Site-specific inhibition of *EcoRI* restriction/modification enzymes by a DNA triple helix

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ABSTRACT

The ability of oligopyrimidines to inhibit, through triple helix formation, the specific protein-DNA interactions of the *EcoRI* restriction and modification enzymes (*EcoRI* and *MEcoRI*) with their recognition sequence (GAATTC) was studied. The oligonucleotides (CTT)₄ and (CTT)₈ formed triplexes in plasmids at (GAA)_n repeats containing *EcoRI* sites. Cleavage and methylation of *EcoRI* sites within these sequences were specifically inhibited by the oligonucleotides, whereas an *EcoRI* site adjacent to a (GAA)_n sequence was inhibited much less. Also, other *EcoRI* sites within the plasmid, or in exogenously added lambda DNA, were not inhibited. These results demonstrate the potential of using triplex-forming oligonucleotides to block protein-DNA interactions at specific sites, and thus this technique may be useful in chromosome mapping and in the modulation of gene expression.

INTRODUCTION

The sequence-specific recognition of DNA by proteins is essential for programmed regulation of cellular functions. Any method which can alter selectively the interaction of a protein at a certain site on DNA, without affecting that same interaction elsewhere on the chromosome, offers the opportunity to modulate biological functions. Triple helix formation may serve this function.

Two types of triplexes in recombinant plasmids and DNA fragments were identified recently: intermolecular triplexes, formed by combining duplex DNA and a single-stranded oligomer (1–8) and intramolecular triplexes (9–15), a non-B DNA structure formed at mirror repeat sequences in supercoiled plasmids. These discoveries renewed interest in the thirty-year-old field of nucleic acid triplexes (16–23). Triple helices form most readily at segments of DNA which have a homopurine·homopyrimidine (pur·pyr) bias. These sequences are overrepresented in eukaryotic genomes (24–26) and often are found near genes and recombination sites (14). The third strand is generally pyrimidine rich, but in some cases may be purine rich. For the triplex with two pyrimidine strands and one purine strand, the extra pyrimidine strand usually binds parallel to the purine strand through Hoogsteen hydrogen bonds (T to A and CH⁺ to G) (1,3,7,20). Cytosine protonation in the third strand

causes these triplexes to be more stable at acidic pH values (pH 5–6). However, intermolecular triplexes exist and were utilized to target cleavage of DNA at neutral pH (1,5,6,8).

We have investigated the ability of pyrimidine oligonucleotides to inhibit *EcoRI* methylation or cleavage of GAATTC sites located in or near pur·pyr tracts. Our data demonstrate that the oligomers form a triple helix, and that they inhibit both of the enzymes specifically at the pur·pyr sites. This approach has implications for both genetic mapping and regulation.

MATERIALS AND METHODS

Plasmids

For pRW1754, the insert 5'(GAA)₄TTC(GAA)₄ was isolated after BamHI digestion of pRW1701 (15) and was cloned into the BamHI site of pBR322. For pRW1703 and pRW1704, the inserts 5'GAATTC(GAA)₈ and 5'(GAA)₉TTC, respectively, both with 5'GATC overhangs, were cloned into the BamHI site of pRW791 (10). Oligonucleotides were synthesized on an Applied Biosystems 380A oligonucleotide synthesizer and purified by HPLC.

Inhibition of Methylation at One of Two *EcoRI* Sites in pRW1754

PstI-linearized pRW1754 (4.4 nM) was incubated with 5 μM (CTT)₄ in pH 5.5 *MEcoRI* buffer (100 mM Tris·acetate, pH 5.5, 100 mM NaCl, 1 mM MgCl₂) (100 μl reaction) for 1h at 15°C. *MEcoRI* (320 U) and S-adenosylmethionine (0.8 mM) were added and at various times 23 μl aliquots were removed and the reaction terminated by phenol extraction followed by ethanol precipitation. The DNA was digested for 2h at pH 7.5 and 37°C with 10 U of *EcoRI* (under these conditions the oligonucleotide does not inhibit *EcoRI*), and then analyzed on a 1% agarose gel.

Inhibition of Restriction at One of Two *EcoRI* Sites in pRW1754

PstI-linearized pRW1754 (7 nM) was incubated with or without 10 μM (CTT)₄ in pH 5.5 *MEcoRI* buffer (11 μl total volume) for 1h at 15°C. This solution was added to 40 μl of pH 6.5 buffer (100 mM Tris·acetate, pH 6.5, 100 mM NaCl, 8 mM MgCl₂) with 100 U *EcoRI* at 15°C. At various times 10 μl aliquots were

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removed, the reaction terminated by the addition of 2 μ l 0.5 M EDTA, and the samples analyzed on a 1% agarose gel.

Inhibition of Restriction at One of Six EcoRI Sites in 50 kbp of DNA

A mixture of lambda DNA (2 μ g) and BglI-linearized pRW1704 (0.8 μ g) was incubated with or without 5 μ M (CTT)₈ in pH 5.5 MEcoRI buffer (12.5 μ l total volume) for 1h at 15°C. This solution was added to 40 μ l of pH 7.0 buffer (100 mM Tris·acetate, pH 7.0, 100 mM NaCl, 8 mM MgCl₂) with 30 U EcoRI at 15°C. Aliquots of 10 μ l were removed at various times, the reaction terminated by the addition of 2 μ l 0.5 M EDTA, and the samples analyzed on a 1% agarose gel.

Inhibition of Methylation at EcoRI Sites Within or Adjacent to a Pur·Pyr Sequence

BglI-linearized pRW1701, 1703 or 1704 (10 nM) was incubated with various concentrations of (CTT)₄ in pH 5.5 MEcoRI buffer (40 μ l reaction) for 1h at 15°C. MEcoRI (120 U) and S-adenosylmethionine (0.8 mM) were added and after 1h the reaction terminated, the DNA digested, and then analyzed by gel electrophoresis, as described above. The percentage of DNA methylated was determined after densitometric analysis of the gels. In each experiment, the percentage of control methylation was calculated as the amount methylated in the presence of the oligomer to the amount methylated in the absence of the oligomer (typically 60–75%).

DMS Protection of the Pur·Pyr Region by (CTT)₄ and (CTT)₈

³²P-labeled fragments containing (GAA)₄TTC(GAA)₄ and (GAA)₉TTC were made from pRW1701 and pRW1704, respectively, after a HindIII and SstI digestion (27). The ³²P fragments (~ 1 nM) were incubated with or without 1 μ M oligo (CTT)₄ or (CTT)₈ in pH 5.5 MEcoRI buffer (total reaction volume 20 μ l) at 15°C for 1h. The DMS reaction was performed at 15°C by the addition of 4 μ l of DMS (diluted 20-fold in water). After 1min the reaction was terminated by 1 μ l 2-mercaptoethanol and ethanol precipitation. The DNA was subjected to piperidine cleavage (100 μ l 1 M piperidine, 20 min at 90°C) and aliquots were used for a 12% denaturing polyacrylamide gel followed by autoradiography. All other materials and methods were as described (10,15,27).

RESULTS AND DISCUSSION

The pur·pyr inserts in the plasmids, a map of pRW1754 (which has the same insert as pRW1701), and potential triple helices are shown in Fig.1. pRW1754 has two EcoRI sites, one is in the center of the pur·pyr insert (GAA)₄TTC(GAA)₄, and the other is the canonical pBR322 site. If the oligomer (CTT)₄ forms a triple helix with the insert (GAA)₄TTC(GAA)₄ (with the maximum number of triads), there will be a three base overlap of the triplex and the EcoRI recognition site (Fig. 1). Another (CTT)₄ molecule can occupy the 12 bp immediately to the right

Fig. 1. A list of the pur·pyr inserts is shown; pRW1701, 1703 and 1704 have EcoRI sites in the center, adjacent to the left end, and at the right end of the pur·pyr segments, respectively. Plasmid pRW1754 contains two EcoRI sites, with one in the center of a pur·pyr segment. E, EcoRI; B, BamHI; P, PstI. Approximate sizes for an EcoRI and PstI restriction digest of pRW1754 are shown in kbp. The triple strand complex between the oligonucleotide (CTT)₄ and the insert (GAA)₄TTC(GAA)₄ is shown also. The filled circles represent Hoogsteen T·A pairs, while the pluses are Hoogsteen pairs between CH⁺ and G. In addition, the potential extent of triplex overlap with the EcoRI sites of pRW1703 and 1704 are shown. The EcoRI sites are underlined.

of the EcoRI site. Similarly, a 3 base overlap of the triplex and the EcoRI site can occur with the insert of pRW1704. However, with pRW1703, triplex formation would be adjacent to, but would not overlap, the EcoRI site (Fig. 1, lower portion).

Site-specific Inhibition of Methylation by (CTT)₄

PstI-linearized pRW1754 was used to determine if the oligomer (CTT)₄ could inhibit methylation at one of two EcoRI sites in a plasmid. If both EcoRI sites in pRW1754 are completely methylated, a 4.4 kbp fragment (linear plasmid) exists after EcoRI digestion. However, if methylation at both sites were inhibited, three fragments would form (0.4, 0.75, and 3.25 kbp). Inhibition only at the pur·pyr site would create two fragments, 3.25 and 1.15 kbp. After treating the plasmid with MEcoRI in the presence of (CTT)₄ (under conditions where the triplex is stable and MEcoRI methylates the DNA completely in the absence of the oligomer, pH 5.5 and 15°C), and then digestion with EcoRI (at pH 7.5 and 37°C, where the triplex is not stable), two predominant fragments of 3.25 and 1.15 kbp were formed (Fig. 2, left panel). Thus, the oligomer specifically inhibited (> 90%) methylation only at the EcoRI site within the pur·pyr segment. To show that the sequence of the oligonucleotide was important for the inhibition, (CT)₆ was substituted for (CTT)₄. At 1 μM, (CTT)₄ gave 80–90% inhibition of methylation at the (GAA)₄TTC(GAA)₄ insert of pRW1701, whereas (CT)₆ gave no inhibition (< 10%) (data not shown).

Site-specific Inhibition of Cleavage by (CTT)₄

To test the ability of the triplex formed by (CTT)₄ to inhibit EcoRI cleavage in the pur·pyr insert of pRW1754, conditions were established where the triplex was stable and EcoRI had sufficient activity to completely digest the DNA in the absence of the oligonucleotide. The oligomer and plasmid were

preincubated at pH 5.5 and the reaction performed at pH 6.5. With no inhibition, or with inhibition at both EcoRI sites, fragments of 3.25, 0.75 and 0.4 or 4.4 kbp, respectively, would be expected. However, if the EcoRI site in the pur·pyr segment was specifically inhibited, the 3.25 and 0.4 kbp fragments would not form; instead they would become a 3.65 kbp fragment. Fig. 2, right panel, shows that (CTT)₄ inhibited EcoRI cleavage (> 90%) only at the pur·pyr site, as fragments of 0.75 and 3.65 kbp were formed. Thus, (CTT)₄ inhibits the reactions of both EcoRI and MEcoRI at a pur·pyr site without affecting their actions elsewhere on a plasmid.

The effect of pH on the inhibition of EcoRI was critical. At lower pH values, EcoRI did not give reproducibly complete cleavage of the DNA. At higher pH values, inhibition at the pur·pyr site was less, probably due to dissociation of the (CTT)₄ from the duplex during the time course of the reaction (0.5–2 h). Increasing the oligomer from 12 to 24 nucleotides increased the triplex stability at neutral pH (see below). Also, preincubation of the oligonucleotide with the duplex at pH 5.5 increased the inhibition, probably because a high percentage of the pur·pyr sites form a triplex at this pH, which then dissociates slowly when shifted to the higher pH of the reaction.

Neutral pH Triplex Formation and EcoRI Inhibition at One of Six Sites

The specificity of inhibition of EcoRI by triplex formation was tested further by mixing pRW1704 [with one EcoRI site in the pur·pyr insert (GAA)₉TTC] with lambda DNA (which has five EcoRI sites). Also, a longer oligonucleotide, (CTT)₈, was used to show that this method is applicable at physiological conditions (pH 7.0). When a mixture of lambda DNA and linear pRW1704 was digested with EcoRI in the absence of the oligomer, eight fragments were formed (six from lambda and two from

Fig. 2 (left panel) Agarose gel analysis showing that (CTT)₄ specifically inhibits methylation by MEcoRI of an EcoRI site within a pur·pyr sequence. The left lane is untreated pRW1754, the center lane is t=0 (no methylase), and the right lane is t=40 min. The other time points (20 and 60 min) gave similar results. (right panel) Agarose gel analysis showing (CTT)₄ can specifically inhibit cleavage by EcoRI at the EcoRI site in the pur·pyr segment of pRW1754. The left lane is untreated pRW1754. The center lane is t=120 min in the absence of (CTT)₄, and the right lane is t=120 min in the presence of (CTT)₄. The other time points (t=30 and 60 min) gave similar results.

Fig. 3. Agarose gel analysis showing that $(CTT)_8$ specifically inhibits EcoRI cleavage at the EcoRI site in the sequence $(GAA)_9TTC$, but does not inhibit any of the five EcoRI sites in lambda DNA. The left lane is lambda DNA and PstI-linearized pRW1704 digested with EcoRI in the absence of $(CTT)_8$. The center lane is undigested lambda DNA and linear pRW1704, and the right lane is lambda DNA and linear pRW1704 treated with EcoRI in the presence of $(CTT)_8$. Approximate sizes (in kbp) of linear and EcoRI digested lambda DNA and pRW1704 are shown on the left and right, respectively. There are two lambda DNA fragments of about 5.6 and 5.8 kbp which comigrate on the gel.

Fig. 4. Inhibition of methylation by $(CTT)_4$ at EcoRI sites within but not adjacent to $(GAA)_n$ repeats. The percentage of DNA methylated was determined for each concentration of oligomer and compared to the reaction without oligomer (defined as 100%). pRW790 (no pur·pyr), Δ ; pRW1701 (center), $\square$; pRW1703 (adjacent), $\blacksquare$; pRW1704 (right end), $\blacktriangle$. (The location of the EcoRI site with respect to the pur·pyr sequence is in parentheses).

pRW1704). In the presence of $(CTT)_8$, all five EcoRI sites of lambda still were cleaved by EcoRI, but pRW1704 remained linear as the single EcoRI site was inhibited ($\sim 90\%$) (Fig. 3).

Fig. 5. Autoradiogram showing DMS protection sites specifically within the pur·pyr regions in the presence, but not in the absence, of the oligomers. The location of the pur·pyr insert is shown to the right of each analysis. The numbers above each lane represent the number of (CTT) repeats in the oligomer used for that reaction, where a dash is no oligomer present. The hyperreactivity at the 3' end of the purine strand was seen also in DMS reactions performed on other sequences which form triplexes (Hanvey and Wells, unpublished results). In the sequence $(GAA)_9TTC$, the G at the 3' of the insert (the G of the EcoRI site) is hyperreactive and not protected. Because the pur·pyr sequence has nine GAA repeats, while the oligomer has only eight CTT repeats, a mixture of protected (when the oligomer occupies repeats 2–9) and hyperreactive (oligomer on repeats 1–8) forms probably occur.

Thus, at pH 7.0 an oligonucleotide, via triple helix formation, blocked a protein-DNA interaction at one specific site in 50 kbp of DNA.

Overlap of Triplex and EcoRI Site is Necessary for Inhibition of MEcoRI

Potentially, a triple helix could cause inhibition of a protein-DNA interaction without overlapping the target site (for example, by inducing an altered conformation in the flanking sequences). Hence, we studied the ability of (CTT)₄ to inhibit the methylation of an EcoRI site adjacent to, but not overlapping, a pur·pyr sequence (pRW1703, Fig. 1). The extent of inhibition was compared to that obtained from EcoRI sites in the center and at the end of similar pur·pyr sequences (pRW1701 and 1704). In the presence of (CTT)₄ there was a significant inhibition of methylation at the EcoRI sites within the pur·pyr inserts of both pRW1701 and 1704 (Fig. 4). The extent of inhibition was essentially identical for both plasmids, with 50% inhibition at about 0.25 μ M. However, (CTT)₄, even at 1 μ M, did not inhibit methylation at the EcoRI site adjacent to the pur·pyr sequence (pRW1703) or at an EcoRI site in a random sequence context (pRW790) (Fig. 4). When the oligomer (CTT)₈ was used, there was some inhibition of methylation in pRW1703 (~70% of control methylation at 0.75 μ M). However, the same extent of inhibition for pRW1704 required ~0.1 μ M (CTT)₈ (data not shown). Thus, these results indicate that a physical overlap of a triplex and the protein recognition site is important (but not absolutely necessary) for effective inhibition of the protein-DNA interaction.

Demonstration of a Triplex by DMS Protection

DMS protection studies were used to show that the interaction of the oligomers with the plasmid inserts is a triple helix, and not some other type of complex (e.g. D loop). Triple helix formation with Hoogsteen pairing in the major groove blocks the N-7 position of G, which is the site methylated by DMS; hence, DMS is a good diagnostic probe for triplexes. This method was used recently to analyze a proposed tetraplex formed by G-rich oligonucleotides (28).

Fig. 5 shows an autoradiogram of a DMS protection experiment in which (CTT)₄ and (CTT)₈ specifically protect Gs in the pur·pyr inserts (GAA)₄TTC(GAA)₄ and (GAA)₉TTC. The Gs in the flanking sequences are not protected. Also, a similar protection at the insert of pRW1703 was observed with both oligomers (data not shown). These results, combined with gel shift experiments, DNaseI protection experiments, and the increased stability of the interactions at lower pH (Hanvey and Wells, unpublished results), strongly indicate that these oligomers are forming an intermolecular triplex with the pur·pyr sequences.

IMPLICATIONS

Triplex formation is an effective mechanism for blocking the actions of DNA binding proteins. Herein, we showed that oligomers enhanced restriction and modification specificity; a triplex blocked the digestion by EcoRI at one of six sites in 50 kbp of DNA. This enhancement of specificity may be even greater in more complex systems, and is limited only by the capacity of an oligomer to form a stable triplex (which is a function of the number of new hydrogen bonds between the oligomer and the DNA target site). The specific recognition of a DNA site by a DNA binding protein involves the formation of new hydrogen bonds, hydrophobic interactions, etc. Likewise, the stabilization of an intermolecular triplex involves similar forces, with two new hydrogen bonds formed per base in the oligomer. Thus, at certain sequences, a triplex may be an effective

alternative to designing custom enzymes for specific interactions. Hence, triple helix formation may be useful as an inhibitor of specific DNA-protein interactions, and thus may be an effective strategy for modulating gene expression *in vitro* and *in vivo* (chemotherapy).

During the final preparation of this manuscript, a paper (29) appeared which reports similar findings.

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