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Inhibition of DNA Binding Proteins by Oligonucleotide-Directed Triple Helix Formation

LOUIS J. MAHER III, BARBARA WOLD, PETER B. DERVAN*

Oligonucleotides that bind to duplex DNA in a sequence-specific manner by triple helix formation offer an approach to the experimental manipulation of sequence-specific protein binding. Micromolar concentrations of pyrimidine oligodeoxyribonucleotides are shown to block recognition of double helical DNA by prokaryotic modifying enzymes and a eukaryotic transcription factor at a homopurine target site. Inhibition is sequence-specific. Oligonucleotides containing 5-methylcytosine provide substantially more efficient inhibition than oligonucleotides containing cytosine. The results have implications for gene-specific repression by oligonucleotides or their analogs.

EUKARYOTIC TRANSCRIPTION IS REGULATED BY THE INTERplay of various protein factors at promoters (*1*). Although transcriptional regulation in prokaryotes may be less complex, prokaryotic repressors can function as negative regulators when bound to heterologous operators in eukaryotic promoters (*2*). This observation suggests that displacement of activating proteins might provide a general strategy for gene-specific repression in eukaryotes. Pyrimidine oligonucleotides bind with sequence-specific dependence to homopurine sites in duplex DNA by triple helix formation and could have sufficient specificity and affinity to compete with site-specific DNA binding proteins for occupancy of overlapping target sites (*3*).

RNA and DNA polymer triple helices were first recognized more than 30 years ago. The structure and sequence requirements for triple helix formation have been investigated in vitro under both physiological and nonphysiological conditions. Poly(U) and poly(A) (polyuridylate and polyadenylate) form a stable 2:1 complex, as do poly(dT-dC) and poly(dG-dA) (polydeoxythymidylate, polydeoxycytidylate, and polydeoxyguanylate-polydeoxyadenylate) (*4*). Specificity arises from base triplets (T-A-T and C+G-C) formed by Hoogsteen base pairing of the second pyrimidine strand with the purine strand of the double helix (*5, 6*). Each base pair (bp) in a homopurine double helical DNA sequence affords two sequence-specific hydrogen bonds for triple helix formation. X-ray diffraction patterns of triple-stranded fibers [poly(A)·2 poly(U) and poly(dA)·2 poly(dT)] suggested an A form RNA-like conformation of the two Watson-Crick base-paired strands, with the third strand bound parallel to the purine strand of the duplex by Hoogsteen hydrogen bonds (*6, 7*). Mixed sequence pyrimidine oligonucleotides equipped with EDTA·Fe(II), the ethylenediamine tetraacetic acid chelate of Fe(II), selectively cleave homopurine sites in large DNA molecules (*3*). These studies confirm that, in the local triple helical complex, the pyrimidine (Hoogsteen) oligonucleotide is bound in the major groove, parallel to the Watson-Crick purine strand (*3*). Oligonucleotide-directed triple helix formation has also been reported in several other contexts (*8–10*). One of the most powerful aspects of the oligonucleotide approach to sequence-specific recognition of double helical DNA is the simplicity of the Hoogsteen hydrogen bonding mode. In a formal sense, a site size of more than 15 bp affords an ensemble of more than 30 discrete hydrogen bonds for sequence-specific recognition of DNA. This specificity is theoretically

L. J. Maher III is in the Division of Biology and the Division of Chemistry and Chemical Engineering, B. Wold is in the Division of Biology, and P. B. Dervan is in the Division of Chemistry and Chemical Engineering, California Institute of Technology, Pasadena, CA 91125.

*To whom correspondence should be addressed.

sufficient for site-specific targeting within human chromosomes, yet may be chemically generalized for a large number of different target sites.

A number of examples of altered DNA conformations consistent with intramolecular triple helix formation have been identified (*11*). Several investigators have also proposed regulation of gene expression in trans by triple helix formation (*12*). In a pioneering study, the binding of RNA homopolymers was shown to inhibit template activity of duplex DNA in the presence of *Escherichia coli* RNA polymerase (*13*). Hogan and co-workers have suggested that purine-rich oligonucleotides will bind specific duplex DNA sequences (*9*). The molecular basis for this recognition has not yet been established, but an in vitro study demonstrated that a purine-rich oligonucleotide [*27* nucleotides (nt)] represses transcription of the human c-*myc* gene (*9*); this finding raises the possibility that there may be at least two different structural patterns for sequence-specific recognition of duplex DNA by oligonucleotides: the pyrimidine motif and the purine motif. The experiments described below focus on the Hoogsteen motif and were designed to test whether pyrimidine oligonucleotide-directed triple helix formation inhibits its sequence-specific DNA binding proteins.

A 21-bp homopurine site was created by site-directed mutagenesis of the murine metallothionein I (MT-I) promoter. This target site for oligonucleotide binding was designed to overlap sequences recognized by restriction endonuclease Ava I, restriction methylase M. Taq I, and transcription factor Sp1. Studies were performed with this construct to document the conditions under which each of these sequence-specific DNA binding proteins is specifically inhibited by oligonucleotide-directed triple helix formation.

Recombinant plasmids and oligonucleotides. Triple helix formation by pyrimidine oligonucleotides was studied in the murine MT-I promoter near the Sp1-A site (*14*). This site is occupied during both basal and metal-induced MT-I transcription in vivo, and is recognized by purified Sp1 transcription factor in vitro (*14*). Two plasmids were used in these experiments. The pMTCAT-TH1 plasmid was created by constructing a 21-bp homopurine sequence overlapping and preserving the Sp1-A site (GGGGCGGTCC) of pMTCAT (Fig. 1A). The resulting oligonucleotide binding site overlaps four of the ten core base pairs of the Sp1-A site (*15*). In addition, a recognition sequence for restriction endonucleases Ava I (CTCGAG), Taq I(TCGA), and restriction methylase M.Taq I (TCGA) was created overlapping the distal terminus of the oligonucleotide binding site in pMTCAT-TH1. Both pMTCAT and pMTCAT-TH1 were designed so that a distant Ava I site and several distant Taq I sites would provide internal controls for triple helix specificity.

The oligonucleotides **1** to **5** were synthesized and tested for binding to the homopurine target site in pMTCAT-TH1 (Fig. 1B). Pyrimidine oligonucleotides **1** to **4** were designed to recognize the target site by Hoogsteen base pairing. The scheme for **1** (20 nt) is shown in Fig. 1C. Oligonucleotide **2** is the same length and sequence as **1** but is modified at the 5′ terminus for site-specific DNA cleavage by incorporation of thymidine covalently linked to EDTA (*3, 16*). Pyrimidine oligonucleotides **3** (20 nt) and **4** (21 nt) were synthesized with 5-methylcytosine substituted for cytosine except at the 3′ terminal residue. Such substitutions have been reported to extend the permissive *p*H range and stability of triple helices (*17, 18*). Purine oligonucleotide **5** (20 nt) was synthesized in

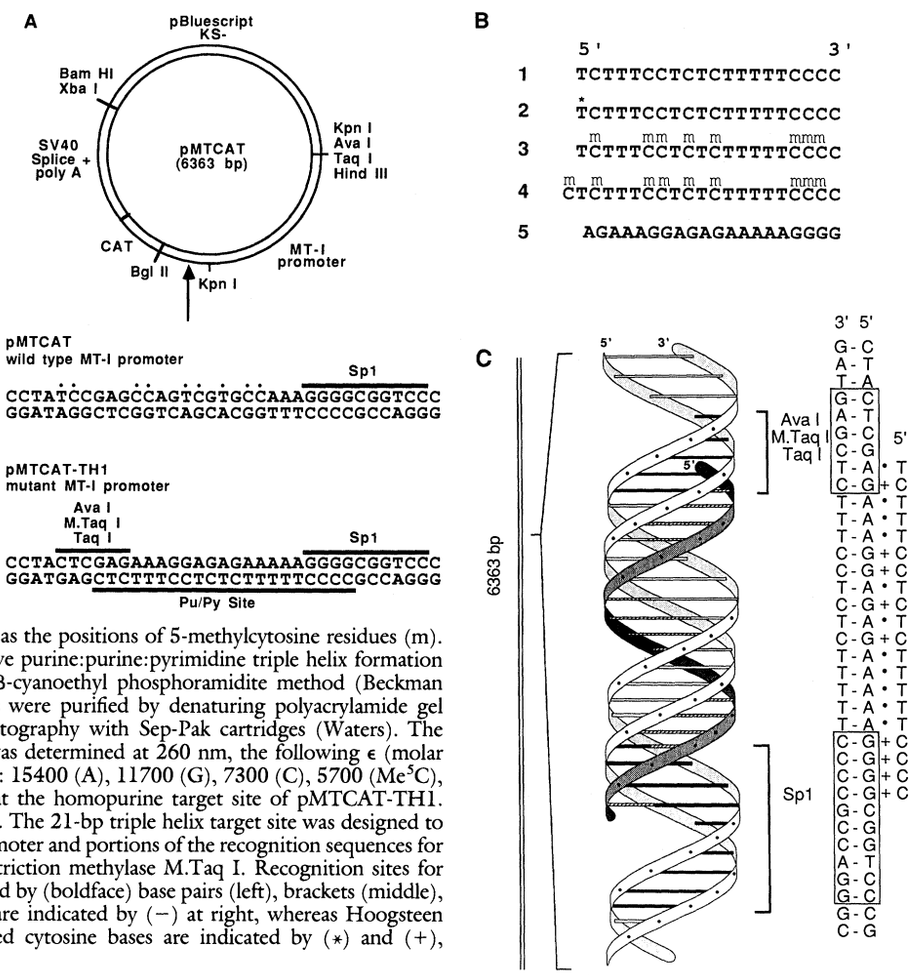

Fig. 1. (**A**) For pMTCAT construction, the chloramphenicol acetyl transferase (CAT) coding sequence of pSV2cat, the splicing and poly(A) addition signals of SV40 (*27*) and the murine metallothionein I (MT-I) promoter (*28*) were placed in the plasmid pBluescript KS- (Stratagene). Site-directed mutagenesis (*29*) was used to create a homopurine sequence in the MT-I promoter of pMTCAT-TH1 (position indicated by arrow). Dots indicate base pairs altered by mutagenesis. Mutations were confirmed by sequencing both DNA strands. (**B**) Nucleotide sequences of synthetic oligodeoxyribonucleotides tested for binding to the homopurine target site of pMTCAT-TH1. The position of a tethered EDTA group (T*) in **2** is indicated (*3, 16*), as well as the positions of 5-methylcytosine residues (m). Oligonucleotide **5** was designed to test an alternative purine:purine:pyrimidine triple helix formation mode. Oligonucleotides were synthesized by the β-cyanoethyl phosphoramidite method (Beckman System 1Plus DNA synthesizer). Oligonucleotides were purified by denaturing polyacrylamide gel electrophoresis and desalted by dialysis or chromatography with Sep-Pak cartridges (Waters). The concentration of single-stranded oligonucleotides was determined at 260 nm, the following ϵ (molar extinction coefficient) values were used for each base: 15400 (A), 11700 (G), 7300 (C), 5700 (Me^5C), and 8800 (T). (**C**) The triple helix formed by **1** at the homopurine target site of pMTCAT-TH1. Plasmid linearization at the Hind III site is depicted. The 21-bp triple helix target site was designed to include 4 of 10 bp of the Sp1-A site of the MT-I promoter and portions of the recognition sequences for restriction endonucleases Ava I and Taq I, and restriction methylase M.Taq I. Recognition sites for sequence-specific DNA binding proteins are indicated by (boldface) base pairs (left), brackets (middle), and boxes (right). Watson-Crick hydrogen bonds are indicated by (−) at right, whereas Hoogsteen hydrogen bonds involving thymine and protonated cytosine bases are indicated by (*) and (+), respectively.

order to test the putative purine triple helix motif (*9*). This oligonucleotide is identical in sequence and polarity to the purine strand of the triple helix target site.

Stable triple helix formation at target site. The specificity of triple helix formation at the homopurine target site of pMTCAT-TH1 was confirmed by means of an affinity-cleaving assay (*3, 16*). In the presence of oxygen and dithiothreitol (DTT), triple helix formation involving oligonucleotide–EDTA·Fe(II) compounds such as **2** is accompanied by double-strand scission of the target DNA (*3*). Local cleavage results from the generation of diffusible radicals at the site of bound oligonucleotide–EDTA·Fe(II) (*16*). Under these conditions, double-strand scission may be limited by oligonucleotide–EDTA·Fe(II) autocleavage and is typically in the 10 to 25 percent range (*16*). Thus, as determined by footprinting studies (see below), target saturation produces only partial target cleavage (Fig. 2). After 30-minute (lane 3) or 180-minute (lane 4) binding reactions at 22°C in the presence of **2** at 1 μ*M*, cleavage of pMTCAT-TH1 was initiated by the addition of DTT. After a 15-hour cleavage reaction, DNA fragments of the expected sizes were observed. There is no evidence of other oligonucleotide binding sites on pMTCAT-TH1. In control experiments, incubation with **2** did not result in cleavage of pMTCAT.

An affinity-cleaving competition assay was used to measure the kinetic stability of the triple helix under these conditions (Fig. 2, lanes 5 to 13). When allowed to bind to pMTCAT-TH1 in the presence of a 20-fold excess of **1**, oligonucleotide–EDTA·Fe(II) **2** did not cleave the target DNA (compare lanes 3 and 5). It was therefore possible to measure the kinetic stability of the triplex involving **2** by adding excess **1** after an initial 30-minute binding reaction, and initiating the cleavage reaction after increasing incubation times. Comparison of lanes 6 to 13 of Fig. 2 indicates no substantial triple helix dissociation for at least 120 minutes at 22°C. This result is consistent with previous kinetic measurements of triple helix stability obtained by gel mobility shift assays (*10*). The apparent stability of the **2** complex suggested that the bound pyrimidine oligonucleotide (21 nt, 22°C, *p*H 6.8) might offer a stable block to DNA site recognition by binding proteins.

Restriction endonuclease protection. Experiments were performed to determine whether oligonucleotides bound to duplex DNA could confer endonuclease resistance to an overlapping restriction site. The endonuclease Ava I recognizes a single 6-bp site (CTCGAG) in pMTCAT and two identical sites in pMTCAT-TH1, one of which overlaps two to three base pairs of the triple helix target site. pMTCAT and pMTCAT-TH1 were treated with Ava I in the presence of 0 to 80 μ*M* concentrations of **1** or **5** (Fig. 3A). Increasing concentrations of **1** had no effect on control plasmid pMTCAT cleavage by Ava I (compare lanes 3, 6, 9, 12, 15, and 18). However, **1** conferred concentration-dependent protection from Ava I cleavage at the pMTCAT-TH1 triple helix target site (compare lanes 4, 7, 10, 13, 16, and 19). Cleavage at the distant Ava I site was unaffected. Endonuclease inhibition did not require prior equilibration of the target DNA with oligonucleotide. Reactions containing both endonuclease and oligonucleotide were initiated by addition of target DNA under conditions permissive for Ava I activity. This result indicates that **1** at 60 to 80 μ*M* concentrations fully protects the target Ava I site from endonuclease for at least 45 minutes at 37°C.

Purine oligonucleotide **5** was tested to determine if an alternative triple helix based on purine:purine:pyrimidine recognition could form at the pMTCAT-TH1 target site. Oligonucleotide **5** had no effect on pMTCAT-TH1 cleavage by Ava I at any of the oligonucleotide concentrations tested in these experiments (Fig. 3A, compare lanes 5, 8, 11, 14, 17, and 20). Deoxyribonuclease I (DNase I) footprinting experiments have confirmed that **5** does not bind to the pMTCAT-TH1 triple helix target site (data not shown). It is of interest to compare this result to a report of apparent triple helix formation by a purine-rich oligonucleotide at a naturally occurring site in the c-*myc* gene (*9*). Although stable A·A·T base triplets have been reported (*19*), the failure of **5** to participate in triple helix formation suggests that mixed A·A·T and G·G·C recognition of homopurine targets is sequence composition dependent. Triple helix formation in the (G-C)-rich c-*myc* gene site may result entirely from G·G·C recognition of the target sequence by a G-rich oligonucleotide (*9, 20*).

The Ava I protection assay was used to compare oligonucleotides **1, 3, 4,** and **5** (Fig. 3B). Substitution of 5-methylcytosine for cytosine in the Hoogsteen strand increases triple helix stability, although the basis for this increased stability is not fully understood (*17, 18*). The cleavage protection curves confirm that oligonucleotides containing 5-methylcytosine are substantially more effective than **1**. A 50 percent endonuclease protection was observed at concentrations of 26 μ*M* for **1**, 0.5 μ*M* for **3**, and 0.25 μ*M* for **4**. This corresponds to molar ratios of oligonucleotide to DNA site of approximately 3×10^4, 5×10^2, and 2.5×10^2, respectively. Thus, the endonuclease inhibition constant for **3** is about 50 times greater than for **1**. Oligonucleotide **4** confers even greater Ava I protection than **3**. This improvement presumably reflects both the longer triple helix (21 compared to 20 base triplets) and the fact that **4** binds to three of the six base pairs of the Ava I site, while **3** binds only two of six. As described above, **5** had no effect at any of the oligonucleotide concentrations tested in these experiments.

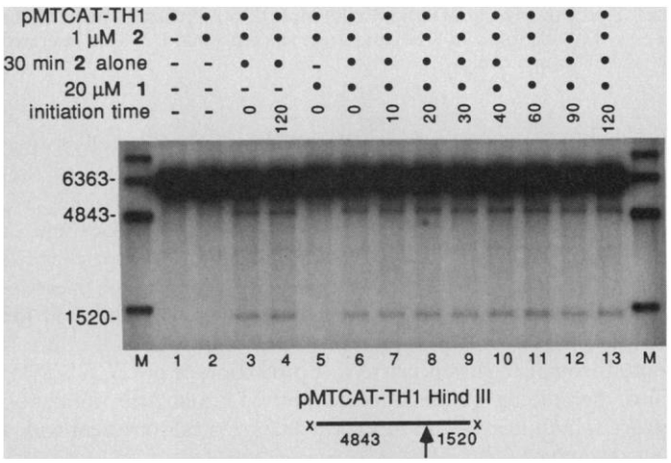

Fig. 2. Stable triple helix formation at the target site of pMTCAT-TH1. Plasmid pMTCAT-TH1 was linearized by digestion with Bam HI and labeled at both termini by incubation with the Klenow fragment of *E. coli* DNA polymerase and α-^{32}P-labeled deoxyribonucleoside triphosphates. Specificity of triple helix formation was measured by oligonucleotide-directed affinity cleavage (*3*). Labeled plasmid (100 ng) was added to 25-μl reaction mixtures containing 25 m*M* tris-acetate, *p*H 6.8, 100 m*M* NaCl, and 1 m*M* spermine. Lanes 3 and 4 included **2** at 1 μ*M* complexed with an equimolar amount of Fe(II). After incubation for 30 minutes at 22°C, cleavage reactions were initiated by addition of 4 m*M* DTT at 0 (lane 3) or 2 hours (lane 4) and were incubated at 22°C for 15 hours. DNA samples were precipitated with ethanol and analyzed by electrophoresis in 0.9 percent agarose with subsequent autoradiography. Standard lanes (M) contain phage λ DNA digested with Hind III. The linearized plasmid structure is indicated below. Positions of radioactive labels are indicated (x). The arrow indicates the **2** binding site on pMTCAT-TH1. A competition assay was used to monitor the rate of oligonucleotide dissociation from the triple helix under the conditions described above (lanes 5 to 13). After standard 30-minute binding reactions in the presence of **2**, a 20-fold molar excess (relative to **2**) of the noncleaving oligonucleotide **1** was added. In lane 5, **1** and **2** were added at the same time. Cleavage reactions were initiated at intervals after the addition of competitor oligonucleotide. The extent of affinity cleavage relative to lane 3 reflects the kinetic stability of the original triple helix.

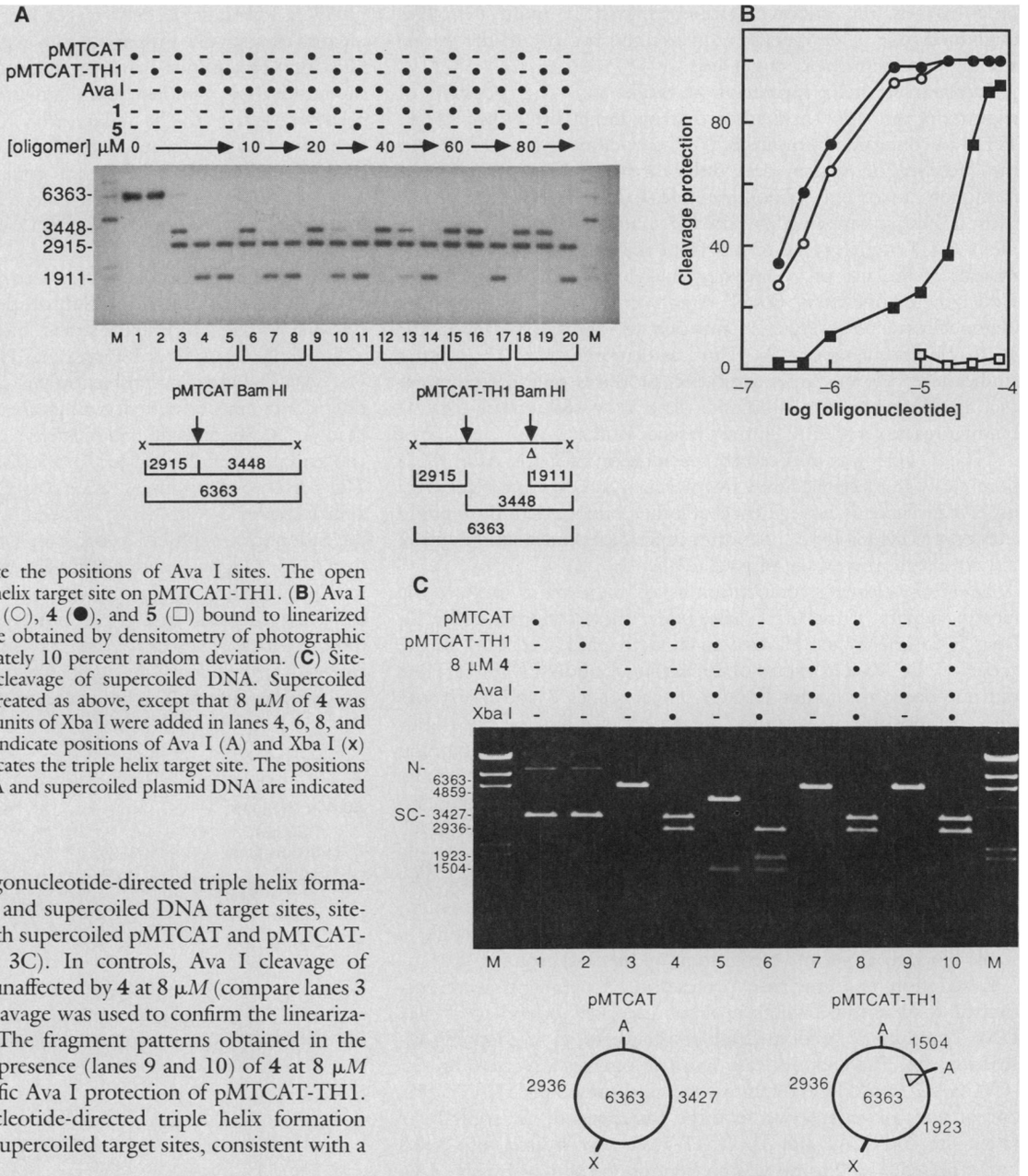

Fig. 3. Restriction endonuclease protection. (**A**) Site-specific inhibition of linear DNA cleavage by Ava I. Plasmid DNA (100 ng, linearized by digestion with Bam HI) was added to 25-μl reaction mixes containing competition buffer [25 m*M* tris-acetate, *p*H 6.8, 70 m*M* NaCl, 20 m*M* $MgCl_2$, 400 μ*M* spermine, bovine serum albumin (BSA) at 100 μg/ml, 10 m*M* β-mercaptoethanol], as well as 1.5 units of Ava I (Pharmacia), and 0 to 80 μ*M* oligonucleotide. Reactions were incubated at 37°C for 45 minutes and analyzed by electrophoresis in 0.8 percent agarose with subsequent autoradiography. Standard lanes (M) contain Hind III–digested phage λ DNA. Plasmid restriction maps are indicated below. Positions of radioactive labels are indicated (x). The filled arrows indicate the positions of Ava I sites. The open arrowhead indicates the triple helix target site on pMTCAT-TH1. (**B**) Ava I inhibition curves for **1** (■), **3** (○), **4** (●), and **5** (□) bound to linearized pMTCAT-TH1. The data were obtained by densitometry of photographic negatives, subject to approximately 10 percent random deviation. (**C**) Site-specific inhibition of Ava I cleavage of supercoiled DNA. Supercoiled plasmid DNA (100 ng) was treated as above, except that 8 μ*M* of **4** was added in lanes 7 to 10, and 10 units of Xba I were added in lanes 4, 6, 8, and 10. Restriction maps (below) indicate positions of Ava I (A) and Xba I (x) sites. The open arrowhead indicates the triple helix target site. The positions of nicked circular plasmid DNA and supercoiled plasmid DNA are indicated (N and SC, respectively).

To ascertain whether oligonucleotide-directed triple helix formation occurs at both relaxed and supercoiled DNA target sites, site-specific Ava I protection with supercoiled pMTCAT and pMTCAT-TH1 was measured (Fig. 3C). In controls, Ava I cleavage of supercoiled pMTCAT was unaffected by **4** at 8 μ*M* (compare lanes 3 and 7). Secondary Xba I cleavage was used to confirm the linearization site (lanes 4 and 8). The fragment patterns obtained in the absence (lanes 5 and 6) or presence (lanes 9 and 10) of **4** at 8 μ*M* confirm complete site-specific Ava I protection of pMTCAT-TH1. Thus, pyrimidine oligonucleotide-directed triple helix formation occurs at both relaxed and supercoiled target sites, consistent with a previous report (*10*).

Restriction methylase protection. To determine whether the observed endonuclease protection results could be extended to other sequence-specific DNA binding proteins, a restriction methylase was studied. In addition to providing a test for the generality of oligonucleotide inhibition of protein binding, site-specific inhibition of a methylase might also be applicable to genomic mapping. In the sequence 5′ TCGA, M.Taq I methylates the N^6 position of adenine and the methylation prevents Taq I endonuclease cleavage at this sequence. A Taq I site partially overlaps the pMTCAT-TH1 triple helix target site. pMTCAT-TH1 was linearized by digestion with Hind III. When digested with Taq I endonuclease, the linearized plasmid is cleaved at four internal sites (Fig. 4, lanes 1 and 2). If pMTCAT-TH1 is methylated by M.Taq I before Taq I treatment, no endonuclease cleavage occurs (lane 3). When pMTCAT-TH1 is methylated by M.Taq I in the presence of **4** at 2.5 to 40 μ*M*, increasing methylation protection, revealed after subsequent Taq I digestion, is observed at the triple helix target site (lanes 4 to 8). This corresponds to molar ratios of oligonucleotide to DNA site of approximately 1×10^3 to 1.7×10^4. The resulting cleavage pattern indicates that triple helix specificity may be conferred on a restriction endonuclease by this technique. This result extends the previous observation that methylation protection is induced by sequence-specific DNA binding proteins (*21*). When implemented in conjunction with an appropriate methylase and endonuclease, transient oligonucleotide-directed triple helix protection may provide a novel method for precise enzymatic dissection of large DNA molecules.

Inhibition of transcription factor binding. To extend the results obtained with prokaryotic DNA modifying enzymes, the effect of triple helix formation on the binding of a eukaryotic transcription factor was studied. Sp1 is a transcriptional activating protein that binds the consensus sequence 5′ G(T)GGGCGGPuPuPy in several eukaryotic promoters (*15*, *22*, *23*). In vivo footprinting studies indicate that the Sp1-A site of the MT-I promoter is occupied during both basal and metal-induced transcription, and is recognized by purified Sp1 protein in vitro (*14*). Complementary DNA clones encoding Sp1 have been isolated, permitting expression of

Sp1 in *E. coli* (*24*, *25*). A polypeptide composed of the 516 carboxyl-terminal amino acids of Sp1 (Sp1-516C) exhibits both DNA binding and transcriptional activating activities (*25*). DNase I footprinting was used to monitor the effect of bound **4** on Sp1-516C binding to its overlapping recognition site in pMTCAT-TH1. Oligonucleotide **4** at 5 to 10 μ*M* caused some nonspecific inhibition of Sp1-516C binding, presumably the result of interactions of **4** with the protein in solution.

The effects of triple helix formation on Sp1-516C binding were therefore studied at concentrations of **4** that did not cause detectable nonspecific inhibition. MT-I promoter restriction fragments of pMTCAT or pMTCAT-TH1 were incubated with or without **4** in a small volume (binding reaction), and the resulting complexes were then diluted in footprinting buffer, incubated in the presence or absence of Sp1-516C, and treated with DNase I (Fig. 5). Incubation of MT-I promoter restriction fragments from pMTCAT or pMTCAT-TH1 with Sp1-516C extract resulted in the formation of a DNase I protected region (footprint) on the purine strand at the Sp1-A site (*26*) (compare lanes 1, 2, 7, and 8). Mock extracts from bacterial cells lacking Sp1 expression vectors did not produce footprints (lanes 6 and 12). When **4** at 12 μ*M* was first incubated in a 4-μl binding reaction and then diluted to 50 μl (**4** at ~1 μ*M*), a DNase I footprint was detected at the triple helix target site of pMTCAT-TH1 (lane 9) but not in pMTCAT (lane 3). DNase I footprinting reveals that bound **4** prevents Sp1-516C binding at the Sp1-A site of pMTCAT-TH1 (compare lanes 8, 10, and 11). Under these conditions, **4** does not nonspecifically inhibit Sp1-516C binding to the pMTCAT control fragment (compare lanes 2, 4, and 5). Thus, inhibition of Sp1-516C binding to pMTCAT-TH1 must result primarily from occlusion of the protein binding site by bound oligonucleotide.

Implications. These experiments assess the extent to which oligonucleotide-directed triple helix formation disrupts DNA-protein interactions in a eukaryotic promoter. A 21-bp homopurine triple helix target site was created in the murine MT-I promoter. The site overlaps recognition sequences for restriction endonuclease Ava I, restriction methylase M.Taq I, and eukaryotic transcription factor Sp1. Pyrimidine oligonucleotides form a specific, stable triple helix at this homopurine target site as judged by DNase I footprinting and affinity cleavage with an oligonucleotide equipped with EDTA·Fe(II). Protection from restriction endonuclease cleavage at this site is conferred by oligonucleotide-directed triple helix formation. Oligonucleotides containing 5-methylcytosine confer substantially more efficient protection than oligonucleotides containing cytosine. Triple helix formation selectively protects the target site

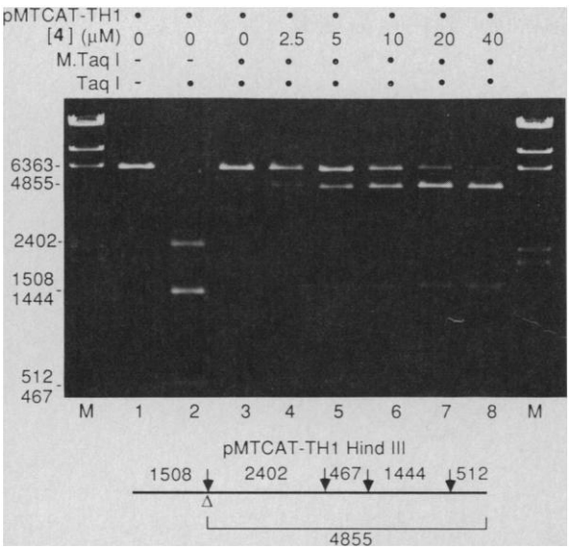

Fig. 4. Restriction methylase protection. Methylation protection by oligonucleotides was analyzed in a two-step procedure. Samples of pMTCAT-TH1 (100 ng, linearized by digestion with Hind III) were assembled in 10-μl reaction mixtures containing competition buffer as well as 80 μ*M* S-Adenosylmethionine (New England Biolabs), **4**, at 0 to 40 μ*M* and 5.4 units of M.Taq I (New England Biolabs). Reaction mixtures were incubated for 1 hour at 44°C under paraffin oil. The methylated DNA was purified by extraction with phenol and precipitation with ethanol. Methylation protection was analyzed by digesting the methylated DNA with 6.75 units of Taq I endonuclease (New England Biolabs) in 25-μl reaction mixtures containing 10 m*M* tris-HCl, *p*H 8.3 (at 25°C), 100 m*M* NaCl, 7 m*M* $MgCl_2$, BSA at 100 μg/ml, and 10 m*M* β-mercaptoethanol. Digestions with Taq I were performed at 70°C for 1 hour under paraffin oil. DNA was analyzed by electrophoresis in 0.8 percent agarose containing ethidium bromide. Standard lanes (M) contain Hind III–digested phage λ DNA. A restriction map of Hind III–linearized pMTCAT-TH1 is shown below. Filled arrows indicate the positions of Taq I sites. The open arrowhead indicates the position of the triple helix target site.

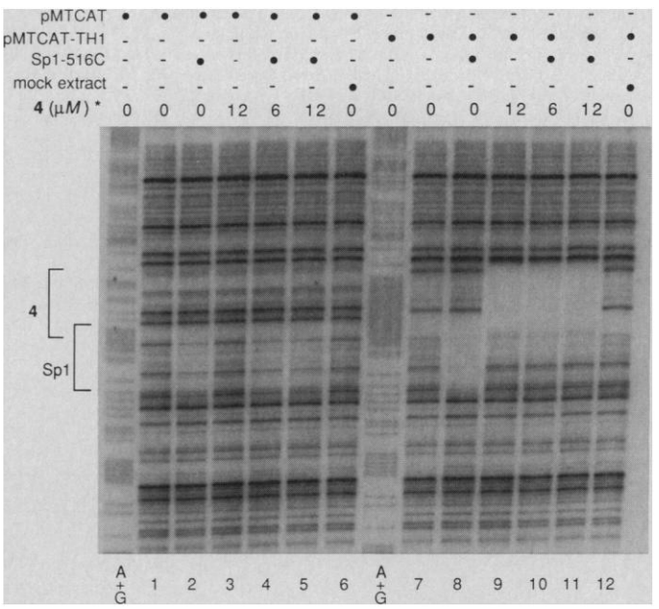

Fig. 5. Inhibition of Sp1 binding. Sp1-516C was expressed in *E. coli* strain C600 Δ*lon* (*30*) by isopropyl-β-D-thiogalactopyranoside (IPTG) induction of plasmid pSp1-516C (*24*). Bacterial extracts were prepared as described (*24*, *25*). Mock extracts were prepared by IPTG treatment of C600 Δ*lon* cells. Fragments (606 bp) obtained by Kpn I and Bgl II digestion of pMTCAT and pMTCAT-TH1 were end-labeled at the Kpn I site by incubation with the Klenow fragment of *E. coli* DNA polymerase and α-^{32}P-labeled deoxyribonucleoside triphosphates. Binding reaction mixtures (4 μl) contained 10 to 20 fmole of [^{32}P]DNA fragment and **4** at 0, 6, or 12 μ*M* in competition buffer. After incubation for 15 minutes at 25°C, binding reaction mixtures were diluted to 50 μl with a solution containing 25 m*M* tris-acetate, *p*H 6.8, 0.5 m*M* EDTA, 10 m*M* $MgCl_2$, 50 m*M* KCl, 0.5 m*M* DTT, 10 percent (v/v) glycerol, 2 percent polyvinyl alcohol, and were cooled to 0°C. Extract (1 μl) from bacterial cells carrying pSp1-516C (4.9-μg of protein) was added to some samples (lanes 2, 4, 5, 8, 10, and 11). Mock Sp1 extract (1 μl, 4.9 μg of protein) was added to other samples (lanes 6 and 12). Triple helix complexes containing **4** were tested in the absence (lanes 3 and 9) or presence (lanes 4, 5, 10, and 11) of Sp1 extract. After a 15-minute incubation at 0°C, 50 μl of a solution containing 10 m*M* $MgCl_2$ and 5 m*M* $CaCl_2$ was added, and the samples were treated for 90 seconds at 0°C with DNase I at a final concentration of 100 ng/ml. Digestions were terminated by addition of 100 μl of a solution containing 200 m*M* NaCl, 20 m*M* EDTA, 1 percent sodium dodecyl sulfate, and transfer RNA at 250 μg/ml. DNA was purified by extraction with phenol and was precipitated with ethanol. DNA samples were analyzed by electrophoresis in 5 percent polyacrylamide sequencing gels containing 7.5*M* urea. Standard lanes (A+G) contain DNA fragments generated by an A+G base-specific chemical cleavage reaction (*31*). The positions of DNase I footprints due to the binding of Sp1-516C or **4** are indicated at the left.

from restriction methylation, and subsequently allows specific restriction endonuclease cleavage at the triple helix target site. Conferring oligonucleotide specificity of more than 15 bp on a restriction endonuclease by triple helix formation may be useful for chromosome mapping. DNase I footprinting indicates that triple helix formation blocks the binding of eukaryotic transcription factor Sp1. These results suggest possible mechanisms for and constraints on the natural regulation of gene function by altered DNA structures in vivo. Oligonucleotide-directed triple helix formation offers a new tool for analyzing protein-DNA interactions in promoters, and in some cases such oligonucleotides or their analogs might be designed to function as artificial gene-specific repressors in vivo.

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26. The affinities of sequence-specific DNA binding proteins such as Sp1 may depend on the context of the conserved DNA recognition site. This dependence is exemplified by the apparent higher affinity of Sp1-516C for its recognition site when the site is adjacent to a homopurine sequence (pMTCAT-TH1) compared to its natural context (pMTCAT).
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