Covalent crosslinks introduced via a triple helix-forming oligonucleotide coupled to psoralen are inefficiently repaired

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ABSTRACT

Triple helix-forming oligonucleotides (TFOs) represent potentially powerful tools to artificially modulate gene activity. In particular, they can be used to specifically introduce a lesion into a selected target sequence: interstrand crosslinks and monoadducts can be introduced via TFOs coupled to psoralen. The efficiency of these strategies depends on the cell ability to repair these lesions, an issue which is still controversial. Here we show, using psoralen-coupled TFOs and the yeast as a convenient cellular test system, that interstrand crosslinks are quantitatively poorly repaired, resulting in an efficient modification of target gene activity. In addition, these lesions result in the introduction of mutations in a high proportion of cells. We show that these mutations are generated by the Error-Prone Repair pathway, alone or in combination with Nucleotide Excision Repair. Taken together, these results suggest that TFOs coupled to psoralen could be used to inactivate a gene with significant efficiency.

INTRODUCTION

Triple helix-forming oligonucleotides (TFOs) represent potentially powerful tools to artificially modulate gene activity. They could have interesting applications both in fundamental research and in therapeutics since, as sequence-specific DNA-binding ligands, they can be directed against selected targets (1). In particular, they have promising applications as anti-retroviral agents and the HIV genome, which includes two copies of a 16 bp sequence that can be recognized by TFOs, the polypurine tract or PPT, is a potential target (2). Two alternative strategies can be proposed to modify gene expression using TFOs. They can be used as competitive inhibitors for sequence-specific DNA-binding ligands such as transcription factors (3,4). Another approach uses TFOs to specifically introduce a lesion in a target gene. In contrast to other strategies using oligonucleotides which interfere with gene expression, functionalized TFOs could promote irreversible effects. TFOs have been used to direct a psoralen molecule to a particular DNA sequence and to introduce a covalent modification (2,5,6). Psoralen is a bifunctional photoactive intercalator which, upon irradiation with near UV light, forms either a monoadduct (where the psoralen is covalently attached to one strand of the target DNA) or an interstrand crosslink (in which the two strands of DNA are linked to the psoralen) (7). The ratio of monoadducts to interstrand crosslinks can be modulated by changing the irradiation wavelength, with monoadducts preferentially formed at longer wavelengths.

A TFO–psoralen conjugate can efficiently inhibit transcription after photoinduced crosslinking, at least using an ectopic transiently transfected promoter (8). The success of such a strategy is highly dependent on the efficiency and the accuracy with which the cell repair machinery will process the lesion. A number of studies have been devoted to determine the cell response to this type of damage (9–20). Taken together, these studies show that psoralen adducts introduced through a TFO are repaired in mammalian cells, but the extent of the repair process is still controversial since some papers have reported a highly efficient repair of these lesions (9,12,13) while others found no repair (21). TFO-targeted psoralen lesions can also result in a high level of mutations, which were analysed in cell lines derived from patients defective in DNA repair such as Fanconi anemia (FA) (17,18) or xeroderma pigmentosum group A (XPA) and xeroderma pigmentosum variant (XPV) cells (18). These studies led to the conclusion that the poorly defined gene(s) mutated in the XPV group was involved in the generation of mutants.

In the present study, we have taken advantage of the fact that the yeast repair machinery is homologous to that of mammals (22) and used the genetically well defined Saccharomyces cerevisiae organism to assess more precisely the parameters of the repair process of site-specific psoralen adducts introduced via a TFO. Our results indicate that the repair efficiency is highly dependent on the fraction of crosslinks versus monoadducts, shedding some light on the controversy over the efficiency of repair of TFO-targeted psoralen lesions: monoadducts do not impair the replication of the plasmid whereas crosslinks are very poorly repaired (of the order of 10%) and the process introduces a high level of mutations in the target sequence. We also analysed the mutagenic processing of crosslinks in yeast strains bearing mutations in genes encoding key proteins of the rad3 epistasis.
group (involved in Nucleotide Excision Repair, NER), the rad6 epistasis group (involved in Error-Prone Repair, EPR) or the rad52 epistasis group (involved in Recombinational Repair, RR) (7). Our results suggest that two different pathways can be used: some of the mutants arise from a combination of NER and EPR, whereas EPR alone, without any involvement of NER, is responsible for the appearance of the rest of the mutants. In addition, these two pathways introduce distinct types of mutations.

**MATERIALS AND METHODS**

### Oligonucleotides

The TFO PSO-T₄CT₃G₀NH₂ (TFO, Fig. 1A) was obtained from Appligene Oncor (Illkirch, France). A 5-methoxy psoralen molecule (5-MOP) was linked via its C5 position to the 5'-end of the oligonucleotide through a hexamethylene linker. The cytosine in the oligonucleotide sequence was replaced by a 5-methyl-cytosine. The 3'-end of the oligonucleotide was modified with a hexylamine group. Unmodified oligonucleotides that were used for constructions, PCR and sequencing were purchased from Eurogentec (Belgium).

### Construction of modified URA3 alleles

The URA3::HIVVTTA and ura3::hiv1taa alleles were constructed by inserting properly designed 42 bp DNA fragments containing the PPT from HIV1 (positions 8662–8677 in HIVBRUCG) after the ATG initiation codon of the URA3 gene. The two modified URA3 alleles were introduced into the multiple cloning site (MCS) of episomal or centromeric *Escherichia coli*–yeast shuttle vectors (23,24; Table 1).

**Table 1. List of plasmids**

<table>
<thead>
<tr>
<th>Name</th>
<th>Yeast marker</th>
<th>URA3 allele</th>
<th>Type</th>
<th>Parental plasmid</th>
</tr>
</thead>
<tbody>
<tr>
<td>pRS2TTA</td>
<td>LEU2</td>
<td>URA3::HIVVTTA</td>
<td>CEN6/ARSH4</td>
<td>pRS315</td>
</tr>
<tr>
<td>pRS1TTA</td>
<td>TRP1</td>
<td>URA3::HIVVTTA</td>
<td>CEN6/ARSH4</td>
<td>pRS314</td>
</tr>
<tr>
<td>YEpTTA</td>
<td>TRP1</td>
<td>URA3::HIVVTTA</td>
<td>2µ</td>
<td>YEplac112</td>
</tr>
<tr>
<td>YEpTAA</td>
<td>TRP1</td>
<td>ura3::hiv1taa</td>
<td>2µ</td>
<td>YEplac112</td>
</tr>
</tbody>
</table>

### Yeast strains

Yeast strains are described in Table 2. CmY826 yeast strain was a kind gift from Dr C. Mann (25). Isogenic yeast strains used for the study of repair pathways were a kind gift from Dr F. Fabre.

**Table 2. Yeast strains**

<table>
<thead>
<tr>
<th>Name</th>
<th>Genotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>CmY826</td>
<td>MATαura3-52 trp1Δ63 leu2Δ1 his3Δ200 lys2-801 ade2-101 bar1::HIS3</td>
</tr>
<tr>
<td>FF18 733</td>
<td>MATαura3-52 trp1-289 leu2-802 his7Δ2 lys1-1</td>
</tr>
<tr>
<td>FF18 739</td>
<td>MATαura3-52 trp1-289 leu2-802 his7Δ2 lys1-1 rad18::LEU2</td>
</tr>
<tr>
<td>FF18 1079-2</td>
<td>MATαura3-52 trp1-289 leu2-802 his7Δ2 lys1-1 rad51::ura5-</td>
</tr>
<tr>
<td>FF18 xxx</td>
<td>MATαura3-52 trp1-289 leu2-802 his7Δ2 lys1-1 rad1::LEU2</td>
</tr>
</tbody>
</table>

**Triplex formation and targeted photoaduct formation**

In a reaction volume of 10 µl, 2.5 µg of plasmid DNA were incubated overnight at room temperature in 10 mM MgCl₂ with or without psoralen-coupled TFOs (5 µM). Irradiation experiments were performed either with a long wavelength UV light (365 nm, 2.5 kJ/m²) using a monochromatic lamp (Bioblock, Illkirch, France) or with visible light using a 150 W xenon lamp and a filter with a 400 nm cut-off (GG-400 filter glass; Oriel). Irradiated samples were diluted to 100 µl with 10 mM Tris, 1 mM EDTA, pH 8.5.

### Restriction analysis

*In vitro* treated plasmids (50 ng) were digested with 15 U of *DraI* and 5 U of *BamHI* for 1 h at 37°C in a *DraI* commercial buffer. In these conditions, TFOs that were not covalently linked to their target by photoreaction of the psoralen did not inhibit the *DraI* restriction enzyme (data not shown). Restriction fragments were separated on a 1% agarose gel. The gel was then soaked for 20 min in a 0.5 µg/ml BET-containing solution and washed twice for 20 min. Image acquisition and bands intensity quantitation were performed using a Bio-Rad bioimager.

### Renaturing gel electrophoresis

Treated plasmids (50 ng) were digested with 10 U of *PstI*, *NdeI* and *NcoI* to generate a 325 bp fragment containing the PPT sequence from HIV1 and a control 180 bp fragment without the PPT. Restriction fragments were 5'-labeled with [γ-32P]ATP using T4 polynucleotide kinase, precipitated and resuspended in 10 µl of water. An equal amount of denaturing loading buffer (20% glycerol, 4 mM EDTA, 150 mM NaOH) was added prior to migration on a vertical 3% NuSieve gel containing 0.5 µg/ml of BET. The gel was then dried and radioactivity quantitated using a Fuji phosphorimager.

**Table 3. Frequency of cells transformed with more than one plasmid**

<table>
<thead>
<tr>
<th>Amount of plasmids pRS315 and pRS314 (ng/ng)</th>
<th>1000</th>
<th>75/25</th>
<th>50/50</th>
<th>25/75</th>
<th>0/100</th>
</tr>
</thead>
<tbody>
<tr>
<td>LEU2* cells</td>
<td>10647</td>
<td>3660</td>
<td>2140</td>
<td>867</td>
<td>0</td>
</tr>
<tr>
<td>TRP1* cells</td>
<td>0</td>
<td>2060</td>
<td>3580</td>
<td>5607</td>
<td>6500</td>
</tr>
<tr>
<td>LEU2* TRP1* cells</td>
<td>0</td>
<td>147</td>
<td>200</td>
<td>47</td>
<td>0</td>
</tr>
<tr>
<td>Co-transformation (%)</td>
<td>0</td>
<td>2.6</td>
<td>3.5</td>
<td>0.7</td>
<td>0</td>
</tr>
</tbody>
</table>

Yeast cells were transformed using the LiAc procedure and plasmids pRS314 and pRS315, which carry, respectively, the TRP1 and LEU2 auxotrophic markers. A total amount of 100 ng of plasmids was used for each transformation. For selection of LEU2* cells, TRP1* cells or LEU2* TRP1* cells, appropriate dilutions of the transformants were plated on the corresponding selection medium. Per cent co-transformation was estimated as the ratio between the number of LEU2* TRP1* cells and the total number of transformants. The given numbers are the mean value between three different transformation experiments.

### Transformation experiments

Yeast cells were transformed by the LiAc procedure (26). Aliquots of 100 ng of plasmid were used for each transformation in order to minimize transformation of multiple plasmid copies, which was determined to be <5% (Table 3). For quantitation of transformation efficiency, a 1/20 dilution of the transformed cells was plated on tryptophan- or leucine-lacking medium, depending on which marker was carried by the plasmid, and grown for 2 days.
at 30°C. Transformation efficiency was calculated as the ratio between the number of transformants obtained with the treated DNA and the number of transformants obtained with the control DNA. Cells bearing a mutated non-functional URA3::HIV1TTA allele were recovered on the same selection medium supplemented with 5-fluoroorotic acid (27) after 3 days at 30°C. ura3::hiv1taa revertants were recovered after 3 days at 30°C on a selection medium lacking uracil. Mutation frequency was calculated as the ratio between the number of mutants and the total number of transformants.

**Fitting of theoretical curves to experimental data**

When considering a sample containing BA% of crosslinks, the residual transformation efficiency, \( T_E \), can be accounted for, in a simple model, by the plasmid molecules that have not been crosslinked, 1 – BA, and by the crosslinked molecules that have been repaired, \( \tau_r \times BA \) where \( \tau_r \) stands for the global repair efficiency:

\[
T_E = 1 - BA + \tau_r \times BA
\]

Quadratic error between experimental data from Figure 3A and theoretical values obtained using the above equation were minimized with \( \tau_r = 9\% \). If \( \tau_m \) stands for the proportion of mutated molecules among the repaired plasmids, the mutation frequency, MF, can be computed as

\[
MF = \tau_m \times \tau_r \times BA / (1 - BA + \tau_r \times BA)
\]

Using \( \tau_r = 9\% \), quadratic error between experimental data form Figure 3D and theoretical values obtained with this equation were minimized with \( \tau_m = 9\% \).

**Molecular analysis of mutants**

DNA from mutants was isolated using standard procedures (28). The plasmid borne URA3 alleles were amplified by PCR with primers specific for each plasmid. PCR products were purified using a QIAquick PCR purification kit and sequenced by using an Applied Biosystems cycle sequencing kit. The sequencing primer was nested.

**RESULTS AND DISCUSSION**

**Experimental strategy**

The approach used to study the repair of site-specific crosslinks introduced through a TFO is described in Figure 1. The PPT from HIV1 (Fig. 1B), a target sequence recognized with high affinity by a previously described TFO (2,6), was inserted in the coding sequence of the URA3 gene in a series of reporter constructs also harboring a distinct marker gene, either LEU2 or TRP1 (Table 1). Previous experiments indicated that insertion of a sequence at the chosen position in the URA3 gene did not significantly impair the activity of the URA3 protein product (data not shown). These plasmids were incubated in vitro with the TFO described in Figure 1A. The TFO is covalently linked to a molecule of psoralen, a bifunctional photoreactive intercalator. Irradiation of the complex target DNA/TFO with visible light (410 nm) resulted in the attachment of the TFO to only one strand of the target DNA (Fig. 1B). After irradiation, the proportion of adducts was estimated using an assay based on the inhibition of a restriction enzyme (DraI, Fig. 1B). In addition, the proportion of interstrand crosslinks was quantified by analysis in renaturing gels. After irradiation, the plasmids were used to transform URA3+ yeast strains (Table 2 and Fig. 1C), under conditions allowing the penetration of one molecule of plasmid per cell (Table 3). Transformants were selected using the LEU2 or TRP1 marker. As crosslinked plasmids penetrated into the cells with an efficiency similar to that observed for control untreated samples (data not shown), the decrease in transformation efficiency was indicative of an inability of the cells to repair the lesions. The analysis of the URA3 phenotype of the transformants allowed quantification of the induced mutation frequency. In order to detect a spectrum of mutations as wide as possible, two types of constructs were employed in the screening of the mutants. In a first approach, the HIV1 sequence was inserted in such a way that it did not disrupt
the URA3 coding frame (Fig. 1D, URA3::HIV1TTA), allowing the detection, on a URA3 counter-selecting medium, of all frameshift mutations and of some of the substitutions that would yield a stop codon. In a second approach, the HIV1 sequence was inserted with a frame such that a stop codon inactivates the URA3 allele (Fig. 1D, ura3::hiv1ltaa). All but one of the possible substitutions at the two targeted thymines would revert this null codon and mutants could be screened on a medium lacking uracil. In order to assess any influence of the mode of replication of the plasmids, these sequences were inserted into a centromeric and an episomal backbone vector. In addition, control plasmids were also constructed, in which the HIV1 PPT was replaced by that of HIV2. The PPT sequence of HIV2 (AAAAAGGGGGAGGA) has four mismatched sites as compared with HIV1; they strongly decrease the affinity of the TFO for the target sequence.

Interstrand crosslinks are poorly repaired

We first compared the efficiency of the repair process of monoadducts versus crosslinks (Fig. 2). Samples of pRS1TTTA plasmids in which the proportion of total lesions were similar (Fig. 2A), but in which the lesions (Fig. 2B) were either monoadducts (TFO+410) or crosslinks (TFO+UV A), were analysed after replication in yeast cells. Results indicated that the introduction of monoadducts did not have any effect on the survival of the plasmid in the cells (Fig. 2C), nor did it result in the appearance of mutations (Fig. 2D). A likely explanation is that TFO-targeted psoralen monoadducts can be excised through NER or avoided during replication through a strand switching mechanism of the DNA polymerase (28) or a recombinational mechanism between the damaged duplex resulting from partial DNA synthesis on the adducted strand and the undamaged duplex resulting from DNA synthesis on the unadducted strand (30).

Indeed, damage avoidance has been shown to be responsible for 92% of the bypass of site-specifically placed AAF adducts in an NER and mismatch repair-deficient yeast strain (31). In previous reports that used the E.coli–mammalian cell SV40 shuttle vector and the bacterial SupF gene, MAs introduced via TFOs have been found to yield slightly more mutations (1–2%) (18). This larger frequency of mutations might be due to the higher number of adducted plasmids transfected in mammalian cells. Moreover, one might not expect transcription-coupled repair of the bacterial SupF gene in mammalian cells, whereas we found such a coupling in our system (F-X.Barre et al., submitted for publication). In any case, monoadducts had little effect in our system. This suggests that in view of gene modulation by TFOs, they would be inefficient.

In contrast, the introduction of a crosslink into the target sequence strongly impaired plasmid survival (Fig. 2C, TFO+UV A). In addition, crosslinks were highly mutagenic (Fig. 2D). Controls included plasmids irradiated in the absence of TFO (Fig. 2C and D) or plasmids incubated with the TFO in the absence of irradiation (data not shown). Experiments performed on the HIV2 PPT did not result in any detectable lesion of the plasmid, nor did it have any effect on transformation efficiency or on the appearance of mutations (data not shown). Finally, experiments performed using the ura3::hivltaa or the URA3::HIV1TTA allele inserted into the episomal (YEp) backbone gave similar results (data not shown). Thus, these data suggest that crosslinks are poorly repaired and that mutations are introduced with a significant frequency. This was further demonstrated when plasmid survival was measured as a function of the proportion of crosslinks (Fig. 3). A sample in which 90% of plasmid pRS2TTA was crosslinked (a result routinely obtained in our conditions) was mixed with a control batch of the same plasmid in various proportions and used to transform yeast cells. The survival of the plasmid was directly correlated with the proportion of crosslinks (Fig. 3C). The relation was linear and the experimental data best fitted with a proportion of repaired crosslinks in the range of 10% (Materials and Methods). The frequency of mutants also correlated with the proportion of crosslinks, although in that case and as expected, the relation (Materials and Methods, equation 2) was not linear (Fig. 3D).

A number of studies (9,12,13) reported that psoralen lesions introduced using a TFO were significantly repaired. In contrast, our results and those of Musso et al. (21) show that triple helix-targeted psoralen lesions can elicit considerable biological effects despite active cellular repair pathways. This discrepancy might come from a lower frequency of RR in our system. Indeed, in our experiments, only one copy of the plasmid was introduced in each cell. However, we wish to emphasize that RR is very efficient in yeast and has some influence on the level of mutagenesis in our system (Fig. 5). The yeast strains used in this study carry a ura3-52 allele, disrupted by a Ty insertion (32), but which is still able to recombine with a linearized fragment containing the URA3::HIV1TTA allele (data not shown). Moreover, non-homologous end-joining repair, a pathway which, like RR, is implicated in resolving DNA double-strand breaks (33), should...
Figure 3. Repair and mutagenesis of plasmids treated with TFOs. Plasmid pRS2TTA was incubated with or without TFO prior to UV A irradiation (TFO+UV A, UV A). In the presence of TFO, ∼95% of plasmids contained a lesion as assayed by Drd restriction analysis (A) and 90% of plasmids were crosslinked, as revealed by the renaturing gel electrophoresis experiment (B). Samples containing intermediate levels of lesions were obtained by diluting the TFO+UV A-treated sample with the UV A-treated sample and they were used to transform CmY826. Three distinct transformations were performed for each sample. Transformation efficiency (C) and mutation frequency (D) are plotted as a function of the per cent of crosslinks.

have been observed in at least one of the assays, with the URA3::HIV1TTA allele. Finally, using modified SupF systems designed for the observation of recombinational events, only a low frequency of TFO-induced intermolecular recombination was found (14). Intramolecular recombination between two tandem repeats of the SupF gene was also low (20) and no double-strand breaks were observed in oocytes (34). We therefore believe that the discrepancy between our results and those obtained in the SupF system (9,13) is not explained by the involvement of RR. Interestingly, our results show that the biological effects of triple helix-targeted lesions are highly dependent on the nature of the targeted damage: in the case of psoralen, crosslinks are mainly responsible for lethality and mutagenicity with little or no effect of monoadducts, in accordance with results obtained using free psoralen in a wide variety of organisms (35,36). We think that this is the reason why a number of studies reported efficient repair of triple helix-targeted psoralen damages: the proportion of crosslinked molecules in the test sample, when it was estimated, was low. Indeed, careful analysis of previously published data (13) suggests an inverse correlation between the percentage of crosslinks and the survival of the plasmid.

Nature of the mutants

We next analysed the mutations induced by the psoralen-coupled TFO in the target sequence of the two URA3 alleles, URA3::HIV1TTA and ura3::hiv1taa (Fig. 1). Plasmids from mutant clones were extracted and sequenced. As expected, mutations were mostly localized at the psoralen intercalation site, a 5′-TpA-3′ located at the extremity of the target sequence (Fig. 4). In the case of the YEpTAA plasmid, biased toward substitutions, they were mostly A→T (at position 18), T→A or T→G (at position 17) transversions. These substitutions are in good concordance with those previously reported in the literature (37). Insertions of G residues were the most frequently observed mutation in the YEpTTA plasmid (Fig. 4), although some multiple insertions (TpG) and some substitutions (at position 16) also occurred. This result was observed in both the centromeric and the 2µ backgrounds. The TTA construct allowed the detection of only one third of all possible base substitutions, but even when taking into account this factor, a higher frequency of insertions was found as compared with previous reports on targeted mutagenesis using free psoralen in a variety of organisms (37,38) or using TFO-targeted psoralen adducts (19). A likely hypothesis is that this is due to the local DNA sequence that, for example, could increase base slippage during the course of polymerization.

The EPR pathway is involved in the generation of mutations

We next characterized the cellular pathway(s) which was used to repair the crosslinks in our system. Results on the survival of the plasmids and on the appearance of mutations obtained in the wild-type strain were compared with those obtained in a variety of mutant cell lines in which one of the repair pathways was impaired (Table 2). None of the mutations in the repair pathways had a significant effect on survival of crosslinked plasmids (data...
A mutation in rad51, encoding the protein that mediates the strand exchange reaction in yeast (7), resulted in an increased number of mutants (Fig. 5A and B, rad51). This result indicated that the RR pathway was involved in some type of error-free repair which competes with the mutagenic process(es), therefore decreasing the generation of mutants as has been proposed in bacteria (44).

Finally, results obtained with the rad1 mutant, a gene necessary for the dual incisions of the NER pathway (7) were strongly dependent upon the type of screen. Using YEpTTA, which allows detection of insertions, the number of mutants was significantly increased in the rad1+ strain (Fig. 5A, rad1). The generated mutations were mainly insertions as in the wild-type strain (Fig. 4). Thus, insertions resulted from EPR without any involvement of NER, as depicted in Figure 5C. However, the number of mutants was significantly decreased when using YEpTAA, a construct biased toward substitutions (Fig. 5B, rad1). This indicates that substitutions resulted from a distinct repair process in the cell: a likely model, as depicted in Figure 5C, is that NER starts the process by excising one of the crosslinked strands, either on both sides of the lesion as shown in bacteria (45) or on one side as recently demonstrated in mammalian cells (46), but cannot process the lesion any further. The lesion is then treated through a translesion mechanism. The predominance of insertions in the case of YEpTTA indicates that the repair process based on NER and EPR is much less mutagenic than the one which does not involve NER. As depicted in Figure 5C, these two pathways seem to compete for crosslinked substrates which likely explains why, in the absence of NER, the total number of mutations (taking into account substitutions and insertions) increases (Fig. 5A, rad1).

In conclusion, our results show that lesions introduced into a target DNA through a TFO can elicit considerable biological effects. However, these effects are highly dependent on the nature of the lesions introduced. In the case of psoralen, TFO-targeted monoadducts have little or no consequences whereas only 10% of TFO-targeted crosslinks are repaired. TFOs could thus be valuable tools in an anti-retroviral strategy since, if all other parameters were optimized, the elimination of 90% of the infected cells could be determinant (47). Furthermore, most of the mutants escaping due to the cell repair machinery are likely to be non-functional since any modification of the PPT sequence is expected to affect survival of the HIV virus.

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