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 chromosome 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 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 target 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 supFG1 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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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

<table>
<thead>
<tr>
<th>Oligonucleotide</th>
<th>Incubation time (h)</th>
<th>Irradiation (J/cm²)</th>
<th>Mutants/total plaques</th>
<th>Mutation frequency (×10⁻⁵)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>–</td>
<td>–</td>
<td>16/208 716</td>
<td>8</td>
</tr>
<tr>
<td>psoAG30</td>
<td>–</td>
<td>–</td>
<td>16/183 624</td>
<td>9</td>
</tr>
<tr>
<td>psoAG30</td>
<td>–</td>
<td>1.8</td>
<td>23/50 540</td>
<td>50</td>
</tr>
<tr>
<td>psoAG30</td>
<td>–</td>
<td>1.8</td>
<td>13/52 208</td>
<td>20</td>
</tr>
<tr>
<td>psoAG30</td>
<td>2</td>
<td>1.8</td>
<td>29/72 626</td>
<td>40</td>
</tr>
<tr>
<td>psoAG30</td>
<td>4</td>
<td>1.8</td>
<td>32/167 625</td>
<td>20</td>
</tr>
<tr>
<td>psoAG30thio</td>
<td>–</td>
<td>–</td>
<td>9/12 980</td>
<td>70</td>
</tr>
<tr>
<td>psoAG30thio</td>
<td>–</td>
<td>1.8</td>
<td>2/11 664</td>
<td>20</td>
</tr>
<tr>
<td>psoAG30thio</td>
<td>2</td>
<td>1.8</td>
<td>9/15 606</td>
<td>60</td>
</tr>
<tr>
<td>psoAG30thio</td>
<td>4</td>
<td>1.8</td>
<td>7/16 298</td>
<td>40</td>
</tr>
<tr>
<td>psoSCR30</td>
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<td>0/20 665</td>
<td>&lt;5</td>
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<td>1.8</td>
<td>4/110 002</td>
<td>4</td>
</tr>
</tbody>
</table>

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 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 photocatalysis 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 ± UVA irradiation, providing further evidence to support the finding that mutagenesis was not dependent on psoralen photoproduction 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 \textit{supFG1} tRNA suppressor gene within a recoverable \textit{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 \textit{supFG1} gene contains a 30 bp triplex target site, whereas the \textit{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\textit{(am)} strain of \textit{E.coli} to detect mutations that occurred in the mouse cells. If no mutation occurred in the \textit{supF} gene, then the amber mutation in the \textit{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 \textit{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 \textit{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 photoproducts 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 \textit{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...
that the psoralen linkage to the TFO could have been degraded in the cells. Thus, we repeated the experiments using a nuclease-resistant modified psoralen-conjugated TFO in which the psoralen–TFO linkage was changed from a phosphodiester to a phosphorothioate linkage. This modified TFO (psoAG30thio; Fig. 1) also contained phosphorothioate linkages between the last 3 nt on the 3’-end. The binding affinity of psoAG30thio (the specific TFO) was in the nanomolar range as assessed by a gel mobility shift assay (28), which was similar to that of psoAG30. When experiments were performed with the phosphorothioate TFO, the mutation frequencies obtained were similar to those of the psoAG30 experiments. Again, the results indicated a 10-fold induction of mutagenesis (Table 1) that was TFO-specific, but not dependent on UV A irradiation. As anticipated from the lack of increased mutation frequencies with UV A irradiation, the psoralen signature mutation (a T·A→T·A transversion at bp 166) was not detected by sequencing analysis of the phosphorothioate TFO-treated mutants (data not shown). Nonetheless, these data consistently suggest that TFOs can enter cells, bind specifically to their chromosomal target sites and induce mutations at those sites.

**Induction of mutagenesis by free psoralen (HMT)**

To investigate further the mechanism of TFO-directed chromosomal targeting in the $supFG1$ gene, we treated cells with unconjugated HMT and assayed for mutagenesis. HMT was toxic to cells at 2 µM (the concentration used as TFO conjugates; Table 1) in the presence or absence of UV A irradiation. To determine a concentration of HMT in combination with UV A irradiation that would be tolerated by the 3340 cells, we varied the concentration of HMT and the dosage of UV A applied to cells from 0.1 to 0.001 µM and 0.18 to 1.8 J/cm$^2$, respectively. Cells did not survive 1.8 J/cm$^2$ at concentrations of HMT ≥0.1 µM. At a UV A dosage of 0.18 J/cm$^2$, cells survived treatment with HMT at 0.1 µM and the mutation frequencies were measured. Under these treatment conditions, the mutation frequencies were 1.5- to 4-fold above the background with an overall frequency nearly 2-fold above background when treatment groups were combined.

**Targeted mutagenesis in the $supF$ gene in LN12 cells**

Based on the induction of mutations by psoAG30 but not psoSCR30, it appeared that the psoAG30-induced mutagenesis was dependent on the ability of psoAG30 to form a triplex helix at the target site. However, to rule out a non-specific effect of psoAG30 on cellular DNA metabolism that might give rise to generalized mutagenesis, we employed the LN12 cell line carrying multiple copies of the $supF$ gene, which lacks the full 30 bp target site (27). The $supF$ gene contains a shorter polypurine run (10 as compared with 30 bp in the $supFG1$ gene) that has been successfully used as a target of triplex-mediated mutagenesis on plasmid DNA in vitro but not in cells (13,18). In those studies a 10mer TFO (psoAG10) was used. Here, we found that neither a 10mer, a 13mer (psoAG13) nor the 30mer (psoAG30) TFO had the ability to induce mutagenesis in the chromosomal $supF$ target gene (Table 2). These results are not surprising since these TFOs all have low binding affinities to the 10 bp target site in the $supF$ gene. These data lend additional support to the notion that psoAG30 mediates directed mutagenesis in a manner dependent on its ability to bind specifically to the $supFG1$ target site.

**DISCUSSION**

**TFO-dependent mutagenesis in a chromosomal target gene**

Triplex technology offers a promising approach to genome modification by directing mutations to specific sites in duplex DNA. This strategy must be applicable to chromosomal sites in living cells to be of general utility and to potentially afford a therapeutic benefit. By targeting the $supFG1$ reporter gene to a chromosomal locus in mammalian cells, we have demonstrated the ability to enhance the frequency of mutations within the triplex target site. Using a 30mer TFO designed to recognize the polypurine run in the $supFG1$ gene, we observed an up to a 10-fold induction of mutagenesis in mouse cells. This novel finding demonstrates the ability to direct mutations to specific sites within mammalian chromosomes using TFOs. However, if this approach is to be of practical utility in modifying a genome, then the mutation
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

<table>
<thead>
<tr>
<th>Oligonucleotide</th>
<th>Incubation time (h) prior to UVA</th>
<th>Irradiation (J/cm²)</th>
<th>Mutants/total plaques</th>
<th>Mutation frequency (x10⁻⁵)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>–</td>
<td>–</td>
<td>1/76 090</td>
<td>3</td>
</tr>
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<td>None</td>
<td>2</td>
<td>1.8</td>
<td>2/50 630</td>
<td>4</td>
</tr>
<tr>
<td>psoAG10</td>
<td>2</td>
<td>1.8</td>
<td>0/55 497</td>
<td>&lt;2</td>
</tr>
<tr>
<td>psoAG13</td>
<td>–</td>
<td>–</td>
<td>0/21 045</td>
<td>&lt;5</td>
</tr>
<tr>
<td>psoAG13</td>
<td>2</td>
<td>1.8</td>
<td>0/18 755</td>
<td>&lt;5</td>
</tr>
<tr>
<td>psoAG30</td>
<td>–</td>
<td>–</td>
<td>1/25 230</td>
<td>4</td>
</tr>
<tr>
<td>psoAG30</td>
<td>2</td>
<td>1.8</td>
<td>1/18 915</td>
<td>5</td>
</tr>
<tr>
<td>psoSCR13</td>
<td>–</td>
<td>–</td>
<td>0/17 240</td>
<td>&lt;6</td>
</tr>
<tr>
<td>psoSCR13</td>
<td>2</td>
<td>1.8</td>
<td>1/22 210</td>
<td>5</td>
</tr>
<tr>
<td>psoMIX30</td>
<td>–</td>
<td>–</td>
<td>1/52 575</td>
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</tr>
<tr>
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<td>1.8</td>
<td>2/58 375</td>
<td>3</td>
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</table>

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 supFG1 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 supFG1 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 supFG1 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 supFG1 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 triplex 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 supFG1 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 psoAG30 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 psoAG30 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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