Sequence-Specific Cleavage of Double Helical DNA by Triple Helix Formation

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Homopyrimidine oligodeoxyribonucleotides with EDTA·Fe attached at a single position bind the corresponding homopyrimidine-homopurine tracts within large double-stranded DNA by triple helix formation and cleave at that site. Oligonucleotides with EDTA·Fe at the 5′ end cause a sequence specific double strand break. The location and asymmetry of the cleavage pattern reveal that the homopyrimidine-EDTA probes bind in the major groove parallel to the homopurine strand of Watson-Crick double helical DNA. The sequence-specific recognition of double helical DNA by homopyrimidine probes is sensitive to single base mismatches. Homopyrimidine probes equipped with DNA cleaving moieties could be useful tools for mapping chromosomes.

THE SEQUENCE-SPECIFIC CLEAVAGE OF DOUBLE HELICAL DNA by restriction endonucleases is essential for many techniques in molecular biology, including gene isolation, DNA sequence determination, and recombinant DNA manipulations (*1, 2*). With the advent of pulsed-field gel electrophoresis, the separation of large pieces of DNA is now possible (*3, 4*). However, the binding site sizes of naturally occurring restriction enzymes are in the range of 4 to 8 base pairs, and hence their sequence specificities may be inadequate for mapping genomes over very large distances. The design of sequence-specific DNA cleaving molecules that go beyond the specificities of the natural enzymes depends on a detailed understanding of the chemical principles underlying two functions: recognition and cleavage of DNA (*5*). Synthetic sequence-specific binding moieties for double helical DNA that have been studied are coupled analogs of natural products (*5*), transition metal complexes (*6*), and peptide fragments derived from DNA binding proteins (*7, 8*).

The DNA cleaving function used in our laboratories is EDTA·Fe(II), which cleaves the DNA backbone by oxidation of the deoxyribose with a short-lived diffusible hydroxyl radical (*5, 9*). The fact that hydroxyl radical is a relatively nonspecific cleaving species is useful when studying recognition because the cleavage specificity is due to the binding moiety alone, not some combination of cleavage specificity superimposed on binding specificity. The most sequence-specific molecules characterized so far, with regard to the natural product analog approach, is bis(EDTA-distamycin)fumaramide, which binds in the minor groove and cleaves at sites containing 9 bp of contiguous A,T DNA (*10*). A synthetic peptide containing 52 residues from the DNA binding domain of Hin protein with EDTA at the amino terminus binds and cleaves at the 13-bp Hin site (*8*). Despite this progress, our understanding of molecular recognition of DNA is still sufficiently primitive that the elucidation of the chemical principles for creating specificity at the ≥15-bp level may be slow when compared to the time scale for and interest in mapping large genomes.

Triple helix formation. We now describe the sequence-specific cleavage of large double helical DNA with modified oligonucleotides that bind in the major groove forming a triple helix structure (Fig. 1). The first triplex of nucleic acids was reported three decades ago (*11*). Poly(U) and poly(A) were found to form a stable 2:1 complex in the presence of $MgCl_2$. After this, several triple-stranded structures were discovered (*12, 13*). Poly(C) forms a triple-stranded complex at *p*H 6.2 with guanine oligoribonucleotides. One of the pyrimidine strands is apparently in the protonated form (*14–16*). In principle, isomorphous base triplets (T-A-T and C-G-C^+) can be formed between any homopurine·homopyrimidine duplex and a corresponding homopyrimidine strand (*17–19*) (Fig. 1). The DNA duplex poly(dT-dC)·poly(dG-dA) associated with poly(U-C) or poly(dT-dC) below *p*H 6 in the presence of $MgCl_2$ to afford a triple-

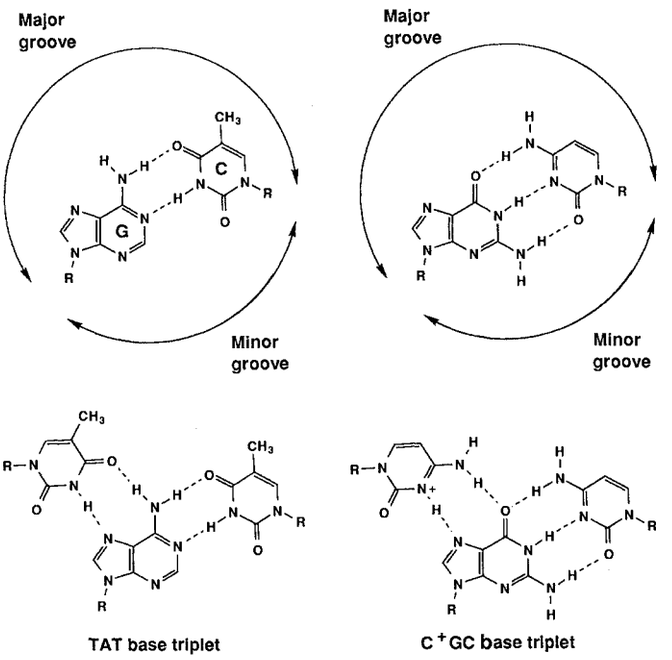

Fig. 1. (**Top**) Watson-Crick base pairs. (**Bottom**) Isomorphous base triplets of TAT and C^+GC. The additional pyrimidine strand is bound by Hoogsteen hydrogen bonds in the major groove to the complementary purine strand in the Watson-Crick duplex.

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stranded complex (*18, 19*). Several investigators proposed an antiparallel orientation of the two polypyrimidine strands on the basis of an *anti* conformation of the bases (*17–19*). The x-ray diffraction patterns of triple-stranded fibers (poly(A)·2 poly(U) and poly(dA)·2 poly(dT)) supported this hypothesis (*20–22*) and suggested an A′ RNA-like conformation of the two Watson-Crick base-paired strands with the third strand in the same conformation, bound parallel to the homopurine strand of the duplex by Hoogsteen hydrogen bonds (*23*). The 12-fold helix with dislocation of the axis by almost 3 Å, the C3′-endo sugar puckering and small base-tilts result in a large and deep major groove that is capable of accommodating the third strand (*24*). A high-resolution x-ray structure of a triple helical DNA or RNA has not yet been reported.

Oligonucleotide-EDTA probes. Oligonucleotides equipped with a DNA cleaving moiety have been described which produce sequence-specific cleavage of single-stranded DNA (*25–28*). An example of this is oligonucleotide-EDTA·Fe hybridization probes

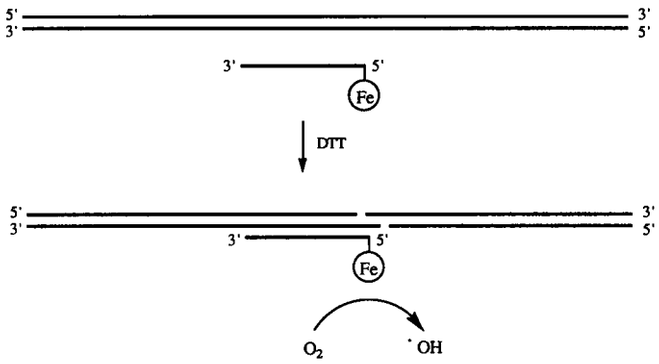

Fig. 2. Oligonucleotide-directed cleavage of double helical DNA by a triple helix–forming DNA-EDTA·Fe probe. One thymidine has been replaced by thymidine with the iron chelator EDTA covalently attached at C-5. Reduction of dioxygen generates localized hydroxyl radical at this position (*26*).

(DNA-EDTA) which cleave the complementary single strand sequence (*26, 27*). A homopyrimidine oligonucleotide equipped with a DNA cleaving moiety should recognize the corresponding complementary sequence of double helical homopurine·homopyrimidine DNA and yield a strand break at the target sequence (Fig. 2). The affinity cleaving method (*5, 26, 29*) with DNA-EDTA allows the effect of reaction conditions, probe length, and single base mismatches on triple helix formation to be analyzed on high-resolution sequencing gels. In addition, the orientation of the third strand as well as the identity of the grooves in right-handed DNA helix occupied by the bound DNA-EDTA probe can be analyzed by high-resolution gel electrophoresis (*5, 26*) (Fig. 2). Finally, the location of triple helices within large pieces of DNA can be mapped by double strand breaks analyzed by nondenaturing agarose gel electrophoresis. Nine homopyrimidine DNA probes, 11 to 15 nucleotides in length, containing a single thymidine with EDTA covalently attached at the 5 position (labeled T* in Figs. 3B, 4B, and 6B) were synthesized for binding and cleavage studies with two different duplex target DNA's. We find that these homopyrimidine-EDTA probes bind the corresponding homopurine sequence of duplex DNA in parallel orientation and, in the presence of Fe(II) and dithiothreitol (DTT), cleave one (or both) strands of the Watson-Crick DNA at that site.

One convenient synthesis of DNA-EDTA probes involves the incorporation of a modified thymidine into an oligonucleotide by chemical methods. This approach allows for automated synthesis and affords control over the precise location of the EDTA moiety at any thymidine position in the oligonucleotide strand (*26*). Oligonucleotides-EDTA **1** to **9** of different length, composition, and EDTA-thymidine position were synthesized in this manner. Each of the nine DNA-EDTA probes was purified by gel electrophoresis.

Orientation and groove location of Hoogsteen strand binding Watson-Crick DNA. Although it is believed that the two homopyrimidine strands in triple helical DNA or RNA are antiparallel, a definite proof is lacking. We examined a double-stranded DNA that

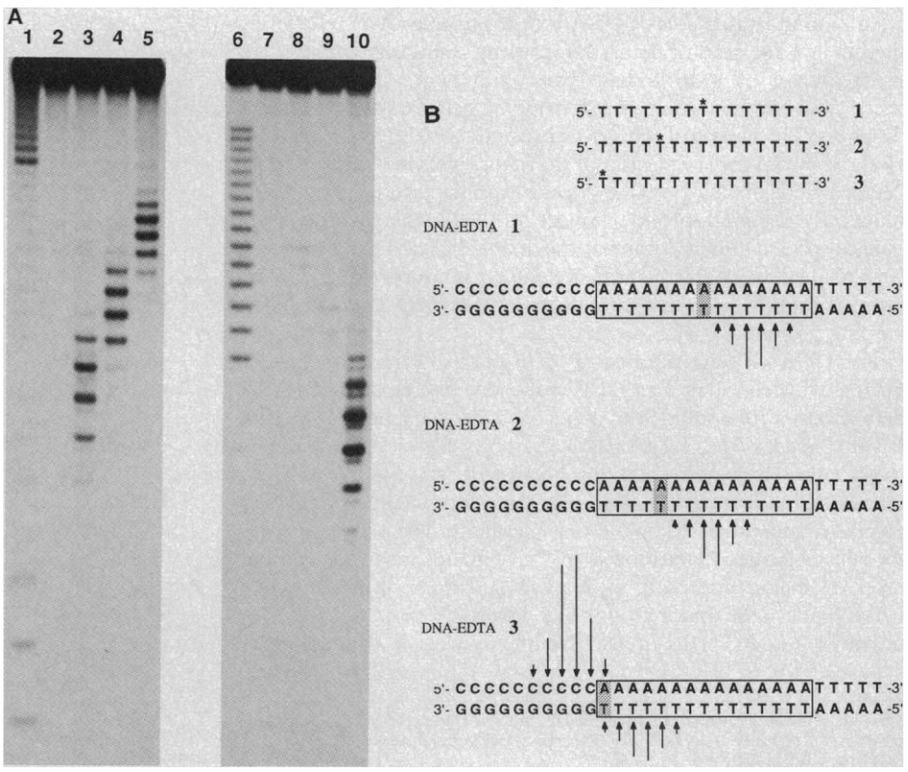

Fig. 3. (**A**) Autoradiogram of the 20 percent Maxam-Gilbert sequencing gel. (Lanes 1 to 5) 5′ end-labeled $d(A_5T_{15}G_{10})$; (lanes 6 to 10) 5′ end-labeled $d(C_{10}A_{15}T_5)$. (Lanes 1 and 6) The Maxam-Gilbert G+A sequencing reactions (*38, 39*). (Lanes 2 and 7) Controls showing the two 5′ labeled 30-bp DNA standards obtained by treatment according to the cleavage reactions in the absence of DNA-EDTA·Fe probes. (Lanes 3 to 5 and 8 to 10) The DNA cleavage products in the presence of DNA-EDTA·Fe probes **1** to **3**, ~0.5 μ*M* (bp) 5′ ^{32}P-labeled DNA, (~10,000 count/min), 10 m*M* tris-HCl, *p*H 7.4, 1 m*M* spermine, 100 m*M* NaCl, 100 μ*M* (bp) sonicated, deproteinized calf thymus DNA, 40 percent (by volume) ethylene glycol, 0.67 μ*M* probe, 25 μ*M* Fe(II), and 1 m*M* DTT; 15 hours, 0°C. (Lanes 3 and 8) DNA-EDTA·Fe **1**; (lanes 4 and 9) DNA-EDTA·Fe **2**; (lanes 5 and 10) DNA-EDTA·Fe **3**. (**B**) $(T)_{15}$-EDTA probes **1** to **3** where T* is the position of the thymidine-EDTA. Histograms of the DNA cleavage patterns derived by densitometry of the autoradiogram shown in (A) (lanes 3 to 5 and 8 to 10). The heights of the arrows represent the relative cleavage intensities at the indicated bases. Arrows are shown if the cleavage intensity at a particular nucleotide was greater than 5 percent compared to the nucleotide cleaved the most efficiently. The box indicates the double-stranded sequence which is bound by the DNA-EDTA·Fe(II) probes **1** to **3**. The Watson-Crick base pair to which T* is Hoogsteen hydrogen-bonded in the triple strand helix is shaded.

contains $(dA{\cdot}dT)_{15}$ as a target sequence which could, in principle, bind the $d(T)_{15}$ probe-strand in parallel or antiparallel orientation. A 30-bp duplex of DNA containing the tract $(dA{\cdot}dT)_{15}$ was labeled separately at the 5′ end of each strand. This was allowed to incubate with $(T)_{15}$-EDTA probes **1** to **3** with the thymidine-EDTA located at oligonucleotide positions 8, 5, and 1, from the 5′ end, respectively. The ^{32}P-labeled DNA was dissolved in buffer containing calf thymus DNA, NaCl, tris, spermine, and ethylene glycol and was mixed with the DNA-EDTA·Fe(II) probes, previously equilibrated with Fe(II) for 1 minute. After incubation at 0°C for 10 minutes, the reactions were initiated by the addition of an aqueous solution of DTT, such that the final concentrations were 10 m*M* tris-HCl (*p*H 7.4), 1 m*M* spermine, 100 m*M* NaCl, 40 percent (by volume) ethylene glycol, 100 μ*M* (bp) of calf thymus DNA, 0.67 μ*M* DNA-EDTA probe, 25 μ*M* Fe(II), and 1 m*M* DTT (*31*). The cleavage reactions were allowed to proceed for 15 hours at 0°C and then stopped by freezing and lyophilization. The resulting cleavage products were separated by electrophoresis on a denaturing 20 percent polyacrylamide gel and visualized by autoradiography (Fig. 3A).

On the $d(T)_{15}$ strand of the Watson-Crick duplex, one major cleavage site is observed for each DNA-EDTA probe **1** to **3** with the maximum cleavage site shifted to the 5′ side of T*. The location of the cleavage patterns on Watson-Crick DNA produced by the probes **1** to **3** with respect to the position of T* reveal the orientation of the DNA-EDTA probe on the duplex DNA (Fig. 3B). The homopyrimidine-EDTA strand binds parallel to the homopurine and antiparallel to the homopyrimidine strands of Watson-Crick DNA. These observations rule out strand displacement (D loop) as the mode of binding. The asymmetry of the cleavage patterns on opposite strands of DNA reveals the identity of the groove in right-handed DNA occupied by EDTA·Fe. An asymmetric cleavage pattern with maximal cleavage shifted to the 5′ or 3′ side on opposite strands corresponds to the diffusible hydroxyl radical being generated in the major or minor groove, respectively. The cleavage patterns shown in Fig. 3B reveal that the DNA-EDTA·Fe(II) probe is located in the major groove of the Watson-Crick DNA.

Homopyrimidine probes **1** and **2**, which bear the EDTA at an internal base position, cleave exclusively the homopyrimidine strand of the target DNA. A model of the triple helix between these homopyrimidine-EDTA·Fe(II) probes and the double helical DNA reveals that the homopurine Watson-Crick strand in the triple helix is protected from the hydroxyl radical by the sugar-phosphate backbone of the Hoogsteen paired strand. In effect, there are now three grooves in the triple helix, and EDTA·Fe is exposed to only one groove (Fig. 1). The nucleotides 3 to 4 bases on the 5′ side of T* in the right-handed triple helix are proximal to the EDTA·Fe(II) and are therefore expected to be cleaved most efficiently. Probe DNA-EDTA·Fe(II) **3**, which carries the cleaving moiety at the 5′ end, should form a triplex with no flanking nucleotides on the 5′side of T*. A homopyrimidine probe with the cleaving function at the 5′ end should generate cleavage on both strands. Indeed, the $d(T)_{15}$-EDTA·Fe(II)**3**, carrying the EDTA at the 5′ end, cleaves both strands of target duplex DNA (Fig. 3B).

Specific cleavage of a DNA restriction fragment. Cleavage by triple helix formation with DNA-EDTA·Fe(II) probes **4** to **9** was examined on a restriction fragment 628 bp in length that contained the sequence d(AAAAAGAGAGAGAGA). This sequence represents a

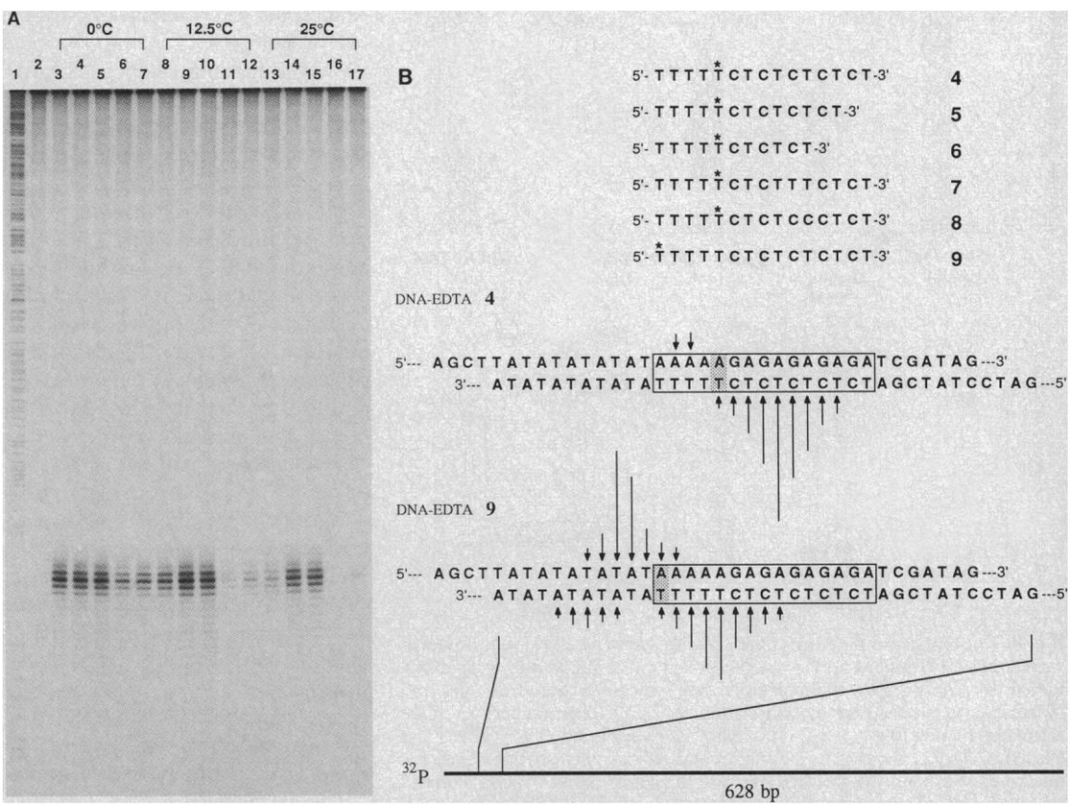

Fig. 4. (**A**) Autoradiogram of the 8 percent Maxam-Gilbert sequencing gel. Eco RI–Bgl I restriction fragment of plasmid pDMAG10 labeled at the 3′ end with ^{32}P. (Lane 1) The Maxam-Gilbert G+A sequencing reactions (*38, 39*). The general conditions for the cleavage reactions were as follows: ~100 n*M* (bp) labeled restriction fragment (~10,000 cpm), 25 m*M* tris-acetate, *p*H 7.0, 1 m*M* spermine, 100 m*M* NaCl, 100 μ*M* (bp) sonicated, deproteinized calf thymus DNA, 20 percent (by volume) ethylene glycol, 1 μ*M* DNA-EDTA probe, 25 μ*M* Fe(II), and 2 m*M* DTT. The cleavage reactions were held for 16 hours at 0°C. For each lane the parameters differing from these general conditions are given below. (Lane 2) Control, minus DNA-EDTA; (lanes 3, 8, and 13) 1 μ*M* DNA-EDTA **6**; (lanes 4, 9, and 14) 1 μ*M* DNA-EDTA **5**; (lanes 5, 10, and 15) 1 μ*M* DNA-EDTA **4**; (lanes 6, 11, and 16) 1 μ*M* DNA-EDTA **7** (Hoogsteen-type TG-mismatch), (lanes 7, 12, and 17): 1 μ*M* DNA-EDTA **8** (Hoogsteen-type CA mismatch). The reactions were run for 16 hours at 0°C (lanes 3 to 7), 12.5°C (lanes 8 to 12) and 25°C (lanes 2 and 13 to 17), respectively. The plasmid pDMAG5 was constructed by inserting the d(AAAAAGAGAGAGAGA)-containing duplex in the large Bam HI–Hind III restriction fragment of pBR322 (*40*). A single site labeled 628-bp Eco RI–Bgl I restriction fragment containing the target sequence was obtained by linearizing pDMAG10 with Eco RI, labeling with ^{32}P (*41, 42*) and cleaving with Bgl I. Electrophoresis on a 5 percent polyacrylamide gel separated the radiolabeled 628-bp fragment from other digest products. (**B**) Sequence of DNA-EDTA probes **4** to **9** where T* is the position of thymidine-EDTA. Histograms of the DNA cleavage patterns derived by densitometry of the autoradiogram from the cleavage of the 628-bp restriction fragment with DNA-EDTA probes **4** and **9**.

mixed homopurine target, which is located 47 nucleotides from the 3′ (and 5′) ^{32}P-label of the DNA fragment. On the 3′ end-labeled DNA strand, carrying the homopyrimidine target sequence, DNA-EDTA·Fe(II) **4** and **9** both produce sequence-specific cleavage patterns shifted to the 5′ side of the T* position (Fig. 4B) consistent with major groove binding. The efficiency of the sequence-specific cleavage of the DNA restriction fragment by DNA-EDTA·Fe(II) **4** is dependent on spermine or $Co(NH_3)_6^{3+}$ concentrations, ethylene glycol, *p*H, and probe concentration (Fig. 5A). In general, the cleavage reactions were carried out as follows: A mixture of DNA-EDTA probe (1 μ*M*) and Fe(II) (25 μ*M*) was combined with the ^{32}P-labeled restriction fragment [~100 n*M* (bp)] in a solution of calf thymus DNA [100 μ*M* (bp)], NaCl (100 m*M*), tris-acetate, *p*H 7.0 (25 m*M* tris), spermine (1 m*M*) and ethylene glycol (20 percent by volume) and incubated for 10 minutes at 0°C. Cleavage reactions were initiated by the addition of 2 m*M* DTT, proceeded 16 hours at 0°C, and stopped by precipitation with ethanol. The reaction products were analyzed on a high-resolution polyacrylamide gel. The histograms of these cleavage patterns were determined by densitometry of the autoradiogram (Fig. 4B).

The cleavage efficiency of probes **4** to **6**, which differ in length (15, 13, and 11 nucleotides), and probes **7** and **8**, which differ in sequence (each contain one Hoogsteen base mismatch in the triple helix complex), was examined under identical conditions at different temperatures. Identical cleavage patterns are observed for the DNA-EDTA·Fe(II) probes **4** to **8**. At 0°C, probes **4** to **6**, which differ in length but have in common T* at position 5 each produce a cleavage pattern of the same intensity. At 25°C probe **6**, which is 11 nucleotides in length, cleaves the target DNA three times less efficiently than probes **5** or **4**, which are 13 and 15 nucleotides in length, respectively. Probes DNA-EDTA **7** and **8**, which contain a single base mismatch at position 10 and 11, generate cleavage patterns of reduced intensity and are temperature-sensitive. Compared to DNA-EDTA probe **4**, the relative cleavage efficiency decreases for the single base mismatch probes **7** from 0.4 (at 0°C) to 0.08 (25°C) and **8** from 0.5 (at 0°C) to 0.13 (25°C) (Fig. 5B).

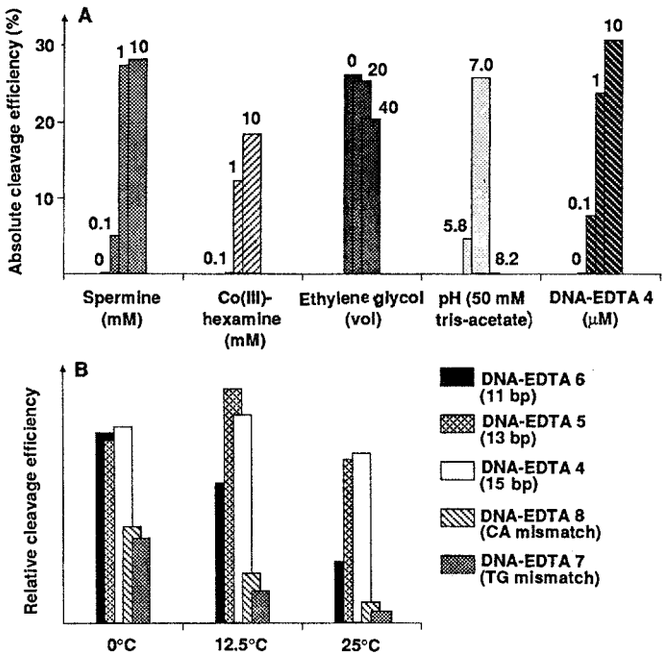

Fig. 5. (**A**) Bar graph presenting the absolute cleavage efficiencies obtained with DNA-EDTA·Fe **4** under various conditions. The values were determined by cutting out the corresponding pieces of the dried gel and measuring its radioactivity by scintillation counting. The numbers given are calculated by dividing the radioactivity of the cleavage site (sum of five most efficiently cleaved nucleotides) with the total radioactivity obtained from the uncleaved fragment, the cleavage site, and the background, which is corrected for the background that resulted from the untreated 628-bp fragment. The remaining values were assigned by correlation of absolute with relative cleavage efficiencies determined by densitometry of the autoradiogram. (**B**) Bar graph presenting the relative cleavage efficiencies (sum of six most efficiently cleaved nucleotides) obtained with DNA-EDTA·Fe **4** to **8** (A) at three temperatures as determined by densitometry. The data are reproducible within ±10 percent of reported values.

Importance of added cations. The importance of added cations for formation of triple-stranded DNA or RNA has been known since the discovery of those structures. To bind double helical DNA, the DNA-EDTA·Fe(II) probe must overcome the repulsion between two anionic chains of the Watson-Crick duplex and its own negatively charged phosphodiester backbone. One way to achieve this is to use multivalent cations (*12, 13*). The naturally occurring polyamines and their derivatives are known to stabilize double and triple helical structures of nucleic acids (*30*). We find optimum cleaving efficiencies for DNA-EDTA·Fe(II) **4** in the presence of millimolar concentrations of spermine or $Co(NH_3)_6^{3+}$. No cleavage occurs in the absence of spermine or $Co(NH_3)_6^{3+}$, which demonstrates the importance of these cations for triple helix formation (Fig. 5A). Spermine appears to be ideal for the stabilization of the triple-stranded complex with DNA-EDTA·Fe(II) probes. It efficiently neutralizes the negative charges of the sugar-phosphate backbones and does not compete with the Fe(II) for the EDTA moiety. No cleavage is observed if $MgCl_2$ or $CaCl_2$ (up to 8 m*M*) is substituted for spermine, which could be due to competition with Fe(II) for the metal chelator EDTA (*9*).

Role of organic solvents. According to x-ray fiber diffraction studies, the three strands of a triple helix occur in an A′ RNA-like conformation (*22*). A conformational transition may be necessary to allow the binding of the DNA-EDTA·Fe(II) probe. It has been established that a B to A conformational change takes place on lowering the relative humidity. This transformation depends on the ratio of (A+T) to (G+C) and can be achieved by addition of various organic solvents to the DNA aqueous solution. The increase in organic solvent concentration should favor the B to A conformational transition, and we might expect that triple helices should form more readily (*24*). As a result, the cleavage due to the DNA-EDTA probe should increase correspondingly. We find that the efficiency of oligonucleotide duplex cleavage by $(T)_{15}$-EDTA·Fe(II) **1** to **3** is increased by a factor of 10 upon addition of ethylene glycol (40 percent by volume). Other organic solvents such as methanol, ethanol, dioxane, or DMF give rise to similar behavior. In the presence of ethylene glycol, DNA-EDTA·Fe(II) probes provide cleavage patterns without detectable background, a result that may be due to radical scavenging by this solvent.

Curiously, the mixed T and C homopyrimidine EDTA·Fe(II) **4** demonstrates different behavior. The addition of ethylene glycol (20 percent by volume) is not necessary and does not increase the cleavage efficiency as found in the case of $(T)_{15}$. One explanation for this difference is that a mixed T,C probe may have a higher affinity than the oligo(T) probe to the corresponding Watson-Crick target sequence because of protonated cytosines required to form the Hoogsteen hydrogen bonds in the triple helix. The alternative explanation is that the target Watson-Crick sequences differ in conformation and one may be more A-like than the other.

The *p*H dependence of cleavage efficiency. Mixed homopyrimidine DNA-EDTA·Fe(II) **4** cleaves the target DNA over a relatively narrow range of *p*H values, producing the maximum cleavage at *p*H 7.0 (*31*) (Fig. 5A). This behavior could be caused by two independent properties of the oligonucleotide-EDTA probes. On one hand, triplex formation requires protonation of cytosines at N-3 in the

third strand to enable the Hoogsteen hydrogen bonds between DNA-EDTA·Fe(II) probes and the target Watson-Crick DNA. It was previously demonstrated that complexes of triple helical nucleic acids, containing cytosines in the homopyrimidine strands, are stable in slightly acidic to neutral solutions and start to dissociate on increasing *p*H (*15, 18*). Therefore it seems not unreasonable that the DNA-EDTA·Fe(II) probes do not bind Watson-Crick DNA in slightly basic solutions (*p*H ≧ 8) and consequently do not produce cleavage under these conditions. In contrast, studies with methidiumpropyl-EDTA·Fe indicate that the cleavage efficiency of EDTA·Fe decreases sharply below *p*H 7 (*9*), presumably as a result either of partial protonation of the EDTA and the resulting loss of Fe(II) or of some *p*H dependence of the cleavage reaction. On the basis of our experience with EDTA cleavage chemistry, we anticipate that at slightly acidic *p*H values, DNA-EDTA·Fe(II) probes do not produce efficient cleavage. Footprinting experiments confirm that the triple helix is forming at acidic *p*H values (*32*).

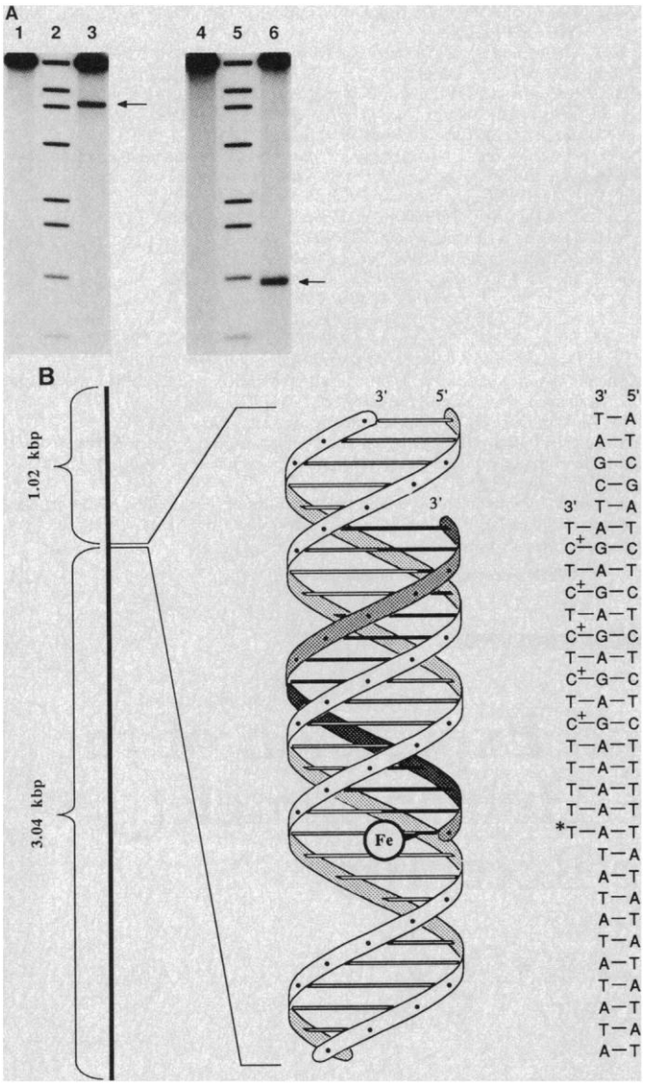

Fig. 6. (**A**) Double strand cleavage of plasmid DNA analyzed on a nondenaturing 0.9 percent agarose gel. (Lanes 1 to 3) Plasmid pDMAG10 linearized with Sty I and labeled at the downstream end of the restriction site with [α-^{32}P]ATP. (Lanes 4 to 6) Same plasmid with the other end-labeled with [α-^{32}P]TTP. Cleavage conditions: ^{32}P-labeled DNA plasmid, 100 m*M* NaCl, 1 m*M* spermine, 25 m*M* tris-acetate, *p*H 7.0, 100 μ*M* (bp) sonicated, deproteinized calf thymus DNA, 5 μ*M* DNA-EDTA·Fe(II) **9**, 25 μ*M* Fe(II), and 2 mM DTT. Cleavage reactions were run for 16 hours at 0°C. (Lanes 1 and 4) Controls containing no DNA-EDTA·Fe(II) **9**. (Lanes 2 and 5) DNA size markers obtained by digestion of Sty I linearized pDMAG10 with Eco RI, Pvu I, Sal I (both ends labeled), and Xmn I labeled with [α-^{32}P]TTP: 4058 (undigested DNA), 3338, 2994, 2368, 1690, 1460, 1064, and 720 bp. (Lanes 3 and 6) DNA-EDTA·Fe(II) probe **9** at 5 μ*M* added. Arrows indicate 3.04-kb (lane 3) and 1.02-kb (lane 6) cleavage fragments. (**B**) (Left) The course resolution cleavage pattern from (A). (Center) Simplified model of triple helix complex between the Hoogsteen-bound DNA-EDTA·Fe(II) **9** at single site within 4.06 kb of plasmid DNA. (Fig. 4B).

Influence of probe length, temperature, and sequence similarity. At 1 μ*M* concentration the DNA-EDTA probe approaches the maximum cleavage efficiency on the 628-bp restriction fragment (Fig. 5A). We chose DNA-EDTA probes 15 nucleotides in length for our initial studies to attain reasonable binding affinities at the double helical target sequence (*33, 34*). Having optimized the cleavage conditions for DNA-EDTA·Fe(II) **4**, we focused on the size dependence for DNA-EDTA·Fe(II) probes to form a triple helix complex with the Watson-Crick DNA. Probes DNA-EDTA·Fe(II) **5** and **6**, which are 13 and 11 nucleotides in size, produce cleavage patterns of similar intensities at 0°C, indicating that homopurine·homopyrimidine sequences as short as 11 nucleotides can specifically bind the 628-bp restriction fragment. The influence of oligonucleotide length becomes more apparent if the cleavage reactions are allowed to proceed at higher temperatures. The DNA-EDTA probes **4** and **5** still cleave the target duplex DNA at 25°C with approximately the same efficiency, whereas the relative intensity of the cleavage pattern produced by the shorter **6** becomes significantly weaker (Figs. 4A and 5B).

In order to test the importance of sequence similarity for triple helix formation and cleavage, we synthesized two probes, DNA-EDTA·Fe(II) **7** and **8**, which contained single base mismatches compared to DNA-EDTA·Fe(II) **4** but had in common the location of T* at position 5. When bound to the double helical target sequence, probes **7** and **8** should give rise to one mismatched base triplet with respect to the Hoogsteen hydrogen bonding. The mismatching bases in the probe strands were chosen so that the corresponding tautomeric or protonated structures of the mismatching pyrimidine base could still allow the formation of isomorphous base triplets. Compared to DNA-EDTA·Fe(II) **4**, both single mismatch probes **7** and **8** generate weaker cleavage patterns at 0°C and the difference becomes more apparent for the cleavage patterns produced at 25°C (Fig. 5B). Probes **7** and **8** cleave the target DNA less efficiently than DNA-EDTA·Fe(II) **4**. This result indicates that a single base mismatch in a DNA-EDTA·Fe(II) probe, 15 nucleotides in length, can lower the cleavage efficiency by at least a factor of 10. Clearly, the sequence-specific recognition of large double helical DNA by DNA-EDTA·Fe(II) probes is sensitive to single base mismatches, an indication of the importance of the correct homopyrimidine probe sequence for the formation of a triple-stranded complex with the target DNA.

Site-specific double strand cleavage of plasmid DNA. The ability of DNA-EDTA·Fe(II) **9** to cause double strand breaks at a homopurine·homopyrimidine insert in large DNA is presented in Fig. 6A. The plasmid pDMAG10 was digested with Sty I restriction endonuclease to produce a linear DNA fragment 4.06 kb in size, which contains the homopurine site d[A_5(AG)$_5$] located 1.0 kb upstream from the restriction site. This affords heterogenous overhangs and each end could be labeled separately with either [α-^{32}P]ATP or [α-^{32}P]TTP according to standard procedures. The ^{32}P end-labeled DNA was allowed to incubate with DNA-EDTA·Fe(II) **9** (5 μ*M*) for 10 minutes at 0°C as previously described, and the cleavage reaction was initiated by addition of DTT (2 m*M*) and run at 0°C for 16 hours. Separation of the cleavage products by agarose gel electrophoresis followed by autoradiography reveals only one major cleavage site producing two DNA fragments, 3.04

and 1.02 kb in size as determined by comparison with comigrating DNA size markers (Fig. 6A, lanes 3 and 6).

Significance of triple helix formation in large DNA. Although triple-stranded structures of polynucleotides were discovered decades ago, the biological significance has remained obscure. Such triplexes were proposed to be involved in processes like regulation of gene expression, maintenance of folded chromosome conformations, chromosome condensation during mitosis, and induction of local conformational changes in B-DNA (*35–37*). The work reported here demonstrates that homopurine-homopyrimidine double helical tracts can be recognized within large DNA by triple helix formation under physiological conditions. Homopyrimidine oligonucleotides and their analogs equipped with efficient DNA cleaving moieties at the 5′ end could become useful tools in chromosome analysis, gene mapping, and isolation. Moreover, as molecular biology defines specific disease states at the DNA level, a chemotherapeutic strategy of "artificial repressors" based on triple helix–forming DNA analogs becomes a possibility.

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43. Supported by grants from the National Institutes of Health (GM 35724) and the Ralph M. Parsons Foundation, and a fellowship (to H.E.M.) from the Swiss National Science Foundation.

8 July 1987; accepted 21 September 1987

Cloning, Sequencing, and Expression of the Gene Coding for the Human Platelet α_2-Adrenergic Receptor

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The gene for the human platelet α_2-adrenergic receptor has been cloned with oligonucleotides corresponding to the partial amino acid sequence of the purified receptor. The identity of this gene has been confirmed by the binding of α_2-adrenergic ligands to the cloned receptor expressed in *Xenopus laevis* oocytes. The deduced amino acid sequence is most similar to the recently cloned human β_2- and β_1-adrenergic receptors; however, similarities to the muscarinic cholinergic receptors are also evident. Two related genes have been identified by low stringency Southern blot analysis. These genes may represent additional α_2-adrenergic receptor subtypes.

A VARIETY OF NEUROTRANSMITTER AND HORMONE RECEPTORS elicit their responses via biochemical pathways that involve transduction elements known as guanine nucleotide regulatory (G) proteins (*1*). Among these are several types of receptors for epinephrine (adrenaline) which are termed adrenergic receptors. These adrenergic receptor subtypes are of particular interest because they are coupled to each of the major second messenger pathways that are known to be linked through G

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