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Sequence-specific cleavage of single-stranded DNA: Oligodeoxynucleotide-EDTA·Fe(II)

(modified nucleosides/DNA probes/oxidative cleavage of DNA)

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ABSTRACT **The synthesis of a DNA hybridization probe 19 nucleotides in length, equipped with the metal chelator EDTA at C-5 of thymidine in position 10 (indicated by T*) is described. DNA-EDTA 1 has the sequence 5′-T-A-A-C-G-C-A-G-T*-C-A-G-G-C-A-C-C-G-T-3′, which is complementary to a 19-nucleotide sequence in the plasmid pBR322. In the presence of Fe(II), O_2, and dithiothreitol, DNA-EDTA 1 affords specific cleavage (25°C, pH 7.4, 60 min) at its complementary sequence in a heat-denatured 167-base-pair restriction fragment. Cleavage occurs over a range of 16 nucleotides at the site of hybridization of 1, presumably due to a diffusible reactive species. No other cleavage sites are observed in the 167-base-pair restriction fragment. The procedure used to synthesize DNA-EDTA probes is based on the incorporation of a thymidine modified at C-5 with the triethyl ester of EDTA. By using routine phosphoramidite procedures, thymidine-EDTA can be incorporated into oligodeoxynucleotides of any desired length and sequence. Because the efficiency of the DNA cleavage reaction is dependent on the addition of both Fe(II) and reducing agent (dithiothreitol), the initiation of the cleavage reaction can be controlled. These DNA-EDTA·Fe(II) probes should be useful for the sequence-specific cleavage of single-stranded DNA (and most likely RNA) under mild conditions.**

The sequence-specific cleavage of DNA has found many applications, such as DNA sequence determinations, chromosome analyses, gene isolation, and recombinant DNA manipulations, but it is limited by the specificities and natural availability of restriction endonucleases (1–3). It has been shown previously that attachment of EDTA·Fe(II) to a DNA-binding molecule creates an efficient DNA-cleaving molecule (4–9). For example, the oligopeptide-EDTA·Fe(II) molecules distamycin-EDTA·Fe(II) and penta-*N*-methylpyrrolecarboxamide-EDTA·Fe(II) cleave double-stranded DNA (25°C, pH 7.4) adjacent to specific A+T-rich regions 5 and 7 base pairs (bp) in length, respectively (6–8). For the cleavage of single-stranded nucleic acid, specificity and flexibility with regard to target sequence could be provided by oligonucleotides equipped with nucleic acid-cleaving functionality (10–12). An oligonucleotide tethered to EDTA·Fe(II) should specifically cleave its complementary DNA sequence at 25°C and pH 7.4 within minutes (Fig. 1). Because the efficiency of the EDTA·Fe(II)-mediated DNA cleavage reactions is enhanced by the addition of reducing agents such as dithiothreitol, the timing of the cleavage event can be controlled (4–9).

We report the synthesis of a DNA hybridization probe 19 nucleotides in length, containing an EDTA-functionalized derivative of thymidine, labeled T* (Fig. 2). DNA-EDTA 1 has the sequence 5′-T-A-A-C-G-C-A-G-T*-C-A-G-G-C-A-C-C-G-T-3′, which is complementary to a 19-nucleotide sequence in a 167-bp restriction fragment from pBR322 plasmid DNA. In the presence of Fe(II), O_2, and dithiothreitol,

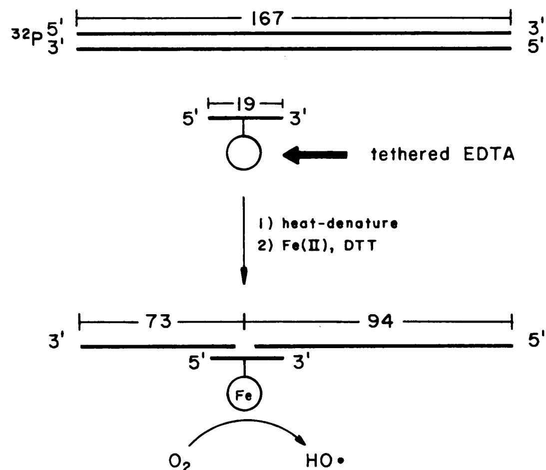

FIG. 1. Oligonucleotide-directed cleavage of DNA by DNA-EDTA·Fe(II) probes. DTT, dithiothreitol.

DNA-EDTA (1) affords cleavage (25°C, pH 7.4, 60 min) at its 19-nucleotide complement in the heat-denatured 167-bp restriction fragment (Fig. 1).

MATERIALS AND METHODS

^1H NMR spectra were recorded at 500 MHz on a Bruker WM 500 spectrometer, using tetramethylsilane or sodium 3-(trimethylsilyl)propanesulfonate (for spectra recorded in ^2H_2O) as an internal reference. Chemical shifts are reported in ppm downfield from tetramethylsilane. Thin-layer chromatography (TLC) was performed with precoated 0.25-mm silica gel 60 F-254 TLC plates (EM Reagents, Darmstadt, F.R.G.). Flash chromatography was performed with EM Reagents silica gel 60 (230–400 mesh). Reagent grade chemicals were used without purification unless otherwise stated. Deoxyuridine and dithiothreitol were purchased from Calbiochem. Protected deoxynucleoside phosphoramidites were prepared by literature procedures (14). K_2PdCl_4 and 10% Pd on C were from Alfa-Ventron (Danvers, MA). $Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$ was from Baker. Aqueous 5′-[α-^{32}P]dATP (3000 Ci/mmol; 1 Ci = 37 GBq) was from Amersham, and aqueous 5′-[γ-^{32}P]ATP (7000 Ci/mmol) was from ICN. Standard NTPs were from Boehringer Mannheim. All enzymes were from New England Biolabs except bacterial alkaline phosphatase and T4 polynucleotide kinase, which were from Bethesda Research Laboratories. Solutions of $Fe(NH_4)_2(SO_4)_2$ and dithiothreitol were freshly prepared. Plasmid pBR322 was grown in *Escherichia coli*, strain HB101, and isolated by standard procedures (13, 15).

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Abbreviations: DMT, 4,4′-dimethoxytrityl; bp, base pairs, DNA-EDTA or 19-mer, oligodeoxynucleotide 5′-T-A-A-C-G-C-A-G-T-C-A-G-G-C-A-C-C-G-T-3′ with an EDTA-modified thymidine at position 10; T*, thymidine modified at C-5 with EDTA; FAB, fast atom bombardment.

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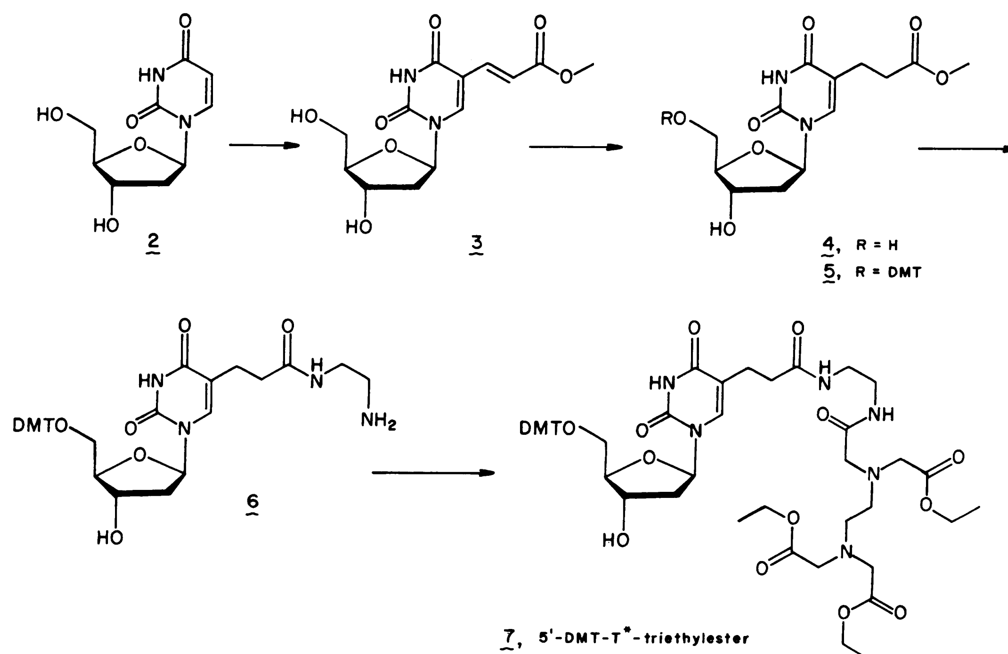

FIG. 2. Scheme for synthesis of modified thymidine 7 equipped with the triethyl ester of EDTA. DMT, 4,4'-dimethoxytrityl.

Nucleoside 3. A solution of 2'-deoxyuridine (2) (2.28 g, 10.0 mmol) and $Hg(OAc)_2$ (3.38 g, 10.6 mmol) in H_2O (13 ml) was stirred at 50°C for 16 hr, after which NaCl (1.5 g, 25 mmol) in H_2O (10 ml) was added with stirring (16). The mixture was concentrated to dryness by rotary evaporation. The remaining white solid was rinsed with methanol (five times, 20 ml each) and diethyl ether (25 ml) in a fritted glass funnel, then dried, yielding the mercurinucleoside (4.58 g, 9.89 mmol; 99% yield): mp 218–219°C (decomposition). To a mixture of 5-chloromercuri-2'-deoxyuridine (1.85 g, 4.00 mmol), methyl acrylate (1.50 ml, 20.0 mmol; freshly distilled) and methanol (50 ml) was added a solution of K_2PdCl_4 (1.30 g, 4.00 mmol) in H_2O (13 ml). After 3.5 hr of stirring the mixture was treated with H_2S for 2 min, then filtered through Celite (Johns-Manville, Denver, CO). The filtrate was concentrated to dryness and purified by flash chromatography (MeOH/EtOAc, 15:85, vol/vol) followed by recrystallization (MeOH/EtOAc, 15:85), to give nucleoside 3 (748 mg, 2.40 mmol; 60% yield): mp 169–170°C. ^1H NMR (^2H_2O): δ 8.25 (1H, s, H_6), 7.37 (1H, d, J = 15.9 Hz), 6.7 (1H, d, J = 15.9 Hz), 6.24 (1H, t, $H_{1'}$), 4.48 (1H, m, $H_{3'}$), 4.07 (1H, m, $H_{4'}$), 3.90–3.80 (2H, m, $H_{5'}$), 3.77 (3H, s, OCH_3), 2.48–2.37 (2H, m, $H_{2'}$). IR (KBr): 3440, 3240, 3080, 2960, 1730, 1670, 1620, 1520, 1440, 1290, and 1100 cm^{-1}. UV (H_2O): 302 nm (ε 19,000). MS: [positive ion fast atom bombardment (FAB)] m/z (relative intensity) 313 (M^+ + 1, 35), 197 (100), 165 (85), 117 (79); (negative ion FAB) m/z 311 (M^+ − 1, 48), 195 (100). Analysis. Calculated for $C_{13}H_{16}N_2O_7$: C, 50.00; H, 5.16; N, 8.97. Found: C, 49.84; H, 5.25; N, 8.77. TLC (MeOH/EtOAc, 15:85): R_f = 0.46.

Nucleoside 4. A solution of nucleoside 3 (780 mg, 2.50 mmol) in MeOH (75 ml) was shaken with 10% Pd on C (117 mg) under H_2 [50 pounds/inch^2 (340 kPa)] for 58 hr, using a Parr apparatus. Filtration and evaporation afforded analytically pure nucleoside 4 (748 mg, 2.38 mmol; 95% yield). ^1H NMR (^2H_2O): δ 7.72 (1H, s, H_6), 6.28 (1H, t, $H_{1'}$), 4.47 (1H, m, $H_{3'}$), 4.03 (1H, m, $H_{4'}$), 3.86–3.75 (2H, m, $H_{5'}$), 3.68 (3H, s, OCH_3), 2.62 (4H, m), 2.42–2.30 (2H, m, $H_{2'}$). IR (film): 3400, 3040, 2930, 1690, 1465, 1440, 1275, 1090, and 1050 cm^{-1}. UV (H_2O): 268 nm (ε 9300). MS: (positive ion FAB) m/z 315 (M^+ + 1, 27), 199 (100), 167 (63), 117 (38); (negative ion FAB) m/z 313 (M^+ − 1, 72), 197 (100). Analysis. Calculated for $C_{13}H_{18}N_2O_7$: C, 49.68; H, 5.77; N, 8.91. Found: C, 49.35; H, 5.96; N, 8.51. TLC (MeOH/EtOAc, 15:85): R_f = 0.41.

Nucleoside 5. A solution of nucleoside 4 (200 mg, 0.64 mmol) and 4,4'-dimethoxytrityl (DMT) chloride (238 mg, 0.70 mmol) in dry pyridine (1.0 ml) was stirred for 4 hr under Ar. Methanol (0.5 ml) was added and after 30 min the mixture was concentrated under reduced pressure to a gum, which was dissolved in CH_2Cl_2 (5 ml), rinsed with H_2O (twice, 5 ml each), and reconcentrated. Flash chromatography ($MeOH/CH_2Cl_2$, 5:95, vol/vol) provided nucleoside 5 (320 mg, 0.52 mmol; 81% yield). ^1H NMR (C^2HCl_3): δ 8.13 (1H, s), 7.49 (1H, s, H_6), 7.39 (2H, d), 7.30–7.22 (7H), 6.82 (4H, d), 6.33 (1H, t, $H_{1'}$), 4.53 (1H, m, $H_{3'}$), 4.01 (1H, m, $H_{4'}$), 3.78 (6H, s, OCH_3), 3.57 (3H, s, OCH_3), 3.42 (2H, m, $H_{5'}$), 2.36 (2H, m, $H_{2'}$), 2.30–2.21 (4H, m), 1.94 (1H, d). MS: (positive ion FAB) m/z 617 (M^+ + 1, 24), 605 (31), 375 (78), 303 (100), 199 (45), 167 (33); (negative ion FAB) m/z 615 (M^+ − 1, 34), 273 (15), 197 (100), 183 (53). Analysis. Calculated for $C_{34}H_{36}N_2O_9$: C, 66.22; H, 5.89; N, 4.54. Found: C, 66.62; H, 5.99; N, 4.66. TLC ($MeOH/CH_2Cl_2$, 5:95): R_f = 0.40.

Nucleoside 7. A solution of nucleoside 5 (320 mg, 0.519 mmol) in freshly distilled ethylenediamine (5.0 ml) was stirred under Ar for 57 hr, at which point silica TLC indicated complete conversion of 5 to a single product (R_f = 0.44 in concentrated aqueous NH_3/MeOH, 5:95, vol/vol). The mixture was reduced under reduced pressure to a gum, redissolved in dioxane, then lyophilized. The resulting powder 6 was stirred with EDTA triethylester-*N*-hydroxysuccinimide ester (1.4 mmol) (6) in dioxane (15 ml) under Ar for 1 hr. The mixture was then stirred with ethanol (5 ml) for 15 min, concentrated, redissolved in CH_2Cl_2 (15 ml), rinsed with H_2O (three times, 10 ml each) and reconcentrated. Flash chromatography (eluting with 100 ml of 5% EtOH in CH_2Cl_2, then 150 ml of 15% EtOH in CH_2Cl_2) afforded 7 (262 mg, 0.257 mmol; 55% yield) as a brittle foam. ^1H NMR (C^2HCl_3): δ 9.67 (1H, bs), 8.44 (1H, m), 7.47 (1H, s, H_6), 7.41 (2H, d), 7.32–7.21 (7H), 6.88 (1H, m), 6.84 (4H, d), 6.36 (1H, t, $H_{1'}$), 4.48 (1H, m, $H_{3'}$), 4.13 (6H, m, OCH_2CH_3), 4.05 (1H, m, $H_{4'}$), 3.78 (6H, s, OCH_3), 3.70 (2H, s), 3.53 (4H, s), 3.39–3.28 (8H), 2.78 (4H, dm), 2.45–2.20 (7H), 1.24 (9H, t, OCH_2CH_3). IR (CCl_4): 3300, 2990, 2920, 1740, 1675, 1605, 1500, 1460,

1440, 1370, 1300, 1270, 1250, 1185, 1135, and 1030 cm^{-1}. UV ($CHCl_3$): 242 nm (ε 15,000), 270 nm (ε 9600). MS: (positive ion FAB) m/z 1003 ($M^+ + 1$, 13), 919 (32), 777 (39), 701 (6), 461 (100), 303 (95), 216 (83); (negative ion FAB) m/z 1001 ($M^+ - 1$, 3), 653 (21), 566 (98), 520 (33), 413 (74), 367 (20), 273 (39), 196 (69), 137 (100). Exact mass (positive ion FAB), calculated for $C_{51}H_{67}N_6O_{15}$ $(M + H)^+$: 1003.4663. Found: 1003.4648. TLC ($EtOH/CH_2Cl_2$, 1:10): $R_f = 0.50$.

Synthesis of 5′-T-A-A-C-G-C-A-G-T*-C-A-G-G-C-A-C-C-G-T-3′ (1). The synthesis of the fully protected 19-mer **1** was accomplished by the phosphoramidite method, using published procedures, beginning with 5′-DMT-T (4 μmol) bound to a silica support (14, 17–19). Nucleoside **7** was coupled in the tenth addition cycle as follows: Nucleoside **7** (80 mg, 79 μmol) was dissolved in $CHCl_3$ (100 μl) and diisopropylethylamine (100 μl), and allowed to react with chloro-*N,N*-dimethylaminomethoxyphosphine (20 μl, 160 μmol) under Ar for 4 hr. The mixture was dissolved in EtOAc (1 ml), rinsed with saturated aqueous NaCl (four times, 1 ml each), dried (Na_2SO_4), and concentrated. The resulting foam (76 mg) was stored under reduced pressure [0.2 torr (27 Pa)] for 24 hr, then was activated with 0.5 M tetrazole in CH_3CN (0.6 ml) and coupled (15 min) to the protected, silica-bound, 5′-detritylated 10-mer C-A-G-G-C-A-C-C-G-T. The subsequent DMT cleavage (5% dichloroacetic acid in toluene) was monitored spectrophotometrically; this procedure indicated a coupling yield of 97% for T*. The remainder of the oligonucleotide synthesis cycles were as previously described (17–19). The penultimate DMT cleavage suggested an overall yield for 19-mer **1** of approximately 50%.

Approximately 1/10 of the fully protected, silica-bound 19-mer **1** was shaken with 5.0 ml of $PhSH/Et_3N$/dioxane (1:2:2, vol/vol) for 1 hr, rinsed (MeOH), then shaken with 1.5 ml of 0.1 M NaOH for 6 hr. The silica was removed, and it showed no color upon treatment with acid, indicating complete detachment of the 19-mer. The supernatant was heated to 50°C for 13 hr, concentrated, treated with glacial HOAc (1.0 ml) for 1.5 hr, reconcentrated, applied to a column of Sephadex G-10-120 (Sigma), and eluted with H_2O. The crude DNA-EDTA probe **1** was lyophilized and purified by electrophoresis (450 V, 22 hr) on a 2-mm-thick 20% polyacrylamide gel (20). The major UV-absorbing band was cut out and eluted with H_2O at 60°C for 24 hr, then passed through Sephadex G-10, affording 27 nmol (5.8 A_{260} units) of purified 19-mer **1**. A sample of the purified oligodeoxynucleotide-EDTA **1**, 5′-end-labeled with T4 polynucleotide kinase and [γ-^{32}P]ATP, was homogeneous by electrophoresis on a 20% polyacrylamide gel. The sequence of **1** was confirmed by Maxam–Gilbert chemical sequencing methods using a 20% polyacrylamide gel (20).

Preparation of Labeled Template DNA Fragment. Plasmid pBR322 DNA was digested with *Eco*RI, then 3′-end-labeled with [α-^{32}P]dATP by using the Klenow fragment of DNA polymerase I (20). A second enzymatic digest, with *Rsa* I, yielded a 3′-end-labeled fragment 167 bp in length, containing the 19-nucleotide complement of the DNA-EDTA probe **1** (13). This restriction fragment was isolated by polyacrylamide gel electrophoresis (20). Cleavage of pBR322 with *Eco*RI and successive treatment with bacterial alkaline phosphatase, [γ-^{32}P]ATP and T4 polynucleotide kinase, then *Rsa* I, yielded the 5′-end-labeled 167-bp fragment (20).

Cleavage Conditions and Analysis. Reaction mixtures (8 μl) containing DNA-EDTA probe **1** (0.6 μM), ^{32}P-end-labeled 167-bp template (2500 cpm, ≈1 nM), 50 mM Tris·HCl at pH 7.4, and 50 mM NaCl were prepared in 1.5-ml Eppendorf tubes. The reaction mixtures were heated to 95°C for 3–4 min and then rapidly chilled in ice water (21). The cleavage reactions were *initiated* by adding aqueous solutions of Fe(II) (1 μl) and dithiothreitol (1 μl), such that the final concentrations were 0.5 μM **1**, 10 μM Fe(II), and 4 mM dithiothreitol. The cleavage reactions were allowed to proceed at 25°C and pH 7.4 for 1 hr, then were terminated by freezing (−78°C) and lyophilization. These samples were suspended in 4 μl of formamide loading buffer, heat-denatured, and loaded on 0.4-mm-thick, 40-cm-long, 8% polyacrylamide (1:20 crosslinked)/50% urea high-resolution sequencing gels. Electrophoresis was conducted at 1200 V for 4.5 hr. Autoradiography of the gels was carried out at −50°C on Kodak X-Omat AR film. Copies (20 cm × 25 cm) of the original autoradiograms were scanned at 485 nm. The relative peak height for each local maximum was equated with the relative cleavage efficiency.

RESULTS

Synthesis of Thymidine-EDTA-Triethylester 7. The synthesis of 5′-DMT-T*-triethylester (**7**) is shown in Fig. 2. 2′-Deoxyuridine (**2**) was converted via its 5-chloromercuri derivative to **3**, using a palladium(II)-mediated coupling reaction with methyl acrylate (16, 22). Hydrogenation of **3** (50 pounds/inch^2 H_2, 10% Pd/C) selectively reduced the exocyclic double bond, affording **4**. The 5′-hydroxyl group of **4** was protected with DMT chloride to give **5** (23). Reaction of **5** with excess ethylenediamine afforded **6**, which was coupled directly with the *N*-hydroxysuccinimide ester of EDTA-triethylester, yielding the protected base 5′-DMT-T*-triethylester, **7**.

Synthesis of Oligodeoxynucleotide-EDTA 1. DNA-EDTA **1** was synthesized by the manual solid-phase phosphoramidite method (14, 17–19). Nucleoside **7** was converted to its 3′-phosphoramidite derivative and used directly in 15-fold excess according to the standard protocols (14, 17–19). DMT cation measurements indicated phosphoramidite coupling efficiencies of 96–99% (97% for T*), although losses were incurred during purification. In the deprotection step, NaOH was substituted for NH_4OH to circumvent possible ammonolysis of the EDTA ethyl esters. Thus, 0.1 M NaOH was employed to cleave the 19-mer from the silica support, hydrolyze the three esters of the tethered EDTA, and deprotect the bases. Final purification of the 19-mer **1** was effected by gel electrophoresis on a 20% polyacrylamide gel. The sequence of DNA-EDTA **1** was verified by chemical sequencing methods (20). The DNA cleavage products containing the T* with the attached EDTA moiety displayed a reduced electrophoretic mobility on a 20% polyacrylamide sequencing gel (data not shown).

Cleavage of DNA. The sequence-specific cleavage of DNA by oligodeoxynucleotide-EDTA·Fe(II) was examined on a 167-bp 3′-end ^{32}P-labeled *Eco*RI/*Rsa* I restriction fragment of plasmid pBR322, containing the 19-base complement to DNA-EDTA **1**. The DNA-EDTA probe **1** (0.5 μM) and 3′-end-labeled 167-bp template (≈1 nM) were combined in 50 mM Tris·HCl, pH 7.4/50 mM NaCl, heated to 95°C for 3–4 min to denature the template, then chilled in ice water to effect hybridization of the probe to the template (21). The cleavage reaction was initiated by addition of Fe(II) (10 μM) followed by dithiothreitol (4 mM), allowed to proceed at 25°C for 1 hr, then stopped by freezing and lyophilization. The reaction products were analyzed by Maxam–Gilbert sequencing gel methods (Fig. 3). Two sites of comparable cleavage, nearly symmetrically distributed about the position of T*, were observed on the labeled template strand (Fig. 3, lane 4). A histogram of the DNA-cleavage pattern obtained from densitometry of the autoradiogram is presented in Fig. 4. Each cleavage site covered seven or eight nucleotides, with maximum cleavage four nucleotides to the 5′ side and three nucleotides to the 3′ side of T*.

Controls. No cleavage occurred when the 167-bp restriction fragment was not heat denatured. No observable cleavage of the denatured 167-bp template occurred in the pres-

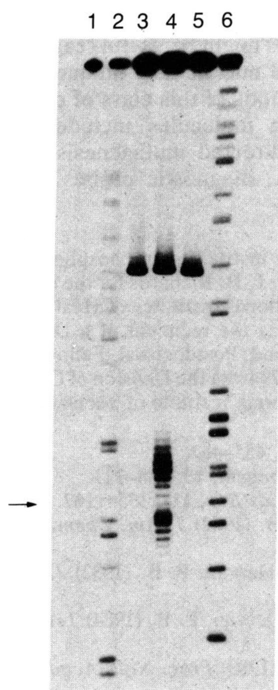

FIG. 3. Autoradiogram of Maxam–Gilbert sequencing gel. Lane 1 is the 3′-end-labeled 167-bp DNA standard. Lanes 2 and 6 are Maxam–Gilbert G reactions on the 3′- and 5′-end labeled 167-bp DNA fragments, respectively. Lane 3 is a control showing the 3′-end-labeled 167-bp fragment under the cleavage reaction conditions, including 10 μM Fe(II) and 4 mM dithiothreitol, but in the absence of **1**. Lanes 4 and 5 are the products of the cleavage reactions [0.5 μM **1**, 10 μM Fe(II), 4 mM dithiothreitol] on the 3′- and 5′-end-labeled 167-bp DNA fragments, respectively. The arrow on the left of the autoradiogram shows the location of A on the template strand opposite T* on the DNA-EDTA probe **1**.

ence of 10 μM Fe(II) and 4 mM dithiothreitol when the DNA-EDTA probe **1** was omitted (Fig. 3, lane 3). DNA-EDTA probe **1** in the presence of Fe(II) (10 μM) but absence of added dithiothreitol does not cleave the denatured DNA restriction fragment. DNA-EDTA **1** in the presence of dithiothreitol (4 mM) but absence of Fe(II) does not cleave the DNA. We conclude from this that the sequence-specific DNA cleavage reaction is dependent on DNA-EDTA probe **1**, Fe(II), and dithiothreitol. Consistent with this observation, cleavage of DNA does not occur with DNA-EDTA/Fe(II)/dithiothreitol in the presence of exogeneous iron chelators such as EDTA at millimolar concentration. Finally, when the reaction was repeated with the denatured 167-bp restriction fragment labeled with ^{32}P on the 5′ end of the DNA strand containing the sequence homologous to **1**, no cleavage was observed (Fig. 3, lane 5).

DISCUSSION

Synthesis of DNA-EDTA. For the synthesis of oligonucleotides containing EDTA, we desired a practical method that would be compatible with known oligonucleotide synthesis methodology. The method should allow the attachment of EDTA at various positions in the oligonucleotide sequence in an unambiguous manner. Aliphatic substitution at C-5 of pyrimidine nucleoside bases can be achieved by palladium-mediated olefination reactions (22, 24, 25). Of several olefinic substrates tested in the Pd(II)-mediated coupling reaction, methyl acrylate and 5-chloromercurideoxyuridine proved most satisfactory, producing **3** in good yield. The 5′-DMT-T*-triethylester (**7**), prepared in three steps from **3**, is a suitable monomer for standard phosphoramidite (and phosphotriester) oligonucleotide synthesis procedures and gives excellent coupling efficiencies.

DNA Cleavage Conditions. The DNA-EDTA probe **1** is first allowed to bind its complementary target sequence from a heat-denatured ^{32}P-end-labeled 167-bp DNA restriction fragment. Fe(II) is added and the cleavage reaction is initiated by the addition of reducing agent (dithiothreitol). After 60 min at 25°C (pH = 7.4) the DNA cleavage products are analyzed by high-resolution gel electrophoresis (Fig. 3). From the autoradiogram we observe cleavage at the site complementary to the DNA-EDTA probe. No other cleavage sites are observed. These results suggest that **1** forms a stable duplex with its complementary sequence, chelates Fe(II), and, in the presence of dioxygen and added reducing agents such as dithiothreitol, effects localized DNA cleavage. No carrier DNA was used in this experiment, and we can only estimate the quantities of template DNA used. If the efficiency for the end-labeling and isolation procedures were high, we estimate the concentration of ^{32}P-labeled 167-bp fragment employed in the reaction conditions would be 1 nM. The concentration of **1** used was 0.5 μM and represents approximately a 500-fold excess of DNA-EDTA·Fe(II) probe to template DNA. At an order of magnitude lower concentrations of **1** the cleavage efficiency decreases. Controls show that the sequence-specific cleavage reaction on the strand containing the complementary sequence is dependent on the presence of the DNA-EDTA probe, Fe(II), and added dithiothreitol. We have found that for optimal cleavage the order of addition is the DNA-EDTA probe and Fe(II) to the substrate DNA, followed by dithiothreitol. Presumably, this sequence of hybridization of the DNA-EDTA·Fe(II) **1** followed by activation with dithiothreitol minimizes the potential problem of autocleavage of the DNA-EDTA·Fe(II) probe before hybridization to the target sequence.

DNA Cleavage Pattern Analysis. The cleavage pattern produced by DNA-EDTA·Fe(II) **1** extends over 16 contiguous nucleotides of the template strand, centered at the position of T*. The cleavage pattern consists of two loci of equal intensity, with maxima occurring four nucleotides to the 5′ side and three nucleotides to the 3′ side of T* (Fig. 4). One possible explanation is that the two cleavage loci arise from two conformations of the tethered EDTA·Fe(II) (Fig. 5) in the major groove of DNA, not quite equidistant from the T* modified base. Each cleavage locus extends over seven or eight nucleotides, suggesting that a diffusible species is responsible for cleavage. From previous studies with the oligo-*N*-methylpyrrolecarboxamide-EDTA·Fe(II) molecules, which bind in the *minor* groove of DNA, each cleavage locus extends over four or five base pairs (5–8). We presume the greater number of cleavage sites produced by DNA-EDTA·Fe(II) **1** reflects the greater range available to a diffusible reactive species generated in the *major* groove of DNA. An alternative explanation for the two cleavage loci is that the tether-EDTA·Fe(II) at T* protects the modified base region from the diffusible reactive species.

Autocleavage. In principle, an oligonucleotide equipped with a chemically reactive moiety has the inherent problem of autocleavage, which could lower the sequence specificity of the DNA-EDTA probe by producing shorter DNA-EDTA fragments. To avoid this problem, the reactive functionality should be masked and activated after synthesis, isolation, and hybridization of the DNA probe to the target complementary sequence. For DNA-EDTA probes we avoid the problem of autocleavage during oligonucleotide synthesis by

FIG. 4. Histogram of the DNA cleavage pattern derived by densitometry of the autoradiogram in Fig. 3 (lane 4). The heights of the arrows represent the relative cleavage intensities at the indicated bases.

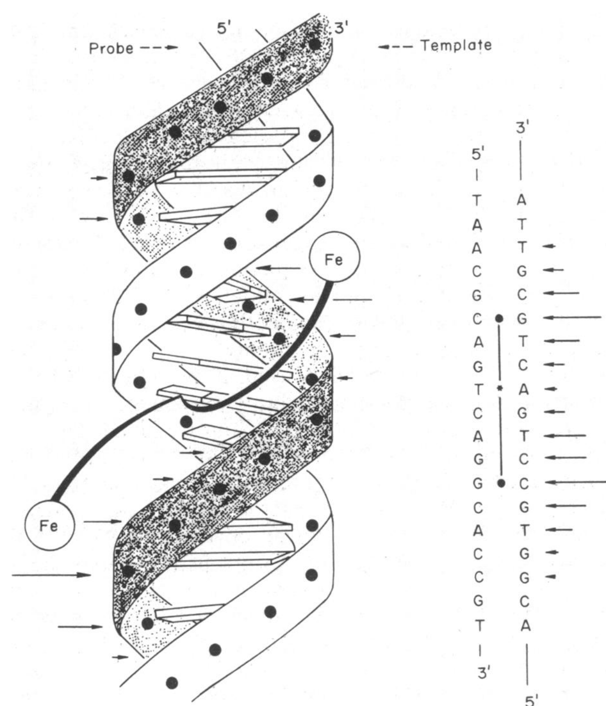

FIG. 5. Two-conformation model for the DNA cleavage pattern visualized on the autoradiogram for the 3′-end-labeled strand from the heat-denatured 167-bp restriction fragment.

keeping the EDTA blocked as the triethyl ester. The EDTA metal-chelating moiety is revealed after the oligonucleotide synthesis is complete and at the last step, which is deprotection of the bases and release of DNA-EDTA from the polymer support. We have found that the DNA-EDTA probe **1**, stored on the polymer in protected form, is fully active when released after 5 months. Controls show that after isolation of the DNA-EDTA probe by electrophoresis on a 20% polyacrylamide gel the purified 19-mer is homogeneous and intact. The DNA-EDTA probe is inactive to either autocleavage or complementary strand DNA cleavage in the absence of Fe(II) and dithiothreitol. This allows prior hybridization of the DNA-EDTA probe [or even DNA-EDTA·Fe(II)] to the target single-stranded DNA. The DNA-EDTA·Fe(II) probe **1** in the presence of dithiothreitol (4 mM) and in the absence of target complementary DNA autocleaves in less than 1 hr. Finally, although DNA-EDTA probes appear stable for weeks in solution, they degrade to smaller fragments over a period of months and are inactive toward complementary strand cleavage, presumably due to autocleavage by trace metal and dioxygen. The probes are best stored in the protected form on the solid support.

Conclusion. A general procedure has been developed for the synthesis of oligodeoxynucleotides equipped with EDTA at specific locations. The sequence-specific cleavage of single-stranded DNA by the DNA-EDTA·Fe(II) probes is initiated by the addition of dithiothreitol. Preliminary work suggests that these DNA-EDTA probes might be useful reagents capable of cleaving single-stranded nucleic acid uniquely at any desired site. Potential applications of this class of oligonucleotide-directed DNA-cleaving molecules include the mapping of large genomes, site-directed mutagenesis, sequence-specific cleavage of RNA, diagnostic probes, and novel chemotherapeutic agents.

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1. Smith, H. O. (1979) *Science* **205**, 455–462.
2. Modrich, P. (1982) *Crit. Rev. Biochem.* **13**, 288–323.
3. Roberts, R. J. (1983) *Nucleic Acids Res.* **11**, r135–r167.
4. Hertzberg, R. P. & Dervan, P. B. (1982) *J. Am. Chem. Soc.* **104**, 313–315.
5. Schultz, P. G., Taylor, J. S. & Dervan, P. B. (1982) *J. Am. Chem. Soc.* **104**, 6861–6863.
6. Taylor, J. S., Schultz, P. G. & Dervan, P. B. (1984) *Tetrahedron* **40**, 457–465.
7. Schultz, P. G. & Dervan, P. B. (1983) *Proc. Natl. Acad. Sci. USA* **80**, 6834–6837.
8. Schultz, P. G. & Dervan, P. B. (1984) *J. Biomol. Struct. Dyn.* **1**, 1133–1147.
9. Hertzberg, R. P. & Dervan, P. B. (1984) *Biochemistry* **23**, 3934–3945.
10. Grineva, N. I. & Karpova, G. G. (1973) *FEBS Lett.* **32**, 351–355.
11. Grineva, N. I., Karpova, G. G., Kuznetsova, L. M., Venkstern, T. V. & Bayev, A. A. (1977) *Nucleic Acids Res.* **4**, 1609–1631.
12. Martin, R. F. & Haseltine, W. A. (1981) *Science* **213**, 896–898.
13. Sutcliffe, J. G. (1979) *Cold Spring Harbor Symp. Quant. Biol.* **43**, 77–90.
14. Beaucage, S. L. & Caruthers, M. H. (1981) *Tetrahedron Lett.* **22**, 1859–1862.
15. Tanaka, T. & Weisblum, B. (1974) *J. Bacteriol.* **121**, 354–362.
16. Bergstrom, D. E. & Ruth, J. L. (1977) *J. Carbohydr. Nucleosides Nucleotides* **4**, 257–269.
17. Dorman, M. A., Noble, S. A., McBride, L. J. & Caruthers, M. H. (1984) *Tetrahedron* **40**, 95–102.
18. Adams, S. P., Kavka, K. S., Wykes, E. J., Holder, S. B. & Galluppi, G. R. (1983) *J. Am. Chem. Soc.* **105**, 661–663.
19. Matteucci, M. D. & Caruthers, M. H. (1981) *J. Am. Chem. Soc.* **103**, 3185–3191.
20. Maxam, A. M. & Gilbert, W. (1980) *Methods Enzymol.* **65**, 499–560.
21. Smith, M., Leung, D. W., Gillam, S., Astell, C. R., Montgomery, D. L. & Hall, B. D. (1979) *Cell* **16**, 753–761.
22. Bergstrom, D. E. & Ogawa, M. K. (1978) *J. Am. Chem. Soc.* **100**, 8106–8112.
23. Schaller, H., Wiemann, G., Lerch, B. & Khorana, H. G. (1963) *J. Am. Chem. Soc.* **85**, 3821–3827.
24. Heck, R. F. (1968) *J. Am. Chem. Soc.* **90**, 5518–5526.
25. Langer, P. R., Waldrop, A. A. & Ward, D. C. (1981) *Proc. Natl. Acad. Sci. USA* **78**, 6633–6637.