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Protection of DNA sequences by triplex-bridge formation

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ABSTRACT

We have demonstrated that the DNA sequence between two triplex-forming polypurine·polypyrimidine (Pu·Py) tracts was protected from DNA modifying enzymes upon formation of triplex DNA structures with an oligodeoxyribonucleotide in which two triplex-forming Pu or Py tracts were placed at the termini (triplex-bridge formation). In model experiments, when two triplex structures were formed between double-stranded DNA with the sequence $(AG)_{17}$–$(N)_{18}$–$(T)_{34}$, and an oligodeoxyribonucleotide, $(T)_{34}$–$(N)_{18}$–$(GA)_{17}$, not only the Pu·Py tracts but also the 18 bp non-Pu·Py sequence in the duplex DNA between the tracts was protected from restriction enzymes, *Hpa*II methylase and DNase I. This protection occurred only when both of the Pu·Py tracts were involved as triplexes. The length of the tracts could be as short as 21 bp, while the difference in length between the non-Pu·Py sequences on the duplex and the oligodeoxyribonucleotide should be within 10 nucleotides. The efficiency of protection was enhanced in the presence of a cationic detergent, cetyltrimethylammonium bromide, during triplex formation. Protection was also observed with another type of the triplex bridge formed between $(G)_{34}$ and $(T)_{34}$ tracts with an oligodeoxyribonucleotide, $(T)_{34}$–$(N)_{20}$–$(G)_{34}$. These findings suggest that the protection of specific DNA sequences from enzymes by triplex-bridge formation can be applied to any DNA sequence by placing it between two triplex-forming sequences.

INTRODUCTION

Protection of DNA sequences from enzymes or association with specific proteins has been considered as a potentially useful means for controlling gene expression (1–3), site-directed cleavage or protection of chromosomal DNA (4–8), and targeted mutagenesis (9). Once well established, this approach could open a new avenue for therapeutics against a number of diseases (1,10) and also for other applications. Using triplex DNA formed with polypurine·polypyrimidine (Pu·Py) sequences in the presence of the third strand, Dervan and co-workers demonstrated that part of the protein recognition DNA sequence which overlaps the Pu·Py sequence was protected from restriction or modification enzymes or a transcription factor (4–6). The technique was further modified by using an alternate-strand triplex (11,12) or a crosslinked triplex (3,13) to increase the variety of Pu·Py sequences to be protected. Another approach using synthetic linkers to increase base specificity and stability of triplex DNA was also developed (14,15). In a quite different approach, Koob and Szybalski showed that the recognition sequence for *lac* repressor was protected from methylation in the presence of the repressor and, after removing the protein, the sequence remained susceptible to methylation-sensitive restriction enzymes, thus enabling the creation of a single specific restriction-sensitive site in the complex genomic DNA (7). However, the requirement of specific DNA sequences for triplex formation or for repressor binding has limited the application of these methods for wider and general use. Attempts to form triplexes at any DNA sequence have been explored by utilizing RecA-mediated triplex DNA formation (8). This approach, however, is still a matter of challenge particularly for its application *in vivo*.

The triplex DNA formed with Pu·Py sequences is classified into three groups. The first and the second groups are Py·Pu·Py and Pu·Pu·Py types where one of the bases in Watson–Crick base-pairs (Pu·Py) is used again as the third base in the triad. T·A·T and C^+·G·C triplets belong to the first group, and G·G·C and A·A·T to the second (16–18). The third group, consisting of G·T·A (19,20), T·C·G (21) and M·G·C (M= N^6-methyl-8-oxo-2′-deoxyadenosine, in place of 5-methylcytosine, ref. 22), has all different bases in the triad. In either case, the same Pu or Py bases in the triad are aligned in the antiparallel orientation, which is the major difference from the triplex structure formed as a recombination intermediate. The triplex DNA is stabilized by environmental factors such as pH, temperature and ionic strength, and by the presence of cofactors such as metal ions (18), basic materials (the present study), or specific proteins (23).

In this paper, we report that DNA sequences between two triplex-forming Pu·Py tracts are protected from several classes of DNA modifying enzymes when triplex structures are formed with an oligodeoxyribonucleotide (hereafter abbreviated as oligonucleotide) in which two triplex-forming tracts are placed at the termini (triplex-bridge formation). This may open a way for the protection of any DNA sequence from modification or

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association with specific proteins. Possible applications of the procedure are also discussed.

MATERIALS AND METHODS

Materials

Oligonucleotides were synthesized by a Millipore Cyclone DNA synthesizer and purified with Milligen Oligo-Pak columns. Cetyltrimethylammonium bromide (CTAB) was purchased from Sigma. Restriction enzymes, *Hpa*II methylase and DNase I were purchased from New England Biolabs (USA) or Takara (Kyoto).

Plasmid construction

The plasmid pGATA1 was constructed by inserting an $(AG)_{17}$ sequence between *Eco*RI and *Sac*I sites, and a $(T)_{34}$ sequence between *Bam*HI and *Hin*dIII sites of the pUC19 vector. Likewise, pGCTA1 and pGAAT4 were constructed by inserting a $(G)_{34}$ or $(AG)_{17}$ sequence between *Eco*RI and *Sac*I sites and a $(T)_{34}$ or $(A)_{34}$ sequence between *Bam*HI and *Hin*dIII sites, respectively.

Triplex formation and protection assay

Approximately 0.5 μg each of the plasmid DNA or M13mp18 (a control) was incubated with oligonucleotides (5 μM or indicated concentrations) in 20 μl of triplex-forming buffer consisting of 10 mM Tris–HCl (pH 7.9), 50 mM NaCl, 10 mM $MgCl_2$ and 1 mM dithiothreitol for 30 min at 37°C (triplex DNA formation). In most of the experiments (see below), the DNA were linearized by *Alw*NI before use.

For restriction enzyme treatment, the mixtures were incubated at room temperature (for *Hin*dIII) or at 14°C (for other restriction enzymes) using the following amount of enzyme (units according to the supplier's instructions): *Eco*RI, 20 U; *Sac*I, 10 U; *Kpn*I, 12 U; *Sma*I, 24 U; *Bam*HI, 2 U and *Hin*dIII, 20 U. The amounts of enzymes used for this assay were the amounts just enough to digest the control M13mp18 DNA completely at indicated temperatures. The reaction was terminated by addition of SDS (0.1%) and the samples were electrophoresed on 0.65% agarose gels and stained with ethidium bromide.

For *Hpa*II methylase reaction, after triplex formation, the reaction mixture was supplemented with 80 μM of S-adenosylmethionine followed by incubation with 4 U of *Hpa*II methylase at room temperature. The mixture was treated with phenol and ethanol, and the DNA was subjected to *Sma*I treatment at 37°C overnight in the presence of 5 μM $(dA)_{34}$. Presence of excess $(dA)_{34}$ abolishes the triplex structure at one end and the *Sma*I site becomes susceptible to digestion.

DNase I footprinting

Substrate DNAs (sense and antisense strands) were prepared by PCR with the template pGATA1 using 5′-end labeled sequencing primers #1233 (positions 500–477 of pUC19, New England Biolabs) and #1224 (352–375). Approximately 0.5 ng of 190 bp PCR product was incubated with the oligonucleotide (TNGA) in 20 mM Tris–HCl (pH 7.5), 10 mM $MgCl_2$ and 100 μM CTAB in 40 μl reaction mixture at 37°C for 30 min. DNase I treatment was then followed by addition of 1 μl of 0.1 M $CaCl_2$ and 60 μl of 10 mM Tris–HCl (pH 7.5), 2.5 mM $CaCl_2$, 10 mM $MgCl_2$, and then 2 μl of DNase I (0.014 U/μl) and incubation at room temperature for 2 min. The reaction was terminated by addition of 100 μl of a solution containing 20 mM EDTA, 0.2% SDS, 0.3 M NaCl and 50 ng/μl yeast tRNA, and the DNA was extracted with phenol, purified with ethanol and, after incubation at 94°C for 90 s, electrophoresed on a 6% polyacrylamide–7 M urea gel. The gel was autoradiographed using Kodak X-Omat film.

RESULTS

Figure 1A depicts the structure of the DNA protected by triplex-bridge formation. Plasmid pGATA1, constructed from the pUC19 vector, has an insert consisting of two triplex-forming 34 bp long Pu·Py tracts, $(AG)_{17}$ and $(T)_{34}$, plus an 18 bp non-Pu·Py sequence sandwiched between them (IBS, in between sequence). The $(AG)_{17}$ tract is flanked by *Eco*RI and *Sac*I sites, and the $(T)_{34}$ by *Bam*HI and *Hin*dIII. *Eco*RI, *Bam*HI and *Hin*dIII sites are located just next to the Pu·Py tracts, while *Sac*I overlaps $(AG)_{17}$ tract. The non-Pu·Py IBS contained recognition sites for *Kpn*I, *Sma*I and *Bam*HI. For triplex formation, pGATA1 was incubated in the presence of Mg^{2+} with an oligonucleotide, TNGA. The oligonucleotide had two triplex-forming Pu or Py sequences, $(GA)_{17}$ and $(T)_{34}$, for simultaneous recognition of $(A)_{34}$ and $(AG)_{17}$, respectively, in the duplex plasmid DNA (pGATA1) at the termini, spanning an 18 bp non-Pu·Py sequence (N) between them which had no homology with its counterpart IBS in the duplex plasmid DNA. It should be noted that the duplex $(AG)_{17}$ was recognized by the $(GA)_{17}$ motif on TNGA while the $(A)_{34}$ strand on the duplex involved parallel binding, therefore antiparallel for the $(T)_{34}$ strand, to the $(T)_{34}$ motif.

To determine whether triplex formation at the two Pu·Py tracts protects not only the Pu·Py sequences but also the IBS from DNA modifying enzymes, the DNA in the mixture after triplex formation was incubated with several restriction enzymes (*Eco*RI, *Sac*I, *Kpn*I, *Sma*I, *Bam*HI and *Hin*dIII). M13mp18, which contains no triplex-forming tracts but has sites for these restriction enzymes was also included in the mixture as a control. The results after gel-electrophoresis of the DNA are shown in Figure 1B. As expected, a substantial proportion of *Sac*I site which overlaps the triplex-forming region in pGATA1 was protected when the plasmid was preincubated with the triplex-forming oligonucleotide TNGA. Restriction sites (*Kpn*I, *Sma*I and *Bam*HI) in the IBS were also protected. Neither the *Eco*RI site, which was located outside the region of the triplex formation, nor the restriction sites on M13mp18 were protected. *Hin*dIII site, on the other hand, showed a substantial protection even though the site is located outside the triplex-forming region (see Discussion). Protection of the IBS was also observed when the original covalently closed circular form of pGATA1 was employed instead of the linearized DNA (Fig. 1C). These findings strongly suggest that triplex formation at the terminal two Pu·Py tracts of the duplex protected the IBS from the restriction enzymes, probably by hindering the access of the enzymes to the sequence by the presence of the N region of the oligonucleotide. The degree of protection was apparently dependent upon the concentration of the oligonucleotide during triplex formation, although the results showed a plateau of the reaction over 5 μM. Figure 1D shows the protection of the *Sma*I site in the IBS and *Sac*I site overlapping one of the Pu·Py tracts of the duplex as a function of the oligonucleotide concentration. Protection of the IBS *Sma*I site required higher concentrations of

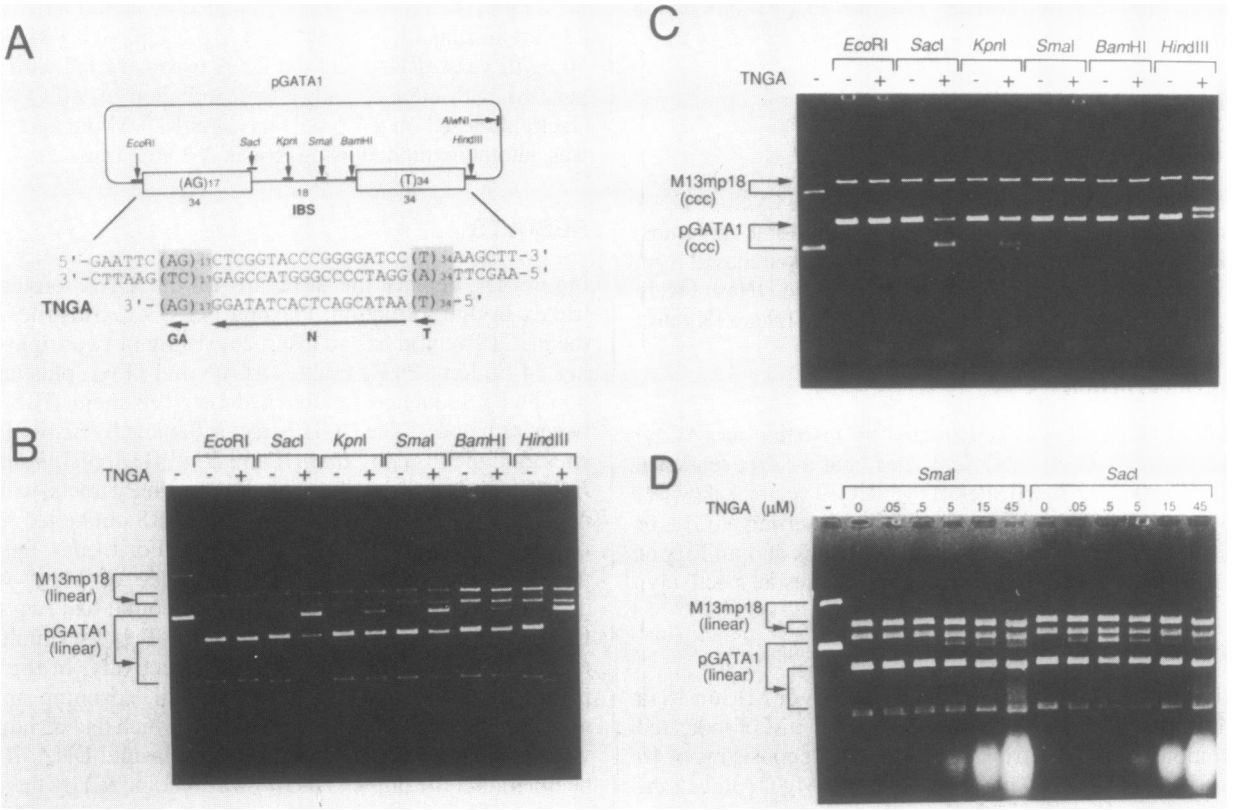

Figure 1. Protection of pGATA1 sequence by triplex bridge formation. (**A**) The structure of pGATA1 is shown with restriction sites and DNA sequences in the vicinity of the two Pu·Py tracts. TNGA consists of 5′-$(T)_{34}$(T), AATACGACTCACTATAGG (N) and $(GA)_{17}$(GA). The regions where triplex structures are formed are boxed. (**B**) The mixture (20 μl) of linearized pGATA1 (0.5 μg) and M13mp18 (0.5 μg) was incubated with TNGA (5 μM) in triplex-forming buffer containing Mg^{2+} at 37°C for 30 min as described in Materials and Methods. After the reaction, a portion of the mixture was treated with *Eco*RI, *Sac*I, *Kpn*I, *Sma*I, *Bam*HI and *Hin*dIII, electrophoresed on agarose gel and visualized by ethidium bromide staining. (**C**) The same as (B) but the original covalently closed circular (ccc) form of pGATA1 and M13mp18 DNA (without *Alw*NI treatment) were employed. (**D**) The same as (B) but the mixture of pGATA1 and M13mp18 was incubated with various concentrations of TNGA, and subsequently treated with *Sma*I or *Sac*I. The concentrations (μM) of TNGA are also shown. In (B) to (D), positions of the original pGATA1 and M13mp18 as well as their restriction products are indicated on the left-side of the figure in which the products are shown by arrows. For details, see Materials and Methods.

the oligonucleotide than that in the triplex-forming tracts. The same tendency was observed with the *Kpn*I site in the IBS and *Hin*dIII site (data not shown). This might be explained by the fact that the access of restriction enzymes to their sites on the duplex were more limited at higher TNGA concentration where the exchange rate of the oligonucleotides on the duplex was higher.

To examine the specificity of the protection by oligonucleotides, pGATA1 was incubated with a series of oligonucleotides in which part of the TNGA sequence was deleted or altered (TNGA2, GA, N, T, NGA, TN, D1, D2 and D3 summarized in Figure 2A), and the mixtures were treated with *Sma*I, *Sac*I or *Hin*dIII. As shown in Figure 2B, among the oligonucleotides with a deletion, protection was observed only with TNGA2 which has shorter (about half-length) triplex-forming Pu and Py tracts than TNGA. The extent of the protection was less efficient with TNGA2 than with TNGA. All other sequences including N which has no triplex-forming tract, and NGA and TN, each with only one triplex-forming tract, failed to protect the IBS in the duplex. On the other hand, protection was reduced when oligonucleotides with a shorter IBS (13, 8 or none for D1, D2 or D3, respectively) were used (Fig. 2C). In contrast, the protection of *Sac*I site which overlaps the Pu·Py tracts of the duplex and *Hin*dIII site was not affected. Protection of the *Sma*I site by the oligonucleotide with a shorter IBS, especially for D1 which showed a control level protection, could be achieved by supplying additional nucleotides from the Pu or Py tracts on TNGA to adjust the length of the IBS. Further shortening the IBS reduced the rate of protection probably because deformation of the potential triplex structure at the IBS region started to occur. These findings suggest that (i) triplex formation at both Pu·Py tracts is required for protection, (ii) the degree of protection is a function of the length of the triplex regions and (iii) the length of the IBS should be equal or close to that of the N region of the oligonucleotide, further supporting the view that the protection of the sequences between the Pu·Py tracts of the duplex is due to triplex-bridge formation with the two Pu·Py tracts. To examine whether the two triplex DNAs behave independently or in a coordinate manner, we mixed 100 nM of ^{32}P-labeled T or GA with various amounts of cold TNGA (Fig. 2D, lanes 3–7) or TN (lanes 8–12) (for T) or TNGA (Fig. 2E, lanes 3–7) or NGA (lanes 8–12) (for GA) to see the competition at $(T)_{34}$ or $(AG)_{17}$ tracts (Figs 2D and E). As shown in the figure, triplex formation at the other end affected the binding of the tract where the two types of the oligonucleotides were competing. The difference of the apparent dissociation constants for TNGA to TN

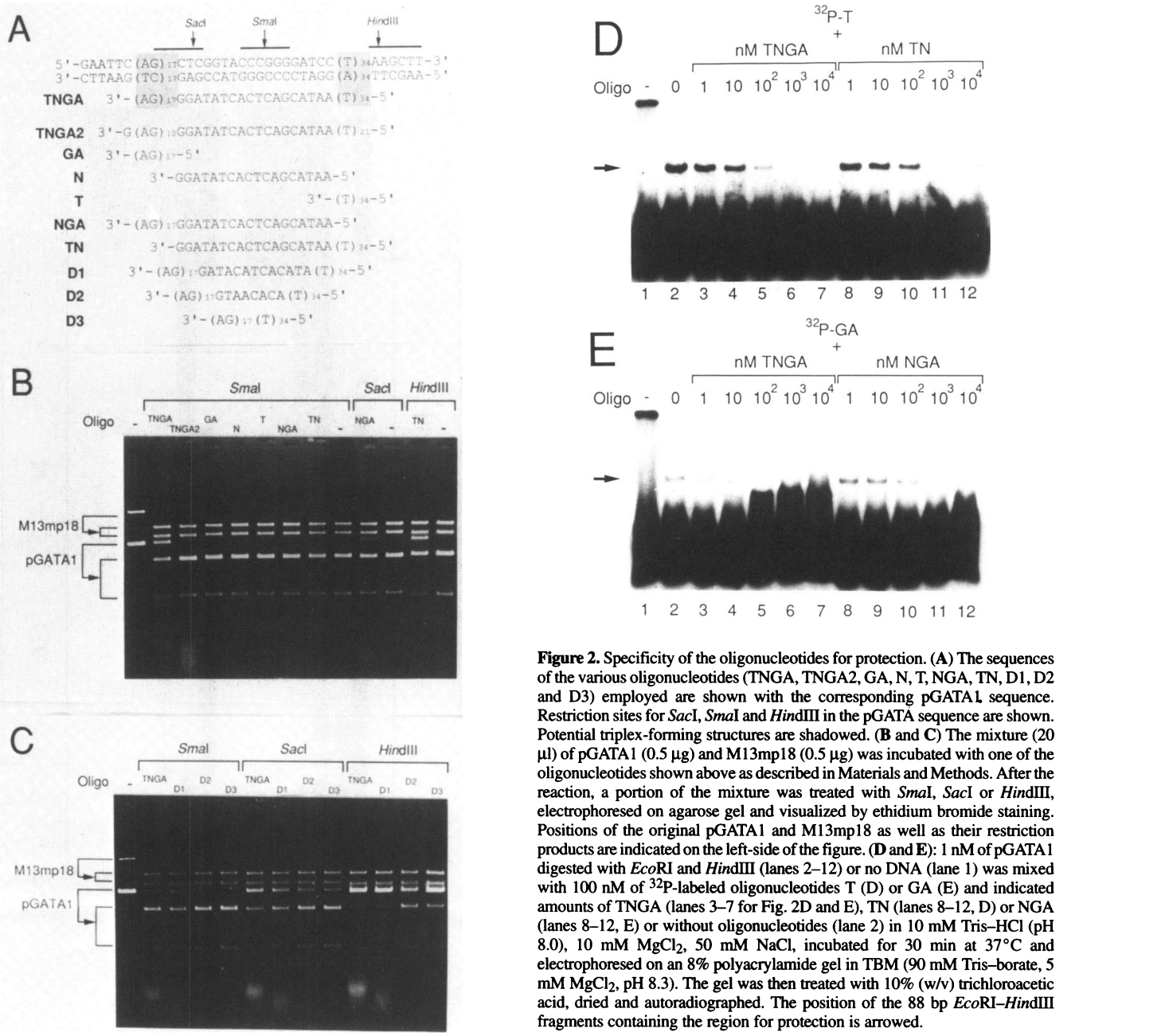

Figure 2. Specificity of the oligonucleotides for protection. (**A**) The sequences of the various oligonucleotides (TNGA, TNGA2, GA, N, T, NGA, TN, D1, D2 and D3) employed are shown with the corresponding pGATA1 sequence. Restriction sites for *Sac*I, *Sma*I and *Hin*dIII in the pGATA sequence are shown. Potential triplex-forming structures are shadowed. (**B** and **C**) The mixture (20 µl) of pGATA1 (0.5 µg) and M13mp18 (0.5 µg) was incubated with one of the oligonucleotides shown above as described in Materials and Methods. After the reaction, a portion of the mixture was treated with *Sma*I, *Sac*I or *Hin*dIII, electrophoresed on agarose gel and visualized by ethidium bromide staining. Positions of the original pGATA1 and M13mp18 as well as their restriction products are indicated on the left-side of the figure. (**D** and **E**): 1 nM of pGATA1 digested with *Eco*RI and *Hin*dIII (lanes 2–12) or no DNA (lane 1) was mixed with 100 nM of ^{32}P-labeled oligonucleotides T (D) or GA (E) and indicated amounts of TNGA (lanes 3–7 for Fig. 2D and E), TN (lanes 8–12, D) or NGA (lanes 8–12, E) or without oligonucleotides (lane 2) in 10 mM Tris–HCl (pH 8.0), 10 mM $MgCl_2$, 50 mM NaCl, incubated for 30 min at 37°C and electrophoresed on an 8% polyacrylamide gel in TBM (90 mM Tris–borate, 5 mM $MgCl_2$, pH 8.3). The gel was then treated with 10% (w/v) trichloroacetic acid, dried and autoradiographed. The position of the 88 bp *Eco*RI–*Hin*dIII fragments containing the region for protection is arrowed.

(and T) or TNGA to NGA (and GA) was about a factor of ten, indicating that the presence of the two triplex-forming tracts act coordinately.

Protection of DNA sequences from DNA modifying enzymes through possible triplex-bridge formation was extended by examining the protection of the IBS against a DNA methylase. After incubation with TNGA, pGATA1 was treated with *Hpa*II methylase, which methylates all CCGG sites including the *Sma*I site (CCCGGG), and then with a restriction enzyme, *Sma*I. *Sma*I is unable to cleave the sequence when the third cytosine residue is methylated. As shown in Figure 3, the CCCGGG sequence in pGATA1 located between the Pu·Py tracts was still partially sensitive to *Sma*I even after *Hpa*II methylase treatment while the same sequence in M13mp18 was completely resistant to *Sma*I digestion. This suggests that triplex formation through the Pu·Py tracts prevented the methylation of the IBS, as observed with restriction enzymes.

We explored various conditions in which the sequences between the triplex-forming Pu·Py tracts were protected to greater degrees from enzymes. Among them, the presence of cetyltrimethylammonium bromide (CTAB), a cationic detergent with a DNA–DNA reassociation-stimulating activity, in the triplex-forming mixture was found to be effective in increasing the degree of protection (24). As shown in Figure 4, the presence of CTAB (100 µM) in the preincubation mixture rendered pGATA1 almost completely resistant to *Sma*I digestion. This is probably derived from a triplex-stabilizing effect through its amphipathic nature (see Discussion).

We also investigated whether protection by triplex-bridge formation was observed throughout the IBS by DNase I

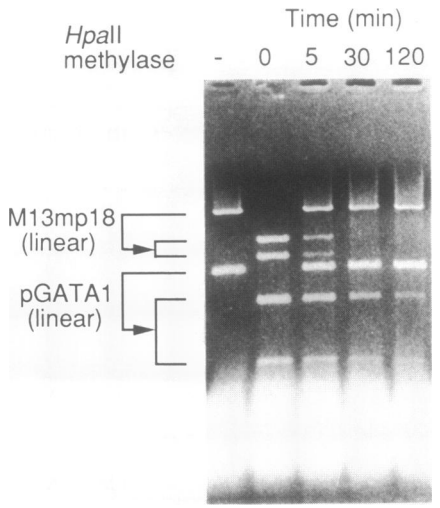

Figure 3. Protection of pGATA1 sequence from *Hpa*II methylase. The mixture (20 µl) of pGATA1 (0.5 µg) and M13mp18 (0.5 µg) was incubated with TNGA (5 µM) for triplex formation as described in Materials and Methods. The mixture was then incubated with *Hpa*II methylase (4 U) and 5-adenosylmethionine (80 µM) at room temperature. At the time indicated in the figure, a portion of the sample was withdrawn. DNA was purified from each sample, subjected to *Sma*I treatment, electrophoresed and visualized by ethidium bromide staining.

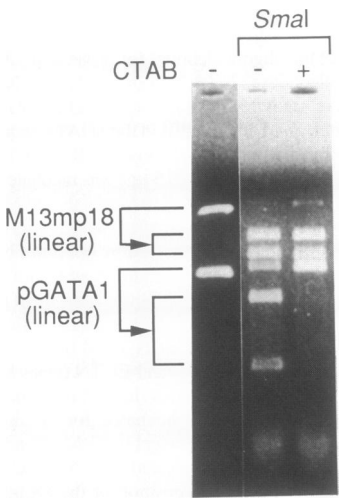

Figure 4. Effects of CTAB on triplex bridge formation. The mixture (20 µl) of pGATA1 (0.5 µg) and M13mp18 (0.5 µg) was incubated with TNGA (5 µM) in triplex-forming buffer in the presence (100 µM) or absence of CTAB. The samples were then incubated with *Sma*I, electrophoresed and stained.

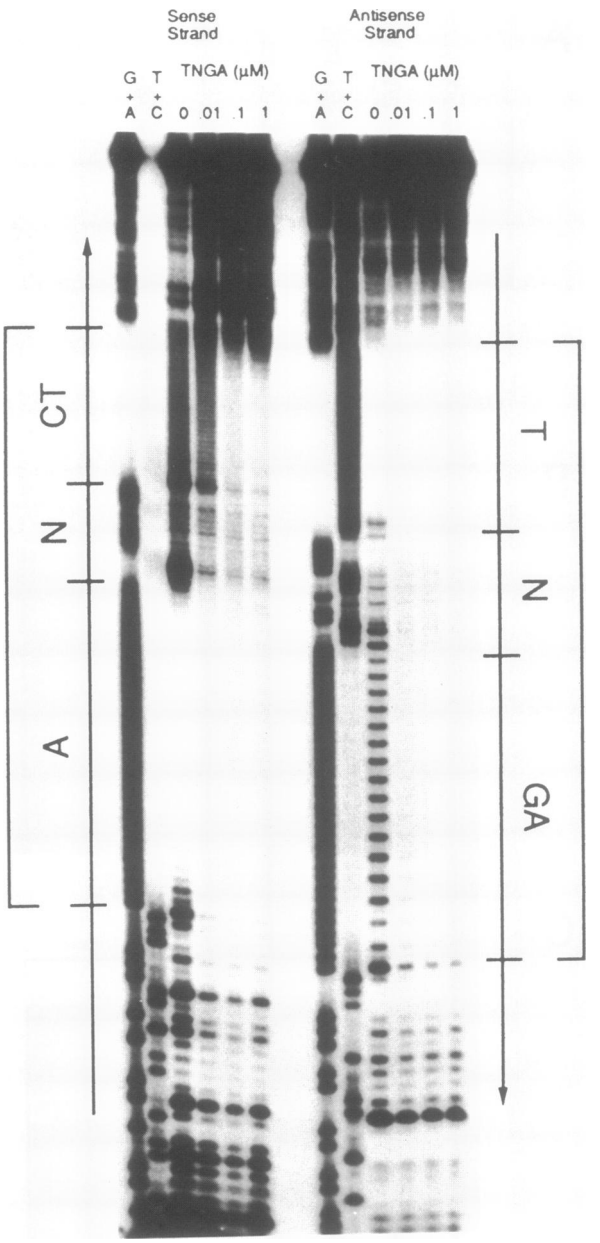

Figure 5. Protection of pGATA1 sequence from DNase I revealed by foot-printing assay. PCR products (0.5 ng) of a part (190 bp) of pGATA1 which were labeled on either (sense and antisense) strand were incubated with various concentrations of TNGA (indicated in the figure) for triplex formation in the presence of CTAB (100 µM). DNase I foot-printing was then performed.

footprinting experiments. From pGATA1, a 190 bp sequence, which included the region of triplex-bridge formation, was amplified by PCR and incubated with different concentrations of TNGA in the presence of CTAB. The mixtures were then subjected to DNase I treatment. As shown in Figure 5, $(GA)_{17}$ tract and the IBS were protected from DNase I even after incubation with TNGA at concentrations as low as 0.01 µM, and the protection was observed throughout the IBS (binding of TNGA at $(T)_{34}$ tract was not demonstrated by this assay, although it has been shown by the binding assay, see Figure 2D).

Triplex-bridge formation and protection of the IBS were also investigated with different combinations and orientations of Pu·Py tracts. We first subjected another plasmid (pGCTA1), which is similar to pGATA1 but in which the triplex forming tracts $(AG)_{17}$ and $(T)_{34}$ in pGATA1 were replaced by $(G)_{34}$ and $(T)_{34}$, respectively, to restriction protection experiments after triplex-bridge formation. As shown in Figure 6A, the 20 bp sequence between $(G)_{34}$ and $(T)_{34}$ was protected when an oligonucleotide, TNG, consisting of a $(T)_{34}$–$(N)_{20}$–$(G)_{34}$ sequence was present in the preincubation mixture. The protection was as effective as that through $(AG)_{17}$ and $(T)_{34}$ tracts in pGATA1. On the other hand, pGAAT4, which includes $(T)_{34}$ and $(GA)_{17}$ tracts separated by the same 18 bp sequence as in

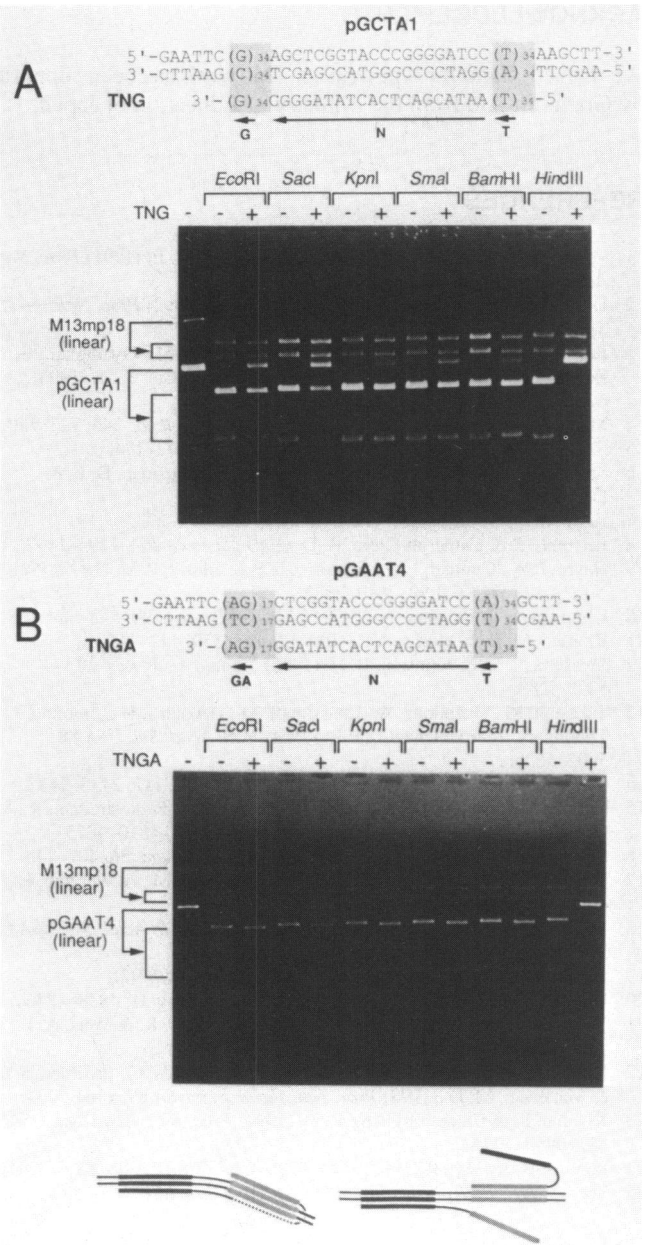

Figure 6. Effects of an alternative combination (A) or orientation (B) of Pu·Py tracts on protection by triplex-bridge formation. (**A**) A triplex-bridge was formed with the linearized plasmid pGCTA1, which has $(G)_{34}$ and $(T)_{34}$ tracts separated by a 20 bp non-Pu·Py sequence, in the presence of the oligonucleotide TNG. (**B**) The experiments were performed as in Figure 1B but with the plasmid (pGAAT4) containing a $(T)_{34}$ tract in the opposite orientation to that in pGATA1. Two possible structures formed between TNGA and pGAAT4 are shown at the lower part.

pGATA1, but whose $(T)_{34}$ tract was placed in the opposite orientation as that in pGATA1, showed no protection (Fig. 6B), indicating that the proper alignment of DNA strands, which is required for triplex DNA formation, is also necessary for the protection. Two possible structures formed between TNGA and pGAAT4 are shown at the bottom of Figure 6B (see Discussion).

DISCUSSION

The present study showed that not only the triplex-forming tracts such as Pu·Py tracts but also intervening non-Pu·Py sequences placed between them were also protected from at least three different types of DNA modifying enzymes (restriction enzymes, a methylase and DNase I) upon formation of a triplex-bridge structure with oligonucleotides having triplex-forming sequences at their termini. As described above (see Fig. 1A), the double-stranded DNA sequences (IBSs) sandwiched between the Pu·Py tracts in the plasmid pGATA1 and the single-stranded oligonucleotide TNGA, had neither homology between them nor a capacity to form triplex DNA, yet the sandwiched double-stranded DNA sequences (IBS) were protected from the enzymes as if they were part of the triplex. Thus the position of the oligonucleotide sequence between the two Pu·Py tracts prevented the access of the enzymes to their recognition sites in the intervening double-stranded DNA sequences. We also observed that linear DNA was protected as efficiently as supercoiled circular DNA (Fig. 1B and C), indicating that no steric constraint on double-stranded DNA is required for protection. Interestingly, we observed different degrees of protection at *Eco*RI and *Hin*dIII sites, although they are equally located next to the triplex-forming tracts (Fig. 1B and C). This apparent differences in the accessibility of *Eco*RI and *Hin*dIII could be explained either by the differences in the stability of triplex DNA at each tract or the differences of the molecular mass of these enzymes.

Since the sequence sandwiched by $(G)_{34}$ and $(T)_{34}$ was also protected as was that between $(AG)_{17}$ and $(T)_{34}$, it is likely that any combination of $(AG)_i$, $(T)_j$ and $(G)_k$, all of which form triplex structures in the presence of Mg^{2+}, can be used to protect the IBS. At present, we do not know exactly how many triplex-forming base pairs are required at the tracts to protect the IBS but Pu·Py tracts as short as 21 bp long were still able to protect the IBS (Fig. 2B).

The region protected by the triplex bridge was 18 or 20 bp long, which is long enough for binding of most sequence-specific binding proteins. Since 18–20 bp is equivalent to approximately two turns of the double helix and protection was observed throughout the sequence (Fig. 5), the third oligonucleotide strand may be wrapped tightly in the major groove of the double helix forming a rigid complex between the duplex and oligonucleotide strand where otherwise no stable hydrogen bonds can be formed. Joining the two Pu·Py tracts over a 20 nucleotide long duplex DNA by a straight line requires a string ~68 Å long (33.8 Å/pitch), while an extended single-stranded 20mer oligonucleotide is 133 Å in length (7.0 Å/phosphate–phosphate distance for $C_{2'}$-*endo* conformation) (25). Therefore, bridging over the 20 bp duplex DNA sequence with a 20mer oligonucleotide without wrapping around the duplex would probably cause a deflection, creating enough space to allow access to proteins. This was probably the case for pGAAT4 (Fig. 6B), where no protection of the duplex was observed when the orientation of one of the Pu·Py tracts was reversed. In the combination of pGAAT4 and the oligonucleotide TNGA, the distance between the two Pu·Py tracts did not match between the plasmid (52 bp) and the oligonucleotide (18 nucleotides long). Two possible structures are shown in Figure 6B (lower part), where two triplex-forming tracts were connected by a single oligonucleotide with a severe deformation in the middle (lower left) or they behaved independently (lower right). From the experiments with shorter IBSs (Fig. 2C), the deformation of

the potentially triplex-forming region started to occur when the difference between the lengths of the IBS and the N region of the oligonucleotides reached 5–10 nucleotides. Although more data should be needed to unravel the precise mechanism and the fine structure that stabilizes the complex formation at the IBS, we tentatively speculate that the complex having additional hydrogen bonds between the duplex and the third strand would have a lower energy level than the structure with two triplexes at both ends and a free conformation in the middle.

Among various conditions and reagents tested, the protection was significantly enhanced when the cationic detergent CTAB was present during triplex formation (Fig. 4). The enhancement was likely due to the cationic charge, which should neutralize the repulsion force between the phosphate groups in the duplex DNA and oligonucleotide, and the presence of the hydrophobic region, which should increase and stabilize the interaction between the detergents (24). CTAB could be replaced by cationic proteins, which may be critical in applying the procedure for *in vivo* protection where CTAB cannot be used.

Conformational protection or modification of specific DNA sequences in huge and complex mammalian genomes by the association of oligonucleotides in general is considered to be one of the important concepts in regulating specific gene expression and also to have potential as therapeutic purposes (1,10). Although triplex DNA, targeted to the region critical for specific gene expression, has been considered very promising, particularly where other methods such as antisense technology cannot be applied, the triplex approach has had one major drawback; DNA sequences to be targeted are limited to those capable of forming triplex DNA structures. The protection of DNA sequences placed between triplex-forming sequences (triplex bridge) presented here may provide a solution to the problem inherent in triplex technology for wider applications. Kessler *et al.* also explored triplex DNA formation for a wider use by increasing the total length of the triplex-forming region with a bidentate structure connected by a synthetic linker (15). In this case, the stability of the triplex as a whole was increased substantially by a straight line with a synthetic linker as an IBS. It should be noted that triplex forming conditions, including Mg^{2+} ion concentrations, employed here are similar to the physiological environment, which is critical for the formation of stable triplex structures *in vivo*.

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