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Targeted Mutagenesis in Mammalian Cells Mediated by Intracellular Triple Helix Formation

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As an alternative to standard gene transfer techniques for genetic manipulation, we have investigated the use of triple helix-forming oligonucleotides to target mutations to selected genes within mammalian cells. By treating monkey COS cells with oligonucleotides linked to psoralen, we have generated targeted mutations in a simian virus 40 (SV40) vector contained within the cells via intracellular triple helix formation. Oligonucleotide entry into the cells and sequence-specific triplex formation within the SV40 DNA deliver the psoralen to the targeted site. Photoactivation of the psoralen by long-wavelength UV light yields adducts and thereby mutations at that site. We engineered into the SV40 vector novel *supF* mutation reporter genes containing modified polypurine sites amenable to triplex formation. By comparing the abilities of a series of oligonucleotides to target these new sites, we show that targeted mutagenesis in vivo depends on the strength and specificity of the third-strand binding. Oligonucleotides with weak target site binding affinity or with only partial target site homology were ineffective at inducing mutations in the SV40 vectors within the COS cells. We also show that the targeted mutagenesis is dependent on the oligonucleotide concentration and is influenced by the timing of the oligonucleotide treatment and of the UV irradiation of the cells. Frequencies of intracellular targeted mutagenesis in the range of 1 to 2% were observed, depending upon the conditions of the experiment. DNA sequence analysis revealed that most of the mutations were T · A-to-A · T transversions precisely at the targeted psoralen intercalation site. Several deletions encompassing that site were also seen. The ability to target mutations to selected sites within mammalian cells by using modified triplex-forming oligonucleotides may provide a new research tool and may eventually lead to therapeutic applications.

Oligonucleotides can bind to duplex DNA and form triple helices in a sequence-specific manner (2, 3, 5, 12, 25, 39). Progress in elucidating the third-strand binding code has raised the possibility of developing nucleic acids as sequence-specific reagents for research and possibly clinical applications. Oligonucleotide-mediated triplex formation has been shown to prevent transcription factor binding to promoter sites and to block mRNA synthesis in vitro and in vivo (4, 9, 11, 17, 18, 21, 26, 29, 33, 41). Such inhibition of expression, however, is transient, depending on the sustained presence of the oligonucleotides. It also depends on the stability of the triple helix, which can be disrupted by transcription initiated at nearby sites (37). To overcome these problems, methods to prolong oligonucleotide-duplex interactions using DNA intercalating or cross-linking agents have been explored in experiments to block transcription initiation or elongation (17, 18, 39, 40).

Instead of using triplex formation to transiently block gene expression, however, we reasoned that it would be advantageous to use triple helix formation to target mutations to specific sites in selected genes in order to produce permanent, heritable changes in gene function and expression (19, 20). In this approach, mutations are targeted to a selected site by linking the triplex-forming oligonucleotide (TFO) to a mutagen so that the sequence specificity of the triplex formation can be imparted to the action of the mutagen.

We describe here experiments to investigate the use of TFO to target mutations to and inactivate selected genes within mammalian cells as an approach to in vivo genetic manipulation. Other techniques for genetic alteration of mammalian cells, such as the replacement or disruption of specific genes by gene transfer and homologous recombination, have provided useful research tools (6). If the third-strand binding code can be extended and if the parameters governing in vivo triplex formation can be determined, oligonucleotide-mediated genetic manipulation may eventually offer the advantages of simplicity, efficiency, and specificity.

In our previous work, we reported targeted mutagenesis of the *supF* mutation reporter gene in bacteriophage lambda vector DNA (20) and simian virus 40 (SV40) vector DNA (19) mediated by in vitro triplex formation and DNA adduct induction, followed by growth of the vectors in bacteria and monkey cells, respectively. In the present work, we report targeted mutagenesis of an SV40 vector mediated by intracellular triple helix formation. We show that site-specific mutations can be produced in viral genomes replicating in monkey cells by treatment of the cells with psoralen-linked oligonucleotides, followed by photoactivation of the psoralen with long-wavelength UV light (UVA). Using a set of modified target sites in the *supF* gene and a series of oligonucleotides, we demonstrate that targeted mutagenesis in our in vivo assay depends on the specificity of the TFO for the target site and on the strength of the oligonucleotide binding. We also report an analysis of both

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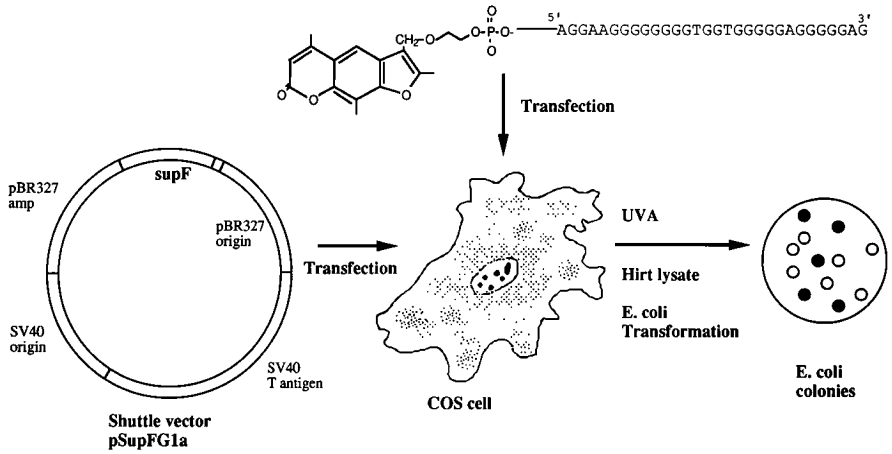

FIG. 1. Experimental strategy for targeted mutagenesis of SV40 DNA mediated by intracellular triple helix formation within COS cells. The SV40 shuttle vector DNA, either pSupFG1 (as shown) or similar constructs containing modified *supF* genes, was transfected into COS cells by electroporation. Separately, the psoralen-conjugated oligonucleotides (pso-AGT30 is shown) were subsequently added to the extracellular growth medium. After time was allowed for oligonucleotide entry into cells and for possible intracellular triple helix formation, the cells were exposed to UVA irradiation to photoactivate the psoralen and generate targeted adducts. Following vector replication, the cells were lysed, and the vector molecules were harvested for transformation into *E. coli* to allow genetic analysis of the *supF* gene. The structure of the tethered psoralen (4′-hydroxymethyl-4,5′,8-trimethylpsoralen attached at the 4′ hydroxymethyl position via a two-carbon linker arm to the 5′ phosphate of the oligonucleotide) is shown.

the time dependence and the concentration dependence of the oligonucleotide-directed targeting within mammalian cells.

MATERIALS AND METHODS

Oligonucleotides and vectors. Psoralen-linked oligonucleotides were obtained from Oligos Etc. (Wilsonville, Oreg.) or were synthesized by J. Flory of the W. M. Keck Biotechnology Resource Center at Yale University by using materials from Glen Research (Sterling, Va.). The psoralen is incorporated into the oligonucleotide synthesis as a psoralen phosphoramidite, resulting in an oligonucleotide linked at its 5′ end via a two-carbon linker arm to 4′-hydroxymethyl-4,5′,8-trimethylpsoralen, as illustrated in Fig. 1. The sequences of the psoralen-conjugated oligonucleotides used in this work are listed in Table 1. All the psoralen-conjugated oligonucleotides were also synthesized in such a way as to contain a 3′ propylamine group (available as an amino-CPG for automated oligonucleotide synthesis from Glen Research) to minimize susceptibility to degradation by 3′ exonucleases (29).

SV40 shuttle vectors pSupFG1, pSupFG2, and pSupFG3 were derivatives of pSP189 (31), and they carried new triplex binding sites within modified *supF* genes engineered into the parent plasmid. The sequences of the putative triplex binding sites in the new *supFG1*, *supFG2*, and *supFG3* genes, as well as that in the original *supF* gene, are listed in Table 1.

Construction of modified *supF* genes. The variant tRNA genes were inserted into a plasmid derived from pSP189, an SV40-based shuttle vector constructed by Parris and Seidman (31). The recipient plasmid was prepared by digestion of pSP189 with *Xho*I and *Eag*I, and the existing *supF* tRNA gene was removed from the rest of the plasmid on a 1% agarose gel. To form the replacement genes, two complementary oligonucleotides, incorporating the desired polypurine and polypyrimidine regions at the 3′ end of the tRNA gene, were synthesized on an Applied Biosystems 390B synthesizer. Following trityl-off synthesis, the oligonucleotides were deprotected at 55°C overnight, the salts were removed on a Sephadex G25 column (Pharmacia NAP-25), and the oligonucleotides were lyophilized. The oligonucleotides were then resuspended in water, annealed in STE (100 mM NaCl, 1 mM EDTA, 10 mM Tris [pH 8.0]) at 85°C, slowly cooled to room temperature, and purified over Sephadex G25. The annealed oligonu-

TABLE 1. Sequences of *supF* target genes[a] and psoralen-conjugated oligonucleotides[b]

Gene or oligonucleotide	Sequence
Genes	
supF	3′ AGGAAGGGGGtggtggtgccggctttaagccatgggccatggg
	5′ TCCTTCCCCCaccaccacggccgaaattcggtacccggtaccc
supFG1	3′ AGGAAGGGGGGGGTGGTGGGGGAGGGGGAGccggctttaagcc
	5′ TCCTTCCCCCCCCACCACCCCCTCCCCCTCggccgaaattcgg
supFG2	3′ AGGAAGGGGGGGGTGGTGGGGGAGGGGGAGGGGGAGGGGAGG
	5′ TCCTTCCCCCCCCACCACCCCCTCCCCCTCCCCCTCCCCCTCC
supFG3	3′ AGGGGAGGGGAGGTGGTGGGGAGGGGAGGGGAGGGGAGGGGAG
	5′ TCCCCTCCCCTCCACCACCCCTCCCCTCCCCTCCCCTCCCCTC
Oligonucleotides	
pso-AG10	5′ pso-AGGAAGGGGG[c]
pso-AGT20	5′ pso-AGGAAGGGGGGGGTGGTGGG
pso-AGT30	5′ pso-AGGAAGGGGGGGGTGGTGGGGGAGGGGGAG
pso-AGTC30	5′ pso-AGTCAGTCAGTCAGTCAGTCAGTCAG
pso-AGT43	5′ pso-AGGAAGGGGGGGGTGGTGGGGGAGGGGGAGGGGGAGGGGGAGG

[a] For each gene, the listed sequences begin at the A · T base pair at position 166. The polypurine-polypyrimidine sites for triple helix formation are indicated in capital letters. The underlined A · T base pair at position 183 in each of the *supF* genes corresponds to the A in the CCA amino acid acceptor site of the mature tRNA.

[b] In addition to the sequences listed, all the psoralen-conjugated oligonucleotides were synthesized in such a way as to contain a 3′ propylamine group to reduce possible degradation by 3′ exonuclease activity. pso-AG10 was designed to bind as a third strand to *supF*. pso-AGT20 and pso-AGT30 were designed to bind to *supFG1*. pso-AGT43 was designed to bind to *supFG2* but not to *supFG3*, which was constructed as a control for comparison with *supFG2*.

[c] pso, psoralen.

cleotides were phosphorylated with T4 polynucleotide kinase, ligated into the plasmid with T4 ligase, purified by ethanol precipitation, and transformed into *Escherichia coli* MBM7070 [*lacZ*(Am)]. Colonies with functional tRNA suppressor genes able to suppress the *lacZ*(Am) mutation in MBM7070 were identified as blue colonies capable of metabolizing X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside). The new genes in selected colonies were sequenced by using the Epicenter Technologies thermal cycle sequencing kit (Madison, Wis.) to confirm that the desired sequence was present.

Triplex binding assays. Third-strand binding to the target duplexes was measured by a gel mobility shift assay. In the case of pso-AGT30 binding to *supFG1*, two complementary 57-mers which contain the sequence corresponding to bp 157 to 213 of *supFG1* were synthesized. Both oligomers were end labeled by using T4 polynucleotide kinase and [γ-^{32}P]ATP. Duplex DNA was prepared by mixing both 57-mers at a ratio of 1:1 in TE buffer (10 mM Tris, 1 mM EDTA [pH 8.0]) and incubating the solution at 37°C for 2 h. A fixed concentration of duplex DNA (10^{-10} M) was incubated with increasing concentrations of the psoralen-linked oligomers in 10 μl of a solution containing 10 mM Tris (pH 7.4), 1 mM spermidine, and 20 mM $MgCl_2$ at 37°C for 2 h. UVA irradiation (1.8 J of broad-band UV light per cm^2 centered at 365 nm; irradiance of 5 mW/cm^2) was used to generate photoadducts and thereby to covalently link the oligomers to their targets. This dose generates approximately 60 to 70% psoralen interstrand cross-links (XL; between the two strands of the target duplex, in addition to the tether connecting the psoralen to the TFO) and 25 to 35% psoralen monoadducts (MA), in the context of the triple helix (data not shown). The samples were mixed with 90 μl of formamide, and a 20-μl aliquot of each sample was analyzed on an 8% polyacrylamide denaturing gel containing 7 M urea. A PhosphorImager (Molecular Dynamics, Sunnyvale, Calif.) was used for quantitation of the reaction products. The concentration at which triplex formation (as indicated by the generation of specific photoadducts) was half-maximal was taken as the equilibrium dissociation constant (K_d). In the case of pso-AG10 binding to *supF*, a 24-bp synthetic duplex target, corresponding to bp 160 to 183 in *supF*, was constructed and end labeled, with binding measured as described above. To evaluate the binding of pso-AGT43 to the site in *supFG2*, a 59-bp duplex target was used.

Mutagenesis protocol. Monkey COS-7 cells were obtained from the American Type Culture Collection (ATCC 1651-CRL). The COS cells, at 70% confluence, were washed with phosphate-buffered saline (PBS)-EDTA, treated with trypsin, and incubated at 37°C for 5 min. The cells were resuspended in Dulbecco modified Eagle medium–10% fetal calf serum and were washed three times by centrifugation at 900 rpm for 5 min (4°C) in a Sorvall RT6000D. The cells were finally resuspended at 10^7 cells per ml. The plasmid DNAs were added at 3 μg of DNA per 10^6 cells, and the cell-DNA mixtures were left on ice for 10 min. Transfection of the cells was performed by electroporation with a Bio-Rad gene pulser at a setting of 25 μF, 250 W, and 250 V in the 0.4-cm-diameter 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 either (i) further diluted and exposed to the oligonucleotides in growth medium at the indicated concentrations while in suspension or (ii) washed, diluted further, and allowed to attach to dishes before oligonucleotide exposure.

The suspension samples were incubated at 37°C with gentle agitation every 15 min. UVA irradiation was administered 2 h later at a dose of 1.8 J/cm^2. 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 2×10^4 cells per cm^2. The cells were harvested 48 h later for vector analysis.

The cells allowed to attach as a monolayer immediately after electroporation were used in two different time course experiments. In one, the cells, as an attached monolayer, were incubated in the presence of the oligonucleotides added to the growth medium of the culture dish at a concentration of 2 μM. At various times following oligonucleotide addition, the cells were washed with PBS and irradiated with UVA light. Fresh growth medium was added, and the cells were harvested 48 h later for analysis. In the other experiment, the cells were left as a monolayer for 12 h, washed again with PBS-EDTA, trypsinized, washed three times with growth medium, and finally exposed to the oligonucleotides in suspension for 2 h, as described above, before UVA irradiation was administered. Again, the cells were collected for shuttle vector isolation 48 h later.

Shuttle vector isolation and analysis. The cells were harvested for vector DNA isolation by 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], 100 μg of RNase A per ml). An equal volume of cell lysis solution (0.2 M NaOH, 1% sodium dodecyl sulfate) was added, followed by 100 μl of neutralization solution (3 M potassium acetate, pH 5.5). A 15-min room temperature (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 *Dpn*I 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 either *E. coli* SY204 [*lacZ125*(Am)] (14, 15) or MBM7070 [*lacZ*(Am)] (30, 31) by electroporation (Bio-Rad; settings, 25 μF, 250 W, and 1800 V; 0.1-cm-diameter cuvette). The transformed *E. coli* cells were plated onto Luria-Bertani plates containing 50 μg of ampicillin per ml, 100 μg of X-Gal per ml, and 1 μM IPTG (isopropyl-β-D-thiogalactopyranoside) and were incubated at 37°C overnight. Mutant colonies containing inactivated *supF* 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 plasmids were isolated for DNA sequence analysis.

DNA sequencing. The purified mutant colonies were picked into 5 ml of L broth containing ampicillin (50 μg/ml) and were incubated at 37°C for 16 to 20 h with shaking at 250 rpm. Cells from 3 ml of culture were collected by centrifugation. Isolation of plasmid DNA was accomplished by using the Wizard plasmid miniprep DNA purification system (Promega, Madison, Wis.). Plasmid DNA was sequenced by using an Applied Biosystems cycle-sequencing kit as directed by the manufacturer. The sequencing primer was chosen to bind to the β-lactamase gene just upstream of the *supF* gene in the vector (30, 31).

RESULTS

Mutagenesis assay. The design of our assay system to study targeted mutagenesis of an SV40 vector in vivo in monkey COS cells is shown in Fig. 1. The SV40 shuttle vector contains the *supF* gene, an amber suppressor tRNA gene of *E. coli*, as the target gene for mutagenesis. Following introduction of the vector DNA into the COS cells by electroporation, the cells are washed and then are incubated, either in suspension or attached to dishes, in the presence of the psoralen-linked oligonucleotides, which are designed to bind to *supF* gene sequences such that the psoralen is delivered to the intended intercalation site at bp 166 to 167. After time is allowed for uptake of the oligonucleotides and for intracellular triplex formation, UVA irradiation is administered to activate the psoralen to form photoadducts, leading to mutations. Another 48 h is allowed for repair and/or replication. The vector DNA is harvested from the cells for genetic analysis of the *supF* gene via bacterial transformation. Prior to transformation, *Dpn*I digestion of the vector DNA is used to eliminate unreplicated vector molecules lacking the mammalian methylation pattern.

Inefficient in vivo targeting by a 10-mer. In our previous in vitro targeted mutagenesis experiments (19), we targeted a 10-bp region of the *supF* gene (bp 167 to 176) in the SV40 vector for triplex formation in the antiparallel motif (2, 3, 23, 32), using a 10-nucleotide psoralen-linked oligomer, pso-AG10. In those experiments, the triplex formation was allowed to occur during a 2-h incubation in vitro in an optimized Mg^{2+}-containing buffer, which was followed by in vitro UVA irradiation and then transfection of the vector-oligonucleotide complex into COS cells. Mutations were generated in the target gene at a frequency of up to 7.3% (19). We subsequently performed experiments in which the SV40 vector and pso-AG10 were cotransfected into the cells without prior coincubation and the cells were subjected to UVA irradiation 2 h later. This protocol yielded targeted mutations in 1.8% of the vector molecules, a frequency within the range of that observed in our in vitro work. However, when we attempted to use pso-AG10 to target mutations to the *supF* gene by treatment of cells already pretransfected with the pSP189 vector, as in the protocol outlined above (Fig. 1), we detected little mutagenesis above the background frequency (0.05% [3 of 6,200 vector molecules] versus a background frequency of 0.02% [3 of 15,100 vector molecules]).

Construction of novel *supF* genes. On the basis of this result and on the basis of the reported binding constants for oligonucleotides of various lengths (10, 35, 36), we hypothesized that the binding affinity for triple helix formation by the 10-mer (pso-AG10) might be insufficient to achieve significant interactions in vivo. To determine the effect of binding affinity on in vivo triplex formation and mutation targeting, we constructed

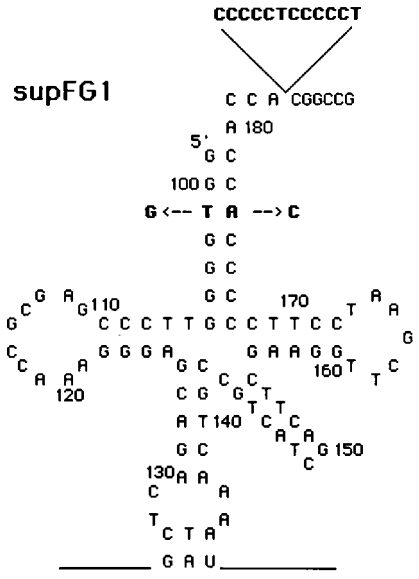

FIG. 2. Strategy for the construction of a modified *supF* gene containing a novel site for triple helix formation. The hypothetical secondary structure of a single DNA strand containing the sequence of the *supF* tRNA is presented. In order to construct *supFG1*, the 93-bp *Xho*I-to-*Eag*I *supF* gene fragment in the pSP189 vector was replaced with a synthetic 105-bp fragment. This fragment was synthesized in such a way as to contain a 12-bp homopurine-homopyrimidine insertion between bp 183 and 184 of the *supF* gene, as indicated. It also incorporated a mutation of the T · A base pair at position 177 to a G · C base pair, along with a compensatory change at position 101 (A · T to C · G) to preserve the structure of the amino acid acceptor stem in the tRNA. The resulting gene, *supFG1*, contained a 30-bp polypyrimidine-polypurine sequence with just two interruptions extending from bp 167 to 196. A psoralen-conjugated polypurine oligonucleotide, pso-AGT30, was designed to bind to the new site in *supFG1* and form the predicted triple helix in the antiparallel motif, as shown.

modified *supF* genes containing new polypurine-polypyrimidine segments. We incorporated them into the vector for use with various oligonucleotides in mutagenesis experiments.

The 93-bp segment in *supF* between unique *Xho*I and *Eag*I sites can be replaced with defined novel sequences. The design of one new *supF* gene, *supFG1*, is illustrated in Fig. 2. To eliminate one interruption at bp 177 in the polypurine-polypyrimidine run, an A · T-to-C · G transversion was incorporated into the synthetic fragment, along with a compensatory T · A-to-G · C change at bp 101 to maintain base pairing in the amino acid acceptor stem of the mature tRNA. In addition, a 12-bp polypurine-polypyrimidine sequence was inserted between bp 183 and 184 to extend the length of the polypurine-polypyrimidine run in the gene to 30 bp. Since the 5′CCA3′ sequence at positions 181 to 183 constitutes the 3′ terminal amino acid acceptor site of the tRNA, the new sequences 3′ to position 183 did not affect the mature tRNA molecule and so did not alter the phenotype of the gene. The new construct contained a 30-bp polypurine site with just two interruptions (Table 1 and Fig. 2).

By a similar method, *supFG2* was also constructed, and it was designed to contain a 43-bp G-rich polypurine site similar to but longer than the site in *supFG1*, also with just two interruptions (Table 1). This site was predicted to be conducive to triple helix formation by a 43-nucleotide, G-rich TFO (pso-AGT43) in the antiparallel motif. With *supF*, *supFG1*, and *supFG2* at hand, we were equipped to embark on an experimental strategy to investigate in vivo targeted mutagenesis by varying not only the putative triplex-forming oligonucleotide but also the triple helix target site. In studying and comparing triplex-directed mutageneses of these tRNA genes, with target sites of 10, 30, and 43 bp, we set out to determine if, in fact, in vivo targeting could be achieved and then to elucidate the requirements for it. An additional gene, *supFG3*, was also created (Table 1). Like *supFG2*, it was designed to contain a 43-bp polypurine site, but with a sequence distinct from that in *supFG2*, enabling further experiments to examine the specificity of the oligonucleotide-mediated targeting.

Triplex binding. To examine triplex formation at the polypurine sites in *supF*, *supFG1*, and *supFG2*, a gel mobility shift assay was used (Fig. 3). Synthetic DNA fragments of either 24 bp (matching bp 160 to 183 in the *supF* gene), 57 bp (matching bp 157 to 213 in *supFG1*), or 59 bp (matching bp 159 to 217 in *supFG2*) were used as duplex targets for triplex formation. The oligonucleotides tested included pso-AG10, pso-AGT30, and pso-AGT43, which were designed to bind in the antiparallel motif to the 10-, 30-, and 43-bp sites in these genes, respectively. pso-AGT20, designed to bind to bp 167 to 186 in *supFG1*, was also tested. Fixed concentrations of radioactively labeled duplex target DNA were incubated with increasing concentrations of the psoralen-linked oligomers. 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 (Fig. 3).

Results of analyses of the binding of pso-AG10 and pso-AGT30 to their respective sites in the *supF* and *supFG1* genes are shown in Fig. 3A and B, respectively. Photoactivation of the triplex molecules with 1.8 J of UVA per cm^2 led to a high proportion of XL (the psoralen-TFO covalently bound to both strands of the duplex target via a psoralen interstrand XL) and to a low proportion of psoralen MA (the psoralen-TFO covalently linked to just one strand of the duplex via psoralen MA formation). The percentage of the sample constituting the sum of the XL and MA bands is proportionate to the extent of triple helix formation. When oligonucleotides that cannot form triplexes at the target site were used in this assay, no XL or MA bands were visualized (data not shown), and so adducts can be taken to be indicative of triplex formation. As the concentration of the triplex-forming oligonucleotide is increased, the proportion of the target duplex bound as either MA or XL, as opposed to unbound target duplex, increases. As can be seen from the autoradiographs and from the corresponding graphical analysis shown in Fig. 3C, the concentration dependence of triplex formation by pso-AG10 is quite different from that of triplex formation by pso-AGT30. A useful measure of triple helix formation is the equilibrium dissociation constant, K_d, which can be estimated as the concentration of TFO at which triplex formation is half-maximal. For pso-AG10 the K_d is 8×10^{-7} M, whereas for pso-AGT30, the K_d is 3×10^{-9} M, a 270-fold difference. The greater affinity for triplex formation by pso-AGT30 is consistent with the results of other studies correlating oligonucleotide length and binding affinity (10, 35, 36), and it places the affinity of pso-AGT30 for binding to *supFG1* in the range of physiologic interactions. Similar analyses revealed K_ds of approximately 10^{-9} M for pso-AGT43 binding to *supFG2* and 8×10^{-9} M for pso-AGT20 binding to *supFG1* (data not shown).

To determine the contribution of the psoralen conjugate to

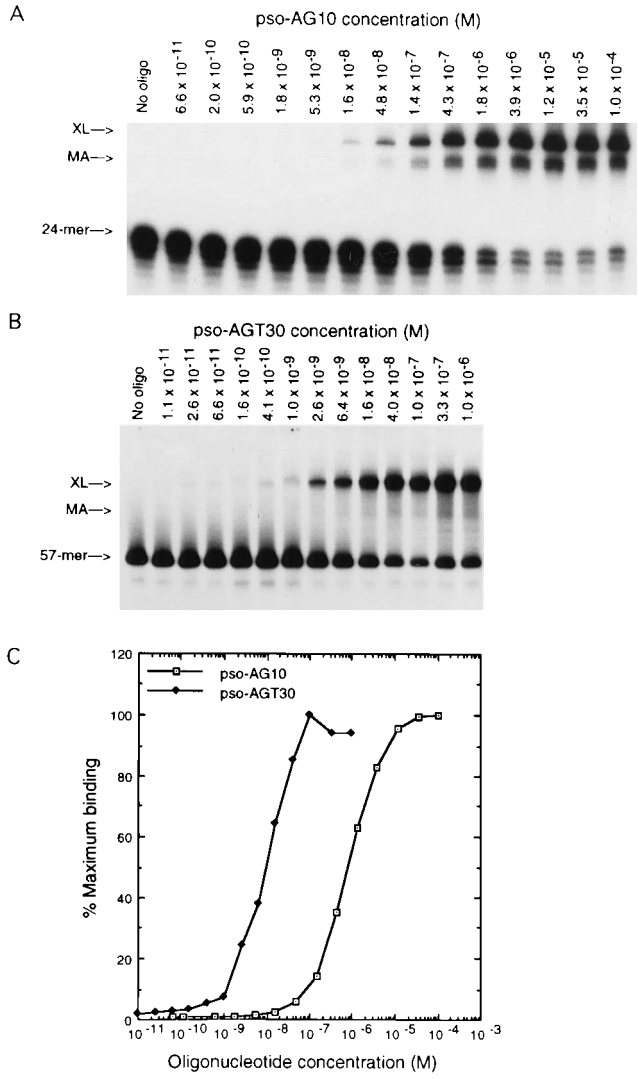

FIG. 3. Comparison of third-strand binding affinities. The binding affinity of pso-AG10 to its target site in the *supF* gene (A) was compared with the binding affinity of pso-AGT30 to its site in the *supFG1* gene (B) over a range of oligonucleotide concentrations. Synthetic 24-bp (A) and 57-bp (B) duplexes (containing the 10- and 30-bp polypurine sites in *supF* and *supFG1*, respectively) were end labeled with ^{32}P and incubated at fixed concentrations with increasing concentrations of either pso-AG10 (A) or pso-AGT30 (B). After 2 h, UVA irradiation (1.8 J/cm^2) was administered to covalently link the psoralen-conjugated oligonucleotides that had bound as third strands to their respective target duplexes. The samples were analyzed by denaturing gel electrophoresis. Third-strand binding is revealed by the bands of reduced mobility corresponding to the psoralen-conjugated oligonucleotide covalently linked either to one strand of the duplex (via a psoralen MA, yielding a two-stranded molecule upon denaturation) or to both strands of the duplex (via a psoralen interstrand XL, forming a three-stranded molecule that persists upon denaturation). The bands corresponding to the 24-mer and the 57-mer represent the denatured single strands from the duplex molecules not bound to the third-strand oligonucleotides. (C) The concentration dependence of the triplex formation by pso-AG10 and that of the triplex formation by pso-AGT30 are graphically compared.

the third-strand binding, we examined triplex formation by AGT30 (without psoralen), using a modified gel mobility shift assay (10). The K_d for triplex formation by AGT30 was 2×10^{-8} M (data not shown), indicating a sevenfold-weaker binding compared with that of pso-AGT30. This difference reflects the thermodynamic benefit of psoralen intercalation.

Targeted mutagenesis in vivo. Using pSupFG1 as a target vector, we carried out experiments to study intracellular mutation targeting by three psoralen-linked oligonucleotides, all of which were designed to form a triple helix with sequences in *supFG1* and to deliver the tethered psoralen to bp 166 to 167 (Table 2). However, the oligomers differed in length and binding affinity for triplex formation (Tables 1 and 2 and Fig. 3). In these experiments, the vector DNA was introduced into the cells by electroporation, the cells were washed, and 1 h later the oligonucleotides were added to the cells in suspension. The cells were irradiated with UVA 2 h after oligonucleotide addition and were transferred to culture dishes for 48 h before rescue of the vector DNA for analysis. Control cells received no oligonucleotide but were irradiated with UVA. The extent of mutagenesis in the *supFG1* gene was seen to depend on the measured strengths of triplex formation by the respective TFOs (Table 2). At concentrations of up to 2 μM, pso-AG10 produced little mutagenesis above the background level. In contrast, pso-AGT30 induced mutations in up to 2.1% of the vector molecules, 70-fold above the background frequency (0.03%). Significant levels of mutagenesis were also produced by pso-AGT20, but they were lower than those seen with pso-AGT30 at the two concentrations tested. These results provide evidence for oligonucleotide-directed mutagenesis within cells, and they help to define the requirements for efficient in vivo triplex formation and mutation targeting.

TABLE 2. Targeted mutagenesis of pSupFG1 within COS cells

Oligonucleotide	K_d for third-strand binding (M)	Concn (μM)	Mutation frequency (%)[a]	No. of mutants/ total[b]
None[c]			0.03	4/14,700
pso-AG10	8×10^{-7}	0.9	0.05	4/8,674
		2.0	0.06	2/3,148
pso-AGT20	8×10^{-9}	0.45	0.3	24/9,525
		2.0	1.2	13/1,073
pso-AGT30	3×10^{-9}	0.45	1.0	51/5,175
		2.0	2.1	48/2,285

[a] The values represent the frequencies of mutations detected in the pSupFG1 vector following electroporation of the vector DNA into COS cells and subsequent treatment of the cells with the indicated oligonucleotides, UVA irradiation, and rescue of the vectors for genetic analysis in bacteria 48 h later.

[b] Number of vector molecules containing mutations per total number of vector molecules as determined on the basis of colony phenotype.

[c] Although no oligonucleotide was added to the control samples, UVA irradiation was administered in parallel with the other samples.

It is theoretically possible that in these experiments some targeted adducts that were not subsequently replicated or repaired before the molecules were transformed into bacteria for analysis were generated in vectors. In such cases, we might have been observing the results of bacterial processing of persistent lesions that were produced in but not processed in the COS cells. Although the *Dpn*I restriction step was designed to eliminate vector molecules that had not replicated in the COS cells, some molecules may have already undergone replication prior to the triplex formation and UVA irradiation. To examine the possible contribution that unprocessed adducts could make to our data, we allowed pso-AGT30 and pSupFG1 to form a triplex in vitro, irradiated the complex with 1.8 J of UVA irradiation per cm^2, and used the sample to directly transform *E. coli* without passage through COS cells. A mutation frequency of only 0.03% (4 of 15,000), which is 70-fold lower than the level of induced mutagenesis seen with pso-AGT30 treatment of the COS cells, was seen. Therefore, the

TABLE 3. Time dependence of targeted mutagenesis within COS cells

Oligonucleotide[a]	Time of UVA irradiation (h after oligonucleotide addition)	Mutation frequency (%)[b]	No. of mutants/ total[c]
None	NA[d]	0.08	3/3,900
pso-AGT30	1	0.2	7/3,275
	2	1.4	25/1,825
	4	0.7	14/2,150
	8	0.1	4/3,850

[a] The oligonucleotide concentration used was 2 μM for all time points.
[b] The values represent the frequencies of mutations detected in the pSupFG1 vector following electroporation of the vector DNA into COS cells, reattachment of the cells as a monolayer, and subsequent incubation of the cells with the oligonucleotide, UVA irradiation of the cells as indicated, and rescue of the vectors for genetic analysis in bacteria.
[c] Number of pSupFG1 vector molecules containing mutations per total number of vector molecules as determined on the basis of colony phenotype.
[d] NA, not applicable. The control sample was not treated with oligonucleotide but did receive UVA irradiation.

TABLE 4. Concentration dependence of the oligonucleotide-mediated targeted mutagenesis within COS cells

Oligonucleotide	Concn (nM)	Mutation frequency (%)[a]	No. of mutants/ total[b]
None	0	0.05	2/3,940
pso-AGT30	1	0.1	3/3,100
	10	0.2	5/2,425
	100	0.2	8/3,625
	450	1.0	51/5,175
	1,000	1.1	37/3,400
	2,000	2.1	178/8,663
	10,000	0.4	8/2,200

[a] The values represent the frequencies of mutations induced at the oligonucleotide concentrations listed. All experimental samples received UVA irradiation at 2 h after addition of oligonucleotide to the cells.
[b] Number of vector molecules containing mutations per total number of vector molecules as determined on the basis of colony phenotype.

targeted mutagenesis observed in our experiments cannot be accounted for by the rescue of unprocessed, lesion-containing vector molecules from the COS cells.

Time course of UVA irradiation. We next sought to investigate the kinetics of intracellular targeted mutagenesis mediated by TFOs. This process depends on the entry of the oligonucleotides into the cells, migration into the nucleus, and specific binding to the triplex target site. In order to achieve targeted mutagenesis via site-specific generation of a psoralen photoadduct, these steps must occur by the time the UVA irradiation is administered. A time course experiment in which the time of UVA irradiation following pso-AGT30 addition to the cells was varied was carried out (Table 3). In this experiment, the cells were electroporated with the pSupFG1 vector, washed, diluted with growth medium, and allowed to attach as a monolayer in the culture dish before addition of the oligonucleotide to the culture medium. (The cells were treated as a monolayer rather than maintained in suspension for this experiment because prolonged incubation of the COS cells in suspension leads to cell aggregation.) UVA irradiation was administered at the indicated times. As the interval between oligonucleotide addition and UVA irradiation was increased, the yield of targeted mutations initially increased, with a frequency as high as 1.4% for the 2-h point. However, at the later time points, the yield of mutations decreased, probably because of the degradation of the oligonucleotide within the cells, even though the oligonucleotides used were synthesized in such a way as to contain a 3′ propylamine to inhibit 3′ exonuclease activity (21, 29). The results indicate that at least 1 to 2 h is required for cellular uptake of the oligonucleotides and for intracellular triplex formation.

Timing of oligonucleotide addition and the effect of chromatin. To test the effect of chromatin on triple helix formation, we transfected cells with pSupFG1, incubated them for 12 h (instead of 1 h) to allow sufficient time for chromatin assembly on the SV40 vector DNA (7), detached them, and exposed them to 2 μM pso-AGT30 for 2 h before UVA irradiation. Mutations were generated in the *supFG1* gene at a frequency of 1.5% (150 of 10,175), which is in the range of frequencies seen when the cells were exposed to 2 μM pso-AGT30 within 1 h after SV40 transfection (1.4 to 2.1%; Tables 2 and 3). This provides evidence that triplex formation can occur within chromatin.

Concentration dependence. The concentration dependence of targeted mutagenesis was also investigated (Table 4). The SV40 vector-containing COS cells were incubated in suspension in the presence of pso-AGT30 at concentrations ranging from 1 nM to 2 μM. UVA irradiation was administered 2 h later. Although we did not directly measure the intracellular oligonucleotide concentrations in these experiments, other studies have reported that treatment of mammalian cells with oligonucleotides produces concentrations within cells that are in the same range as or even higher than the extracellular concentrations (21, 29). A low level of mutagenesis, just slightly above the background level, was observed when the extracellular oligonucleotide concentration was in the range of 1 to 100 nM. Significant mutagenesis was seen only at concentrations in the range of 450 nM to 2 μM (mutation frequencies from 1.0 to 2.1%). It might have been expected that concentrations in the range of 1 to 100 nM would be effective in targeting mutations, since the K_d for triplex formation by pso-AGT30 and *supFG1* was determined by measurement to be approximately 3×10^{-9} M. However, this measurement was performed in vitro in an optimized Mg^{2+}-containing buffer and in the absence of K^+, which can inhibit antiparallel triplex formation (8, 28). Cheng and Van Dyke (8) reported that physiologic levels of K^+ can reduce triplex formation by a factor of at least 6. This effect may explain, in part, why extracellular TFO concentrations in the range of 1 μM or more are needed for significant levels of targeted mutagenesis in vivo. However, much higher concentrations may not necessarily lead to further improvements. We also tested a concentration of 10 μM pso-AGT30. At this high concentration, some cellular toxicity, presumably due to the participation of the psoralen in secondary photoreactions with cellular macromolecules, was observed following UVA irradiation. The frequency of mutations observed in this case fell to 0.4%, probably because of this toxicity.

Sequence analysis. The DNA sequences of 21 mutations induced in the *supFG1* gene by pso-AGT30 are presented in Fig. 4. These mutations were generated in 15 separate experiments, including the time and concentration dependence analyses. To avoid siblings, a particular mutation was counted only once within each experiment. Of the 21 mutations, 15 (71%) were T · A-to-A · T transversions at bp 166 (the predicted psoralen intercalation site), while 2 were point mutations at nearby base pairs and 4 were deletions of various sizes spanning the triplex target site. The observed frequency of deletions may be an underestimate, since larger deletions involving

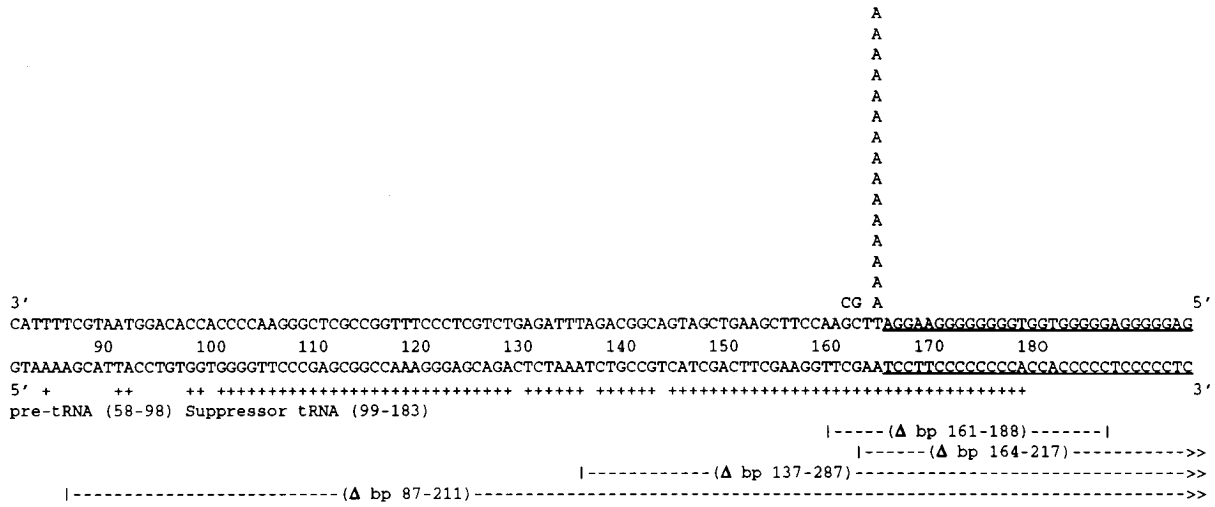

FIG. 4. Sequence analysis of mutations targeted in vivo within COS cells to the *supFG1* gene in the SV40 vector by the psoralen-conjugated, triple helix-forming oligonucleotide pso-AGT30. Point mutations produced by pso-AGT30 and UVA treatment of the vector-containing cells are indicated above each base pair, with the listed base representing a change from the sequence in the top strand. Deletion mutations are presented below the *supFG1* sequence; they are indicated by the dashed lines. The + signs below the sequence are sites in the *supF* gene at which mutations are known to produce a detectable phenotype change (24), demonstrating that the use of *supF* in this assay does not bias detection of mutations at any particular site. The underlined nucleotides delineate the site targeted for triple helix formation.

sequences surrounding the *supFG1* gene would inactivate genes essential for vector replication and so molecules undergoing such deletions would not be detected in the assay. These results demonstrate the base pair specificity of the intracellular mutation targeting and suggest that the psoralen adducts can generate either specific point mutations or deletions at the target site.

Specificity: the effect of partial homology on triplex formation. To test the effect of the degree of homology on triplex formation, we compared targeted mutageneses involving the four target genes and the three oligonucleotides shown in Table 5. The respective sequences of these genes and nucleotides are shown in Table 1.

The construction of the target genes and the design of the oligonucleotides allowed us to examine the effects of partial homologies that were disrupted by mismatches in two ways. For some of the oligonucleotide-target site pairs, the partial homologies were interrupted by scattered mismatches throughout the putative triple helix-forming domain (pso-AGT43 and *supFG3* and pso-AGTC30 and *supFG1*). For the others, the homologies for potential triple helix formation were disrupted by mismatches arising from sequences at the 3′ end of the oligonucleotide (pso-AGT43 and *supFG1* and pso-AGT30 and *supF*).

pso-AGT43 was able to effectively target mutations to *supFG2*, to which it has almost complete homology for triplex formation (only 2 mismatches), but it generated very little mutagenesis above the background frequency in either *supFG1* or *supFG3* (13 and 12 mismatches, respectively). Hence, the number of mismatches plays a major role in this in vivo assay for triplex-directed mutagenesis. In the examples tested, both terminal and internal mismatches prevented significant in vivo interactions, even though as many as 30 or 31 of 43 nucleotides in pso-AGT43 fit the third-strand binding code for triplex formation with the *supFG1* and *supFG3* genes. Partial homologies of this degree are therefore not sufficient to mediate significant mutation targeting in this assay.

To further consider the effect of terminal mismatches on the stability of triple helix formation, we directly measured the binding of pso-AGT43 to the 57-bp fragment containing the

TABLE 5. Site specificity of the oligonucleotide-mediated targeted mutagenesis

Oligonucleotide	Target gene	No. of mismatches/ total no. of base pairs[a]	Position of mismatches	Mutation frequency (%)[b]	No. of mutants/ total[c]
pso-AGT43	*supFG2*	2/43	Internal	1.4	223/16,425
pso-AGT43	*supFG1*	13/43	3′ terminal	0.1	7/6,887
pso-AGT43	*supFG3*	12/43	Internal	0.06	4/6,725
pso-AGT30	*supFG1*	2/30	Internal	2.1	178/8,663
pso-AGT30	*supF*	13/30	3′ terminal	≤0.02	0/5,300
pso-AGTC30	*supFG1*	20/30	Internal	0.03	3/8,740

[a] The values represent the fraction of base mismatches between the listed oligonucleotide and the designated target gene in the antiparallel motif for triple helix formation. The following are considered matches: G for a G · C base pair and either A or T for an A · T base pair. Any other combination of third-strand nucleotide and duplex base pair is considered a mismatch. The relevant sequences are listed in Table 1.

[b] The values represent the frequencies of induced mutations observed by using oligonucleotide concentrations of 2 μM, with UVA irradiation administered at 2 h after addition of oligonucleotide to the cells.

[c] Number of vector molecules containing mutations per total number of vector molecules as determined on the basis of colony phenotype.

30-bp polypurine site in *supFG1* (Fig. 3). We found that the binding was 30-fold weaker than the binding of pso-AGT30 to the same site (K_d of 1×10^{-7} M versus 3×10^{-9} M; data not shown). Hence, we conclude that the terminal mismatches between pso-AGT43 and *supFG1* destabilize triple helix formation sufficiently to prevent any significant in vivo targeting of *supFG1* by pso-AGT43.

In conceptually similar experiments, we also compared the ability of pso-AGT30 to target the *supFG1* gene (with which it is specifically designed to form a triplex) with its ability to target the original *supF* gene (to which it has homology in the case of only 10 of 30 nucleotides for triplex formation). We found that pso-AGT30 can target mutations to *supFG1* but that it is not effective in inducing mutations in the original *supF* gene.

As another control, we tested whether an oligonucleotide of 30 nucleotides containing a mixture of all four bases, pso-AGTC30, could induce mutations in *supFG1*. This oligonucleotide would not be expected to form a triple helix with *supFG1*, since it had 20 mismatches and only 10 matches in the binding code for antiparallel triplex formation with bp 167 to 196 of *supFG1*. We found no mutagenesis above the background level with this oligonucleotide. In contrast, pso-AGT30 produced mutations in *supFG1* at a frequency of 2.1%. Both oligonucleotides are 30 nucleotides in length, but they vary in nucleotide sequence and consequently in affinity for triplex formation with *supFG1*. Overall, these results demonstrate the specificity of the triplex-mediated intracellular targeting.

Interestingly, the data shown in Table 5 also indicate that the 43-mer (pso-AGT43) was not superior to the 30-mer (pso-AGT30) for generating targeted mutagenesis in vivo. The frequency of targeted mutations induced by pso-AGT43 in *supFG2* was, in fact, slightly lower than that seen with pso-AGT30 and *supFG1*. This is in contrast to the increase in the frequency of targeted mutations observed in going from pso-AG10 to pso-AGT20 to pso-AGT30 (Table 2). The 43-nucleotide-long and very G-rich pso-AGT43 may be subject to increased levels of K^+-driven self-association to form G-quartets (1, 34), limiting its effectiveness within the cells. However, this remains to be established.

DISCUSSION

The results presented here demonstrate the possibility of using a triple helix-forming oligonucleotide to generate targeted mutations within mammalian cells. Linkage of the oligonucleotide to a mutagen, psoralen, confers sequence specificity on the action of the psoralen, which is delivered to the selected site via intracellular triple helix formation. Using an oligonucleotide that binds strongly as a third strand to the target gene, we observed targeted mutagenesis in vivo at frequencies in the range of 1 to 2%.

A modified *supF* mutation reporter gene, *supFG1*, containing a 30-bp polypurine site optimized for triplex formation was constructed. The use of this new gene in an SV40 vector allowed investigation of parameters governing intracellular triplex formation and mutation targeting. By comparing three oligonucleotides, all designed to form triplexes with *supFG1* and to deliver psoralen to positions 166 to 167 but varying in length and K_d for triplex formation, it was determined that an oligomer with a K_d in the range of 10^{-9} M is required for significant intracellular interactions.

Other factors that might affect triplex formation under physiologic conditions, such as oligonucleotide base composition and backbone structure, could be tested by using this type of assay. Since selected sequences within and adjacent to the *supF* gene can be manipulated without disrupting suppressor activity, additional target sites designed to specifically examine other aspects of intracellular triplex formation could be constructed. The modification of sequences within the *supF* target gene has also been used to study sequence context effects on UV mutagenesis in human cells (30).

Sequence analysis of the mutations targeted by pso-AGT30 revealed a high specificity for T · A-to-A · T transversions at the predicted psoralen intercalation site. Of 21 mutations, 15 (71%) were T · A-to-A · T transversions at that site. Two other point mutations (C · G-to-G · C transversions) were also seen at positions 163 and 164. These mutations at nearby bases may arise from perturbations in polymerase fidelity caused by the targeted psoralen adducts. However, the relative contributions of bypass replication and excision repair in generating the ultimate mutations from the targeted premutagenic adducts in this system have yet to be elucidated.

The several deletion mutations, clustered around the targeted site, may arise from the site-specific psoralen XL between the two strands of the target duplex, in a process mediated by strand breaks. However, this remains to be determined. Hence, although the triplex-targeted mutations are highly specific for the target site, there may be some variability in the mechanism by which the adducts are processed and in the kinds of mutations which are produced. If strand breaks are generated in the repair of the triplex-directed adducts, then the triplex targeting strategy could be used not just to induce mutations directly but also to create recombinogenic substrates. It is conceivable that the production of single or double strand breaks at the site of the triplex-directed psoralen adduct might enhance homologous recombination at that site, in a process of repair-coupled recombination.

The specificity of the triplex-mediated targeting is demonstrated not only in the base pair specificity of the mutagenesis but also in the failure of partially homologous TFOs to generate mutations in the target genes. pso-AGT43 successfully targeted mutations to *supFG2*, to which it binds well as a third strand. However, this oligonucleotide was ineffective in targeting mutations to either *supFG1* or *supFG3*, genes to which it has substantial but incomplete homology for triplex formation. Similar results were found in the inability of pso-AGT30 to target mutations to the unmodified *supF*. However, this latter result was not surprising, since pso-AGT30 has only 13 nucleotides of homology to the site in *supF*. On the basis of our work with pso-AG10, which binds well to the 10-bp polypurine site in *supF* in vitro but did not induce mutations in *supF* within COS cells in vivo, we would have predicted that partial homology consisting of 13 of 30 bp would be insufficient for significant in vivo interactions. Nonetheless, the fact that pso-AGT30 effectively targeted mutations to *supFG1* but not to *supF* serves as an additional control supporting the interpretation that the observed mutagenesis was, in fact, targeted by sequence-specific triple helix formation.

The results of the time course experiment (in which the time of UVA irradiation after oligonucleotide addition was varied) indicate that the processes of oligonucleotide entry into cells and of intracellular triplex formation occur over at least 1 to 2 h. These results are consistent with direct measurements of oligonucleotide uptake by peripheral blood mononuclear cells, which showed near-maximal uptake within 2 h after oligonucleotide exposure (29). Higher levels of mutagenesis might be seen at later time points if oligonucleotide uptake and stability could be enhanced. For example, conjugation of cholesterol to the 3′ end has recently been shown to improve cellular uptake of oligonucleotides (21). We did modify the oligonucleotides with a 3′ propylamine group, since this has been reported to

reduce 3′ exonuclease activity and enhance oligonucleotide stability (29). However, additional modifications may increase the activity of the oligonucleotides in vivo.

In our mutagenesis assay, the highest frequency of targeted mutations was seen when the concentration of oligonucleotide added to the culture medium was 2 μM. This concentration is somewhat lower than the concentrations of triplex-forming oligonucleotides reported to be required for inhibition of gene expression in mammalian cells (21, 29, 33). In those experiments, concentrations in the range of 10 to 100 μM were used. The fact that targeted mutations were seen in our work at oligonucleotide concentrations too low to produce inhibition of gene expression may in the first place reflect differences in assay sensitivity, since the mutagenesis assay is more sensitive than standard methods of measuring gene expression. As shown above, we can measure mutation frequencies as low as 0.1%. In contrast, 0.1 or even 1% inhibition of gene expression would be impossible to detect. Secondly, the different concentration dependencies in these experiments may reflect mechanistic differences between transcription inhibition and targeted mutagenesis by TFOs. Inhibition of gene expression requires the persistent presence of the triple helix to block transcription factors. It has been shown that such triple helices are labile and that they can be disrupted by transcriptional activity at nearby promoters (37). It is therefore not surprising that, in the reported experiments to block gene expression, high concentrations of oligonucleotides were needed in order to inactivate a significant proportion of the promoter sites by third-strand formation in the steady state. However, to induce a targeted mutation, such prolonged persistence of the triplex helix is not necessary. The mutagen must be delivered to the target site to generate the DNA damage, but after that the maintenance of the triple helix is not required.

The frequencies of targeted mutations observed in this work, in the range of 1 to 2%, were lower than the frequencies (6 to 7%) that we observed following transfection into COS cells of vector molecules containing preformed triplex-directed psoralen adducts that were generated in vitro (19). Clearly, forming a triple helix in vitro under controlled buffer conditions is an easier task than doing so in vivo with exogenously added oligonucleotides, which must be taken up by the cells, enter the nuclei, and bind to the target site under suboptimal conditions. In fact, it is encouraging that a targeted mutation frequency of even 1 to 2% was observed in this work. With modifications to enhance oligonucleotide uptake, stability, and triplex formation under physiologic conditions, it may be possible to improve upon the present results.

Nonetheless, the work presented here provides strong evidence for the ability of exogenously added oligonucleotides to form triple helices at sites within cells. Oligonucleotides designed to form triple helices at promoter sites have been reported to specifically inhibit expression of certain genes within cells (21, 33), but questions as to whether some of these effects were mediated by site-specific triplex formation or by interactions of the oligonucleotides with other cellular components that might influence gene expression have been raised (28). The basis for these concerns is the observation that high K^+ ion concentrations, such as those found within cells, can inhibit antiparallel triplex formation (8, 28). It is likely that intracellular conditions do influence the efficiency of intermolecular triple helix formation, but our finding of base pair-specific mutations precisely at the intended triple helix target site argues that triple helix formation by the psoralen-conjugated oligonucleotides did, in fact, occur at the targeted site within the cells. It would be difficult to imagine how interaction of the oligonucleotides with transcription factors or other molecules could mediate such a precise mutagenesis.

The similar frequencies of targeted mutagenesis seen whether the cells were treated with the oligonucleotides at 1 h or at 12 h after electroporation with the vector DNA further support the argument that the targeted mutagenesis that we observed was mediated by intracellular triplex formation. In the 12-h experiment, the cells were electroporated with the SV40 vector DNA, washed, grown for 12 h as a monolayer in culture, trypsinized, diluted, and extensively washed before the oligonucleotides were added to the culture medium. Under these conditions, it would be unlikely that any vector DNA would persist in the extracellular medium at the time of oligonucleotide treatment. In addition, by 12 h after SV40 transfection, chromatin assembly on the vector molecules would be expected to occur. Hence, this result suggests not only that the triplex formation occurred within the cells, but also that it occurred at a target site within chromatin.

The ability of selected oligonucleotides to enter cells, form site-specific triple helices, and mediate targeted mutagenesis may provide a useful research tool for in vivo genetic manipulation of cells. It may also be useful as a method to study DNA damage and repair pathways and as a technique to probe genome structure. Theoretically, targeted mutagenesis and inactivation of selected genes might also eventually have therapeutic applications. However, the general applicability of this approach will depend on the extension of the third-strand binding code and the development of nucleotide analogs so that triple helix formation is not limited to polypurine sequences. Much work in this regard is under way (13, 16, 22, 27, 28, 38). Further experiments to develop a better understanding of cellular repair and replication of the triplex-directed lesion are also needed. In addition, the work reported here was performed with a highly constrained experimental model system, and targeted mutagenesis of a chromosomal gene by this approach has yet to be demonstrated.

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