

Chromosomal mutations induced by triplex-forming oligonucleotides in mammalian cells

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ABSTRACT

Specific recognition of a region of duplex DNA by triplex-forming oligonucleotides (TFOs) provides an attractive strategy for genetic manipulation. Based on this, we have investigated the ability of the triplex-directed approach to induce mutations at a chromosomal locus in living cells. A mouse fibroblast cell line was constructed containing multiple chromosomal copies of the λ supFG1 vector carrying the *supFG1* mutation-reporter gene. Cells were treated with specific (psoAG30) or control (psoSCR30) psoralen-conjugated TFOs in the presence and absence of UVA irradiation. The results demonstrated a 6- to 10-fold induction of *supFG1* mutations in the psoAG30-treated cells as compared with psoSCR30-treated or untreated control cells. Interestingly, UVA irradiation had no effect on the mutation frequencies induced by the psoralen-conjugated TFOs, suggesting a triplex-mediated but photoproduct-independent process of mutagenesis. Sequencing data were consistent with this finding since the expected T·A→A·T transversions at the predicted psoralen crosslinking site were not detected. However, insertions and deletions were detected within the triplex binding site, indicating a TFO-specific induction of mutagenesis. This result demonstrates the ability of triplex-forming oligonucleotides to influence mutation frequencies at a specific site in a mammalian chromosome.

INTRODUCTION

A promising approach to targeting a specific DNA sequence is through formation of triplex DNA. By delivering reagents to specific sites on chromosomes in living cells it is theoretically possible to modify a mammalian genome (1). The recognition of a specific dsDNA target by a single-stranded triplex-forming oligonucleotide (TFO) was demonstrated over a decade ago (2). Since that time TFOs have been used to inhibit protein binding to DNA (3,4), to inhibit gene expression (5–9), to inhibit replication (10,11), to direct site-specific DNA damage (2,12–14), to enhance recombination (K.M.Vasquez, PhD thesis, 1996; 15,16) and to induce site-specific mutagenesis (13,17–19).

TFOs conjugated to a DNA-damaging agent can direct damage to a single site within megabases of DNA (20,21). The specificity afforded by TFOs is derived from the hydrogen bonding patterns between the single-stranded TFO and the double-stranded DNA target (22). Purine TFOs bind in the major groove of the underlying target duplex in an anti-parallel fashion via reverse Hoogsteen hydrogen bonds, forming stable triple helices at physiological pH.

By utilizing triplex technology to target site-specific mutations, permanent, heritable changes in gene function and expression can be achieved. Examples of this have been demonstrated in our laboratory using the *supF* reporter gene (13,17–19). These studies showed that a 30mer TFO targeted to the *supFG1* reporter gene stimulated mutation ~10-fold, while the same TFO with psoralen linked to the 5'-end stimulated mutation nearly 100-fold on plasmid DNA in mammalian cells (13). Interestingly, triplex-directed mutagenesis was not detected in repair-deficient cells, indicating a requirement for both nucleotide excision repair and transcription-coupled repair to generate mutations. It is proposed that the mutagenesis induced by triplex formation is mediated by error-prone, abortive repair at the triplex site (19). Although these extrachromosomal results are encouraging, it was our goal to take the next step with this approach by demonstrating triplex-mediated mutagenesis on the chromosome in mammalian cells.

The work reported here describes the initial steps toward the development of a new approach to genome modification via targeted mutagenesis and its application to the *supFG1* reporter gene in a chromosomal context in mouse cells. We report that TFOs can induce specific mutations within a chromosomal reporter gene. Transgenic mice were generated containing multiple copies of λ vector DNA containing the *supFG1* gene, and transformed cell lines (3340) were established from these mice (23). To test whether mutations could be induced in a chromosomal target gene via triplex formation, 3340 cells were incubated with TFOs, UVA irradiated and assayed for mutagenesis by phage vector rescue and analysis. Mutations were induced in *supFG1* at a frequency nearly 10-fold above background and were localized to the triplex binding site. This result demonstrates the ability of TFOs to influence mutation frequencies at a specific site in a mammalian chromosome and supports the potential therapeutic application of TFOs.

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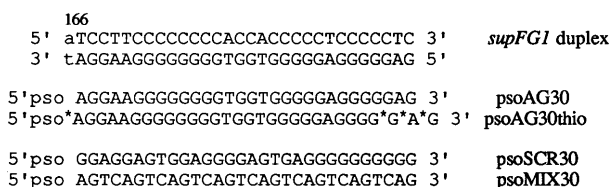


Figure 1. *SupFG1* triplex target site and corresponding anti-parallel TFOs. Nucleotide sequences of the *supFG1* target site duplex and TFOs are depicted in the predicted binding orientation. PsoAG30thio contained phosphorothioate linkages in the four positions indicated by asterisks. All TFOs were modified with a 4'-hydroxymethyl-4,5',8-trimethylpsoralen group at the 5'-end. PsoAG30, psoSCR30 and psoMIX30 were modified with a 3'-OPO₂OCH₂CHOH-CH₂NH₃⁺ group. The control TFO, psoSCR30, was designed with the same base composition as psoAG30, but a scrambled sequence. PsoMIX30 was a mixed sequence control TFO.

MATERIALS AND METHODS

Oligonucleotide synthesis and purification

The sequences and modifications of oligodeoxyribonucleotides used are shown in Figure 1. Oligodeoxyribonucleotides were provided by Codon Pharmaceuticals (Gaithersburg, MD). All oligonucleotides contained a propanolamine group at the 3'-end (3'-OPO₂OCH₂CHOHCH₂NH₃⁺; Glen Research, Sterling, VA), unless otherwise noted. Oligonucleotides were synthesized on an automated Expedite DNA synthesizer from Perseptive Biosystems (Framingham, MA) using standard solid-phase chemistry.

Cell mutagenesis protocol

The mouse fibroblast cell line (3340) containing ~15 copies of λ supFG1 shuttle vector DNA was established from a 3340 *supFG1* mouse skin biopsy as previously described (23). The cells were maintained and treated in medium (DMEM supplemented with 20% fetal calf serum) containing G418 at 0.2 mg/ml (Life Technologies, Gaithersburg, MD). TFOs (at a final concentration of 2 μ M) were added in complete culture medium to the cells and incubation was carried out for the indicated times at 37°C. Following incubation, UVA irradiation was administered at a dose of 1.8 J/cm² (unless otherwise noted). Cells were collected for shuttle vector rescue and analysis 2–4 days later. Genomic DNA was isolated from the cells as described (24). The DNA was incubated with λ *in vitro* packaging extracts for λ vector rescue from the genomic DNA as described (25).

RESULTS

Experimental design

In order to study the mutagenic potential of triplex-directed lesions in mammalian cells, we utilized a mouse fibroblast cell line (3340) containing multiple copies of λ supFG1 shuttle vector DNA in a chromosomal locus (23). The *supFG1* gene contains a 30 bp triplex target site and encodes a tRNA that suppresses amber mutations. Using packaging extracts, the vector DNA can be isolated from the mouse genomic DNA into phage particles for subsequent mutational analysis (23,26,27). We chose the *supFG1* gene as our triplex target because site-directed mutagenesis at this gene induced by TFOs, both *in vitro* and on plasmid DNA in mammalian cells, has been well established (13,19). The 4'-hydroxymethyl-4,5',8-trimethylpsoralen (HMT)-conjugated

TFOs used in this study were designed to bind to the 30 bp polypurine site in the *supFG1* gene in an anti-parallel fashion such that the psoralen is positioned at the 5'-ApT psoralen crosslinking site at bp 166–167 (Fig. 1). PsoAG30 binds its target duplex specifically and with high affinity ($K_d = 10^{-9}$ M; 13), while the control TFO, psoSCR30, shows no binding to the duplex target up to 10⁻⁶ M.

The experimental design is shown in Figure 2. Monolayer cells were incubated for 2–4 h with TFOs to allow cellular uptake and triplex formation to occur. The cells were then subjected to UVA irradiation to form photoadducts in the duplex DNA. Incubation was continued for an additional 48 h to provide an opportunity for the repair and/or replication machinery to generate mutations. The vector DNA was then isolated for genetic analysis.

TFO-induced mutagenesis on the chromosome

In order to demonstrate triplex formation on a chromosomal target, we treated 3340 mouse cells with TFOs in the presence and absence of UVA irradiation (1.8 J/cm²) and then assayed for induced mutations. The results indicated a modest, but reproducible, oligonucleotide-dependent induction of mutation. The specific TFO, psoAG30, induced mutations in *supFG1* at a frequency nearly 10-fold greater than that of the control TFO, psoSCR30. Surprisingly, UVA irradiation produced no enhancement in the mutation frequencies (Table 1).

Table 1. Targeted mutagenesis of the chromosomal *supFG1* gene in 3340 mouse cells by psoralen-modified TFOs

Oligonucleotide	Incubation time (h) prior to UVA	Irradiation (J/cm ²)	Mutants/total plaques	Mutation frequency ($\times 10^{-5}$)
None	–	–	16/208 716	8
None	–	1.8	16/183 624	9
psoAG30	–	–	23/50 540	50
psoAG30	–	1.8	13/52 208	20
psoAG30	2	1.8	29/72 626	40
psoAG30	4	1.8	32/167 625	20
psoAG30thio	–	–	9/12 980	70
psoAG30thio	–	1.8	2/11 664	20
psoAG30thio	2	1.8	9/15 606	60
psoAG30thio	4	1.8	7/16 298	40
psoSCR30	–	–	0/20 665	<5
psoSCR30	2	1.8	4/110 002	4

The TFOs (2 μ M final concentration) were added to medium containing G418 (200 μ g/ml) and incubated at 37°C for the times indicated. Cells were then irradiated with 1.8 J/cm² UVA and allowed to recover for 2–4 days prior to collection for DNA isolation. The frequency of mutations in the *supFG1* gene was calculated by dividing the number of clear mutant plaques by the total number of plaques counted.

As an additional control for UVA-independent mutagenesis, cells were treated with psoralen-modified TFOs and immediately irradiated with 1.8 J/cm² UVA. This treatment should cause photoactivation of the psoralen derivative prior to the binding of the TFO to its target site (Table 1), therefore preventing TFO-directed psoralen crosslinking. The mutation frequency was similar to those treated with psoAG30 \pm UVA irradiation, providing further evidence to support the finding that mutagenesis was not dependent on psoralen photoproduct formation. Sequencing data from the mutant phage (Fig. 3) were consistent with this finding,

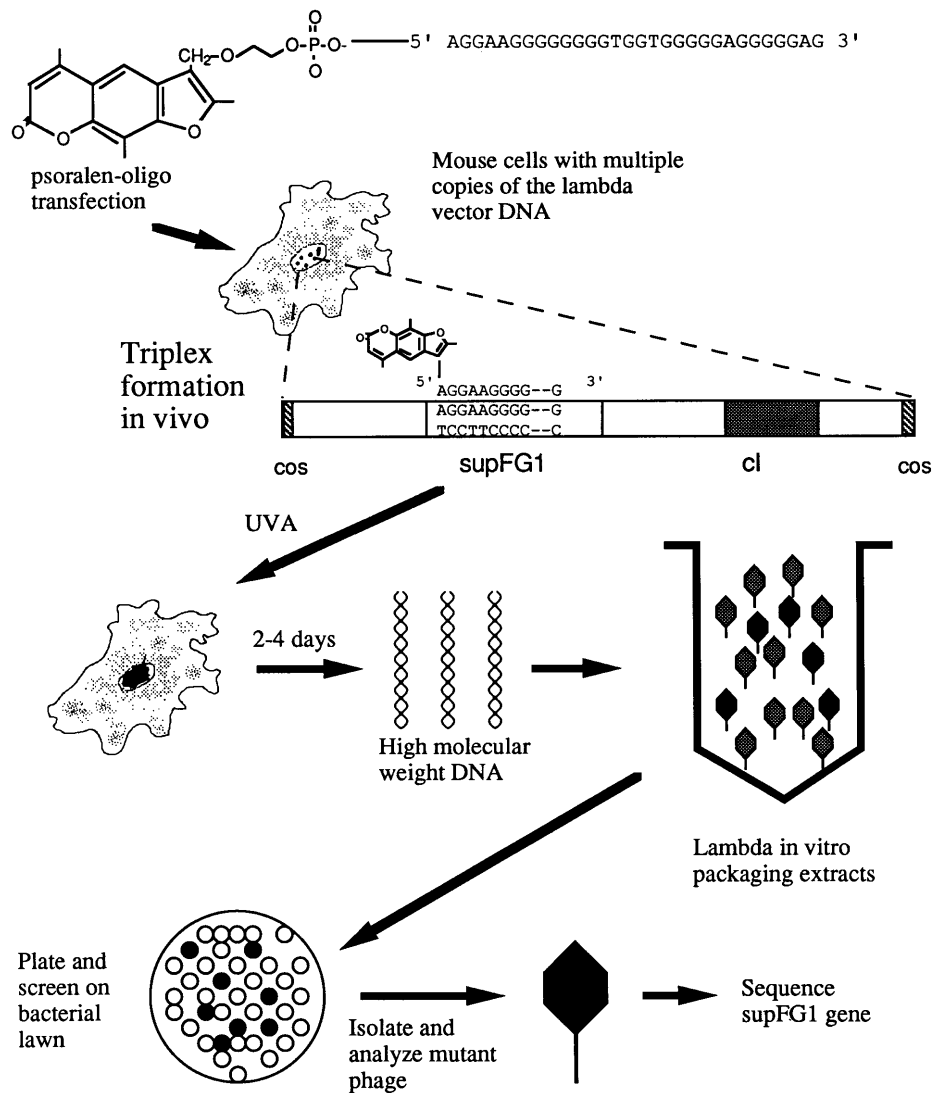


Figure 2. Experimental system for detecting chromosomal mutations targeted by TFOs in mouse cells. The mouse fibroblast cell line, 3340, was established from transgenic mice containing multiple copies of the *supFG1* tRNA suppressor gene within a recoverable λ phage shuttle vector integrated on the chromosome. Following addition of psoralen-modified TFOs to the medium, cells were UV irradiated to activate the psoralen, time was allowed for mutations to form and genomic DNA was isolated and analyzed. The *supFG1* gene contains a 30 bp triplex target site, whereas the *supF* gene contains a 10 bp triplex target site. The vector DNA can be isolated, excised and packaged into viable phage particles for analysis in a *lacZ(am)* strain of *E.coli* to detect mutations that occurred in the mouse cells. If no mutation occurred in the *supF* gene, then the amber mutation in the β -galactosidase gene will be suppressed and plaques will be blue in the presence of IPTG and X-Gal. If, however, a mutation occurred in the *supF* gene, the amber mutation will not be suppressed and the resulting plaque will be white.

in that none showed the expected T·A→A·T transversion at bp 166, the predicted psoralen crosslinking site (13). The DNA sequences of a subset of the mutations induced by psoAG30 + UVA (Fig. 3A) and psoAG30 - UVA (Fig. 3B) generated in several experiments are presented in Figure 3. The sequencing data provided further evidence for TFO-induced mutagenesis on the chromosome since most of the mutations analyzed were located in the triplex binding site with single base pair insertions within the eight G:C base pair tract predominating. While the majority of the mutants sequenced consisted of single base pair insertions, large deletions (>250 bp) surrounding the *supFG1* sequence were detected by PCR analysis in ~30% of TFO-treated mutants (data not shown).

Taken together, these data suggest that the TFO binds its target site on the chromosome to provoke repair and/or replication errors, but that psoralen photoadducts either are not formed, are not recognized by the repair/replication machinery in the context of the triple helix or are subject to a repair process that correctly repairs the site of photodamage but leads to frequent mutations in the adjacent mononucleotide repeat sequence.

Chromosomal targeting of *supFG1* using modified TFOs

Since no T·A→A·T transversions were detected at bp 166 (the expected outcome of a triplex-directed psoralen crosslink) from mutants obtained from psoAG30 + UVA treatment, we reasoned

frequency at which this occurs must be increased. Experiments with this aim are currently in progress.

Table 2. Targeted mutagenesis of the chromosomal *supF* gene in LN12 mouse cells by psoralen-modified TFOs

Oligonucleotide	Incubation time (h) prior to UVA	Irradiation (J/cm ²)	Mutants/total plaques	Mutation frequency (×10 ⁻⁵)
None	–	–	1/76 090	3
None	2	1.8	2/50 630	4
psoAG10	2	1.8	0/55 497	<2
psoAG13	–	–	0/21 045	<5
psoAG13	2	1.8	0/18 575	<5
psoAG30	–	–	1/25 230	4
psoAG30	2	1.8	1/18 915	5
psoSCR13	–	–	0/17 240	<6
psoSCR13	2	1.8	1/22 210	5
psoMIX30	–	–	1/52 575	2
psoMIX30	2	1.8	2/58 375	3

The TFOs (2 μM final concentration) were added to medium containing G418 (200 μg/ml) and incubated at 37°C for the times indicated. Cells were then irradiated with 1.8 J/cm² UVA and allowed to recover for 2–4 days prior to collection for DNA isolation. The frequency of mutations in the *supF* gene was calculated by dividing the number of clear mutant plaques by the total number of plaques (clear + blue) counted.

We have recently reported that peptide nucleic acids (PNAs) are also capable of inducing mutagenesis at the *supFGI* gene in the same line of mouse cells used in this study (25). PNA oligonucleotides contain an uncharged polyamide backbone (29) which forms very stable triplex structures. The mechanism by which PNAs form triple helical structures differs from that of TFO triplexes in that PNAs mediate a strand displacement event whereas TFOs bind in the major groove of the intact duplex (29). Both TFOs and PNAs were capable of producing a 10-fold increase in induction of mutagenesis at their target sites. Although the mutation frequencies were similar, interestingly the spectrum of mutations differed (below).

Triplex-induced mutagenesis is not dependent on UVA irradiation

In previous studies, we reported that TFOs can induce mutations in the *supFGI* gene in an extrachromosomal context (19). This effect was repair-dependent, and the proposed mechanism was one of 'gratuitous repair' (30,31) where transcription was blocked at the triplex site leading to gratuitous and potentially error-prone repair. Interestingly, mutagenesis was induced by TFOs in the presence and absence of psoralen crosslinking which indicated that error-prone repair can be induced even in the absence of chemical damage to the DNA (19). Our chromosomal results are consistent with these extrachromosomal reports. However, the lack of enhancement of mutagenesis in the presence of UVA was unexpected. The data suggest that the TFOs did bind to the chromosomal target site, but that psoralen was not crosslinked to the DNA target duplex or, if crosslinks were formed, they had no additional effect on the mutation frequency under our experimental conditions.

The lack of a requirement for a damaging agent in the induction of triplex-directed mutagenesis is encouraging since if triplexes alone can afford the desired effect, it may not be necessary to conjugate a mutagen to the TFO. This is especially advantageous for therapeutic applications, where use of potential mutagens may be undesirable. In addition, effective administration of UVA could be a formidable challenge in intact animals.

Sequence analysis indicates a slippage mechanism in the poly(G) run induced by TFO treatment

Although the frequency of mutation induction was similar with both specific TFOs and PNAs, the mutation spectra differed. The majority of mutants sequenced were found in the poly(G) tract (eight consecutive G residues) within the *supFGI* triplex binding site with both TFO and PNA treatment. This result is not surprising since the poly(G) sequence may be especially prone to strand slippage events due to the ability of this tract to stabilize dislocation and misalignment of the helix during repair and/or replication (32,33). Inhibition of DNA polymerase during repair or replication by either the TFO or PNA bound to the target site may induce template misalignment, leading to strand slippage events, consistent with the base pair insertions and deletions detected in the poly(G) run. Both TFOs and PNAs are clearly provoking instability in the poly(G) tract within the triplex binding site, but their pathways may differ. Evidence for this is provided by the sequence analysis of the mutants. While the majority of the TFO-induced mutants consisted of single or double base pair insertions (70%), the PNA-induced mutants were fairly evenly dispersed among single base pair insertions (43%), deletions (21%) and substitutions (36%). Another notable difference is that large deletions (>250 bp) in the region surrounding the *supFGI* sequence were produced by TFO but not PNA treatment. In previous studies of triplex-directed mutagenesis of episomal, SV40-based reporter constructs, a significant proportion of deletions was also seen (13,19). The source of TFO-induced deletions is not clear, but may be derived from gaps produced at the triplex site during an attempt to repair the triplex lesion. Clearly the triple helices formed by the long polypurine TFOs and the short PNA clamps provide different substrates for the repair machinery.

Induction of mutagenesis by psoAG30 is greater than that of HMT alone

As expected, the frequency of mutations induced by the potent mutagen HMT was lower than that of psoAG30 treatment. Moreover, unconjugated HMT was more toxic to the cells than the HMT-TFO conjugate. This suggests that the TFO was able to confer specificity to the activity of HMT in the cells. Surprisingly, mutants were found in the poly(G) run, but the mutation spectra differed from either the TFO-induced or PNA-induced mutants (data not shown). These results indicate that the polypurine tract in the *supFGI* triplex site may be an unstable site prone to slippage errors during repair or replication when chromosomally integrated into the mouse genome. Hence, the observation that HMT treatment also induced slippage errors in the poly(G) run provides the basis for understanding the apparent lack of an effect of UVA irradiation on psoAG30-induced mutagenesis: damage generated within or near this site, regardless

of type, provokes DNA repair during which the instability of the poly(G) tract is manifest and dominates the mutation pattern.

Triplex-induced mutagenesis requires high-affinity binding

To provide additional evidence for triplex-induced mutagenesis, we compared mutation frequencies in cells containing the 30mer polypurine site (*supFG1* in 3340 cells) with those of the 10mer site (*supF* in LN12 cells). In contrast to results obtained with *psaAG30* in 3340 cells, treatment of LN12 cells produced no increase in mutation frequencies (Table 2). This result was not unexpected since the LN12 cells carried the low-affinity binding site and, therefore, targeted mutagenesis was not detected. This result also argues against a general effect of *psaAG30* on repair or replication in the absence of high-affinity triplex formation (e.g. an aptamer effect or interaction with enzymes involved in DNA repair or replication). It also provides additional evidence to support a mechanism of induced mutagenesis that is dependent on the binding of the TFO to its specific target site on the chromosome in mammalian cells.

Summary

The work reported here provides evidence for a triplex-induced stimulation of mutagenesis at a chromosomal locus in mammalian cells. Efforts are currently underway to enhance the cellular uptake and stability of TFOs, to produce modified TFOs with improved triplex formation on the chromosome, to assess the cell cycle dependence of triplex formation and mutagenesis and to increase chromosomal target site accessibility. Ultimately, if triplex-mediated DNA modification at a specific site in a chromosomal context can be achieved, many useful purposes could be served, including regulation of gene expression and direct gene inactivation, as well as sensitization of sites for gene replacement.

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