Potassium-resistant triple helix formation and improved intracellular gene targeting by oligodeoxyribonucleotides containing 7-deazaxanthine

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Received September 4, 1996; Revised and Accepted December 3, 1996

ABSTRACT

Triple helix formation by purine-rich oligonucleotides in the anti-parallel motif is inhibited by physiological concentrations of potassium. Substitution with 7-deazaxanthine (c\textsuperscript{7}X) has been suggested as a strategy to overcome this effect. We have tested this by examining triple helix formation both \textit{in vitro} and \textit{in vivo} by a series of triple helix-forming oligonucleotides (TFOs) containing guanine plus either adenine, thymine, or c\textsuperscript{7}X. The TFOs were conjugated to psoralen at the 5\textquotesingle end and were designed to bind to a portion of the \textit{supF} mutation reporter gene. Using \textit{in vitro} gel mobility shift assays, we found that triplex formation by the c\textsuperscript{7}X-substituted TFOs was relatively resistant to the presence of 140 mM K\textsuperscript{+}. The c\textsuperscript{7}X-containing TFOs were also superior in gene targeting experiments in mammalian cells, yielding 4- to 5-fold higher mutation frequencies in a shuttle vector-based mutagenesis assay designed to detect mutations induced by third strand-directed psoralen adducts. When the phosphodiester backbone was replaced by a phosphorothioate one, the \textit{in vitro} binding of the c\textsuperscript{7}X-substituted TFOs was not affected, but the efficiency of \textit{in vivo} triple helix formation was reduced. These results indicate the utility of the c\textsuperscript{7}X substitution for \textit{in vivo} gene targeting experiments, and they show that the feasibility of the triplex anti-gene strategy can be significantly enhanced by advances in nucleotide chemistry.

INTRODUCTION

Triple helix-forming oligonucleotides (TFOs) bind in the major groove of duplex DNA containing polypurine/polypyrimidine stretches and form hydrogen bonds with the target homopurine sequence (1–3). Pyrimidine-rich TFOs bind parallel to the purine strand of the duplex and form T:A:T and G:G:C base triplets by reverse Hoogsteen hydrogen bonding (4). Due to the requirement for protonation of cytosines, the pyrimidine-rich motif requires a slightly acidic pH, and therefore its use under physiological conditions has been limited (1). Recently, novel cytosine analogs have been synthesized which reduce the pH dependence, but these have not yet been tested in cells (5). In contrast, triple helix formation by purine-rich TFOs is pH-independent, and for this reason they have been more frequently used for \textit{in vivo} applications (6–8).

Nevertheless, several reports have shown that triplex formation by guanine-rich oligonucleotides is diminished by physiological concentrations of monovalent cations, particularly K\textsuperscript{+} (9,10). This inhibition has been attributed, in part, to the aggregation of the purine-rich oligonucleotides due to the formation of guanine quartets (11,12), potentially restricting the effectiveness of purine-rich TFOs as anti-gene reagents.

To overcome this K\textsuperscript{+} effect, several strategies have been employed. In one, some of the G residues within a G-rich TFO were replaced with 6-thioguanine (10,13,14). Although this modification reduces G-quartet formation, it also lessens the overall binding affinity of the third strand. Similarly, the replacement of the N-7 of guanine with carbon, creating 7-deazaguanine, eliminates the ability of the TFO to form G-quartets but also decreases the capacity of the oligonucleotide to form triple helices (15). However, TFOs containing 7-deazaxanthine (c\textsuperscript{7}X) in place of A or T in the anti-parallel triple helix motif (potentially forming c\textsuperscript{7}X:A:T triplets), have shown high third strand binding affinity in \textit{in vitro} experiments under physiological K\textsuperscript{+} and Mg\textsuperscript{++} concentrations and at pH 7.2 (15). The c\textsuperscript{7}X substitution therefore appeared promising in \textit{in vitro} assays, but its effectiveness within cells remained to be determined.

In this study, we have examined the utility of oligonucleotides containing the c\textsuperscript{7}X substitution for anti-parallel triple helix formation both \textit{in vitro} and \textit{in vivo} in gene targeting experiments within mammalian cells. We have carried out a systematic comparison of the binding affinities of a series of modified psoralen-linked, G-rich TFOs to a G:C bp-rich target sequence under physiological K\textsuperscript{+} concentrations. In addition, we have

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Psalen-linked triplex-forming oligonucleotides, pso-AG13, pso-TG13 and pso-TG13(S) (Fig. 1) were synthesized at Gilead as described previously (15). The c'X is incorporated into the oligonucleotides as a phosphorothioate group at the 3' end for exonuclease resistance (16). TFOs with this backbone have previously been shown to form triple helices in the presence of the above buffer containing 140 mM KCl. UVA irradiation (1.8 J/cm² of broad band UVA light centered at 365 nm, irradiance of 5 mW/cm²) was used to generate photodadducts and thereby covalently link the oligomers to their targets (20). This dose generates ~60–70% psoralen interstrand cross-links (between the two strands of the target duplex, in addition to the tether connecting the psoralen to the TFO) and 25–35% psoralen monoadducts, in the context of the triple helix (21).

Because another factor for in vivo applications of oligonucleotides is their stability under physiological conditions, we also tested the same series of TFOs with phosphorothioate internucleoside linkages, a modification which has been shown to confer nuclease resistance (16). TFOs with this backbone have previously been shown to form triple helices in the urine anti-parallel motif (17). However, we show that phosphorothioate TFOs are less effective for intracellular gene targeting than are TFOs with a standard phosphodiester backbone carrying a propylamine group at the 3' end of duplex DNA (10⁻¹⁰ M) was incubated with increasing concentrations of the psoralen-linked oligomers in 10 µl of 10 mM Tris (pH 7.4), 1 mM spermidine, and 20 mM MgCl₂ at 37°C for 2 h. The experiment was repeated in the presence of the above buffer containing 140 mM KCl. UVA irradiation (1.8 J/cm² of broad band UVA light centered at 365 nm, irradiance of 5 mW/cm²) was used to generate photodadducts and thereby covalently link the oligomers to their targets (20). This dose generates ~60–70% psoralen interstrand cross-links (between the two strands of the target duplex, in addition to the tether connecting the psoralen to the TFO) and 25–35% psoralen monoadducts, in the context of the triple helix (21).

They were mixed with 90 µl of formamide, and a 20 µl aliquot of each sample was analyzed on an 8% polyacrylamide denaturing gel containing 7M urea. A phosphor-imager (Molecular Dynamics, Sunnyvale, CA) was used for quantitation of the reaction products. The concentration at which triplex formation was half-maximal (as indicated by the generation of specific photodadducts) was taken as the equilibrium dissociation constant (Kₐ) (7,22).

**Gene targeting and mutagenesis protocol**

Monkey COS-7 cells were obtained from the ATCC (1651-CRL). The COS cells at 70% confluence were washed with PBS-EDTA, treated with trypsin, and incubated at 37°C for 5 min. The cells were resuspended and washed 3 times in Dulbecco’s modified Eagle’s medium/10% fetal calf serum. The cells were finally resuspended at 1×10⁶ cells/ml. The plasmid DNAs were added at 3 µg DNA/10⁶ cells and the cell/DNA mixtures were left on ice for 10 min. Transfection of the cells was performed by electroporation using a Bio-Rad gene pulser at a setting of 25 µF/250 W/250 V in the 0.4 cm cuvette. Following electroporation, the cells were kept on ice for 10 min. The cells were diluted with growth medium, washed, and transferred to 37°C for 30 min. At this point, the cells were further diluted and exposed to the oligonucleotides in growth medium while in suspension.

The suspension samples were incubated at 37°C with gentle agitation every 15 min. UVA irradiation was given 2 h later at a dose of 1.8 J/cm² (irradiance of 5 mW/cm²). A window glass filter was used to eliminate contaminating UVB radiation. All samples, including control cells not exposed to oligonucleotides, received UVA irradiation. The cells were further diluted in growth medium and allowed to attach to plastic dishes at a density of 2×10⁴ cells per cm². The cells were harvested 48 h later for vector analysis.

**Shuttle vector isolation and analysis**

The cells were harvested for vector DNA isolation using a modified alkaline lysis procedure. The cells were detached by trypsinization, washed, and resuspended in 100 µl of cell resuspension solution (50 mM Tris–HCl, 10 mM EDTA, pH 8.0;
Figure 2. Gel mobility shift analysis of triplex formation by pso-AG13. The binding affinity of pso-AG13 to its target site in the supFG1 gene was analyzed over a range of oligonucleotide concentrations, as indicated, in (a) the absence and (b) presence of 140 mM KCl in the binding buffer. (c) Graphical comparison of the effect of 140 mM KCl on triplex formation. (MA, monoadduct; XL, interstrand crosslink).

Figure 3. Analysis of triplex formation by pso-AG13(S) in (a) the absence and (b) presence of 140 mM K+ (S) indicates phosphorothioate internucleoside linkages. See Figure 2 legend for additional details.

100 μg/ml RNase A). An equal volume of cell lysis solution (0.2 M NaOH, 1% SDS) was added, followed by 100 μl of neutralization solution (3 M potassium acetate, pH 5.5). A 15 min RT incubation was followed by centrifugation in a microcentrifuge for 10 min. The supernatant was extracted with an equal volume of phenol/chloroform (1:1) once, and the DNA was precipitated with 2.5 volumes of ethanol at −70°C for 10 min. The DNA was collected by centrifugation for 10 min, washed with 70% ethanol once, and allowed to air dry for 5 min at RT. The DNA was digested with DpnI and RNase A at 37°C for 2 h, extracted with phenol/chloroform, and precipitated with ethanol. The DNA pellet was dissolved in 10 μl of TE buffer, and 1 μl of the sample of vector DNA was used to transform Escherichia coli SY204 [lacZamber] (23) by electroporation (Bio-Rad, setting 25 μF/250 W/1800 V, using a 0.1 cm cuvette). The transformed E.coli cells were plated onto LB plates containing 50 μg/ml of ampicillin, 100 μg/ml of X-gal, and 1 μM IPTG and were incubated at 37°C overnight. Mutant colonies containing inactivated supFG1 genes unable to suppress the amber mutation in the host cell β-galactosidase gene were detected as white colonies among the wild type blue ones. The mutant colonies and the total colonies were counted. The mutant colonies were purified and the
RESULTS

Triplex formation in the presence of K⁺

To examine the effect of physiological potassium concentrations on triplex formation by a series of modified oligonucleotides (Fig. 1), a gel mobility shift assay was used. Synthetic DNA fragments of 24 bp (matching bp 160 to 183 in the supF gene) were used as targets for triplex formation (Fig. 1). Fixed concentrations of radioactively labeled duplex target DNA were incubated with increasing concentrations of the psoralen-linked oligomers (Fig. 1), both in the presence of optimum binding buffer and in the presence of binding buffer containing 140 mM KCl (Figs 2–7). The binding reactions were carried out for 2 h, a time period previously found to be sufficient to approach equilibrium for third strand binding by purine TFOs in the anti-parallel motif (24,25). UVA irradiation was used to generate photoadducts and thereby to covalently link the TFOs to their targets, ensuring that subsequent manipulation of the samples would not alter the apparent binding. The samples were then analyzed by denaturing gel electrophoresis and autoradiography (Figs 2–7).
Table 1. Oligonucleotide-mediated targeted mutagenesis of an SV40-based shuttle vector, pSupFG1, within COS cells

<table>
<thead>
<tr>
<th>Oligonucleotide</th>
<th>Kd (M) for third-strand binding without K+</th>
<th>Relative binding in the presence of K+ (%</th>
<th>No. of mutants/total</th>
<th>Mutation frequency (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>n.a.</td>
<td>n.a.</td>
<td>4 / 9526</td>
<td>0.04</td>
</tr>
<tr>
<td>pso-AG13</td>
<td>1 x 10^{-8}</td>
<td>28</td>
<td>25 / 8932</td>
<td>0.28</td>
</tr>
<tr>
<td>pso-AG13(S)</td>
<td>5 x 10^{-9}</td>
<td>37</td>
<td>16 / 6840</td>
<td>0.23</td>
</tr>
<tr>
<td>pso-TG13</td>
<td>2 x 10^{-8}</td>
<td>51</td>
<td>16 / 7180</td>
<td>0.22</td>
</tr>
<tr>
<td>pso-TG13(S)</td>
<td>1 x 10^{-8}</td>
<td>55</td>
<td>21 / 7054</td>
<td>0.30</td>
</tr>
<tr>
<td>pso-XG13</td>
<td>8 x 10^{-9}</td>
<td>95</td>
<td>118 / 8660</td>
<td>1.36</td>
</tr>
<tr>
<td>pso-XG13(S)</td>
<td>5 x 10^{-9}</td>
<td>100</td>
<td>34 / 11 410</td>
<td>0.30</td>
</tr>
</tbody>
</table>

The equilibrium dissociation constant (Kd) for third strand binding (column 2) was estimated as the concentration of TFO at which apparent binding was half maximal, as judged by gel mobility shift assay (Figs 2–7). The relative binding in the presence of K+ (column 3) was calculated as the ratio of third strand binding with and without K+ at a TFO concentration of 10^{-6} M. The values in the third and fourth columns represent the frequency of mutations in the supFG1 gene in the SV40 shuttle vector induced by third strand targeted psoralen adducts in monkey COS cells. After electroporation of the pSupFG1 vector into COS cells, the cells were incubated with the indicated oligonucleotides, followed by irradiation with 1.8 J/cm² of UV A (365 nm). After 48 h, the vectors were rescued for genetic analysis in bacteria. Control samples (None) were irradiated but were not incubated with any oligonucleotide.

n.a., not applicable.

Results of binding analyses of TFOs pso-AG13, pso-AG13(S), pso-TG13 and pso-TG13(S) are shown in Figures 2a–5a. Photoactivation of the triplex molecules with 1.8 J/cm² of UVA (irradiance of 5 mW/cm²) led to both XL (the psoralen-TFO covalently bound to both strands of duplex target via a psoralen interstrand cross-link) and to psoralen MA (the psoralen-TFO covalently linked to just one strand of the duplex via psoralen monoadduct formation). This dose of UVA was previously found to provide near-maximum photoproduct yields, since higher doses can lead to photoisomerization and photodegradation of the psoralen (21). The percentage of the sample constituting the sum of the XL and MA bands represents the overall efficiency of cross-linking, which in turn is proportionate to the extent of triplex helix formation under the near-equilibrium conditions. Both pso-TG13 and pso-TG13(S) were seen to form triplexes as efficiently as pso-AG13 and pso-AG13(S) (compare Figs 2a and 3a with Figs 4a and 5a). As a means to quantitate binding affinity, the equilibrium dissociation constant (Kd) for each TFO was estimated as the concentration at which cross-linking (and therefore third strand binding) was half-maximal. The KdS were found to be in the range of 10^{-8} M for all four oligomers, based on the quantitation of the binding data presented in Figures 2c–5c (see also Table 1). This is consistent with the ability of both A:A:T and T:A:T triplets to form by reverse Hoogsteen hydrogen bonding in the anti-parallel triplex helix motif (4).

When the in vitro triplex binding reaction was carried out in the presence of a buffer containing physiological K+ concentration (140 mM KCl), there was a sharp decrease in triplex formation as evidenced by the reduced levels of XL and MA products in the gel shift analyses (Figs 2b–5b). Graphical comparisons of the binding data in the presence and absence of K+ are presented in Figures 2c–5c. Inhibition of triplex formation by the presence of K+ was observed for oligonucleotides containing either phosphodiester (pso-AG13 and pso-TG13) or phosphorothioate [pso-AG13(S) and pso-TG13(S)] internucleoside linkages. In addition, both the A and G and the T and G containing TFOs showed diminished third strand binding in the presence of K+. The T and G TFOs, however, appeared to be slightly more resistant to the inhibitory effect of K+ on triple helix formation (compare Figs 2 and 3 with Figs 4 and 5; see also Table 1).

Oligonucleotides containing 7-deazaxanthine form stable triplexes in the presence of K+

Oligonucleotides pso-XG13 and pso-XG13(S), containing phosphodiester and phosphorothioate internucleoside linkages, respectively, were synthesized such that the A residue of the purine TFO, pso-AG13, was substituted with c7X (Fig. 1) (15). These modified TFOs were also tested in gel mobility shift assays, as described above. As shown in Figures 6a and 7a, these TFOs bound the target sequence with KdS in the 10^{-9} M range, a 10-fold greater binding affinity than for TFOs pso-AG13, pso-AG13(S), pso-TG13 and pso-TG13(S) (Figs 2–5). When the triplex binding reactions were carried out in the presence of 140 mM KCl, the binding was only slightly diminished (Figs 6 and 7, b and c). For pso-XG13, the apparent Kd was reduced somewhat by K+, but the total extent of third strand binding at a 10^{-6} M concentration was approximately equal in the presence or absence of 140 mM KCl (Figs 5c and 6c; Table 1). In the case of pso-XG13(S), third strand binding was not affected by the presence of K+ (Fig. 6 and Table 1).

Gene targeting in vivo

We used the SV40-based shuttle vector, pSupFG1, in an assay designed to compare the ability of the six variously modified psoralen-linked oligonucleotides to mediate intracellular mutation targeting. All of these oligonucleotides were designed to bind as third strands to base pairs 167–179 of the supFG1 gene in the vector and to deliver the tethered psoralen to intercalate between base pairs 166 and 167 (7). In these experiments, the vector DNA was introduced into the cells by electroporation, the cells were washed, and 40 min later the oligonucleotides were added to the cells in suspension. After incubation with the oligonucleotides for 2 h, the cells were irradiated with UVA light and then transferred to culture dishes for 48 h before rescue of the vector DNA for analysis (7). Control cells received no oligonucleotide but were
irradiated with UVA (Fig. 8). In the absence of oligonucleotide
treatment, a background mutation frequency of 0.04% was
observed (Table 1). When the cells were treated with either
pso-AG13, pso-AG13(S), pso-TG13, or pso-TG13(S), there was
a 5- to 8-fold increase in mutation frequency above the
background, ranging from 0.22 to 0.30%. When pso-XG13 was
used in the assay, a mutation frequency of 1.36% was observed,
a 34-fold increase above the background and ~5-fold above that
induced by the other TFOs. However, pso-XG13(S), which has
the same c7X modification as pso-XG13 but contains phosphoro-
thioate internucleoside linkages, gave a mutation frequency of
only 0.30%, comparable to the TFOs lacking the c7X modifica-
tion (Table 1).

In previous work, we found that a non-specific, mixed
sequence psoralen-oligonucleotide did not induce mutagenesis
above the background level in this assay, whereas a 30mer A and
G containing, phosphodiester TFO, pso-AG30, (which binds to
the polypurine site in supFG1 with a $K_d$ of $3 \times 10^{-9}$ M) induced
mutations at a frequency of 2.1% under conditions similar to the
ones used here (7).

The DNA from a representative number of pso-XG13-induced
mutants was isolated and subjected to sequencing to locate the site
of mutation within the supFG1 gene. As found in the previous
work with pso-AG30 (7,26,27), the mutations generated by the
pso-XG13 were targeted to the psoralen intercalation site at bp
Figure 8. Assay for gene targeting of pSupFG1 by intracellular triple helix formation within COS cells. The plasmid DNA was transfected into COS cells by electroporation followed by the addition of the various psoralen-conjugated TFOs to the extracellular growth medium. After allowing time for oligonucleotide entry into cells and for intracellular triple helix formation, the cells were exposed to UV A irradiation to photoactivate the psoralen and generate targeted adducts. Following vector replication, the cells were lysed, and the plasmid DNA was isolated for transformation into E.coli to allow genetic analysis of the supFG1 gene. The structure of the tethered psoralen (4′-hydroxymethyl-4,5′,8-trimethylpsoralen) attached via a two-carbon linker arm to the 5′ phosphate of the oligonucleotide is shown.

166–167 in the supFG1 gene (data not shown). These results indicate the ability of variously modified 13mer TFOs to form site-specific triplexes within cells and induce targeted mutations.

Lack of photoreactivity of the 7-deazaxanthine substitution
Since UV A was used in our experiments to generate the targeted psoralen adducts, we tested the possible photoreactivity of the c7X analog, itself. We synthesized two 13mers, XG13 and AG13, lacking 5′ psoralen modification. Both oligomers bound as third strands to the target duplex, as determined by non-denaturing gel mobility shift assays (not shown). However, no covalent modification of the target duplex by either TFO could be detected upon irradiation with 1.8 J/cm² of UV A, as judged by gel mobility shift assays under denaturing conditions (i.e. the same assay as shown in Figs 2–7; data not shown). Hence, the c7X analog does not appear to be photoreactive, at least under the conditions used in our experiments.

DISCUSSION
In the present work, we have carried out a comparison of A- and G-, T- and G-, and X- and G-containing TFOs with either phosphodiester or phosphorothioate backbones. Our results indicate that the c7X modification not only enhances triplex formation in vitro under physiological K⁺ concentrations but also improves intracellular triplex formation, as measured in a gene targeting assay. These findings are consistent with the previous observation of improved in vitro binding by c7X-containing TFOs (15).

It should be noted, however, that Olivas and Maher found only modest K⁺ resistance upon c7X substitution (3 c7X’s and 11 G’s in a 14mer) (10). Instead, they saw superior K⁺ resistance with 6-thioguanine substitution of selected G residues. It is possible that the c7X substitution is sequence-dependent with respect to its effects on triplex affinity and K⁺ resistance. This possible sequence dependence, however, remains to be elucidated.

In light of the results of Olivas and Maher (10), and given our highly G-rich TFO sequence (which would appear to be especially prone to G-quartet formation), it might have been predicted that substitution of just three residues with c7X would be insufficient to achieve the level of K⁺ resistant binding that we found. However, it may be that the c7X substitution not only strongly inhibits G-quartet formation, but also it may serve to minimize the formation of other unusual structures, such as homoduplexes, which can compete with triplex formation by A- and G-containing TFOs (28).

Although the phosphorothioate-modified, c7X-containing TFO, pso-XG13(S), showed good binding in vitro in the presence of K⁺ (Fig. 7), it did not exhibit enhanced intracellular activity. This may be due to non-specific interactions with cellular proteins, as have been reported for phosphorothioate-containing oligonucleotides (29,30), thereby limiting the effective intracellular concentration of the TFO.

In previous work, we found that a psoralen-linked 10mer TFO (pso-AG10) was unable to induce targeted mutagenesis in vivo, and this lack of activity correlated with a weak binding affinity to the reporter gene target site (Kd = 8 × 10⁻⁷ M) (7). In contrast, our present work indicates that a 13mer (either pso-AG13 or pso-TG13) can bind with sufficient affinity to mediate intracellular triplex formation, as evidenced by the mutagenesis experiments. The frequency of intracellular targeted mutagenesis induced by pso-AG13, however, was ∼10-fold lower than that previously obtained with pso-AG30 (2.1%) (7). This difference is in keeping with the relative binding affinities of the TFOs: Kd
However, with the c\textsuperscript{7}X substitution, the frequency of targeted gene sequences. Although substitutions such as c\textsuperscript{7}X do not extend long polypurine/polypyrimidine stretches that are rare in most the third strand binding code so as to reduce the requirement for mediation by the 30mer.

In recent years, significant effort has been devoted to expanding the third strand binding code so as to reduce the requirement for long polypurine/polypyrimidine stretches that are rare in most gene sequences. Although substitutions such as c\textsuperscript{7}X do not extend the binding code, they do improve binding in existing motifs and therefore allow shorter polypurine sequences to serve as feasible in vivo target sites. The general applicability of the triplex anti-gene strategy will depend on further modifications along these lines to enhance binding affinity in vivo and also on the development of novel nucleotide analogs to enable third strand binding to mixed sequences.

ACKNOWLEDGEMENTS

We thank M.M. Sediman, M. Raha, G. Wang, J. George, F.P. Gasparro, T. Yeasky, L. Cabral, R. Franklin, and S.J. Baserga for their assistance. This work was supported by the Leukemia Society of America, the Charles E. Culpeper Foundation, the American Cancer Society (CN128) and the NIH (CA64186). A.F.F. is an Anna Fuller Foundation Fellow.

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