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Factors influencing the extent and selectivity of alkylation within triplexes by reactive G/A motif oligonucleotides

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ABSTRACT

G/A motif triplex-forming oligonucleotides (TFOs) complementary to a 21 base pair homopurine/homopyrimidine run were conjugated at one or both ends to chlorambucil. These TFOs were incubated with several synthetic duplexes containing the targeted homopurine run flanked by different sequences. The extent of mono and interstrand cross-linking was compared with the level of binding at equilibrium. Covalent modification took place within a triple-stranded complex and usually occurred at guanine residues in the flanking double-stranded DNA. The efficiency of alkylation was dependent upon the sequence of the flanking duplex, the solution conditions, and the rate of triplex formation relative to the rate of chlorambucil reaction. Self-association of the TFOs as parallel duplexes was demonstrated and this did not interfere with triple strand formation. With an optimal target, cross-linking of the triplex was very efficient when incubation was carried in a physiological buffer supplemented with the triplex selective intercalator coralyne.

INTRODUCTION

Triplex-forming oligonucleotides (TFOs) bind sequence specifically to homopurine runs in double-stranded DNA and represent a promising strategy for regulating gene expression (1–6). Three recognition motifs have been described based on the use of C^+/T, G/A or G/T oligonucleotides (ODNs). *In vitro* studies have demonstrated that TFOs can interfere with normal functioning of genes or viral genomes. Homopurine runs are frequently found in promoter regions (7) and triple strand formation at such sites can prevent transcription initiation (8–11). Triplexes can also arrest transcription elongation provided that the complex is extremely stable (12,13) or the TFO is covalently linked to the non-coding DNA strand (14,15).

Many triplexes formed by unmodified TFOs have low to moderate stability under physiological conditions and this represents a serious obstacle to their practical use. Neutral pH impairs C^+/T motif triplexes by suppressing protonation of third strand cytidine residues (16). Potassium ion destabilizes triplexes in mixed-valence salt solutions (17,18) and additionally promotes G-tetrad formation by G/T motif TFOs (19). With association constants often on the order of 10^6–10^8 M^{-1}, the free energy of triplex formation is similar to that observed for interaction of minor groove binding agents with poly(dA–dT) (20). Yet a minor groove binder such as netropsin only interacts with 4–5 base pairs of duplex (20) whereas a TFO usually interacts with homopurine runs 10–25 bases in length.

Modified TFOs which bind to DNA with high affinity and then cross-link to it would eliminate concerns about complex stability. More specifically, covalent linkage of the complex would prevent spontaneous dissociation or enzymatic displacement of the TFO. While considerable progress has been achieved using modified backbones, base analogs and appended conjugate groups to improve physical triplex stability (1–6), less effort has been expended on cross-linking technology. Psoralen derivatives have frequently been conjugated to TFOs (21). Upon irradiation of the triplex with near UV light, these agents photo-react with thymidines in the flanking duplex. Unfortunately, use of light activated reagents *in vivo* is problematic. Several chemically reactive agents have been appended to TFOs and shown to cross-link within a pyrimidine motif triplex. These include 2-chloroethylamine (22), aziridine (23), *N*-bromoacetyl (24), binuclear platinum (25) and transplatin (26) reagents.

We have been studying TFOs conjugated to chlorambucil (27). This nitrogen mustard is used in cancer chemotherapy. When linked to the end of a pyrimidine motif TFO by a hexylamine linker, chlorambucil reacts with N-7 of guanines in DNA flanking the triplex. In this investigation, we have employed four synthetic duplexes that share a common A-rich homopurine run flanked by different sequences. Triplex formation by several G/A-containing TFOs has been compared with levels of alkylation elicited by the same TFOs when conjugated to chlorambucil. The data show that strategies which enhance triplex stability, such as an acridine

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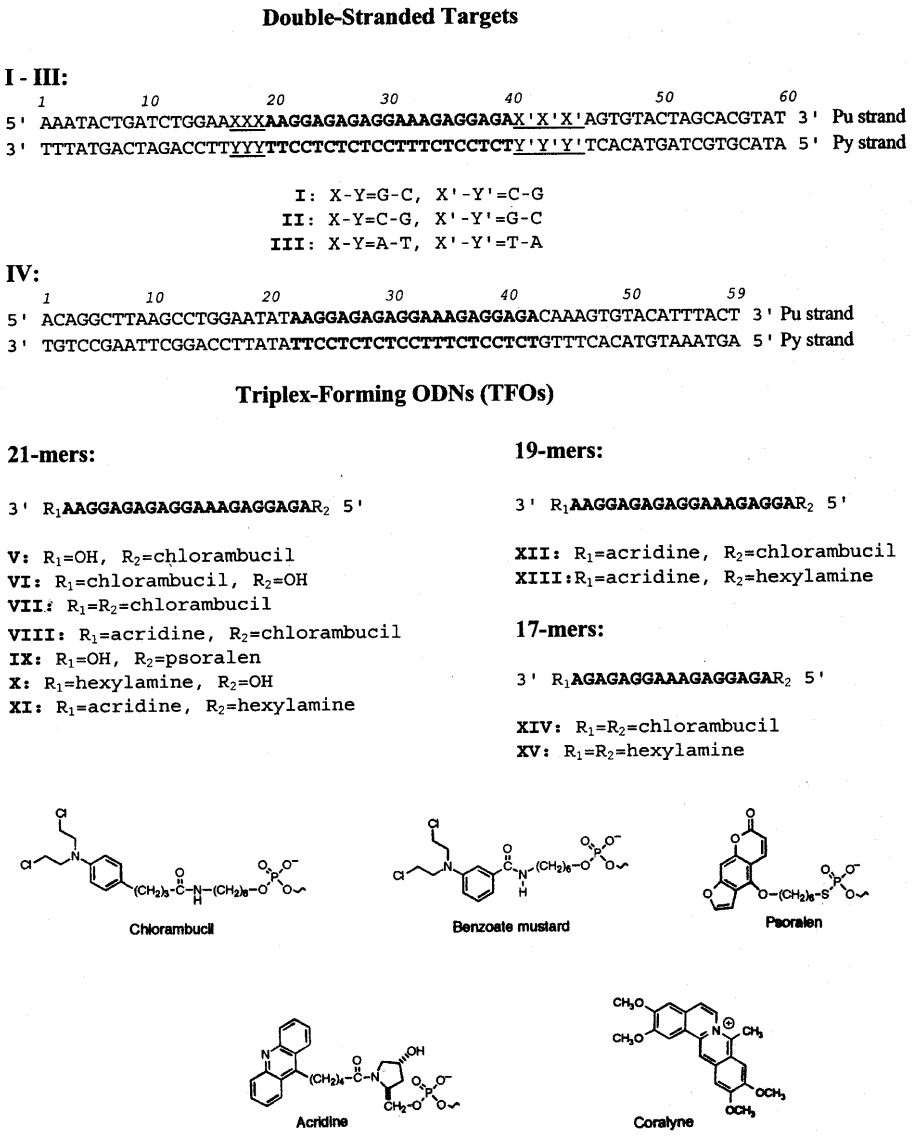

Figure 1. Oligonucleotides and reactive groups used in this study. Chlorambucil was conjugated to ODNs containing one or two terminal hexylamine groups. In one experiment, the chlorambucil group in TFOs **V–VIII** was replaced by the benzoate mustard.

group conjugated to the TFO or the presence of free coralyne (28), promote efficient alkylation of DNA within a triplex.

MATERIALS AND METHODS

Oligodeoxynucleotides

An Applied Biosystems Model 380B DNA synthesizer was used to prepare oligonucleotides on a 1 µmol scale. The trityl-on ODNs were purified by reverse phase HPLC, detritylated, and analyzed for concentration and purity as described earlier (29). The 5′-aminohexyl modification was introduced using *N*-monomethoxytrityl-hexanolamine phosphoramidite. 3′-Terminal hexylamine, acridine and hexanol groups were incorporated into ODNs using modified controlled pore glass supports (29). The method of Takasugi *et al.* (30) was used to conjugate 5-(6-iodohexyloxy)psoralen to an ODN bearing a 5′-thiophosphate group. Aminohexyl modified ODNs were conjugated through an amide linkage of chlorambucil {4-[bis(2-chloroethyl)amino]benzenebutanoic acid} or benzoate mustard {3-[bis(2-chloroethyl)amino]benzoic acid}. The conjugation chemistry will be described elsewhere. Structures of the conjugate groups are shown in Figure 1. ODNs linked to a nitrogen mustard were stored in aqueous solution at –70°C. Stock solutions were thawed and kept on ice prior to use.

Aqueous stability of nitrogen mustard–TFO conjugates

The rates of hydrolysis at 37°C of the chlorambucil and benzoate mustard conjugates were determined using an HPLC assay. One hundred microlitres of a 0.1 mM solution of the conjugate of interest was prepared in 20 mM HEPES, pH 7.2. In parallel experiments, 10 mM $MgCl_2$ and 140 mM KCl were added. The stock solution was immediately aliquoted into five Eppendorf tubes that were submerged in a 37°C bath. For the chlorambucil

conjugates, aliquots were removed at 30, 45, 60, 90 and 120 min. For the benzoate mustard conjugates, aliquots were removed at 2, 4, 6, 8 and 12 h. The aliquots were immediately frozen (–20°C), and thawed just prior to HPLC analysis. Ten microlitres of each sample was injected on a 4.6 × 150 mm Rainin Microsorb C18 column and eluted using a gradient of 5–65% acetonitrile in 0.1 M triethylammonium acetate (pH 7.5) over 20 min. ODN products were detected by UV absorbance at 260 nm and data was analyzed using Rainin Dynamax software. Intact conjugates eluted at ~10 min and a mixture of degradation products eluted at 5–8 min. After integration, the percent intact conjugate was plotted versus time and half-life for disappearance of starting conjugate was determined from the line of best fit.

End-labeling of ODNs

Unreactive ODNs (10 pmol) were kinased at 37°C for 30 min with excess [γ-^{32}P]ATP (20 pmol; specific activity >6000 Ci/mmol; Dupont-NEN) using T4 polynucleotide kinase (United States Biochemical/Amersham). Labeled ODN was purified on a Nensorb 20 column (Dupont). Quantitative recovery of the ODN was assumed when calculating the specific activity. To preserve the chlorambucil group appended to TFO **VI**, this ODN was kinased for 2 h on ice and purified on a chilled Nensorb 20 column. The eluted product was concentrated to <100 µl by repeated butanol extractions on ice and precipitated by addition of 1.5 ml of cold 2% $LiClO_4$ in acetone. After pelleting, the ODN was washed with cold acetone, briefly dried in a Speedvac, and dissolved in 30 µl of cold water.

Formation and cross-linking of triple-stranded complexes

Target duplex was formed by adding labeled purine-rich strand or labeled pyrimidine-rich strand to a 2-fold molar excess of the unlabeled complementary strand in 20 mM HEPES, pH 7.2, 10 mM $MgCl_2$, 1 mM spermine and 0 or 140 mM KCl (Buffer A or B, respectively). Strands were denatured 2 min at 95°C and then annealed 30 min at 37°C. Labeled duplex (20 or 200 nM) was incubated in a final volume of 25 µl with 1 µM TFO for 6 h (24 h for benzoate mustard–TFO conjugates) at 37°C in Buffer A or B. In one experiment triplexes were formed using 1 µM labeled TFO as the limiting reagent and 2 µM duplex. When indicated the buffer was supplemented with 40 µM coralyne chloride (Sigma). A 10-fold aqueous stock of this compound was freshly prepared once a month and stored at 4°C.

Triple strand formation by unreactive TFOs was determined by gel mobility shift analysis. Reactions were mixed with an equal volume of loading dye containing 20% Ficoll type 400 and buffered salts as in the sample. Aliquots of 5 µl were loaded onto a non-denaturing 10% polyacrylamide gel and electrophoresed for 8 h at 200 V and 6°C in 90 mM Tris–borate, pH 8.0, 5 mM $MgCl_2$. Cross-linked products generated by reactive TFOs were analyzed by denaturing polyacrylamide gel electrophoresis. Reactions were mixed with a half volume of loading dye containing 8 M urea. Aliquots of 3 µl were electrophoresed on an 8% sequencing gel at 55°C. Bands were visualized by autoradiography of the dried gel and quantified using a BioRad GS-250 phosphorimager.

Mapping of alkylation sites

Reaction products were electrophoresed as usual under denaturing conditions. After autoradiography of the wet gel, bands of interest were excised and extracted overnight at 37°C in 5 ml of 0.1 M Tris–HCl, pH 7.7, 10 mM triethylamine, 1 mM EDTA. ODNs were isolated on a Nensorb 20 column and dissolved in 10 µl of water. Sites of alkylation were converted into nicks by adding 90 µl of 11% piperidine and heating at 95°C for 30 min. Cleavage products were precipitated with ethanol, dried in a SpeedVac and dissolved in 10 µl of loading dye. Sites of alkylation were mapped relative to Maxam–Gilbert sequencing ladders (31) on an 8% denaturing polyacrylamide gel.

Photo-reactions

Triplexes formed with TFO **IX** under standard conditions were irradiated in a Model 100 near UV reaction chamber (HRI Research). The photo-reaction was complete in under 3 min.

RESULTS

Experimental strategy

Human DQβ1*0302 is an HLA allele (GenBank accession no. K01499) which predisposes carriers to insulin-dependent diabetes mellitus. The first intron of this gene contains a 21 base A-rich homopurine run notable in having no more than two guanines or three adenines in a row. In physiological buffer this homopurine run is best targeted by G/A motif TFOs (Gamper *et al.*, unpublished results). Here we synthesized several chlorambucil-bearing G/A motif TFOs and characterized their reaction with four short duplexes containing the 0302 homopurine run flanked by different sequences (Fig. 1). Double-stranded targets **I** and **II** contain flanking G–C and C–G base pairs while target **III** contains flanking A–T and T–A base pairs. These targets were chosen to elucidate the reaction preferences of a conjugated chlorambucil group. Duplex **IV** replicates the flanking sequence present in the 0302 allele, although in subsequent work we have shown that the reported sequence is incorrect (Belousov *et al.*, unpublished results).

Different reactive G/A motif TFOs were employed in this study (Fig. 1). The reactive group was directed to the major groove of the flanking duplex by 21mers conjugated to chlorambucil on the 5′ end (**V**), the 3′ end (**VI**) or both ends (**VII**). TFO **VIII** contained both 3′ acridine and 5′ chlorambucil groups. Other 21mers were conjugated to a less reactive benzoate mustard or to psoralen. Acridine and psoralen were expected to enhance triple strand formation by intercalating into the triplex–duplex junction (32). In addition, psoralen provided the opportunity to photochemically link the triplex strands upon exposure to near UV. Shorter TFOs bearing one (**XII**) or two (**XIV**) chlorambucil groups were also used to target duplex **IV**. These TFOs were designed to alkylate a guanine residue in the uncomplexed portion of the homopurine run. By this strategy, it was expected that TFO **XIV** (a 17mer with two chlorambucil groups) would alkylate both strands of the duplex.

Modification of targets I–III

An analysis of the reaction products generated by incubating TFOs **V–VIII** with target **I** is presented in Figure 2. An incubation time of 6 h was chosen to ensure complete alkylation, given a

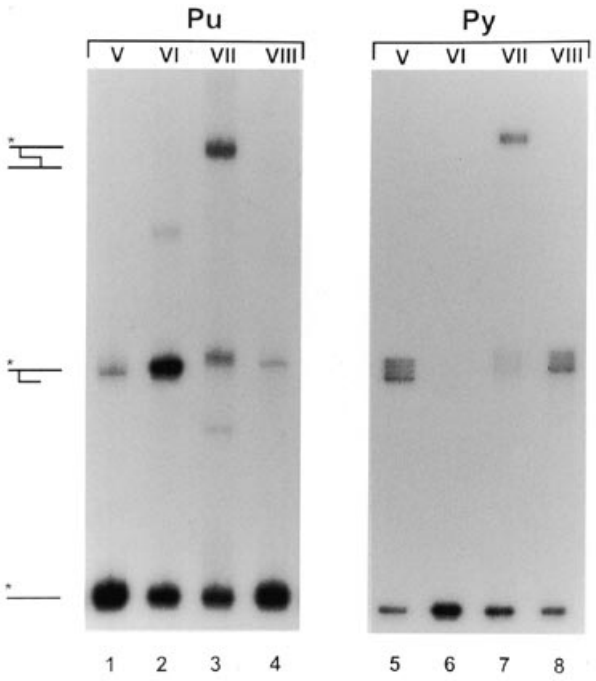

Figure 2. Alkylation of duplex **I** by TFOs **V–VIII**. Reactions were conducted at 37°C in Buffer A using a 50:1 molar ratio of TFO to duplex. Parallel reactions were carried out with duplex (20 nM) which was 5′ end-labeled (site denoted by an asterisk) in the purine-rich strand (Pu) or the pyrimidine-rich strand (Py). After 6 h aliquots were analyzed by denaturing PAGE.

report that chlorambucil-bearing TFOs react with a half-life of ~45 min at 37°C (27). Mono alkylated complexes were generated by all four TFOs. **V** and **VIII** primarily alkylated the pyrimidine-rich strand of the target while **VI** only alkylated the purine-rich strand. **VII** reacted with both strands and yielded interstrand cross-linked product as well. The patterns of reaction are consistent with alkylation of guanine bases in the flanking duplex. Limited reaction of **V** and **VIII** with the purine-rich strand of the target is probably due to fraying of the triplex and alkylation of guanine bases within the homopurine run (Kutyavin *et al.*, unpublished results). A similar reaction could result in both chlorambucil groups of **VII** alkylating the purine-rich strand of the target. This may explain the origin of the weak band in lane 3 which runs ahead of the mono alkylated product.

Table 1. Percent binding and alkylation of duplex **I** by TFOs **V–VIII**[a]

TFO	Molar ratio 5:1				Molar ratio 50:1			
	0 mM KCl		140 mM KCl		0 mM KCl		140 mM KCl	
	binding[b]	XL[c]	binding	XL	binding	XL	binding	XL
V	36	23^{Py}	19	11^{Py}	45	56^{Py}	31	9^{Py}
VI	36	17^{Pu}	19	10^{Pu}	45	47^{Pu}	31	4^{Pu}
VII	36	27^{bis}	19	3^{bis}	45	41^{bis}	31	2^{bis}
VIII	47	50^{Py}	40	22^{Py}	51	55^{Py}	47	16^{Py}

[a]Incubations were carried out for 6 h at 37°C in Buffer A or B using the indicated molar ratio of TFO to duplex.
[b]Determined using TFOs **X** and **XI**.
[c]XL, alkylation of purine-rich strand (Pu), pyrimidine-rich strand (Py) or both strands (bis) of **I**.

The extent to which the four TFOs bound to and cross-linked target **I** was determined at molar ratios of 5:1 and 50:1 (TFO:duplex) in buffer with KCl concentrations of 0 (Buffer A) or 140 mM (Buffer B) (Table 1). Control experiments demonstrated that 6 h was sufficient time to achieve equilibrium when using 1 μM unreactive TFO to drive the reaction. Triplex formation by unreactive TFOs was incomplete under all conditions. In general, raising the molar ratio of TFO to duplex, reducing the KCl concentration, or conjugating an acridine group to the TFO enhanced triple strand formation. Binding levels varied from 19% when using a 5:1 molar ratio in buffer containing 140 mM KCl to 45% when using a 50:1 molar ratio in buffer lacking KCl. The inhibitory effect of K^+ probably reflects the preference of triplexes for multivalent counterions (17,18). By conjugating an acridine group to the TFO, binding levels under the same conditions increased to 40 and 51%, respectively. Separate experiments showed that conjugation of an acridine group to the 3′ end of the TFO increased the association constant for triple strand formation by 10-fold in Buffer A and 30-fold in Buffer B (data not shown).

Modification of target **I** by reactive TFOs **V–VIII** exhibited the same trends as described for triplex formation but usually at a lower level. We note that Table 1 only lists the percentage yield of the most abundant mono alkylated product for TFOs bearing a single chlorambucil group and entirely omits this information for TFO **VII**. Assuming that linkage of chlorambucil to a TFO does not alter its affinity for the homopurine run, we can estimate from the data in Table 1 the fraction of triplex in which at least one strand of the target was linked to a reactive TFO. This value defines the efficiency of alkylation. At a molar ratio of 5:1 in Buffer A or B, 47–64% of hybridized **V** or **VI** alkylated one strand of the target. The acridine group linked to **VIII** doubled the mono alkylation efficiency of chlorambucil in Buffer A but had no effect in Buffer B. At the same molar ratio interstrand (bis)alkylation of the triplex by **VII** was 75% efficient in Buffer A and 16% efficient in Buffer B. In both instances, the level of bis cross-linking was well above that predicted if independent alkylation by the two tethered chlorambucil groups is assumed. We propose that fixation of the triplex by mono cross-linking increases the likelihood of a second alkylation event.

Uniformly high levels of alkylation were observed when the reactions were conducted at a 50:1 molar ratio in buffer lacking KCl. **V**, **VI** and **VIII** generated levels of mono cross-linking equal to or greater than the equilibrium binding levels and **VII** was nearly as efficient in bis cross-linking. Kinetic data which follow suggest that a rapid and most likely reversible duplex–triplex equilibrium contributed to the high levels of alkylation observed under these conditions. When the same reactions were conducted in the presence of 140 mM KCl, levels of mono and bis cross-linking were reduced to a greater extent than levels of binding, leading to reaction efficiencies <35 and 10%, respectively.

The major sites at which TFOs **V** and **VI** alkylated duplexes **I–III** were mapped by cleavage analysis. Since the TFO resides in the major groove of DNA, the tethered chlorambucil group was expected to alkylate the N-7 position of adenine and guanine. Modeling showed that the linker arm was too short to allow reaction of chlorambucil with minor groove determinants. Purines alkylated at N-7 can be detected by strand scission following depurination. The efficiency and location of mono cross-linking in the respective duplexes is presented in Figure 3. For comparison, the efficiency of bis cross-linking by TFO **VII**

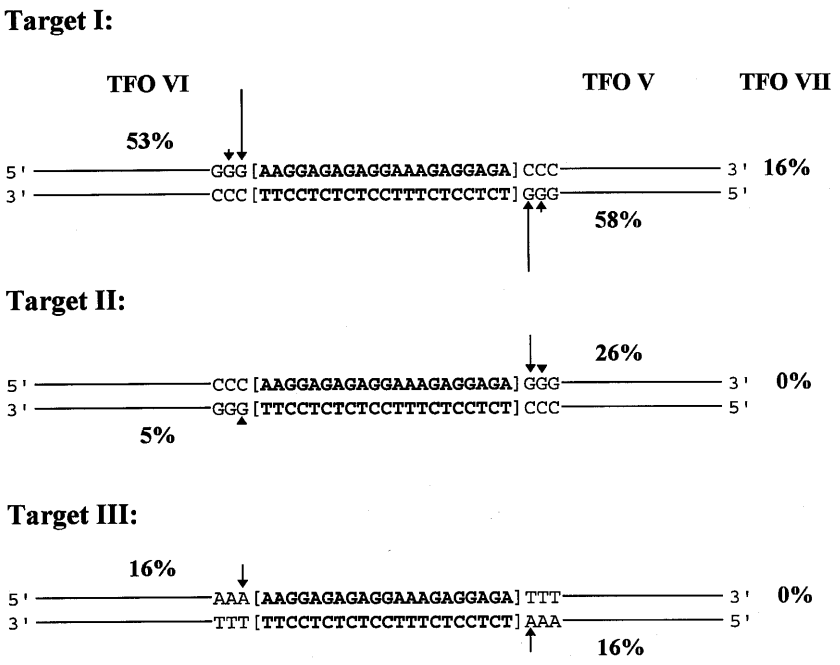

Figure 3. Location and extent of alkylation in targets **I–III**. TFOs **V** and **VI** were incubated with targets **I–III** (molar ratio of 5:1) for 6 h at 37°C in Buffer B. For each reaction, the major cross-linked product was isolated by denaturing PAGE and sites of alkylation were determined. Mono-alkylation efficiencies (defined as percent modification of the indicated triplex strand) are shown for **V** and **VI**. For comparison, the efficiency of bis alkylation by **VII** is listed to the right of the targets. Arrows denote the alkylated bases and their relative efficiencies of modification.

is included. In all three targets, alkylation was restricted to purine bases within 2 base pairs of the triplex. No evidence was obtained for alkylation of cytidine or thymine bases. Modification of guanine bases was more efficient than adenine bases. This can be attributed to the greater nucleophilicity of guanine N-7 relative to adenine N-7. Positional effects clearly modulated the reactivity of guanine bases. Reaction was greatest in target **I** where this base was located 5′ to the triplex on either strand of the flanking duplex. In target II, where the guanine bases were 3′ to the triplex, reaction was diminished.

Self-alkylation and hydrolysis of TFO VI

G/A-containing TFOs have been reported to base pair with one another as presumptive parallel-stranded homoduplexes (33). To determine whether the G/A motif TFOs used here self-associate, we incubated 5′ end-labeled **X** with a 50-fold excess of **VI** in buffer with different salt compositions. Analysis of the reaction mixtures by denaturing PAGE showed a slow moving band in those samples which had been incubated in the presence of 10 mM $MgCl_2$ (Fig. 4A). The position of this band in the gel is consistent with a cross-linked dimer of **X** and **VI**. Slower moving bands indicative of cross-linking within multimeric complexes were absent. The site of alkylation on **X** was determined by electrophoresing the labeled complex on a sequencing gel after cleavage with hot piperidine (Fig. 4B). Position of the labeled fragments relative to A+G and G sequencing ladders demonstrates that alkylation occurred on the two guanine bases nearest to the 3′ end of **X**. This reaction pattern is consistent with a parallel-stranded homoduplex.

The reaction profile of **VI** was examined by incubating 5′ end-labeled TFO under different conditions and then analyzing the reaction products on a denaturing gel (Fig. 4C). Incubation of **VI** in Buffer B for 6 h at 37°C generated a set of unresolved reaction products together with a slower moving band attributed to the cross-linked TFO dimer. The reaction products probably resulted from hydrolysis, self-modification and alkylation of low molecular weight nucleophiles such as spermine. Approximately 13% of the TFO ran as a cross-linked dimer. In the presence of 0.5 M pentaethylenehexamine a major band corresponding to an adduct between **VI** and this nucleophile was generated. When **VI** was incubated in the presence or absence of 140 mM KCl with a 2-fold excess of unlabeled duplex **I**, 22 or 42% of the TFO was cross-linked to the purine-rich strand of the duplex. The amount of TFO cross-linked as a dimer was reduced to 3–5% in the presence of duplex.

Relative rates of triplex formation and DNA alkylation

To better understand the dynamics of DNA cross-linking by chlorambucil-bearing TFOs, we investigated the kinetics of TFO binding and chlorambucil activation. Triplex formation was monitored by mixing psoralen-bearing TFO **IX** with target **I** under standard conditions and removing aliquots for irradiation. When exposed to near UV light, the tethered psoralen group reacts with thymine bases in the pyrimidine-rich strand of the target at or near the triplex–duplex junction (see Fig. 1). In this study psoralen was used instead of chlorambucil because the photo-reaction is both rapid and controlled. The time courses in Figure 5 lead us to conclude that triple strand formation, as reflected in the generation of mono cross-linked complex, proceeded with a half-life of less than 5 min. As might be anticipated, triplexes formed more rapidly in Buffer A than in Buffer B.

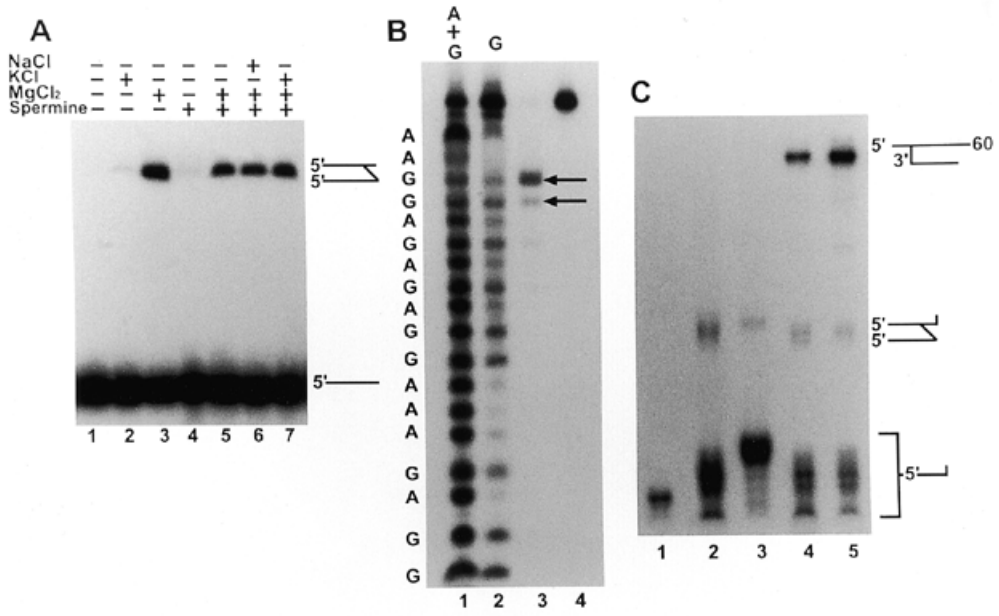

Figure 4. TFO **VI** self-reacts within a parallel homoduplex. (**A**) Counterion requirements for interstrand alkylation. 5′ end-labeled **X** (20 nM) was incubated with a 50-fold excess of **VI** for 6 h at 37°C in 20 mM HEPES, pH 7.2, supplemented with the indicated salts. Aliquots were analyzed by denaturing PAGE. (**B**) Site of interstrand alkylation in the parallel duplex. Cross-linked dimer from the preceding reaction was isolated, treated with hot piperidine, and run in a sequencing gel (lane 3) alongside A+G (lane 1) and G (lane 2) ladders prepared from **X**. Lane 4 contains **X**. The arrows denote cleaved fragments. (**C**) Reaction profiles of **VI** under different conditions. **VI** was end-labeled and incubated at 1 μM concentration for 6 h at 37°C in Buffer B (lane 2), in water with 0.5 M pentaethylenehexamine (lane 3), in Buffer B with 2 μM **I** and in Buffer A with 2 μM **I**. Aliquots were analyzed on an 8% polyacrylamide–7 M urea gel. Lane 1 contains **X**.

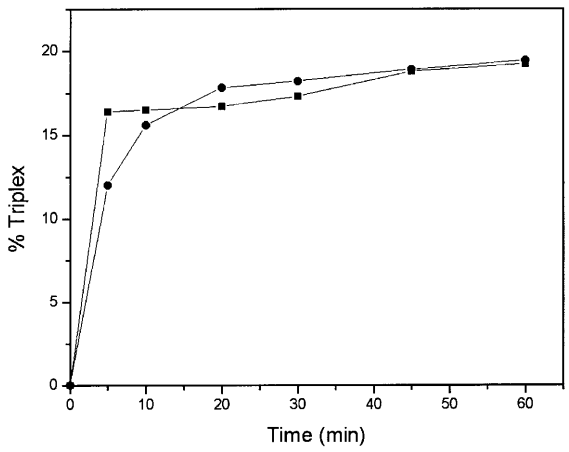

Figure 5. Kinetics of triple strand formation. TFO **IX** (1 μM) was added to duplex **IV** (200 nM) in Buffer A (■) or B (●) and triplex formation was carried out at 37°C. Aliquots were removed at the indicated times and irradiated for 3 min at 4°C with near UV light. Samples were analyzed by denaturing PAGE. Modification of the labeled pyrimidine-rich strand of the target functioned as an indicator of triplex formation.

Nitrogen mustards such as chlorambucil react through an intermediate aziridinium ion and exhibit half-lives which are dependent upon molecular structure, solution conditions and temperature. An indirect assay estimated the reactive half-life of chlorambucil-bearing TFOs to be ~45 min at 37°C (27). We have re-evaluated this half-life at 37°C using a more direct HPLC based assay and found it to be 25 min in pH 7.2 HEPES buffer and 27 min in the same buffer supplemented with 10 mM $MgCl_2$ and 140 mM KCl. When chlorambucil was replaced by benzoate mustard (see Fig. 1), the half-life of the reactive TFO increased to 162 and 216 min, respectively. We note that the increase in half-life in the presence of chloride can be attributed to a reversible reaction between the aziridinium and chloride ions (34). Conversely, self-alkylation of the TFO appears to irreversibly consume aziridinium ion since the free mustards reacted more slowly than the conjugates (data not shown).

Enhancement of triplex formation and DNA alkylation by coralyne

The reduced levels of alkylation observed under physiological conditions prompted us to investigate whether addition of coralyne to the buffer could enhance G/A motif triplex formation and chlorambucil induced cross-linking. Coralyne has been shown to stabilize long pyrimidine motif triplexes through preferential intercalation into the triple strand (28). Our results indicate that the same is true for oligomeric G/A motif triplexes. In Figure 6A the extent of physical binding and mono-crosslinking of TFOs **X** and **V** to target **I** is plotted as a function of coralyne concentration in the incubation buffer. Saturating concentrations of coralyne (>10 μM) increased binding by 4-fold and alkylation by 8-fold. In the presence of coralyne mono-crosslinking of **V** to the pyrimidine-rich strand of the triplex was nearly quantitative.

The effect of coralyne on mono and bis alkylation was further evaluated by reacting TFOs **V–VIII** with target **I** under physiological conditions (Fig. 6B). Buffer B was supplemented with 40 μM coralyne and a molar ratio of 5:1 was employed. When compared with otherwise identical reactions conducted in the

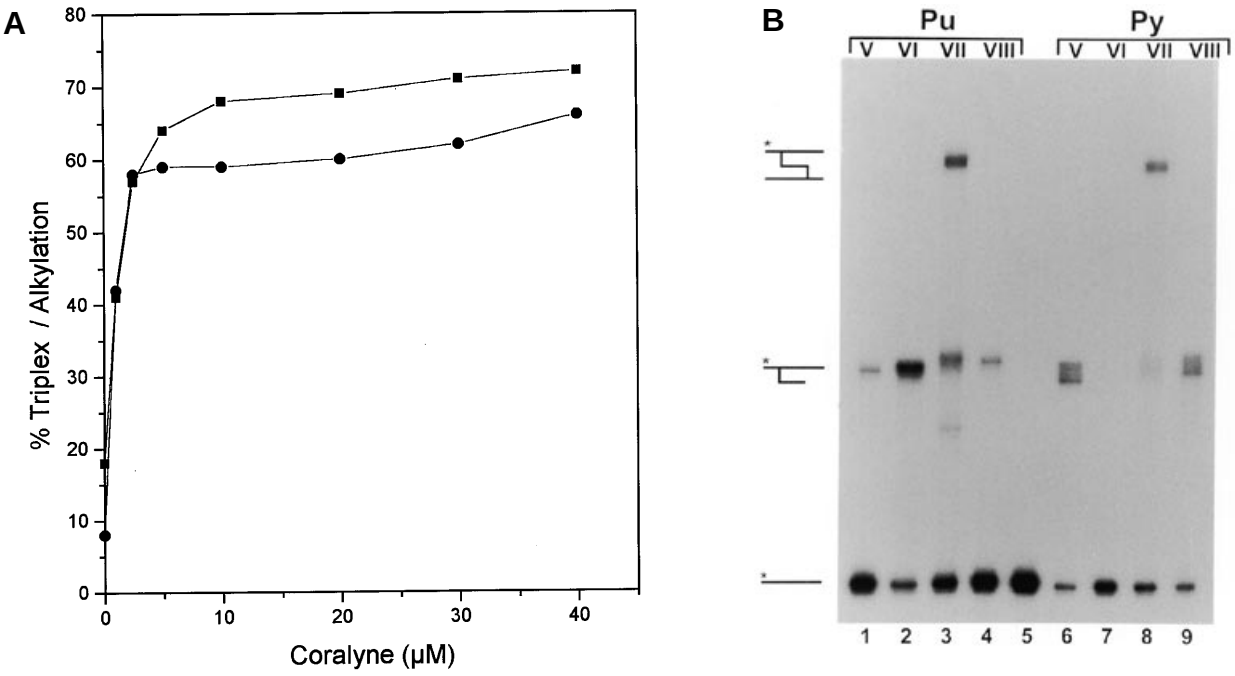

Figure 6. Coralyne promotes triple strand formation and alkylation. (**A**) Levels of binding and mono alkylation as a function of coralyne concentration. Incubations of TFOs **V** and **X** with duplex **I** (molar ratio 5:1) were conducted for 6 h at 37°C in Buffer B containing the indicated concentrations of coralyne. Binding (■) and alkylation (●) levels were determined by gel electrophoresis. (**B**) Cross-linking of TFOs **V–VIII** to duplex **I** in the presence of 40 μM coralyne. Reactions were conducted for 6 h at 37°C in Buffer B using a molar ratio of TFO to duplex of 5:1. Concentration of **I** was 200 nM. Aliquots were analyzed on a denaturing gel. The duplex was end-labeled in the purine-rich (Pu) or pyrimidine-rich (Pu) strand. A control reaction lacking TFO was added to lane 5.

absence of coralyne (Table 1), the level of reaction was significantly greater. Mono alkylation by **V** and **VI** increased at least 6-fold while interstrand alkylation by **VII** increased 10-fold. Reaction of **VIII** increased 2.5-fold over the already enhanced level of cross-linking made possible by a tethered acridine group. Alkylation efficiency as defined earlier was >85% for **V** and **VI** and ~50% for **VII**.

Comparison of DNA alkylation by TFOs conjugated to chlorambucil or benzoate mustard

Conjugation of TFOs to nitrogen mustards other than chlorambucil permits us to alter the rate at which the TFO alkylates DNA. This could prove useful when optimizing the activity of these conjugates in cell culture, where the reactive TFO must survive the time required for uptake. To determine whether chlorambucil-bearing TFOs are predictive of the reaction patterns elicited by other nitrogen mustard conjugates, we compared the levels of mono and bis alkylation of duplex **I** by TFOs **V–VIII** when the latter were conjugated to chlorambucil or benzoate mustard. The benzoate mustard conjugates were incubated with duplex for 24 h instead of 6 h to assure >98% reaction. Although the results in Table 2 show differences between each pair of TFOs, the aggregate levels of modification for the two classes of TFOs are very similar.

Table 2. Percent alkylation[a] of duplex **I** by TFOs **V–VIII**: comparison of chlorambucil versus benzoate mustard[b]

TFO	Chlorambucil	Benzoate
V	63^{Py}	46^{Py}
VI	63^{Pu}	80^{Pu}
VII	31^{bis}, 16^{Py}	33^{bis}, 12^{Py}
VIII	58^{Py}	45^{Py}

[a]Alkylation took place on the purine-rich strand (Pu), the pyrimidine-rich strand (Py) or on both strands (bis) of **I**.
[b]Incubations were carried out for 6 (chlorambucil conjugate) or 24 h (benzoate conjugate) at 37°C in Buffer B with 40 μM coralyne. The molar ratio of TFO to duplex was 5:1.

Modification of target IV

With the exception of one C–G base pair, the homopurine run in target IV is bracketed by T–A and A–T base pairs. As a consequence TFO **VII** did not effectively bis cross-link to the duplex (Table 3). In an attempt to circumvent this limitation, we tested two truncated versions of the standard TFO hoping to react with guanine residues in the homopurine run. Table 3 summarizes the binding and cross-linking results. Sites of alkylation were mapped by piperidine cleavage and are reported below. **XII** is a 19mer with 5′-chlorambucil and 3′-acridine groups. In Buffer B a 5-fold excess of this TFO alkylated a guanine base near the

3′ end of the homopurine run (G-41) with very high efficiency based on the equilibrium level of triple strand (Table 3). These levels of binding and mono alkylation are nearly equivalent to those obtained using the 21mer **VIII**. A still shorter 17mer (**XIV**) was conjugated to chlorambucil on both ends so that a guanine base on each strand of the duplex could be targeted (G-25 on the purine-rich strand and G-17 on the pyrimidine-rich strand). As shown in Table 3, this TFO yielded 25% bis alkylation and 29% mono alkylation even though the physical triplex itself was too unstable to survive non-denaturing gel electrophoresis.

DISCUSSION

The results presented here are consistent with the premise that alkylation of DNA by chlorambucil-bearing TFOs only occurs within a triple-stranded complex. The same conditions which favor triple strand formation (such as addition of coralyne, elimination of KCl, or a high molar ratio of TFO to duplex) also favor alkylation. Conversely, neither binding nor cross-linking was observed when $MgCl_2$ was removed from the buffer or when the chlorambucil-bearing TFO was not complementary to the homopurine run (data not shown). The absence of non-specific alkylation indicates that physical binding of the conjugate to DNA is mediated by the TFO with little or no contribution from the chlorambucil group. Like simple intercalating agents (32), chlorambucil lacks sufficient DNA binding affinity to interact on its own when conjugated to an ODN.

Following triplex formation, the hexylamine linker positions the chlorambucil moiety in the major groove of the flanking duplex where reaction with guanine N-7 is favored. Evaluation of other linker arms differing in length and composition might improve the level of cross-linking. Furthermore, linkers of sufficient length might permit modification of minor groove determinants.

The enhancement of binding by coralyne is the first report of this agent stabilizing a G/A triplex. In limited testing, the triplex selective intercalators benzopyridoindole (35) and naphthylquinoline (36) failed to stabilize purine motif triple strands. The structure of coralyne may be uniquely suited for intercalation into anti-parallel triplexes, since this ligand also enhances the stability of G/T triplexes (Gamper *et al*., unpublished results). By contrast, coralyne interacts weakly with double-stranded DNA (28) and does not interfere with or alter the reaction of chlorambucil with the flanking duplex or the intercalation of a tethered acridine group into the triplex–duplex junction (data not shown).

Table 3. Percent binding and alkylation of duplex **IV** by TFOs **VII**, **VIII**, **XII** and **XIV**[a]

TFO	Binding[b]	Alkylation[c]
VII	62	$<1^{bis}$, 10^{Pu}
VIIII	63	54^{Py}
XII	50	54^{Pu}
XIV	0	25^{bis}, 29^{Pu}

[a]Incubations were carried out for 6 h at 37°C in Buffer B with 40 μM coralyne. The molar ratio of TFO to duplex was 5:1.
[b]Determined using TFOs **X**, **XI**, **XIII** and **XV**, respectively.
[c]Alkylation took place on the purine-rich strand (Pu), the pyrimidine-rich strand (Py) or on both strands (bis) of **IV**.

In the presence of coralyne, the alkylation efficiency was usually >85% of the triplex formed when reactive TFOs were incubated with duplex **I**. We hypothesize that under these conditions, the triple strand is less likely to fray at the ends. As a consequence the tethered chlorambucil group spends more time in close proximity to the flanking duplex and the probability of alkylation is increased.

The level of cross-linking observed with a chlorambucil-bearing TFO was highly dependent upon the sequence flanking the homopurine run. When tethered to a bound TFO, chlorambucil alkylated bases in either strand of the flanking duplex up to two base pairs from the triplex–duplex junction. The N-7 position of guanine was the preferred site of alkylation, while the N-7 position of adenine was a secondary site. By reducing the length of the TFO, guanines within the homopurine run could be targeted. This option expands the number of potential alkylation sites and creates additional opportunities for bis cross-linking.

Under favorable conditions the level of alkylation of duplex **I** sometimes exceeded the equilibrium binding level. This could be explained if the tethered chlorambucil group reacted slowly within the context of a rapid and reversible equilibrium between duplex and triplex. In such a situation DNA alkylation would remove triplex from the equilibrium and lead to the formation of additional physical triplex. Furthermore, triplexes containing a non-reactive TFO (due to non-productive reaction of the chlorambucil) could eventually exchange with reactive TFO. We have shown that under certain conditions the rate of triplex formation is rapid relative to the half-life of the tethered mustard. Recognizing that physical triplex formation is incomplete at equilibrium, it follows that dissociation of the triplex must also be relatively rapid. These conditions meet the requirements for ‘chemical pumping’ described above and probably occur to some extent in most of the reactions described here.

In the presence of $MgCl_2$ the G/A-containing TFOs described here also self-associated as parallel-stranded homoduplexes. At 37°C these duplexes are relatively unstable and do not interfere with triple strand formation (33; Gamper *et al*., unpublished observations). However, interstrand cross-linking of the homoduplex by chlorambucil prevents interaction of the linked TFOs with DNA. The same is probably true for reactive TFOs which undergo self-alkylation in the monomeric state. Our results indicate that triple strand formation is rapid enough to minimize the inhibitory effect of these competing reactions.

Another variable influencing the level of modification is the half-life of the reactive group. Different mustards cyclize to aziridinium ions and hence react with nucleophiles at different rates. This provides flexibility in preparing TFO conjugates that exhibit different half-lives. Comparison of cross-linking by TFOs conjugated to chlorambucil and benzoate mustard showed that the level of DNA alkylation did not significantly differ as long as the reactions were allowed to go to completion.

In summary, we have demonstrated targeted alkylation of DNA under physiological conditions using G/A motif TFOs bearing a chlorambucil group. Coralyne was an essential component of the reaction buffer for efficient alkylation. With an appropriate target, alkylation was primarily limited by the extent of triple strand formation and not by non-productive reaction of the tethered chlorambucil group. TFO conjugates of the type described here represent promising candidates for evaluating the antigene concept in cell culture.

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REFERENCES

1 Cheng, Y.-K. and Pettitt, B.M. (1992) *Prog. Biophys. Mol. Biol.*, **58**, 225–257.
2 Thuong, N.T. and Helene, C. (1993) *Angew. Chem. Int. Ed.*, **32**, 666–690.
3 Mirkin, S.M. and Frank-Kamenetskii, M.D. (1994) *Annu. Rev. Biophys. Biomol. Struct.*, **23**, 541–576.
4 Nielsen, P.E. (1995) *Annu. Rev. Biophys. Biomol. Struct.*, **24**, 167–183.
5 Plum, G.E., Pilch, D.S., Singleton, S.F. and Breslauer, K.J. (1995) *Annu. Rev. Biophys. Biomol. Struct.*, **24**, 319–350.
6 Sun, J.-S., Garestier, T. and Helene, C. (1996) *Curr. Opin. Struct. Biol.*, **6**, 327–333.
7 Wells, R.D., Collier, D.A., Hanvey, J.C., Shimizu, M. and Wohlrab, F. (1988) *Fed. Am. Soc. Exp. Biol. J.*, **2**, 2939–2949.
8 Cooney, M., Czernuszewicz, G., Postel, E.H., Flint, S.J. and Hogan, M.E. (1988) *Science*, **241**, 456–459.
9 Maher, L.J. 3rd, Dervan, P.B. and Wold, B. (1992) *Biochemistry*, **31**, 70–81.
10 Grigoriev, M., Praseuth, D., Robin, P., Hemar, A., Saison-Behmoaras, T., Dautry-Varsat, A., Thuong, N.T., Helene, C. and Harel-Bellan, A. (1992) *J. Biol. Chem.*, **267**, 3389–3395.
11 Ebbinghaus, S.W., Gee, J.E., Rodu, B., Mayfield, C.A., Sanders, G. and Miller, D.M. (1993) *J. Clin. Invest.*, **92**, 2433–2439.
12 Escude, C., Giovannangeli, C., Sun, J.-S., Lloyd, D.H., Chen, J.-K., Gryaznov, S.M., Garestier, T. and Helene, C. (1996) *Proc. Natl. Acad. Sci. USA*, **93**, 4365–4369.
13 Giovannangeli, C., Perrouault, L., Escude, C., Nguyen, N. and Helene, C. (1996) *Biochemistry*, **35**, 10539–10548.
14 Duval-Valentin, G., Thuong, N.T. and Helene, C. (1992) *Proc. Natl. Acad. Sci. USA*, **89**, 504–508.
15 Young, S.L., Krawczyk, S.H., Matteucci, M.D. and Toole, J.J. (1991) *Proc. Natl. Acad. Sci. USA*, **88**, 10023–10026.
16 Lee, J.S., Johnson, D.A. and Morgan, A.R. (1979) *Nucleic Acids Res.*, **6**, 3073–3091.
17 Singleton, S.F. and Dervan, P.B. (1993) *Biochemistry*, **32**, 13171–13179.
18 Cheng, A.-J. and Van Dyke, M.W. (1993) *Nucleic Acids Res.*, **21**, 5630–5635.
19 Scaria, P.V., Shire, S.J. and Shafer, R.H. (1992) *Proc. Natl. Acad. Sci. USA*, **89**, 10336–10340.
20 Zimmer, C. and Wahnert, U. (1986) *Prog. Biophys. Mol. Biol.*, **47**, 31–112.
21 Takasugi, M., Guendouz, A., Chassignol, M., Decout, J.L., Lhomme, J., Thuong, N.T. and Helene, C. (1991) *Proc. Natl. Acad. Sci. USA*, **88**, 5602–5606.
22 Vlassov, V.V., Gaidamakov, S.A., Zarytova, V.F., Knorre, D.G., Levina, A.S., Nikonova, A.A., Podust, L.M. and Fedorova, O.S. (1988) *Gene*, **72**, 313–322.
23 Shaw, J.-P., Milligan, J.F., Krawczyk, S.H. and Matteucci, M. (1991) *J. Am. Chem. Soc.*, **113**, 7765–7766.
24 Povsic, T.J. and Dervan, P.B. (1990) *J. Am. Chem. Soc.*, **112**, 9428–9430.
25 Gruff, E.S. and Orgel, L.E. (1991) *Nucleic Acids Res.*, **19**, 6849–6854.
26 Colombier, C., Lippert, B. and Leng, M. (1996) *Nucleic Acids Res.*, **24**, 4519–4524.
27 Kutyavin, I.V., Gamper, H.B., Gall, A.A., and Meyer, R.B. (1993) *J. Am. Chem. Soc.* **115**, 9303–9304.
28 Lee, J.S., Latimer, L.J.P. and Hampel, K.J. (1993) *Biochemistry*, **32**, 5591–5597.
29 Gamper, H.B., Reed, M.W., Cox, T., Virosco, J.S., Adams, A.D., Gall, A.A., Scholler, J.K. and Meyer, R.B. (1993) *Nucleic Acids Res.*, **21**, 145–150.
30 Takasugi, M., Guendouz, A., Chassignol, M., Decout, J.L., Lhomme, J., Thuong, N.T. and Helene, C. (1991) *Proc. Natl. Acad. Sci. USA*, **88**, 5602–5606.
31 Maxam, A.M. and Gilbert, W. (1977) *Proc. Natl. Acad. Sci. USA*, **74**, 560–564.
32 Sun, J.S., Francois, J.-C., Montenay-Garestier, T., Saison-Behmoaras, T., Roig, V., Thuong, N.T. and Helene, C. (1989) *Proc. Natl. Acad. Sci. USA*, **86**, 9198–9202.
33 Noonberg, S.B., Francois, J.-C, Garestier, T. and Helene, C. (1995) *Nucleic Acids Res.*, **23**, 1956–1963.
34 Chatterji, D.C., Yeager, R.L. and Gallelli, J.F. (1982) *J. Pharm. Sciences*, **71**, 50–54.
35 Escude, C., Sun, J.-S., Nguyen, C.H., Bisagni, E., Garestier, T. and Helene, C. (1996) *Biochemistry*, **35**, 5735–5740.
36 Cassidy, S.A., Strekowski, L. and Fox, K.R. (1996) *Nucleic Acids Res.*, **24**, 4133–4138.