining within 14 megabase pairs of total yeast genomic DNA, nor was there evidence of random degradation of total genomic DNA by the piperidine treatment (Figure 8A). No cleavage products were detected on DNA from a yeast strain lacking a target site or in the absence of oligonucleotide **5** (Figure 8). The cleavage efficiency was quantitated by hybridization with the *HIS4* marker specific to the 110-kb product (Figure 8B). A double-strand-cleavage efficiency of 90% was observed at the target site after two 20-h reaction cycles, and no low-efficiency secondary cleavage sites were detected on chromosome III. A slightly lower cleavage efficiency (85%) was observed if only one cleavage cycle was performed. These efficiencies for single-site cleavage of chromosome III are in sharp contrast to oligonucleotide–EDTA·Fe mediated cleavage which results in an overall yield of 6%.[5j] Moreover, the specificity of cleavage appears higher. Single-site cleavage predominates with *N*-bromoacetyloligonucleotide **5** whereas several secondary binding sites are observed with the oligonucleotide–EDTA·Fe mediated cleavage reaction. The specificity and efficiency of the oligonucleotide-directed double-strand alkylation and cleavage of megabase DNA are comparable to that achieved by triple-helix-mediated enzymatic cleavage.[5k]

Concluding Remarks. Oligonucleotide-directed alkylation of double-helical DNA has been examined at inverted adjacent binding sites on DNA of different sizes: restriction fragments, plasmids and yeast. The high specificity of the double-strand-cleavage reaction is derived from the requirement of two adjacent inverted oligonucleotide binding sites that each contained a G two base pairs to the 5′-side of the triple-helical complex. The cleavage reaction resulting from depurination afford DNA termini that were ligatable with fragments generated from restriction enzyme digestion. This *nonenzymatic* approach affords double-strand-cleavage yields comparable to those attained by triple-helix-mediated *enzymatic* cleavage of DNA. Because oligonucleotide-directed recognition of double-helical DNA by triple-helix formation is one million times more specific than restriction enzymes, it is not unthinkable that quantitative reactions targeted to single atoms in the human genome (3 billion base pairs) should be possible by strictly chemical methods.

Experimental Section

^1H NMR spectra were recorded at 400 MHz on a JEOL-GX 400 NMR spectrometer. Chemical shifts are reported in parts per million downfield from tetramethylsilane. IR spectra were recorded on a Shimadzu IR-435 infrared spectrometer. High-resolution mass spectra (HRMS) were recorded using electron ionizations (EI) or fast atom bombardment (FAB) techniques at the Midwest Center for Mass Spectrometry at the University of Nebraska or the Mass Spectrometry Laboratory at the University of California, Riverside. Collidine, 9-fluorenylmethyl chloroformate, and 2-cyanoethyl-*N*,*N*-diisopropylchlorophosphoramidite were purchased from Aldrich and used without further purification. Diisopropylethylamine (Aldrich) was distilled from CaH_2 prior to use. DMF, methylene chloride, and dioxane were obtained as anhydrous solvents from Fluka. Restriction enzymes were obtained from New England BioLabs and used with the manufacturer's supplied buffers. Polynucleotide kinase, T4 DNA ligase, and the Klenow fragment of DNA polymerase were obtained from Boehringer Mannheim. Radioactive nucleotides were purchased from Amersham. Prep-a-Gene matrix was purchased from Bio-Rad. Plasmids were purified using Maxi-plasmid purification kits from Qiagen. Dideoxy-sequencing kits were obtained from US Biochemicals. Yeast strain SEY6210 was obtained from S. Emr. The HIS4 marker used from Southern blotting was the gift of R. Schekman. Cobalt(III) hexamine trichloride was obtained from Kodak. Incert LMP agarose was obtained from FMC Biochemicals. Nytran charge modified Nylon-66 membrane (0.45 μm) was obtained from Schleicker & Schnell. Biochemical manipulations were carried according to standard procedures[23] unless otherwise noted.

Deoxyribonucleoside 2. To a solution of aminonucleoside **1**[20] (820 mg, 1.27 mmol) in 5 mL of dioxane was added collidine (500 μL, 3.9 mmol) and the solution was chilled to 0 °C. 9-Fluorenylmethyl chloroformate (670 mg, 2.6 mmol) in 5 mL of dioxane was added slowly and the

(25) (a) Keesey, J. K.; Bigelis, R.; Fink, G. R. *J. Biol. Chem.* **1979**, *254*, 7427. (b) Deshaies, R. J.; Schekman, R. *J. Cell Biol.* **1987**, *105*, 633.

(26) Note that the 230 kb cleavage product from chromosome III is of similar size to chromosome I of yeast.

reaction was allowed to warm to room temperature. After 20 min, the reaction mixture was frozen (77 K), lyophilized, and purified by column chromatography (2% MeOH in CH_2Cl_2) to afford 1.02 g (92% yield) of **2**. ^1H NMR ($CDCl_3$): δ 7.72 (m, 2 H), 7.55 (m, 2 H), 7.39–7.13 (m, 14 H), 6.80 (q, 4 H, J = 9 Hz), 6.35 (t, 1 H, J = 7 Hz), 4.48 (m, 1 H), 4.37 (d, 2 H, J = 6 Hz), 4.15 (t, 1 H, J = 7 Hz), 4.04 (m, 1 H), 3.74 (s, 6 H), 3.38 (m, 2 H), 3.20–3.06 (m, 4 H), 2.37 (m, 2 H), 2.18 (m, 4 H). IR (KBr) cm^{-1}: 3307, 3065, 2933, 1701, 1508, 1448, 1251, 1177, 1091, 1032, 909, 829, 759, 714. HRMS (FAB) calculated for $C_{50}H_{51}O_{10}N_4$ ($M + H^+$): 867.3607. Found: 867.3580.

Phosphoramidite 3. Diisopropylethylamine (350 μL, 2 mmol) and 2-cyanoethyl *N,N*-diisopropylchlorophosphoramidite[21] (100 μL, 448 mmol) were added to a solution of deoxyribonucleoside **2** (150 mg, 176 μmol) in 3 mL of methylene chloride, and the reaction mixture was allowed to stir for 3 h. The reaction was quenched with ethanol (0.5 mL) and diluted with ethyl acetate. The organic layer was washed with aqueous $NaHCO_3$, H_2O, and twice with brine and dried (Na_2SO_4). Concentration followed by flash chromatography (500:15:5 methylene chloride:isopropyl alcohol:triethylamine) afforded 110 mg (60% yield) of **3**. ^1H NMR ($CDCl_3$): δ 8.40 (bs, 1 H), 7.72 (t, J = 7.3 Hz, 2 H), 7.55 (m, 3 H), 7.13–7.39 (m, 13 H), 6.79 (m, 4 H), 6.33 (m, 1 H), 5.49 (m, 1 H), 4.59 (m, 1 H), 4.39 (d, 2 H, J = 6.6 Hz), 4.15 (m, 1 H), 4.09 (m, 1 H), 3.77 (s, 6 H), 3.54 (m, 2 H), 3.52–3.65 (m, 4 H), 3.24–3.12 (m, 4 H), 2.57 (t, J = 6.1 Hz, 1 H), 2.37 (t, J = 6.1 Hz, 1 H), 2.26 (m, 2 H), 2.06–1.98 (m, 4 H), 1.14–1.00 (m, 12 H). IR (KBr) cm^{-1}: 3385, 3065, 2966, 2930, 1715, 1508, 1458, 1251, 1180, 1081, 1032, 979, 830, 760, 743, 700. ^{31}P NMR ($CDCl_3$): δ 149.1, 148.8. HRMS calculated for $C_{59}H_{67}N_6O_{11}P_1$ ($M{\cdot}Na^+$): 1089.4536. Found: 1089.4503.

Oligodeoxyribonucleotide Amine 4. Oligodeoxyribonucleotide **4** was synthesized by automated methods, and deprotected with concentrated NH_4OH at 55 °C for 20 h. After lyophilization, oligonucleotide **4** was isolated by gel electrophoresis (20% denaturing polyacrylamide gel), eluted from the excised band with 200 mM NaCl, 1 mM EDTA for 24 h at 37 °C, and dialyzed against Millipore Ultra-pore water. Its concentration was determined by UV (ϵ_{260} = 8800 cm^{-1} M^{-1} for T and T^E, ϵ_{260} = 5700 cm^{-1} M^{-1} for ^{Me}C, ϵ_{280} = 6400 cm^{-1} M^{-1} for T, and ϵ_{280} = 8300 cm^{-1} M^{-1} for ^{Me}C).

***N*-Bromoacetyloligonucleotide 5.** To a solution of 10 nmol of oligonucleotide **4** in 10 μL of 200 mM borate buffer, pH 8.9, was added an equal volume of a solution of 250 mM *N*-hydroxysuccinimidyl bromoacetate in DMF. After 10 min the solution was analyzed by HPLC (Vydac anion exchange, 4.6 × 200 mm column). Oligonucleotide **5** was eluted in 20 mM Na_2HPO_4, 20% acetonitrile buffer, using a gradient of 0.4–0.8 M NaCl over 60 min. Elution times were 15.4 min for oligonucleotide **4** and 18.2 min for the *N*-bromoacetyloligonucleotide **5**. The product peak was collected and extracted with butanol to remove the acetonitrile and salts were removed using a 3 mL Sephadex G-50-80 spun column. The eluent was precipitated with ethanol and washed with 70% ethanol. The oligonucleotide **5** was diluted in water to an appropriate concentration and stored at −20 °C until needed.

Cloning of Plasmids pUCLEU2C and pUCSfiI. The plasmid pUCLEU2C was obtained by ligating a duplex of the inverted repeat oligonucleotide 5′-TCGACTTTTCTTTCCTTTTCTTTTACTAGT-AAAAGAAAAGGAAAGAAAAG-3′ with pUCLEU2 previously digested with XhoI (Figure 4). Plasmid pUCSfi was obtained by forming a duplex from the oligonucleotides 5′-GATCGGCCTCT-AGGGCCCTGCA-3′ and 5′-GGGCCCTAGAGGCC-3′ and ligating into BamHI and PstI digested pUC19.[23] The insert retained in-frame translation of the *lacZ* gene so that α-complementation could subsequently be used for subcloning into the SfiI site. Transformation was performed using *E. coli* strain XL-1 blue (Stratagene). Plasmid DNA from individual clones was analyzed by digestion with SpeI (pUCLEU2C) or SfiI (pUCSfiI) and confirmed by dideoxy sequencing. Large-scale plasmid DNA was purified using a Qiagen maxi-purification column.

Analysis of Cleavage Reaction Products at Nucleotide Resolution. pUCLEU2C was radiolabeled by first digesting with Nar I, to produce 5.6- and 1.1-kb fragments. The DNA was labeled at the 5′-end using T4 polynucleotide kinase and γ-^{32}P ATP and labeled at the 3′-end using the Klenow fragment to incorporate α-^{32}P-dCTP and α-^{32}P-dGTP. After labeling, unincorporated nucleotides were removed using a 1-mL Sephadex G-50-80 spun column. The DNA was digested with EcoRI, producing bands 4.5, 1.1, 0.9, and 0.2 kp in size. The 1.1-kp band, containing the target sequence, was separated by 5% polyacrylamide gel electrophoresis, excised, and eluted at 37 °C using a solution of 200 mM NaCl, 1 mM EDTA. The DNA was purified by filtration through a 0.45 μM filter, butanol extraction, and two ethanol precipitations.

Radiolabeled DNA was allowed to react in 20-μL reactions containing 0.8 mM $Co(NH_3)_6^{3+}$, 10 mM HEPES, pH 7.4, and 100 nM *N*-bromoacetyloligonucleotide **5**. After 24 h, the reaction was precipitated with the addition of NaOAc and EtOH, washed with 70% ethanol, and treated with 1.0% piperidine at 90 °C for 30 min. The samples were lyophilized, suspended in formamide buffer, and loaded onto a 6% denaturing polyacrylamide gel for analysis. Cleavage efficiencies were determined by quantitative analysis of storage-phosphor autoradiograms using a PhosphorImager (Model 400S) and Image Quant (Molecular Dynamics) software.

Rate constants for the reaction of *N*-bromoacetyloligonucleotide **5** with each strand were determined by running 10 parallel reactions and quenching reactions by EtOH precipitation at the desired time intervals. After piperidine treatment and gel electrophoresis, PhosphorImaging was used to determine the fraction of intact DNA. Plotting ln (fraction of intact DNA) vs time yielded linear plots from which rate constants were derived.[17]

Double-Stranded Cleavage of Plasmid DNA. Plasmid pUCLEU2C was linearized with Hind III and radiolabeled using Klenow of DNA polymerase I and α-^{32}P-dATP, resulting in a 6.7-kb DNA fragment labeled at both ends with the target site located approximately an equal distance from each end. The DNA was incubated in a solution (final volume 20 μL) containing 0.8 mM $Co(NH_3)_6^{3+}$, 10 mM HEPES, pH 7.4, and 100 nM *N*-bromoacetyloligonucleotide **5** for 24 h at 37 °C. The DNA was precipitated with ethanol, washed in 70% ethanol, dried, and treated with 100 μL of 0.1% piperidine, 100 mM NaCl, 10 mM Tris·HCl, pH 7.4, 10 mM EDTA at 55 °C for 12 h. The DNA was loaded onto a 0.8% agarose gel and electrophoresed for 5 h at 100 V. Cleavage efficiencies were quantitated by phosphor-storage analysis as above.

Enzymatic Ligation of Double-Strand Cleavage Products. pUCLEU2C DNA was digested with EcoRI to completion and subjected to cleavage with *N*-bromoacetyloligonucleotide **5**. The resulting 450- and 600-bp fragments were separated by gel electrophoresis and purified with Prep-a-Gene matrix according to manufacturer protocol. pUCSfi DNA (5 μg) was digested with SfiI and EcoRI, and the 2.8-kb product gel was separated and purified as above. Ten nanograms of pUCSfiI DNA in 10 μL of T4 DNA ligase buffer (0.5 mM ATP, 20 mM Tris·HCl, pH 7.4, 5 mM $MgCl_2$, 5 mM dithiothreitol, 50 μg/mL BSA) was added to 2 molar equiv of either of the oligo/EcoRI fragments from pUCLEU2C, heated to 55 °C for 5 min to denature all cohesive ends, cooled to 16 °C, and allowed to react with 10 units of T4 DNA ligase for 4 h. The ligation mixture was used to transform *E. coli* (XL1-Blue, Stratagene) and transformants selected on media containing ampicillin (50 μg/mL) and X-gal and IPTG for α-complementation.[23] DNA from the resulting white colonies was isolated and sequenced by dideoxynucleotide chain termination using the USB Sequenase 2.0 kit.

Homologous Recombination and Production of Alternate Yeast Strain. Haploid yeast strain SEY6210 (*MATαleu2-3 112 ura3-52·his 3-Δ200 trp·Δ901 lys2-801 suc2-Δ9 GAL*) was grown in a 200-mL liquid culture to an OD_{600} of 0.7, harvested by centrifugation at 50 °C, washed in sterile H_2O, centrifuged, resuspended in 40 mL of 0.1 M LiOAc, 10 mM Tris·HCl, pH 8.0, 1 mM EDTA, and incubated at 30 °C for 1 h. Cells were again harvested by centrifugation and resuspended in 2 mL of LiOAc solution, and 50 μL of the resulting competent cells was aliquoted into sterile microcentrifuge tubes. PstI digested pUCLEU2C (5 μg) was added to the yeast solution and incubated at 30 °C for 1 h. The cells were heat shocked for 5 min at 37 °C and harvested by brief centrifugation. The supernatant was removed, and the cells were resuspended in 125 μL of sterile H_2O and plated on yeast minimal media plates lacking leucine. Recombinants were detected after 2 days at 30 °C, and selected colonies were plated on a second minimal media plate, grown an additional 2 days at 30 °C, and screened for proper insertion of the oligonucleotide target sites adjacent to the *LEU2* gene by PCR amplification and DNA hybridization.

PCR amplification was performed using two oligonucleotides that hybridize approximately 250 bp on either side of the oligonucleotide insertion site (Xho-235 5′-TCTATTACATTATGGGTGGTATGTT-3′ and Xho+250 5′-GGTCAAGATATTTCTTGAATCAGGC-3′). Chromosomal DNA was prepared from 0.5 mL of yeast liquid culture by glass bead lysis followed by PCR amplification with Taq DNA polymerase (Perkin Elmer Cetus) according to manufacturer protocol. The amplified products were (500 bp) digested with either XhoI or SpeI. Proper insertion of the oligonucleotide target sites would yield a PCR product cleaved only with SpeI, whereas an improperly modified strain would be cleaved with XhoI. Intact chromosomal DNA from SpeI cut transformants was prepared in an agarose matrix,[27] separated on a pulsed-field gel, and screened for unique insertion of the *LEU2* gene within chromosome III by hybridization with the random primer labeled EcoRI/KpnI fragment from pUCLEU2C. DNA from a yeast strain meeting these criteria was then used in alkylation reactions with oligonucleotide **5**.

(27) Strobel, S. A.; Dervan, P. B. *Methods in Enzymology*, in press.

Cleavage of Yeast Chromosomal DNA. DNA from yeast strains SEY6210 (no target site) and SEY6210C (+ target site) was isolated in low melting point agarose.[27] Agarose plugs containing yeast strain SEY6210C and SEY6210 were cut to a thickness of approximately 2 mm (volume approximately 80 μL) and placed in 2-mL microcentrifuge tubes. The plugs were washed four times with 900 μL of 1 mM $Co(NH_3)_6^{3+}$, 20 mM HEPES, pH 7.2, and 5 mM EDTA (triplex/alkylation buffer). After removal of the last wash solution, the plugs were incubated with 120 μL of triplex/alkylation buffer, *N*-bromoacetyloligonucleotide **5** was added to a final concentration of 1 μM, and the plugs were incubated at 37 °C. After 20 h, the plugs were washed 4 × 30 min in 900 μL of 10 mM Tris·HCl (pH 9.5), 10 mM EDTA, allowing the *N*-bromoacetyloligonucleotide to fully diffuse out of the agarose. Each of the plugs were then washed with 900 μL of triplex/alkylation buffer (4 × 15 min) and incubated with 120 μL of triplex/alkylation buffer and *N*-bromoacetyloligonucleotide **5** as before. After this second 20-h incubation, the agarose embedded DNA was washed with 900 μL of 0.1% piperidine (3×), 100 mM NaCl, 10 mM Tris·HCl, pH 8.0, 10 mM EDTA. After removal of the final wash solution, the DNA was heated in 900 μL of the piperidine solution described above at 55 °C for 12 h. The plugs were transferred directly without melting to a 0.5 × TBE 1.0% agarose gel and products separated by pulsed field gel electrophoresis using a BioRad Chef-DR II system. Electrophoresis was performed at 200 V for 24 h, with switch times ramped from 10 to 40 s over the first 18 h and from 60 to 90 s over the last 6 h. The gel was stained with ethidium bromide and photographed under UV excitation.

Hybridization. The DNA was fragmented for efficient transfer to a Nytran charge modified Nylon-66 membrane by a 40-s exposure of the gel with a 254-nm UV transilluminator. The gel was soaked in 1.0 M NaCl, 0.5 M NaOH for 30 min to denature the DNA and then equilibrated in 1.5 M NaCl, 0.5 M Tris·HCl, pH 7.4, to neutralize the gel. The DNA was transferred using a Stratagene Pressure Control Station in 6xSSPE. *HIS4* hybridization was performed with a 250-bp SalI/EcoRI fragment derived from HIS4-SUC2 fusion plasmid YCp503.[25] The fragment was labeled using random primer hybridization with degenerate 6mers and α-^{32}P-dCTP.[23,28] The membrane was prehybridized with 5 mL of a solution containing 6× SSPE, 10× Denhardt's, 1% SDS, and 50 mg/mL Salmon sperm DNA solution at 42 °C for 2–4 h. After removal of this solution, the membrane was washed with 2 mL of 6× SSPE, 50% formamide, 1% SDS, and 50 mg/mL salmon sperm DNA solution at 42 °C for 30–60 min. The labeled probe was denatured by incubation at 37 °C for 5 min with 1/10 volume of 0.1 M NaOH and incubated with the membrane in 2 mL of 6× SSPE, 50% formamide, 1% SDS, and 50 mg/mL salmon sperm DNA solution at 42 °C overnight. The blot was washed four times in 25 mL of 1× SSPE buffer, 1% SDS at 42 °C and exposed to film, and cleavage efficiencies were quantitated using PhosphorImaging.

Acknowledgment. We are grateful for generous support from the National Institutes of Health (GM-35724 and HG-00329), a Parsons predoctoral fellowship to T.J.P., and a Howard Hughes Medical Institute predoctoral fellowship to S.A.S.

(28) Feinberg, A. P.; Vogelstein, B. *Anal. Biochem.* **1983**, *132*, 6.
(29) Iverson, B. L.; Dervan, P. B. *Nucleic Acids Res.* **1987**, *15*, 7823.
(30) Maxam, A. M.; Gilbert, W. *Proc. Natl. Acad. Sci. U.S.A.* **1977**, *74*, 560.

^{15}N Isotope Effects on Nonenzymatic and Aspartate Transcarbamylase Catalyzed Reactions of Carbamyl Phosphate

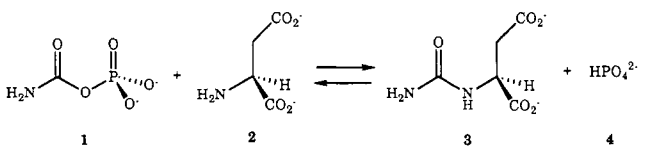
Grover L. Waldrop, Jeffrey L. Urbauer, and W. W. Cleland*

Contribution from the Institute for Enzyme Research, University of Wisconsin, Madison, Wisconsin 53705. *Received February 28, 1992*

Abstract: Kinetic isotope effects at the amide nitrogen of carbamyl phosphate were measured to determine whether catalysis by aspartate transcarbamylase proceeds through a cyanic acid or tetrahedral intermediate. Decomposition of the mono- and dianion of carbamyl phosphate was used as models for reaction of carbamyl phosphate proceeding through a tetrahedral adduct or cyanic acid intermediate, respectively. The ^{15}N kinetic isotope effects for decomposition of the mono- (1.0028) and dianion (1.0105) of carbamyl phosphate were sufficiently different to permit a distinction to be made between a cyanic acid intermediate or tetrahedral adduct. The intrinsic ^{15}N kinetic isotope effects for the aspartate transcarbamylase catalyzed reaction were determined with an active site mutant of aspartate transcarbamylase in which histidine 134 was replaced with alanine (1.0027) and with the wild-type enzyme with cysteine sulfinate as substrate in place of aspartate (1.0024). In both of these systems a full ^{13}C intrinsic isotope effect has been previously observed (Waldrop et al. *Biochemistry*, in press; Parmentier et al. *Biochemistry*, in press). The similarity of these isotope effects to the isotope effect for the decomposition of the carbamyl phosphate monoanion demonstrates that cyanic acid is not an intermediate in the enzyme catalyzed reaction. The observed ^{15}N kinetic isotope effect for the wild-type enzyme with a low level of aspartate as substrate was 1.0014, a value consistent with the previously measured commitment for carbamyl phosphate (Parmentier et al. *Biochemistry*, in press). ^{31}P NMR analysis of phosphate from reactions run in 50% $H_2{}^{18}O$ showed that catalysis by aspartate transcarbamylase involves C–O bond cleavage, thus ruling out carbamic acid as a potential intermediate. We conclude that catalysis by aspartate transcarbamylase proceeds via a tetrahedral adduct.

Aspartate transcarbamylase (ATCase) catalyzes the transfer of the acyl moiety of carbamyl phosphate (CbmP) (1) to the amino group of aspartate (2) to yield carbamylaspartate (3) and inorganic phosphate (4). In *Escherichia coli*, this reaction is the first committed step in pyrimidine biosynthesis and as such ATCase is subject to feedback inhibition by CTP, the end product of the pathway, and activation by ATP, the end product of the parallel purine biosynthetic pathway.[1] ATCase is a dodecamer organized as two catalytic trimers and three regulatory dimers. The regulatory and catalytic subunits can be separated by treatment of the holoenzyme with mercurials.[2] The isolated catalytic trimers

Scheme I



1 2 3 4

remain catalytically competent, and the regulatory dimers retain the ability to bind ATP and CTP.[2] While the activity of the holoenzyme is sigmoidal with respect to the concentrations of both CbmP[3] and aspartate,[1] the catalytic trimer exhibits Michaelis–

(1) Gerhart, J. C.; Pardee, A. B. *J. Biol. Chem.* **1962**, *237*, 891–896.
(2) Gerhart, J. C.; Schachman, H. K. *Biochemistry* **1965**, *4*, 1054–1062.

0002-7863/92/1514-5941$03.00/0