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Triple helix formation with purine-rich phosphorothioate-containing oligonucleotides covalently linked to an acridine derivative

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ABSTRACT

Purine-rich (GA)- and (GT)-containing oligophosphorothioates were investigated for their triplex-forming potential on a 23 bp DNA duplex target. In our system, GA-containing oligophosphorothioates (23mer GA-PS) were capable of triplex formation with binding affinities lower than (GA)-containing oligophosphodiesters (23mer GA-PO). The orientation of the third strand 23mers GA-PS and GA-PO was antiparallel to the purine strand of the duplex DNA target. In contrast, (GT)-containing oligophosphorothioates (23mer GT-PS) did not support triplex formation in either orientation, whereas the 23mer GT-PO oligophosphodiester demonstrated triplex formation in the antiparallel orientation. GA-PS oligonucleotides, in contrast to GT-PS oligonucleotides, were capable of self-association, but these self-associated structures exhibited lower stabilities than those formed with GA-PO oligonucleotides, suggesting that homoduplex formation (previously described for the 23mer GA-PO sequence by Noonberg *et al.*) could not fully account for the decrease in triplex stability when phosphorothioate linkages were used. The 23mer GA-PS oligonucleotide was covalently linked via its 5′-end to an acridine derivative (23mer Acr–GA-PS). In the presence of potassium cations, this conjugate demonstrated triplex formation with higher binding affinity than the unmodified 23mer GA-PS oligonucleotide and even than the 23mer GA-PO oligonucleotide. A (GA)-containing oligophosphodiester with two phosphorothioate linkages at both the 5′- and 3′-ends exhibited similar binding affinity to duplex DNA compared with the unmodified GA-PO oligophosphodiester. This capped oligonucleotide was more resistant to nucleases than the GA-PO oligomer and thus represents a good alternative for *ex vivo* applications of (GA)-containing, triplex-forming oligonucleotides, allowing a higher binding affinity for its duplex target without rapid cellular degradation.

INTRODUCTION

Several strategies using short oligonucleotides can be used to regulate gene expression (1). In the 'antisense' strategy, the oligonucleotide is bound to a single-stranded sequence in a specific mRNA and inhibits translation of the mRNA into protein. In the 'antigene' strategy, the oligonucleotide is targeted to an oligopurine–oligopyrimidine sequence in a DNA double helix (2). The formation of a local triple helix may inhibit transcription of a specific gene, either by preventing binding of regulatory transcription factors (3–8) or by directly interfering with the RNA polymerase machinery at the initiation or elongation steps (9–11). Triple helix formation may also inhibit progression of DNA polymerases and affect DNA replication *in vitro* (12–16).

Pyrimidine-rich or purine-rich oligonucleotides recognize the oligopurine–oligopyrimidine target sequences of double-helical DNA by forming specific base triplets. The involvment of Hoogsteen hydrogen bonds in T·A*T and C·G*C$^+$ base triplets allows binding of an oligopyrimidine third strand in a parallel orientation with respect to the oligopurine sequence of the DNA double helix (2).

The requirement for an acidic pH in order to protonate cytosines within the pyrimidine-rich (C·G*C$^+$, T·A*T) triplex binding motif has led to the development of other triplex binding motifs based upon C·G*G and T·A*T (GT oligonucleotides) or C·G*G and T·A*A triplets (GA oligonucleotides) (17–19). Formation of triple helices involving purine-rich third strands has been shown to be pH independent and stabilized by multivalent cations. Triplex formation with (GA)-containing oligonucleotides involves reverse Hoogsteen hydrogen bond formation. GA oligonucleotides are bound to DNA in an antiparallel orientation with respect to the oligopurine sequence of the duplex (2,19,20). (GT)-containing oligonucleotides bind to the oligopurine sequence of the DNA duplex in an orientation that depends on both the number of GpT and TpG steps and the length of the G and T tracts (21,22).

The use of oligonucleotides to regulate gene expression *in vivo* is limited by the rapid extracellular and intracellular nuclease-mediated degradation of oligomers in biological media. To augment oligonucleotide stability, several non-natural oligonucleotide analogues with modified backbone structures have been synthesized (2). The effects of several backbone modifications of triplex-

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forming oligonucleotides were previously studied. For example, oligodeoxyribopyrimidines containing α anomers and 2′-*O*-methyl-oligoribopyrimidines could be used as nuclease-resistant triple helix-forming oligonucleotides with similar or higher binding affinities than unmodified oligopyrimidines (23–27). In contrast, pyrimidine-containing methylphosphonate or phosphorothioate oligonucleotides have lower triplex binding affinities than those obtained with their corresponding unmodified oligomers (28–33). It was shown that the triplex binding affinity of some triplex-forming oligonucleotides with only a few phosphorothioate linkages decreased inversely with the number of phosphorothioate bonds in the oligopyrimidine (31,33,34). Triplex-forming oligopyrimidines where several phosphodiester linkages were replaced with phosphorothioates could inhibit restriction endonuclease-mediated cleavage of DNA (35) or T7 RNA polymerase-mediated transcription (33,34), but with less efficiency than the unmodified oligopyrimidines.

Purine-rich triplex-forming oligonucleotides containing methylphosphonate or phosphorothioate linkages or α-nucleotides have been studied for their triplex binding affinities (32,36–40). Recently, it was shown that an $(A,G)_8$ methylphosphonate oligonucleotide targeted to a single-stranded mRNA could efficiently block protein synthesis via the formation of a triple-helical complex involving two $(A,G)_8$ oligomethylphosphonates bound to the pyrimidine target sequence of the mRNA (36,40). Triplex formation with (GT)-containing, triplex-forming oligonucleotide phosphorothioates has recently been described (32,37,38). The binding affinities of these oligonucleotides for their double-stranded target were similar or slightly higher than those of the unmodified oligomers, depending on the presence of polyamines in the incubation buffer (37). More recently, it was demonstrated that a highly G-rich (GA)-containing oligophosphorothioate could bind to duplex DNA via triplex formation (39).

The present report uses standard *in vitro* experimental techniques to determine whether (GA)-containing, triplex-forming oligonucleotides with phosphorothioate linkages can be used in the ‘antigene’ strategy. A 23 bp oligopurine–oligopyrimidine sequence of the human insulin-like growth factor I gene (IGF-I) was used as a target. It is shown that (GA)-containing oligophosphorothioates form triple-helical complexes with the 23 bp target, but binding affinities are lower as compared with oligophosphodiesters. Covalent linkage of an intercalating agent to the (GA)-containing phophorothioate oligonucleotide increases the binding affinity of the nuclease-resistant oligonucleotide to the target sequence. No triplex was observed with (GT)-containing oligophosphorothioates, in contrast to previous reports describing triplex formation on different sequences (32,37,38).

We have previously demonstrated that a (GA)-containing 23mer oligophosphodiester could self-associate to form a homoduplex which could compete with triplex formation (20,26). The (GA)-containing phosphorothioate oligonucleotide could also self-associate, but melting curves suggest that self-association is not sufficient to fully explain the destabilization observed with (GA)-containing phosphorothioates.

MATERIALS AND METHODS

Oligonucleotides

Purified phosphodiester and phosphorothioate oligodeoxynucleotides were purchased from Eurogentec (Belgium). Their absorbance was measured at 50°C to determine concentrations using molar extinction coefficients at 260 nm as previously described (20,26). The molar extinction coefficients of phosphorothioate oligomers were assumed to be identical to those for phosphodiester oligonucleotides.

Purified acridine–phosphorothioate oligonucleotide conjugates were also obtained from Eurogentec. Acridine phosphoramidite or acridine-CPG (Glen Research) was used to label the 5′- (23mer Acr–GA-PS) or 3′-end (23mer GA-PS–Acr) of the GA oligophosphorothioate respectively. Before utilization, the acridine–oligonucleotides were purified by gel filtration on G25 Sephadex minicolumns in order to eliminate free acridine contaminant. The quality of acridine–oligonucleotide conjugates was then confirmed by denaturing gel electrophoresis (20% acrylamide) using both the UV shadow effect of the oligonucleotide with irradiation at 254 nm on chromatography paper containing a fluorescent indicator (TLC plastic sheet silica gel 60 F254; Merck) and the fluorescence of the incorporated acridine with irradiation at 365 nm. Dark bands at 254 nm (corresponding to the bases of the oligomers) co-migrated with yellow bands at 365 nm (corresponding to fluorescence of the acridine). With this control assay, the 23mers GA-PS–Acr and Acr–GA-PS exhibited only one band that migrated more slowly than the dark band of the 23mer GA-PS, demonstrating that the phosphorothioate oligonucleotides were 100% linked to the acridine derivative. The concentrations of acridine-containing oligomers were determined using molar extinction coefficients at 425 nm (ε_{425} = 8500 $M^{-1}cm^{-1}$) or at 260 nm (ε_{260} = 85 000 $M^{-1}cm^{-1}$) (26). With purified acridine–oligonucleotide conjugates both determinations gave similar concentrations.

Degradation kinetics of oligonucleotides in tissue culture medium

Oligonucleotide degradation was assessed at 37°C by incubating 10 nM ^{32}P 5′-end-labelled and 1 µM unlabelled 23mers GA-PO or GA-PS in 1 ml Ham’s F-10 culture medium containing 10% fetal calf serum that had first been heat inactivated for 30 min at 60°C. At successive time points, 100 µl of each sample were phenol extracted, ethanol precipitated and electrophoresed on 7 M urea–20% polyacrylamide gels. The extent of degradation of the full-length oligonucleotide was determined by quantitative analysis on a Molecular Dynamics phosphorimager.

Triplex formation studies

Gel retardation assays were performed using the protocols previously described (20,26). Briefly, increasing concentrations of the triplex-forming oligonucleotide were incubated with 10 nM 42mer duplex (5′-end-labelled on the purine-rich strand) under the buffer conditions described in the figure legends. Electrophoresis was performed at 37°C on a non-denaturing 10% polyacrylamide gel containing 10 mM $MgCl_2$ and 50 mM HEPES, pH 7.2.

UV absorption spectroscopy

Melting experiments were performed on a Kontron Uvikon 940 spectrophotometer equipped with a thermoregulated cuvette holder. Samples were heated (or cooled) at a rate of 0.2°C/min with absorbance readings at 260 and 540 nm taken every 5 min. Samples were maintained at each temperature extreme for an

additional 15 min. Absorbance readings at 540 nm were subtracted from readings at 260 nm. Melting temperatures T_m were estimated as the value at which the first derivative of absorbance versus temperature (d*A*/d*T*) reached a maximum.

RESULTS AND DISCUSSION

Oligonucleotides and target

A 23 bp oligopurine–oligopyrimidine sequence located within a 42 bp double-stranded DNA (duplex in Fig. 1) was used as the target sequence to investigate triplex formation with purine-rich phosphorothioates. This 23 bp sequence corresponds to a region of the human insulin-like growth factor I gene (IGF-I) (41). We have previously used this sequence as a target for pyrimidine-rich and purine-rich triplex-forming oligonucleotides (20,24,26). The 23mers GT-PO and GA-PO were unmodified, whereas the 23mer GA-4PS/PO represents a phosphodiester oligodeoxynucleotide 'capped' with two phosphorothioate linkages at each extremity (Fig. 1). The 23mers GA-PS and GT-PS were diastereoisomeric mixtures of phosphorothioate oligomers. A GA-containing oligonucleotide with stereoregular (all Rp) phosphorothioate linkages (23mer GA-PS/R) was synthesized using the enzymatic method previously described (32,42). In the 23mers Acr–GA-PS and GA PS–Acr, an intercalator (2-methoxy-6-chloro-9-amino-acridine) was linked via a linker of six carbons to the 5′- or 3′-phosphate of the diastereoisomeric mixture of (GA)-containing phophorothioate oligonucleotides (Fig. 1).

Table 1. Degradation kinetics of triplex-forming oligonucleotides in cell culture media containing 10% heat-inactivated fetal calf serum (see Materials and Methods)

Oligonucleotides	$t_{1/2}$ (h)
23mer GA-PO	0.75
23mer GA-PS	20
23mer GA-4PS/PO	4.5
23mer GA-PS/R	1.5
23mer GA-PS–Acr	26

Nuclease resistance of (GA)-containing triplex-forming oligonucleotides

In order to check the nuclease resistance of phosphorothioate-containing oligonucleotides, we performed a kinetic assay of oligonucleotide degradation at 37°C in tissue culture medium which is known to contain nucleases. Half-lives ($t_{½}$) for the full-length oligonucleotides are given in Table 1. As expected, the oligonucleotides composed entirely of phosphorothioate linkages (23mers GA-PS and GA-PS–Acr) were the most resistant to nuclease degradation. Capping both extremities of phosphodiester oligonucleotides with phosphorothioate linkages (23mer GA-4PS/PO) increased the nuclease resistance of the oligonucleotide in culture medium as previously described (43). The stereoregular (all Rp) oligonucleotide phosphorothioate, 23mer GA-PS/R, was more stable than unmodified oligophosphodiester (23mer GA-PO), but less stable than the capped oligonucleotide (23mer GA-4PS/PO) or the mixture of diastereoisomers (23mer GA-PS) (Table 1). This nuclease sensitivity of a stereoregular phosphorothioate (all Rp) synthesized via an enzymatic method confirmed results previously described (42).

42 base-pair double-stranded target (duplex):

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5' TAACTTTGCCAGAAGAGGGAGAGAGAGAGAAGGCAAATGTTC  3' 42mer-R1
3' ATTGAAACGGTCTTCTCCCTCTCTCTCTCTTCCGTTTACAAG  5' 42mer-Y1
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23mer triplex-forming oligonucleotides:

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     3' AGAAGAGGGAGAGAGAGAGAAGG 5'  23mer GA-PO
     3' AGAAGAGGGAGAGAGAGAGAAGG 5'  23mer GA-PS

     3' AGAAGAGGGAGAGAGAGAGAAGG 5'  23mer GA-PS/R (all Rp)
     3' AGAAGAGGGAGAGAGAGAGAAGG 5'  23mer GA-4PS/PO

     3' AGAAGAGGGAGAGAGAGAGAAGG-ACR 5'  Acr-23mer GA-PS
3'ACR-AGAAGAGGGAGAGAGAGAGAAGG 5'  23mer GA-PS-Acr

     3' TGTTGTGGGTGTGTGTGTGTTGG 5'  23mer GT-PO
     3' TGTTGTGGGTGTGTGTGTGTTGG 5'  23mer GT-PS
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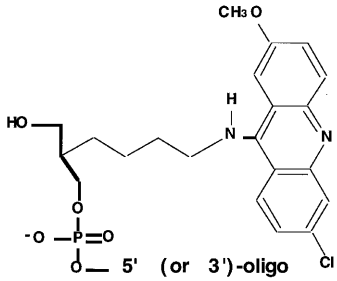

Figure 1. Sequences of triplex-forming oligonucleotides and their double-stranded target. The duplex fragment 42mer R1/42mer Y1 corresponds to a 42 bp sequence of the human IGF-1 gene with the polypurine–polypyrimidine region in bold (26). All the (GA)- and (GT)-containing 23mer oligonucleotides used in this report were designed to bind in an antiparallel orientation relative to the purine strand of the duplex. The 23mers GA-PS and GT-PS correspond to diastereoisomeric mixtures of phosphorothioates. All diastereoisomeric phosphorothioate linkages are underlined. The 23mer GA-4PS/PO is an oligophosphodiester capped with two phosphorothioate linkages at both extremities. The 23mer GA-PS/R (all Rp) corresponds to an oligonucleotide containing stereoregular (all Rp) phosphorothioate linkages (underlined with a dotted line). The acridine derivative was linked to 23mer GA phosphorothioates via their 5′- or 3′-end, giving 23mers Acr–GA-PS and GA-PS–Acr respectively. The chemical structure of the linker used in acridine–oligonucleotide conjugates is shown.

Triplex formation with (GA)-containing but not with (GT)-containing oligophosphorothioates

Figure 2A–C illustrates the triplex binding affinity of 23mers GA-PO and GT-PO and their phosphorothioate counterparts, GA-PS and GT-PS. In the presence of 100 mM NaCl and 10 mM $MgCl_2$ and after 48 h incubation, the 23mers GA-PO and GT-PO were bound to their duplex target at 37°C with apparent dissociation constants (K_d) of 0.6 and 5 µM respectively (Fig. 2A and C) (20). As previously described (20,26), both oligonucleotides bind to their target sequence in an antiparallel orientation. Both diastereoisomeric mixtures of phosphorothioate 23mers GA-PS and GT-PS were analysed for their triplex binding capability. Only the (GA)-containing oligophosphorothioate, 23mer GA-PS, formed a triple-helical complex with a higher dissociation constant than the 23mer GA-PO (Fig. 2B and Table 2) (the K_d for 23mer GA-PS was ~25 µM). The 23mer GT-PS did not form a triplex even at 50 µM (Fig. 2C). The same result was obtained under a variety of experimental conditions (the presence of sodium, potassium or spermine) and when the 23mer GT-PS

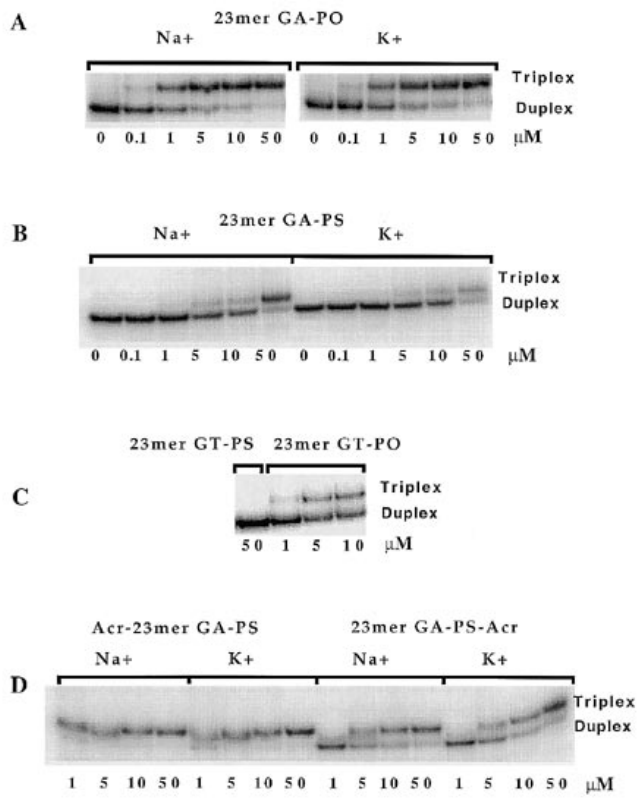

Figure 2. Gel retardation assay comparing triplex binding at 37°C of (GA)- and (GT)-containing oligophosphodiesters and oligophosphorothioates conjugated (or not) to an acridine via their 5′- or 3′-end to the 42mer R1/Y1 duplex. 10 nM radiolabelled double-stranded target and increasing concentrations of triplex-forming oligonucleotides, 23mer GA-PO (**A**), 23mer GA-PS (**B**), 23mer Acr–GA-PS and 23mer GA-PS–Acr (**D**) were first incubated for 48 h in 10 mM $MgCl_2$, 0.5 µg/µl tRNA, 50 mM HEPES, pH 7.2, 10% sucrose and 100 mM NaCl (Na^+) or 140 mM KCl (K^+). The 23mers GT-PO and GT-PS (**C**) were incubated for 48 h in the sodium-containing buffer. Samples were then analysed by electrophoresis at 37°C in a running buffer containing 10 mM $MgCl_2$ and 50 mM HEPES, pH 7.2.

was targeted to a duplex with reverse orientation. In the few examples described in the literature, triplex binding affinities of GT phosphorothioates and GT phosphodiesters were found to be quite similar (32,37). In these two reported examples, GT-rich oligonucleotides were targeted to a polypurine sequence that contained guanine blocks (three or four guanines). In contrast, our sequence has a repetition of GpA (6-fold) that could be unfavourable for GT oligonucleotide binding (the K_d for 23mer GT-PO was higher than for 23mer GA-PO; 20) and even less favourable for GT phosphorothioates.

The stereoregular (all Rp) oligophosphorothioate (23mer GA-PS/R) did not form a triple-helical complex when assayed at 10 µM under the conditions described in Figure 2B for 23mer GA-PS (data not shown). The (GA)-containing oligonucleotide capped with phosphorothioate linkages, 23mer GA-4PS/PO, exhibited the same binding affinity for duplex as the all-phosphodiester oligonucleotide, 23mer GA-PO. The apparent dissociation constant of 23mer GA-4PS/PO was 0.8 µM after 48 h incubation, while that of 23mer GA-PO was 0.6 µM (Table 2). These results suggest that two phosphorothioate linkages at both ends do not significantly destabilize triple-helical complexes, as previously observed with pyrimidine-rich oligonucleotides (33,34).

Table 2. Apparent dissociation constants (K_d) of triplex-formation with oligonucleotides

Oligonucleotide ligands	Apparent K_d ($\times 10^6$ M)	
Monovalent cation	Na^+	K^+
23mer GA-PO	0.6	1.6
23mer GA-PS	25	34
23mer GA-4PS/PO	0.8	–
23merGA-PS–Acr	4.8	6
Acr-23mer GA-PS	0.65	0.7

Apparent K_d shown in this table was estimated as the added oligonucleotide ligand concentration which dispatched 50% of the radiolabeled target (42mer-R1/Y1) from a double-stranded to a triple-stranded state based upon gel mobility assays performed after an incubation at 37°C for 48 h in the presence of 20 mM HEPES pH 7.2, 10 mM $MgCl_2$ and 100 mM NaCl (Na^+) or 140 mM KCl (K^+).

In all the experiments described in Figures 2 and 3, the gel retardation assays were performed with a labelled duplex at 10 nM concentration and increasing concentrations of third strand oligonucleotide. Due to self-association of (GA)-containing oligonucleotides (see below and ref. 20), we also tried to use a radiolabelled third strand at nanomolar concentration and increasing concentrations of cold duplex, as previously reported for (GA)-containing oligophosphodiesters (20). However, in the presence of magnesium ions, the GA-PS oligonucleotides generated multiple bands, probably related to interactions with Mg^{2+} within the polyacrylamide gels, and this precluded any analysis of triplex formation. In the absence of magnesium ions, when these migration problems did not occur, no triplex was formed, as previously observed (20).

It should be noted that absorption spectroscopy could not be used to study triplex formation because no hypochromism seems to be associated with triplex formation for (GA)-containing oligonucleotides, i.e. no change in absorbance is observed even when triplex is detected by other methods, such as gel shift assays. This property has been previously described for other sequences (44). This might be ascribed to the high stacking efficiency of (GA)-containing oligonucleotides, which might not change measurably when triplex is formed. In several cases the transitions observed in melting curves of triplexes involving (GA)-containing oligonucleotides are due to melting of self-associated structures of the third strand oligonucleotide rather than to triplex dissociation (see below and Arimondo *et al.*, manuscript in preparation).

Different gel mobilities of triple helical complexes

Figure 3 shows the different gel mobilities of triple-helical complexes formed with modified oligonucleotides. The length of the oligonucleotides used in these experiments was checked on denaturing gel electrophoresis (data not shown). All the pyrimidine-rich oligodeoxynucleotides, 23mers TC, TC* (with 5-methylcytosines replacing cytosines) and α-TC (α anomer of 23mer TC) formed triple-helical complexes that exhibited the same gel mobility on a 10% polyacrylamide gel at pH 6 (Fig. 3). This

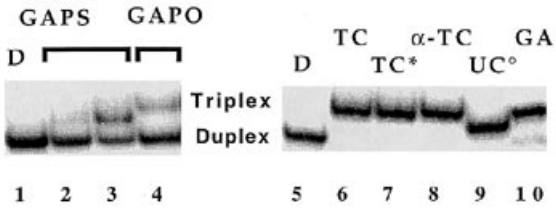

Figure 3. Triplex-forming oligonucleotides with different structures form triple helical complexes that exhibit differences in gel migration. Incubation was performed overnight at 37°C with 10 nM radiolabelled duplex target and triplex-forming oligonucleotides in a buffer containing 0.1 M NaCl, 0.5 µg/µl tRNA, 20 mM HEPES, pH 7.2, 10 mM $MgCl_2$, 10% sucrose (lanes 1–4) or 0.1 M NaCl, 50 mM phosphate buffer, pH 6, 5 mM $MgCl_2$, 0.5 mM spermine, 0.5 µg/µl tRNA, 10% sucrose (lanes 5–10). Gel electrophoresis was performed at 37°C in 50 mM HEPES, pH 7.2, 10 mM $MgCl_2$ (lanes 1–4) or 50 mM MES, pH 6, 10 mM $MgCl_2$ (lanes 5–10). The purine-rich oligonucleotide used in lanes 2 and 3 was 23mer GA-PS (5 and 10 µM), with 23mer GA-PO (0.1 µM) in lane 4. Pyrimidine-rich oligonucleotides (all at 50 µM) in lanes 6–9 are 23mers previously studied for their triplex-forming capabilities (20,24,26). TC is a 23mer containing thymines and cytosines that is designed to bind in a parallel orientation with respect to the purine strand of the duplex target (lane 6) (24). TC* has the same sequence as the 23mer TC but cytosine is replaced by 5-methylcytosine (lane 7). α-TC corresponds to the α anomer analogue of 23mer TC but synthesized in reverse orientation (lane 8). α-TC binds to the duplex in an antiparallel orientation with respect to the purine strand of the target (26). UC° is a 2′-*O*-methyl-UC oligonucleotide with the same binding target as the oligodeoxyribonucleotide 23mer TC (lane 9) (24). Lane 10 is the 23mer GA-PO at 10 µM (20). In lanes 1 and 5, D corresponds to the radiolabeled 42mer R/Y duplex alone.

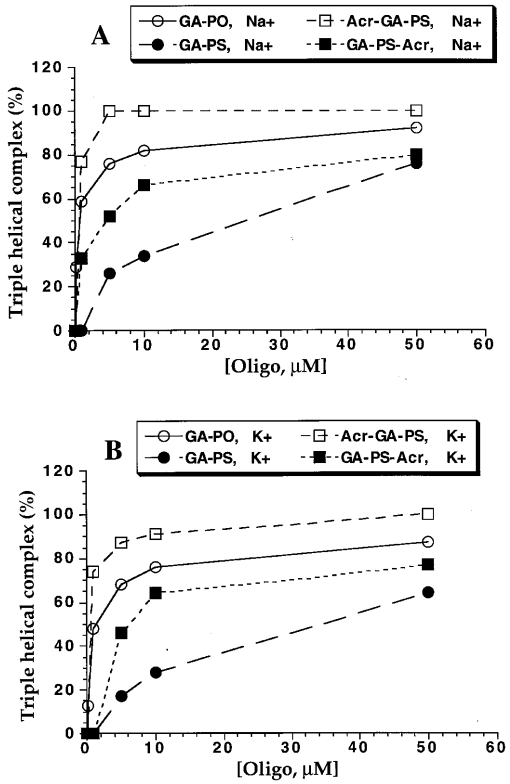

Figure 4. Binding curves of GA-containing oligonucleotides with the target duplex. They were obtained using data from electrophoretic mobility shift titrations shown in Figure 2. All samples were incubated for 48 h at 37°C in a buffer containing 50 mM HEPES, pH 7.2, 0.1 µg/µl tRNA, 10% sucrose, 10 mM $MgCl_2$ with 100 mM NaCl (Na^+) or 140 mM KCl (K^+). Electrophoresis was performed at 37°C in 50 mM HEPES, pH 7.2, 10 mM $MgCl_2$. The extent of formation of triple helical complex was determined by quantitative analysis with a Molecular Dynamics phosphorimager.

mobility also corresponds to the gel mobility observed for the triplexes formed with (GA)- and (GT)-containing oligonucleotides. The GA phosphorothioate and the 2′-*O*-methyl-UC oligoribonucleotide formed triple-helical complexes that migrated faster on non-denaturing 10% polyacrylamide gel (compare lanes 3 and 9 to the other lanes in Fig. 3). This peculiar behaviour should be taken into account when analysing triplex binding capabilities of different oligonucleotides.

(GA)-containing oligophosphorothioate–acridine conjugates as triplex-forming oligonucleotides

The covalent linking of an intercalating agent, such as an acridine derivative, to the 5′- or 3′-end of an oligonucleotide increases its binding affinity toward either single-stranded (45) or double-stranded targets (2,46–49). When the target is double-stranded DNA, stabilization occurs by intercalation of the acridine ring at the triplex/duplex junction (2,46).

In order to stabilize the triple-helical complexes obtained with 23mer GA-PS, an acridine derivative was covalently linked to the (GA)-containing oligophosphorothioate at either its 5′- (23mer Acr–GA-PS) or 3′-end (23mer GA-PS–Acr). It was noticeable that the acridine-linked oligophosphorothioates exhibited the same gel mobility for their triple-helical complexes as those formed by oligophosphorothioates (Fig. 2). After 48 h incubation at 37°C with 140 mM KCl, the apparent dissociation constant of 23mer Acr–GA-PS was 0.7 µM, while the apparent dissociation constants of 23mers GA-PO and GA-PS were 1.6 and 34 µM respectively (Table 2). Linking of acridine to the 5′-end of the GA phosphorothioate increased the binding affinity of the oligonucleotide to the duplex target by 40- to 50-fold (Table 2 and Fig. 4). Less stabilization was provided when the acridine was attached to the 3′-end (23mer GA-PS–Acr). A 5- to 6-fold increase in stability was observed (Table 2). In the presence of 140 mM KCl at 37°C the affinity of 5′-substituted 23mer Acr–GA-PS was about twice as high as that of 23mer GA-PO.

These results demonstrated that covalent linkage of an acridine to oligophosphorothioates increased the stability of triple-helical complexes but also that the additional stability depended on the position of the acridine. The acridine tethered to the 5′-end of the GA phosphorothioate gave better affinity than that tethered to the 3′-end via the same linker. The length of the linker might be appropriate for good intercalation when acridine is linked to the 5′-end of the GA-PS oligonucleotide, but not when acridine is linked to the 3′-end. Further work is needed to optimize the linker tethering an intercalating agent to a (GA)-containing oligonucleotide.

Kinetics of binding of oligophosphorothioates on target duplex

The kinetics of triple helix formation by the oligophosphorothioates and the 42mer duplex were investigated by gel shift assay. The extent of triple helix formation was determined after incubation of the duplex target with 10 or 50 µM oligonucleotide at 37°C for 2, 24 and 48 h (Fig. 5). After 2 h incubation, the 23mer

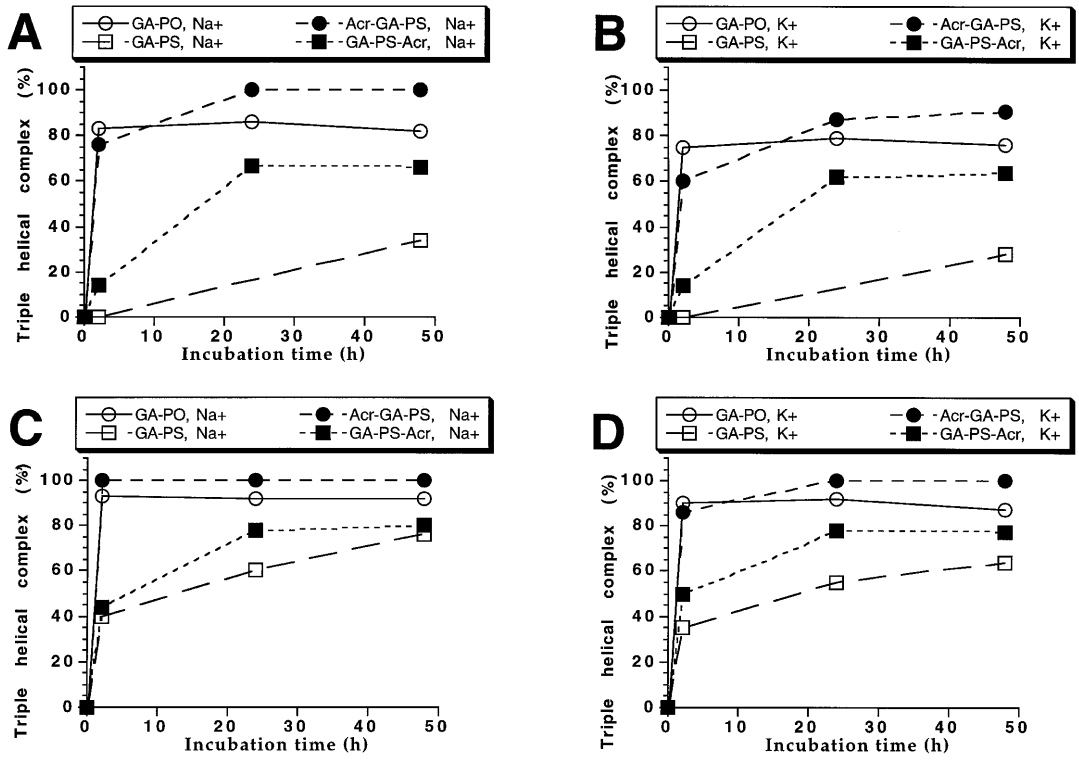

Figure 5. Kinetic studies of triplex formation with GA-phosphodiester and GA-phosphorothioate oligonucleotides. Triplex-forming oligonucleotides [10 (**A** and **B**) or 50 µM (**C** and **D**)] were incubated with radiolabelled duplex target for 2, 24 and 48 h in the buffer conditions described in Figure 2 [with 100 mM NaCl (**A** and **C**) or 140 mM KCl (**B** and **D**)] and then analysed by gel shift assay. The extent of triplex formation was determined and plotted versus the incubation time.

GA-PO at 50 µM was completely bound to the duplex, whereas only 40% of the 23mer GA-PS at the same concentration was bound (Fig. 5C). After 48 h and even after 72 h (data not shown), we never observed complete formation of triplex with the 23mer GA-PS at 50 µM, suggesting the existence of secondary structures or aggregates that trapped the oligophosphorothioate and competed for triple-helix formation.

The 23mer GA-PS–Acr behaved as the 23mer GA-PS and bound to the duplex more slowly than the (GA)-containing oligophosphodiester, 23mer GA-PO. After 2 h incubation, only 40% of 23mer GA-PS–Acr at 50 µM was bound to the duplex (Fig. 5C). Nevertheless, after 72 h, 80% of the oligonucleotide was bound (data not shown), suggesting that either the acridine derivative increased the binding affinity and the association kinetics or the structure formed by 23mer GA-PS was destabilized with 23mer GA-PS–Acr.

When the acridine was covalently linked to the 5′-end of the GA phosphorothioate, the complexes formed more rapidly than with the unmodified 23mer GA-PS. The 23mer Acr–GA-PS at 50 µM was completely bound to duplex DNA after 2 h incubation (Fig. 5B), emphasizing the importance of the position of the acridine in the oligophosphorothioate for triplex formation.

Kinetics of triplex formation were analysed when oligonucleotides were incubated with 140 mM K^+ or 100 mM Na^+ monocations (Fig. 5A–D). The replacement of sodium cations (at 100 mM) by potassium cations (at 140 mM) slightly decreased the binding affinity of (GA)-containing oligophosphodiesters and oligophosphorothioates for the duplex target (Table 2 and Fig. 5). This destabilizing role of potassium ions has been previously described in the literature for guanine-rich (37,50,51) and pyrimidine-rich, triplex-forming oligonucleotides (52). No such effect was seen for the 5′-acridine-substituted oligonucleotide.

(GA)-containing oligophosphorothioates self-associate and form homoduplexes

UV absorption spectroscopy was used to determine if GA oligophosphorothioates form homoduplexes in solution that could compete with triplex formation, as previously observed with the 23mer GA-PO (20). The GA-containing oligophosphodiester, 23mer GA-PO, forms homoduplex structures that could be monitored via melting experiments (20). Figure 6 shows the melting curves obtained in the presence of 10 mM $MgCl_2$ and 100 mM NaCl with the oligophosphodiester 23mers GA-PO and GA-4PS/PO and the oligophosphorothioate 23mers GA-PS and Acr–GA-PS. All curves were reversible with no apparent hysteresis. The structures of 23mer GA-PO and the phosphorothioate-capped 23mer GA-4PS/PO had similar T_m values (Table 3), suggesting that the presence of phosphorothioate linkages at both extremities in 23mer GA-4PS/PO did not interfere with homoduplex formation. The (GA) repeats, which are responsible for homoduplex formation (20,53,54), keep a phosphodiester backbone in the 23mer GA-4PS/PO.

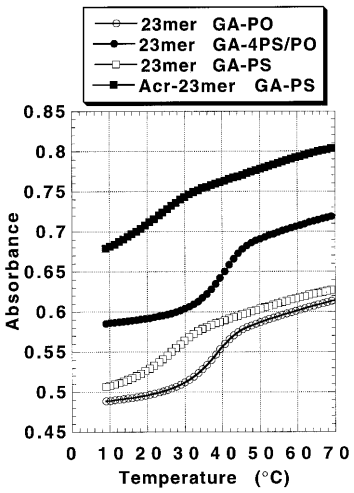

Figure 6. Self-association of purine-rich oligophosphorothioates analysed by UV absorption spectroscopy. Samples contained 10 mM $MgCl_2$, 100 mM NaCl, 20 mM sodium cacodylate, pH 7.2, with 2 µM 23mers GA-PO (open circle), GA-PO/4PS (filled circle), GA-PS (open square) and Acr–GA-PS (filled square).

Table 3. Melting temperatures of self-associated structures formed by GA-phosphodiester and GA-phosphorothioate-containing oligonucleotides

Oligonucleotides	T_m (Na^+) (°C)	T_m (K^+) (°C)
23mer GA-PO	37 ± 1	35 ± 1
23mer GA-PS	24 ± 1	25 ± 1
23mer GA-4PS/PO	38 ± 1	38 ± 1
23mer Acr–GA-PS	24 ± 1	23 ± 1
23mer GA-PS–Acr	26 ± 1	27 ± 1
23mer GT-PS	NA	–

All samples contained 2 µM oligonucleotide, 10 mM sodium cacodylate, pH 7.2, 10 mM $MgCl_2$ plus 100 mM NaCl (Na^+) or 140 mM KCl (K^+).
NA, no transition detected.

The 23mer GA-PS and the acridine-containing oligophosphorothioates exhibited self-associated structures that were less stable than that of the 23mer GA-PO. The T_m values at 2 and 10 µM for the 23mer GA-PS incubated under the conditions described in Figure 6 were 24 and 26°C respectively (data not shown). These results indicate that phosphorothioate d(A,G) repeats present in the oligonucleotides could form self-associated structures, but with less stability as compared with the oligophosphodiester.

Similar observations were also made with oligonucleotides with other modified sugar–phosphate backbones. (GA)-containing α-oligonucleotides with d(GA) repeats form homoduplex structures that are less stable than those formed with β-oligonucleotides (26). It was recently shown that a $d(GA)_8$ oligomer with a methylphosphonate backbone was also unable to form homoduplexes (40), in contrast to the $d(GA)_8$ oligophosphodiester.

Covalent linkage of an acridine derivative to the 5′- or 3′-end of the oligophosphorothioate did not markedly change the T_m value of the self-associated structures, indicating that the presence of acridine did not increase the stability of the self-associated structures (Table 3). As shown in Table 3, the structures of oligophosphorothioates and oligophosphodiesters monitored by UV spectroscopy were not changed in the presence of potassium ions. In order to analyse the behaviour of the complexes in the presence of multivalent cations, we performed melting experiments with the same oligonucleotides in the presence of 0.2 mM spermine and 10 mM $MgCl_2$ (data not shown). Under these conditions the self-associated structures of 23mers GA-PO and GA-4PS/PO were stabilized as previously observed (20), whereas the phosphorothioate-containing oligonucleotides precipitated when cooling the cuvettes.

No melting transition could be observed with (GT)-containing oligophosphodiesters and oligophosphorothioates (Table 3), suggesting that the transition observed with GA-containing oligonucleotides was due to the presence of (GA) repeats, as previously described (20,26).

CONCLUSION

We have shown in this report that nuclease-resistant (GA)-containing oligophosphorothioates are capable of triplex formation. The triple-helical complexes formed with the GA phosphorothioates are less stable than those formed with GA phosphodiesters. In order to increase the binding affinity of triplex-forming oligonucleotides, an intercalating agent can be attached to the oligonucleotides. Here we have demonstrated that covalent linking of acridine to the GA phosphorothioate oligonucleotide stabilized the triplex. Stabilization depended on the position of the acridine within the oligonucleotide. Acridine linked to its 5′-end increased stability of the triplex much more than when it was linked to the 3′-end with the same linker. 5′-Linked acridine–GA phophorothioate exhibited an apparent association constant slightly higher than the unmodified GA phosphodiester. We have also demonstrated that GA phosphorothioates containing (GA) repeats formed self-structures, as did their GA phosphodiester counterparts. These self-structures are less stable than those formed by GA phosphodiesters and could not fully explain the lower binding affinity for triplex formation as compared with the GA phosphodiester. The fact that we never observed complete formation of triplex with the 23mer GA-PS suggested the existence of other structures than those detected by UV spectroscopy. Further experiments should give more information about these unidentified structures.

Contrary to other published reports, (GT)-containing phosphorothioates were completely unable to bind to duplex DNA via triple helix formation under a variety of experimental conditions. One possible explanation of our results is the presence of alternating d(GA) repeats and the absence of long stretches of guanines in the sequence that we have investigated.

An alternative to fully modified triplex-forming oligonucleotides could be the use of capped oligonucleotides either in the pyrimidine (31,33,34) or in the purine motif, provided that these capped oligonucleotides exhibit exonuclease resistance and are not completely degraded by endonucleases. Unmodified (GA)- and (GT)-containing, triplex-forming oligophosphodiesters have already been used to inhibit gene expression in cellular systems via triplex formation. Non-specific effects have recently been

described for antisense phosphorothioates that contain long stretches of guanines (55,56). It should be noted that if GA-rich phosphorothioates are used as triplex-forming oligomers, appropriate controls should be designed to clearly demonstrate the specificity of the observed effect. To conclude, this report adds to the growing database of non-natural oligonucleotides that can be used to form triplexes. General rules to decide which kind of modified oligonucleotides should be chosen are difficult to draw at the present stage, as the sequence context always plays a crucial role in triplex formation.

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REFERENCES

1 Hélène,C. and Toulmé,J.J. (1990) *Biochim. Biophys. Acta*, **1049**, 99–125.
2 Thuong,N.T. and Hélène,C. (1993) *Angew. Chem. Int. Edn English*, **32**, 666–690.
3 Cooney,M., Czernuszewicz,G., Postel,E.H., Flint,S.J. and Hogan,M.E. (1988) *Science*, **241**, 456–459.
4 Postel,E.H., Flint,S.J., Kessler,D.J. and Hogan,M.E. (1991) *Proc. Natl. Acad. Sci. USA*, **88**, 8227–8231.
5 Grigoriev,M., Praseuth,D., Robin,P., Hemar,A., Saison-Behmoaras,T., Dautry-Varsat,A., Thuong,N.T., Hélène,C. and Harel-Bellan,A. (1992) *J. Biol. Chem.*, **267**, 3389–3395.
6 Grigoriev,M., Praseuth,D., Guieysse,A.L., Robin,P., Thuong,N.T., Hélène,C. and Harel-Bellan,A. (1993) *Proc. Natl. Acad. Sci. USA*, **90**, 3501–3505.
7 Grigoriev,M., Praseuth,D., Guieysse,A.L., Robin,P., Thuong,N.T., Hélène,C. and Harel-Bellan,A. (1993) *C.R. Acad. Sci. Paris Life Sci.*, **316**, 492–495.
8 Ing,N.H., Beekman,J.M., Kessler,D.J., Murphy,M., Jayaraman,K., Zendegui,J.G., Hogan,M.E., O'Malley,B.W. and Tsai,M.J. (1993) *Nucleic Acids Res.*, **21**, 2789–2796.
9 Young,S.L., Krawczyk,S.H., Matteucci,M.D. and Toole,J.J. (1991) *Proc. Natl. Acad. Sci. USA*, **88**, 10023–10026.
10 Duval-Valentin,G., Thuong,N.T. and Hélène,C. (1992) *Proc. Natl. Acad. Sci. USA*, **89**, 504–508.
11 Escudé,C., Giovannangéli,C., Sun,J., Lloyd,D., Chen,J., Gryaznov,S., Garestier,T. and Hélène,C. (1996) *Proc. Natl. Acad. Sci. USA*, **93**, 4365–4369.
12 Samadashwily,G.M., Dayn,A. and Mirkin,S. (1993) *EMBO J.*, **12**, 4975–4983.
13 Samadashwily,G. and Mirkin,S. (1994) *Gene*, **149**, 127–136.
14 Giovannangéli,C., Thuong,N.T. and Hélène,C. (1993) *Proc. Natl. Acad. Sci. USA*, **90**, 10013–10017.
15 Guieysse,A.L., Praseuth,D., François,J.C. and Hélène,C. (1995) *Biochem. Biophys. Res. Commun.*, **217**, 186–194.
16 Hacia,J.G., Dervan,P.B. and Wold,B.J. (1994) *Biochemistry*, **33**, 6192–6200.
17 Durland,R.H., Kessler,D.J., Gunnell,S., Duvic,M., Pettitt,B.M. and Hogan,M.E. (1991) *Biochemistry*, **30**, 9246–9255.
18 Pilch,D.S., Levenson,C. and Shafer,R.H. (1991) *Biochemistry*, **30**, 6081–6087.
19 Beal,P.A. and Dervan,P.B. (1991) *Science*, **251**, 1360–1363.
20 Noonberg,S.B., François,J.C., Garestier,T. and Hélène,C. (1995) *Nucleic Acids Res.*, **23**, 1956–1963.
21 Sun,J.S., De Bizemont,T., Duval-Valentin,G., Montenay-Garestier,T. and Hélène,C. (1991) *C.R. Acad. Sci. Paris Sér. III*, **313**, 585–590.
22 De Bizemont,T., Duval-Valentin,G., Sun,J.-S., Bisagni,E., Garestier,T. and Hélène,C. (1996) *Nucleic Acids Res.*, **24**, 1136–1143.
23 Shimizu,M., Konishi,A., Shimada,Y., Inoue,H. and Ohtsuka,E. (1992) *FEBS Lett.*, **302**, 155–158.
24 Escudé,C., François,J.C., Sun,J.S., Ott,G., Sprinzl,M., Garestier,T. and Hélène,C. (1993) *Nucleic Acids Res.*, **21**, 5547–5553.
25 Liquier,J., Letellier,R., Dagneaux,C., Ouali,M., Morvan,F., Raynier,B., Imbach,J.L. and Taillandier,E. (1993) *Biochemistry*, **32**, 10591–10598.
26 Noonberg,S.B., François,J.C., Praseuth,D., Guieysse-Peugeot,A.L., Lacoste,J., Garestier,T. and Hélène,C. (1995) *Nucleic Acids Res.*, **23**, 4042–4049.
27 Sun,J.S. and Lavery,R. (1992) *J. Mol. Recognition*, **5**, 93–98.
28 Kibler-Herzog,L., Kell,B., Zon,G., Shinozuka,K., Mizan,S. and Wilson,W.D. (1990) *Nucleic Acids Res.*, **18**, 3545–55.
29 Kibler-Herzog,L., Zon,G., Whittier,G., Mizan,S. and Wilson,W.D. (1993) *Anti-Cancer Drug Design*, **8**, 65–79.
30 Latimer,L.J.P., Hampel,K. and Lee,J.S. (1989) *Nucleic Acids Res.*, **17**, 1549–1561.
31 Kim,S.-G., Tsukahara,S., Yokoyama,S. and Takaku,H. (1992) *FEBS Lett.*, **314**, 29–32.
32 Hacia,J.G., Wold,B.J. and Dervan,P.B. (1994) *Biochemistry*, **33**, 5367–5369.
33 Xodo,L., Alunni-Fabbroni,M., Manzini,G. and Quadrifoglio,F. (1994) *Nucleic Acids Res.*, **22**, 3322–3330.
34 Alluni-Fabbroni,M., Manfioletti,G., Manzini,G. and Xodo,L.E. (1994) *Eur. J. Biochem.*, **226**, 831–839.
35 Tsukahara,S., Kim,S. and Takaku,H. (1993) *Biochem. Biophys. Res. Commun.*, **196**, 990–996.
36 Reynolds,M.A., Arnold,L.J., Almazan,M.T., Beck,T.A., Hogrefe,R.I., Metzler,M.D., Stoughton,S.R., Tseng,B.Y., Trapane,T.L., Ts'o,P.O.P. and Woolf,T.M. (1994) *Proc. Natl. Acad. Sci. USA*, **91**, 12433–12437.
37 Musso,M. and Van Dyke,M.W. (1995) *Nucleic Acids Res.*, **23**, 2320–2327.
38 Tu,G., Cao,Q. and Israel,Y. (1995) *J. Biol. Chem.*, **270**, 28402–28407.
39 Svinarchuk,F., Debin,A., Bertrand,J.-R. and Malvy,C. (1996) *Nucleic Acids Res.*, **24**, 295–302.
40 Trapane,T.L., Hogrefe,R.I., Reynolds,M.A., Kan,L. and Ts'o,P.O.P. (1996) *Biochemistry*, **35**, 5495–5508.
41 Kim,S., Lajara,R. and Rotwein,P. (1991) *Mol. Endocrinol.*, **5**, 1964–1972.
42 Tang,J., Roskey,A., Li,Y. and Agrawal,S. (1995) *Nucleosides Nucleotides*, **14**, 985–990.
43 Ghosh,M.K., Ghosh,K. and Cohen,J.S. (1993) *Anti-Cancer Drug Design*, **8**, 15–32.
44 Jetter,M.C. and Hobbs,F.W. (1993) *Biochemistry*, **32**, 3249–3254.
45 Asseline,U., Toulmé,F., Thuong,N.T., Delarue,M., Montenay-Garestier,T. and Hélène,C. (1984) *EMBO J.*, **3**, 795–800.
46 Sun,J.S., François,J.C., Montenay-Garestier,T., Saison-Behmoaras,T., Roig,V., Thuong,N.T. and Hélène,C. (1989) *Proc. Natl. Acad. Sci. USA*, **86**, 9198–9202.
47 Collier,D.A., Thuong,N.T. and Hélène,C. (1991) *J. Am. Chem. Soc.*, **113**, 1457–1458.
48 Orson,F.M., Kinsey,B.M. and McShan,W.M. (1994) *Nucleic Acids Res.*, **22**, 479–484.
49 Fox,K.R. (1995) *FEBS Lett.*, **357**, 312–316.
50 Olivas,W.M. and Maher,L.J. (1995) *Nucleic Acids Res.*, **23**, 1936–1941.
51 Olivas,W.M. and Maher,L.J. (1995) *Biochemistry*, **34**, 278–284.
52 Singleton,S.F. and Dervan,P.B. (1993) *Biochemistry*, **32**, 13171–13179.
53 Rippe,K., Fritsch,V., Westhof,E. and Jovin,T.M. (1992) *EMBO J.*, **11**, 3777–3786.
54 Evertsz,E.M., Rippe,K. and Jovin,T.M. (1994) *Nucleic Acids Res.*, **22**, 3293–3303.
55 Rando,R.F., Ojwang,J., Elbaggari,A., Reyes,G.R., Tinder,R., McGrath,M.S. and Hogan,M.E. (1995) *J. Biol. Chem.*, **270**, 1754–1760.
56 Guvakova,M.A., Yabukov,L.A., Vlodavsky,I., Tonkinson,J.L. and Stein,C.A. (1995) *J. Biol. Chem.*, **270**, 2620–2627.