Targeted Gene Knockout by 2'-O-Aminoethyl Modified Triplex Forming Oligonucleotides*

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Forming Oligonucleotides

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Triplex forming oligonucleotides (TFOs) are of interest because of their potential for facile gene targeting. However, the failure of TFOs to bind target sequences at physiological pH and Mg2+ concentration has limited their biological applications. Recently, pyrimidine TFOs with 2'-O-aminoethyl (AE) substitutions were shown to have enhanced kinetics and stability of triplex formation (Cuenoud, B., Casset, F., Husken, D., Natt, F., Wolf, R. M., Altmann, K. H., Martin, P., and Moser H. E. (1998) Angew. Chem. Int. Ed. 37, 1288–1291). We have prepared psoralen-linked TFOs with varying amounts of the AE-modified residues, and have characterized them in biochemical assays in vitro, and in stability and HPRT gene knockout assays in vivo. The AE TFOs showed higher affinity for the target in vitro than a TFO with uniform 2'-O-Me substitution, with relatively little loss of affinity when the assay was performed in reduced Mg2+. Once formed they were also more stable in “physiological” buffer, with the greatest affinity and stability displayed by the TFO with all but one residue in the AE format. However, TFOs with lesser amounts of the AE modification formed the most stable triplexes in vivo, and showed the highest HPRT gene knockout activity. We conclude that the AE modification can enhance the biological activity of pyrimidine TFOs, but that extensive substitution is deleterious.

Reagents that recognize and bind specific genomic sequences in mammalian cells would have broad application for promoter suppression, gene knockout, target validation, and as probes of chromosome structure. Thus the interaction of third strands and intact DNA duplexes to form stable DNA triplexes (1) has been of interest for many years (2–6). Some of the properties of these structures make triplex forming oligonucleotides (TFOs)† attractive candidates for development as gene targeting reagents. Triplex formation, which occurs on polypurine:polypyrimidine stretches, these elements are over-represented in the mammalian genome (16), and most genes contain appropriate target sequences. Furthermore, data from different experimental systems indicate that both extrachromosomal and chromosomal target sites in living cells are accessible to TFOs (17–19).

However, despite 40 years of research, there remain a number of impediments to the successful employment of TFOs as gene targeting reagents. Some of these obstacles reflect the properties of the oligonucleotides. Depending on the nature of the target either purine or pyrimidine TFOs can be used, but there are problems associated with each motif. Under physiological conditions purine TFOs are often subject to self-structure formation, which is incompatible with triplex formation (20, 21). Cytosine residues in pyrimidine motif TFOs must be protonated at N-3 in order for hydrogen bond formation at this position. The pKₐ of cytosine is 4.5, and most pyrimidine TFOs are relatively inactive at physiological pH. Furthermore, triplex formation by TFO in both motifs is often sharply dependent on Mg2+ concentration (22). Most binding studies use 5–10 mM Mg2+, which is likely to be higher than the free intracellular concentration. Obviously, if TFOs are to become effective gene targeting reagents, they, at a minimum, must be active at physiological pH and Mg2+ concentration.

Some progress has been made toward overcoming these problems, particularly in the pyrimidine motif. Replacement of cytosine by 5-methylcytosine partially alleviates the pH restriction on triplex formation (23, 24). Following the observation that pyrimidine triplexes with RNA third strands are more stable than the complex with the corresponding DNA third strand (25–27), it was shown that triplex stability could be enhanced when the TFOs contained 2'-O-methyl (OMe)-modified sugars (28–30). Structural studies indicate that this modification stabilizes the C3'-endo,2'-exo configuration of the sugar. This is the optimal conformation for triplex formation by pyrimidine oligonucleotides, and provokes the least perturbation of the underlying duplex (31).

Recently, additional sugar modifications have been described which considerably extend the stability of pyrimidine motif triplexes (32). Among these the 2'-O-aminoethyl (AE) substitution produced a 3.5 °C increase in thermal stability per modification, measured at pH 7.0. NMR analysis indicated that a hydrogen bond formed between the positively charged amino

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residue and an oxygen of the negatively charged phosphate of the purine strand of the duplex. The sugar conformation was C3'-endo,2'-exo and the underlying duplex showed only slight distortion (33). This substitution also conferred high nuclease resistance on the TFO (32, 34). Although the AE TFOs were not tested in bioassays their properties would seem consistent with activity in cell-based assays.

We are developing TFOs as gene targeting reagents, and have demonstrated specific gene knockout in mammalian cells using a TFO linked to psoralen (18). Consequently oligonucleotide modifications that have the potential to solve some of the problems mentioned above are of considerable interest. Here we describe the preparation and characterization of psoralen-linked TFOs with different degrees of 2'-AE substitution. We have characterized these in biochemical assays in vitro, and in stability and gene knockout assays in mammalian cells in vivo. We find that these TFOs have enhanced affinity for their target sequences in vitro, and some, but not all, are active in bioassays in vivo.

MATERIALS AND METHODS

Reagents—The 5'-O-[4,4'-dimethoxytrityl]-5-methyluridine-2'-O-methyl-3'-O-[(4,4'-cyanophenyl-N,N-diisopropyl)phosphoramidite, the 5'-O-[4,4'-dimethoxytrityl]-5-methyluridine-2'-O-methyl-3'-O-succinimidyl-N'-hexanamido-N'-propyl-controlled pore glass support, and anhydrous acetonitrile (low water) were purchased from Glen Research, Inc., Sterling, VA. The N'-acetyl-5'-O-[4,4'-dimethoxytrityl]-5-methylcytidine-2'-O-methyl-3'-O-[(4,4'-cyanophenyl-N,N-diisopropyl)phosphoramidite and 6'-[4'-[(hydroxymethyl)-4,5,8-trimethylpsoralen]hexyl-1-O-[(4,4'-cyanophenyl-N,N-diisopropyl)phosphoramidite were purchased from Glen Research, Inc., Sterling, VA.

Synthesis of Phosphoramidites—The synthesis of 5'-O-[4,4'-dimethoxytrityl]-5-methyluridine-2'-O-(2-aminoethoxy)-3'-O-[(4,4'-cyanethyl-N,N-diisopropyl)phosphoramidite followed the shorter route reported previously (35). Starting from the 2'-anhydro-5-methyluridine the 2'-ring was opened by the complex generated from trimethylisopropoxide and N-(2-hydroxyethyl)phthalimide. Subsequent 5'-dimethoxytritylation, removal of the phthalimide group by hydrazine, and the protection of the liberated amine by trifluoroacetyl, followed by phosphorylation on the 3'-hydroxy gave the required phosphoramidite. For the synthesis of 5'-O-[N-(methylpyrrolidin-3-yl)-4,4'-dimethoxytrityl]-5-methylcytidine-2'-O-(2-aminoethoxy)-3'-O-[(4,4'-cyanethyl-N,N-diisopropyl)phosphoramidite, an earlier procedure (33) was followed starting from N'-benzoyloxymethyl-5'-3'-O-1,1,3,3-tetraisopropyl-1,3-disisoxyl-5-methyluridine. The synthesis involved generation of the 2'-azidoethyl group through standard procedures with the initial alkylation of the starting material. With the azido group as the intermediate protection, the 4'-amine was replaced by an amino group by triazolyltetrazolium/ammonolysis. Protection of the N'-amino group with 4'-methylpyrrolidin amide, reduction of the 2'-azidoethyl group and its protection followed by 5'-dimethoxytritylation and 3'-phosphitylation gave the required phosphoramidite.

Synthesis of TFOs—The oligonucleotides were synthesized on controlled pore glass supports (500 A) using an Expedite 8909 synthesizer. All protected nucleoside phosphoramidites were dissolved in anhydrous acetonitrile at a concentration of 0.05 M. The nucleoside (2'-AE) phosphoramidite, the azide and its protection followed by 5'-O-methylpyrrolidine amidine, reduction of the 2'-aminoethyl group supported triplex formation (20 mM Hepes, pH 7.2, 10 mM MgCl2), the preferred target for psoralen intercalation and cross-linking (for a discussion of the general strategy for construction of functional variant supT mutation marker genes see Ref. 37). In vivo stability assays were performed as described previously (36). Briefly, the plasmid was incubated with individual Pso-TFOs under conditions that supported triplex formation (20 mM Hepes, pH 7.2, 10 mM MgCl2, 2 μM TFO, 0.6 pmol of plasmid), unbound TFO removed, and the plasmid-TFO complexes were electrophoresed into Cos-1 cells. At various times after electrophoration the cells were exposed to UVA to activate the psoralen. The cells were plated for an additional 48 h, during which time the psoralen cross-links were repaired and/or mutagenized, and the plasmid replicated. Progeny plasmids were then harvested, treated with DpnI to remove nonreplicated input plasmids (38), and introduced into the Escherichia coli indicator strain MM 7070 (39). The bacteria were spread on indicator plates containing isopropyl-thiO-β-D-galactopyranoside and 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside and the frequency of white or light blue colonies, which contained the supF mutation, was determined by plaque assays (36). Because the mutation frequency was a measure of the frequency of triplexes at the time of UVA treatment. Each experiment was done at least three times.

Cells and HPRT Mutagenesis Protocol—Chinese hamster ovary cells were grown in Dulbecco's modified Eagle's medium supplemented with...
penicillin and streptomycin and 10% fetal bovine serum. Prior to an HPRT knockout experiment cells were cultured in HAT medium (10^-4 M hypoxanthine, 5 x 10^-6 M aminopterin, 10^-3 M thymidine) for 1 week to remove pre-existing HPRT-deficient cells. Cells were suspended at 10^6/ml in complete medium and Pso-TFOs added to 5 μM. The cell/TFO mixture was then electroporated (Bio-Rad) at a setting of 110 volts, 960 microfarads, followed by incubation at room temperature for 3 h, and exposure in the Rayonet chamber to UVA light for 3 min at 1.8 J/cm². The cells were plated in complete medium for 8–10 days with 2–3 passages, and then placed in selective medium depleted of hypoxanthine and containing 20 μM thioquanine (200,000 cells/100-mm dish). Cells were also plated in selective medium without thioquanine to determine plating efficiency. After 10 days resistant colonies were counted and picked for expansion and DNA analysis.

A similar protocol was observed when the frequency of cells with mutations in APRT (adenosine ribosyltransferase) was determined. Chinese hamster ovary cultures were cleared of APRT-deficient cells by growth in azaserine. Selections for APRT colonies were done in medium containing azan-denine.

RESULTS

TFOs and Targets—The sequence and substitution patterns of the Pso-TFOs in this study are displayed in Fig. 1A. The TFOs were designed to form a triplex with a target sequence found in intron 4, immediately adjacent to Exon 5 in the Chinese hamster HPRT gene (18). We prepared TFOs designed to target the 17-base uninterrupted polypurine:polypyrimidine element which ends in a T and is followed by an A, providing an appropriate site for psoralen cross-linking. We were interested in determining the biochemical and biological activity of TFOs with different amounts and distribution of 2'-AE substitution. Accordingly we prepared a TFO with all but one position containing the AE substitution (AE-01), or with 6 AE residues at the 3' end (AE-02), or with 4 AE residues at the 3' and 5' ends (AE-03). The remainder of each oligonucleotide contained 2'-OMe sugars. In addition, we prepared a TFO uniformly modified with 2'-OMe sugars (PS-01).

We constructed a shuttle vector plasmid, psupF12, with a variant, but functional, supF mutation marker gene containing the hamster HPRT target placed in the 5’ pre-tRNA region of the gene with the psoralen cross-link site embedded in the first two bases of the mature tRNA gene (Fig. 1B) (see Levy et al. (37), for the construction strategy). Psoralen cross-links placed by the TFO in these positions cause mutations during repair and replication of the plasmid in mammalian cells (17, 36). The cross-link site was also positioned in a unique XbaI site that allowed the presence or absence of a cross-link to be determined by restriction enzyme protection.

Psoralen-conjugated TFOs—Psoralen is a linear furocoumarin which reacts via a [2 + 2] cycloaddition, in concert with long wave UV light (UVA), to form photodadducts primarily with thymidines at 5'-TA-3' sites in double stranded DNA. This reaction is highly regio- and stereospecific, forming the interstrand cross-link as the major product under the conditions employed in our experiments. Conservation of the lactone ring of psoralen is vital for preserving its cross-linking activity. The unsaturated lactone ring of psoralen can be susceptible to hydrolysis at elevated temperatures by harsh aqueous bases like the ammonium hydroxide commonly used for deprotecting oligonucleotides. Consequently it was important to identify deprotection conditions that preserved the psoralen while removing the trifluoroacetyl groups on the 2’-O-aminoethyloxyl, the acetly group on N° of the 2’-O-methyl-5-methylcytidine, and the N° methylpyrrolidine amide group on N°° of the 2’-O(2-aminoethyl)-5-methylcytidine.

Oligodeoxyribonucleotides with psoralen tethered to their 5’ terminus have been deprotected by concentrated ammonium hydroxide treatment (55 °C, 16 h) (40). However, with AE oligos under these deprotection conditions, even with reduced exposure times (55 °C, 1 h), we recovered quite heterogeneous products as analyzed by HPLC (Fig. 2). Different liquid phase deprotection conditions were tried (tert-butylamine:methanol:water (1:1:2) (41), 10% 1,8-diazabicyclo[5.4.0]undec-7-ene in ethanol (42), and sequential treatment with ammonia at room temperature, followed by ethylenediamine (43)) but none were satisfactory. Subsequently we tried a novel gas phase deprotection protocol using anhydrous methylamine in an enclosed steel chamber (44). The chromatogram (Fig. 2) of the oligonucleotide following this treatment showed a homogeneous profile...
with a major peak. The oligonucleotide (AE-03 as an example in Fig. 2) was purified by RP-HPLC and its capillary zone electrophoresis chromatogram showed a single peak (Fig. 2). The matrix-assisted laser desorption-time of flight spectrometry data was consistent with the theoretical value and an intact psoralen. Triplexes were formed with the TFOs and duplex DNA containing the HPRT target sequence and an XbaI site corresponding to the 5' end of the TFO. Psoralen activity was measured by monitoring the resistance to XbaI digestion of duplex DNA following triplex formation, photoactivation, and removal of non-cross-linked TFO. TFOs prepared by gas phase deprotection consistently displayed very high levels of DNA cross-linking (Fig. 2).

**TFO Affinity**—The affinity of each TFO for the HPRT target was determined by a restriction enzyme protection assay (“Materials and Methods”) (35, 45). These assays were performed in buffers containing 10 mM MgCl₂. Representative binding curves (for PS-01 and AE-03) are shown in Fig. 3A, and the half-maximal values for all the TFOs shown in the bar diagram (Fig. 3B). The $K_D$ values ranged from 112 (PS-01) to 15 mM (AE-01).

Although commonly employed for these determinations, 10 mM Mg²⁺ is unlikely to accurately reflect the concentration of free Mg²⁺ in cells. Consequently we repeated the assays with PS-01 and AE-03 in 1 mM MgCl₂ (Fig. 3C). The results demonstrated the anticipated decline in affinity with PS-01 ($K_D = 319$ nM). However, the affinity of AE-03 was essentially unchanged relative to the measurement in 10 mM MgCl₂.

**Triplex Stability in Vitro**—In earlier biochemical studies triplexes formed by TFOs with AE modifications were shown to be more stable than those formed by unmodified equivalents (32). We measured the stability, in vitro, of the triplexes formed on the HPRT target by preparing them with each of the TFOs in incubations in which the TFOs were at saturating concentrations (“Materials and Methods”). Under these conditions essentially all plasmid molecules contained triplexes. Following removal of unbound oligonucleotide, the plasmid-triplex complexes were incubated at 37 °C in a physiological buffer (140 mM KCl, 1 mM MgCl₂, 10 mM Hepes, pH 7.2) that included a 10-fold excess of a duplex trap oligonucleotide containing the target sequence. At various times after the start of the incubation, samples were exposed to UVA and then the extent of triplex formation in each sample determined by restriction protection. The results (Fig. 4) showed that all the triplexes formed by the AE TFOs were extremely stable over the time course, with 99% of the AE-01 triplex present after 8 h. The AE-02 and AE-03 triplexes were also quite stable with 93 and 94% maintained after 8 h. The least stable was the PS-01 triplex with a decline to 83% in the same time period. These data were consistent with previous results (32), and encouraged the expectation that triplexes formed by the AE oligonucleotides would be more stable in vivo than those formed by the PS-01 TFO with only the 2'-OMe modification.

**Triplex Stability in Vivo**—We have described an approach to the measurement of triplex stability in vivo based on a shuttle vector mutation assay (36). The assay reports the presence of a triplex in the nuclear compartment that supports replication and mutagenesis of DNA carrying psoralen cross-links. Triplexes were formed by incubation of saturating levels of a TFO with the supF12 plasmid containing the embedded HPRT triplex target sequence adjacent to a TA step appropriate for psoralen cross-linking (Fig. 1B) (see “Materials and Methods”). Unbound TFOs were removed, and the TFO-plasmid triplex complexes were electroporated into Cos-1 cells. At the indicated times the cells were exposed to UVA to activate the psoralen. After an additional 48 h the progeny plasmids were harvested and the frequency of plasmids with mutations in the supF gene determined in a microbiological screen. Mutagenesis was dependent on psoralen cross-linking, which in turn was dependent on the bound pso-TFO. Thus the mutation frequency measurement reflected the frequency of plasmids with pso-TFOs bound in triplexes at the time of photoactivation. The plot of mutation frequency (normalized to the 0 time value) versus time of photoactivation gives a picture of the decay of the triplexes in the replication compartment. We found that the triplexes formed by AE-02 and AE-03 (both with $t_{1/2} = 105$ min) were about 1.5 times as stable in vivo as the PS-01 triplex ($t_{1/2} = 72$ min). In contrast, the stability of the AE-01 triplex was substantially less than the other TFOs ($t_{1/2} = 25$ min) (Fig. 5). These results indicated that there was not a simple correlation between the stability of a triplex formed by a particular TFO, as measured in vitro, and the stability in vivo of the triplex formed by the same TFO.

**Activity of AE TFOs in HPRT Knockout Assay in Vivo**—The activity of the Pso TFOs was then measured in an HPRT gene knockout assay in Chinese hamster ovary cells. The TFOs were introduced into the cells by electroporation and the psoralen activated by exposure to UVA (“Materials and Methods”). Con-
trol experiments showed that no mutagenesis occurred without UVA treatment. The cells were carried in culture for 8–10 days to allow pre-existing enzyme to decay, and then were placed in selective medium. Each TFO was tested in at least three independent experiments. The Ps-01 TFO showed only marginal activity relative to the mock-transfected control cultures (Fig. 6A), similar to our previous experience with this oligonucleotide (18). However, both the AE-02 and AE-03 TFOs were active, with AE-03 (0.04%) consistently showing greater activity. Somewhat surprisingly, the AE-01 TFO was no more active than PS-01. A scrambled version of the AE-03 TFO with the same distribution of AE substitution (Sc AE-03, Fig. 1A), but with 8 mismatches, was inactive.

The activity of the AE-02 and AE-03 TFOs against the intended HPRT target prompted us to analyze the activity against another selectable marker, the gene for APRT. This gene also has polypurine:polypyrimidine tracts, some linked to psoralen target sites (46). We reasoned that if the activity of the AE-02 and AE-03 TFOs were simply a function of nonspecific interactions with these kinds of sequence elements then they would show activity against the APRT gene. However, we found that all the TFOs were inactive in the assay (Fig. 6B). These results argued that the activity of the AE-02 and AE-03 TFOs was specific to the HPRT gene.

We then analyzed the sequences of some of the mutant...
**Requirement for non-physiological levels of Mg\textsuperscript{2+}**

The pH problem has been somewhat ameliorated by the replacement of cytosine by 5-Me-cytosine (23). However, the realization that RNA-like third strands (as defined by an oxygen at the 2′ position) formed more stable triplexes than the 2′-deoxy equivalents (25) led to the use of the 2′-OMe substitution in pyrimidine TFOS (28–30). Although TFOS with this modification clearly showed enhancements (Fig. 2), the binding of our PS-01 TFO was diminished in physiological Mg\textsuperscript{2+}, and was essentially inactive in our knockout experiments despite the uniform presence of 2′-OMe modified sugars (18) (this report). Significant biological activity was observed, in our earlier study, only when an intercalator was introduced into the oligonucleotide (18). Those results underscored the need to extend the activity of pyrimidine TFOS containing 2′-OMe sugars, but also indicated that appropriate chemical modifications could confer biological activity. Consequently it was of considerable interest to ask if TFOS with 2′-AE sugars would show enhanced biological activity relative to their counterparts carrying only 2′-OMe sugars.

It was crucial for these analyses that the TFOS with AE substitutions carry fully functional psoralen moieties. TFOS with inactive psoralen would compete with fully active molecules and thus reduce the overall efficacy of the reagents. We found that protocols used previously for deportation of AE oligonucleotides gave heterogeneous products and appreciable loss of psoralen activity. We eventually determined that gas phase deprotection with anhydrous methylamine produced a largely homogeneous product with excellent psoralen activity (44). While methylamine is a stronger base than the ammonia used in conventional procedures, the deprotection reactions were performed at room temperature, which reduced the probability of hydrolysis of the psoralen lactone ring.

**TFOS Design**—The Pso TFOS described here contained 2′-AE sugars in three different formats. AE-01 was uniformly substituted save for the 3′ residue (for synthetic reasons). This TFO was analogous to the fully modified 15-mer characterized in the earlier study by Cuenoud et al. (32). That TFO displayed a dramatic enhancement of target affinity and stability of the resultant triplex. AE-02 contained 6 contiguous 2′-AE residues at the 3′ end and was designed with the expectation that this organization would confer enhanced binding as well as resistance to 3′-exonuclease activity (48). The AE-03 TFO retained the motif of a 3′ patch of AE combined with a 5′ patch. Based on the previous work (32) we anticipated that the AE TFOS would have greater affinity for their targets than the 2′-OMe equivalent and this was substantiated in the in vitro experiments (Fig. 2). Furthermore, the 2′-AE modification appeared to reduce the requirement for Mg\textsuperscript{2+} for triplex formation, which characterized the TFO with only the 2′-OMe substitution. In addition, the AE TFOS formed triplexes that were more stable in physiological buffer at 37 °C. Remarkably stable purine motif triplexes have been described (14, 15) and the triplexes formed by the AE TFOS appear to be comparably stable. The results of these biochemical analyses suggested that, while the AE TFOS as a group were clearly superior to PS-01, there was little to distinguish one from the other, and we expected them to display similar activity in the biological assays.

**TFOS Bioactivity**—We tested the TFOS in two bioassays, one that measured triplex stability, the other gene targeting activity.

**DISCUSSION**

Generally, pyrimidine motif TFOS with deoxyribose sugars have been characterized by relatively weak affinity for their duplex targets at physiological pH and Mg\textsuperscript{2+} concentration (2). The pH problem has been somewhat ameliorated by the replacement of cytosine by 5-Me-cytosine (23). However, the requirement for non-physiological levels of Mg\textsuperscript{2+} still requires a satisfactory solution. The realization that RNA-like third strands (as defined by an oxygen at the 2′ position) formed more stable triplexes than the 2′-deoxy equivalents (25) led to the use of the 2′-OMe substitution in pyrimidine TFOS (28–30). Although TFOS with this modification clearly showed improvement in conventional assays *in vitro*, we found that the binding of our PS-01 TFO was diminished in physiological Mg\textsuperscript{2+}, and was essentially inactive in our knockout experiments despite the uniform presence of 2′-OMe modified sugars (18) (this report). Significant biological activity was observed, in our earlier study, only when an intercalator was introduced into the oligonucleotide (18). Those results underscored the need to extend the activity of pyrimidine TFOS containing 2′-OMe sugars, but also indicated that appropriate chemical modifications could confer biological activity. Consequently it was of considerable interest to ask if TFOS with 2′-AE sugars would show enhanced biological activity relative to their counterparts carrying only 2′-OMe sugars.

It was crucial for these analyses that the TFOS with AE substitutions carry fully functional psoralen moieties. TFOS with inactive psoralen would compete with fully active molecules and thus reduce the overall efficacy of the reagents. We found that protocols used previously for deportation of AE oligonucleotides gave heterogeneous products and appreciable loss of psoralen activity. We eventually determined that gas phase deprotection with anhydrous methylamine produced a largely homogeneous product with excellent psoralen activity (44). While methylamine is a stronger base than the ammonia used in conventional procedures, the deprotection reactions were performed at room temperature, which reduced the probability of hydrolysis of the psoralen lactone ring.

**TFOS Bioactivity**—We tested the TFOS in two bioassays, one that measured triplex stability, the other gene targeting activ-
ity. The plasmid based stability assay measures the persistence of a preassembled triplex in the nuclear compartment that supports plasmid replication and mutagenesis of the TFO-pсорaleran cross-link. The triplex is exposed to the ionic environment as well as to the proteins and enzymes of this compartment. In our earlier study we found that triplex stability could be extended by lowering the temperature of the cells prior to photoactivation (36), and we suggested that temperature-sensitive factors could destabilize triplexes in vivo. These might include helicases (49), the enzymology of RNA and DNA synthesis, chromatin assembly and modulation factors, and the proteins and enzymes involved in DNA repair (50). This is speculative and it is clear that we lack an understanding of the ionic and protein effectors of triplex stability in the nucleus. However, two conclusions can be drawn from our experiments. The first is that it is possible to extend triplex stability in this compartment by modification of the TFO, as demonstrated by AE-02 and AE-03. This is encouraging because it suggests that, whatever the nature of the destabilizing factors, they can be countered, at least partially, by changes in the oligonucleotide chemistry. The second, somewhat unexpected given the data in Fig. 4, was that too much of the AE modification was deleterious (see below).

The results of the knockout assay showed that TFOs AE-02 and AE-03 were active, unlike PS-01. The PS-01 TFO had the weakest affinity for the target sequence in 10 mM Mg2+ concentration, and showed a marked decline in activity when the Mg2+ was reduced to 1 mM. In contrast, TFOs with 2’-AE substitutions formed triplexes at low concentrations (this report), or even in the absence, of Mg2+ (32, 33). It is generally believed that a TFO must be effective at low concentrations of Mg2+ if it is to be active in vivo (51). Thus among the explanations for the activity differences between PS-01 and AE-02 and AE-03, it is likely that triplex formation in low Mg2+ is an key distinction. The importance of Mg2+ for triplex formation by conventional oligonucleotides is well established (22), and the contribution of oligonucleotide modifications to improved TFO activity in reduced Mg2+ has been emphasized in recent publications (51, 52). TFOs with a phosphoramidate backbone have been extensively characterized and shown to form very stable triplexes in 10 mM Mg2+. However, in contrast to the AE TFOs, triplex formation in the absence of Mg2+ was diminished (51), and these TFOs appear to show only modest bioactivity (19).

The activity in vitro, in low levels of Mg2+, does not explain the striking biological failure of the AE-01 TFO, which was almost completely substituted with 2’-AE sugars. This TFO is an analogue of one described previously, which bound its target in the absence of Mg2+ (32). Although AE-01 had the greatest affinity and formed very stable triplexes in vitro, it performed poorly in both assays of activity in vivo. We suggest that there are at least two explanations. The first is based on our observations of this oligonucleotide during the preparation and purification. We had difficulty recovering material and found that it was adherent to glass, columns etc. Apparently the extensive aminoethoxy substitution altered the properties of the molecule and we think it likely that the oligonucleotide may have been bound by cellular membranes, proteins, nonspecifically to DNA, etc., greatly reducing the effective TFO concentration in the nucleus.

In the case of the stability assay in vivo, the triplexes were preformed and would have been carried into the nucleus by the transfection of the plasmid. If the action of cellular proteins and enzymes underlies triplex instability then, apparently, the AE-01 triplex was a better substrate for these activities than the triplexes formed by the other TFOs. The 2’-AE modification makes two contributions to the biochemistry of a TFO. In addition to the interaction between the amine and an oxygen in the purine strand of the duplex, this modification also preorganizes the C3’-endo, 2’-exo conformation of the sugar (33). This structure has been shown to be preferred in pyrimidine triplexes (31). In studies with oligonucleotides containing sugars locked in this conformation it has been shown that the local conformation of a single locked sugar can influence the conformation of unmodified sugars in the adjacent four nucleotides (53). If this is also true for the AE sugars then the combination of the conformational influence on the immediate and adjacent residues may produce a TFO that is perhaps too constrained, unable to make subtle structural adjustments that may be required for triplex stability in vivo. Whatever the actual reason(s) for the poor activity of the AE-01 TFO our data do indicate that extensive modification is not productive. It will be of interest to determine if the extent of other sugar analogues that improve TFO binding in vitro (51, 54) must also be limited in TFOs intended for biological applications.

The end point of the HPRT knockout assay is a mutation frequency. This reflects a summation of events, most with negative effect, that influence the probability of a targeted mutation in HPRT. The frequency can be no greater than the number of cells with triplexes in place at the time of photactivation of the psoralen. This will be a function of the TFO transfection efficiency, the intrinsic accessibility of the target sequence at the time of TFO entry, and the frequency and stability of triplexes that form on accessible targets. It is likely that many binding events are unregistered because they fail to persist to the time of UVA treatment. Following photoactivation the oligonucleotide-psoralen cross-link can be a substrate for the machinery of DNA repair, whose action may have no mutational consequences. Thus the mutation frequency is an underestimate, perhaps considerable, of the binding activity of a TFO in vivo. We expect that the development of efficient and robust gene knockout protocols will involve manipulation of the cell biology to enhance target accessibility and mutagenic processing of the targeted DNA damage. In addition, we believe that further optimization of the AE format TFOs, perhaps in combination with other sugar and base modifications (54–57), will produce TFOs with potent biological activity.

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