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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 cerevisae* 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

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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-$T_4CT_4G_6$-NH_2 (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*::*HIV1TTA* 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 initiaton 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

Name	Yeast marker	*URA3* allele	Type	Parental plasmid
pRS2TTA	*LEU2*	*URA3*::*HIV1TTA*	CEN6/ARSH4	pRS315
pRS1TTA	*TRP1*	*URA3*::*HIV1TTA*	CEN6/ARSH4	pRS314
YEpTTA	*TRP1*	*URA3*::*HIV1TTA*	2µ	YEplac112
YEpTAA	*TRP1*	*ura3*::*hiv1taa*	2µ	YEplac112

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

Name	Genotype
CmY826	MAT**a** *ura3-52 trp1Δ63 leu2Δ1 his3Δ200 lys2-801 ade2-101 bar1*::*HIS3*
FF18 733	MAT**a** *ura3-52 trp1-289 leu2*$^-$ *his7-2 lys1-1*
FF18 739	MAT**a** *ura3-52 trp1-289 leu2*$^-$ *his7-2 lys1-1 rad18*::*LEU2*
FF18 1079-2	MAT**a** *ura3-52 trp1-289 leu2*$^-$ *his7-2 lys1-1 rad51*::*ura3*$^-$
FF18 xxx	MAT**a** *ura3-52 trp1-289 leu2*$^-$ *his7-2 lys1-1 rad1*::*LEU2*

Triplex formation and targeted photoadduct formation

In a reaction volume of 10 µl, 2.5 µg of plasmid DNA were incubated overnight at room temperature in 10 mM $MgCl_2$ with or without psoralen-coupled TFOs (5 µM). Irradiation experiments were performed either with a long wavelength UV light (365 nm, 2.5 kJ/m^2) 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 *Dra*I and 5 U of *Bam*HI for 1 h at 37°C in a *Dra*I commercial buffer. In these conditions, TFOs that were not covalently linked to their target by photoreaction of the psoralen did not inhibit the *Dra*I 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 *Pst*I, *Nde*I and *Nco*I 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-^{32}P]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 phosphoimager.

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

	Amount of plasmids pRS315 and pRS314 (ng/ng)				
	100/0	75/25	50/50	25/75	0/100
LEU2$^+$ cells	10 647	3660	2140	867	0
TRP1$^+$ cells	0	2060	3580	5607	6500
LEU2$^+$ *TRP1*$^+$ cells	0	147	200	47	0
Co-transformation (%)	0	2.6	3.5	0.7	0

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-functionnal *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 τ_r stands for the global repair efficiency:

$$T_E = 1 - BA + \tau_r \times BA \quad \mathbf{1}$$

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

$$MF = \tau_m \times \tau_r \times BA/(1 - BA + \tau_r \times BA) \quad \mathbf{2}$$

Using τ_r = 9%, quadratic error between experimental data form Figure 3D and theoretical values obtained with this equation were minimized with τ_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). Irradiation at 365 nm resulted in the introduction of an interstrand crosslink between the two strands of the target DNA (Fig. 1B). After irradiation, the proportion of adducts was estimated using an assay based on the inhibition of a restriction

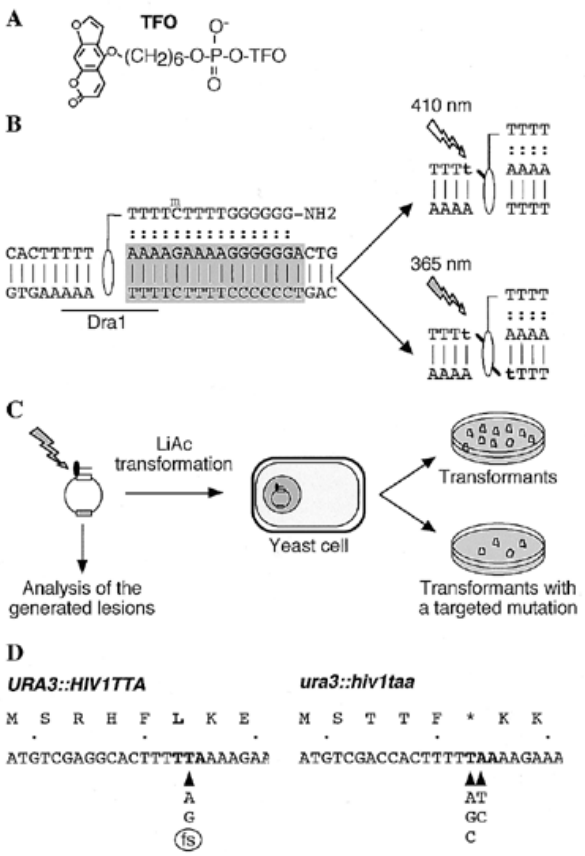

Figure 1. Experimental strategy. (**A**) Schematic representation of the TFO–psoralen conjugate (TFO) used in this study. (**B**) Triple helix formation on the PPT of HIV1. The psoralen molecule is intercalated at a 5′-TpA-3′ site. Upon irradiation, it forms covalent links with either one (monoadduct, 410 nm) or two (crosslink, 365 nm) of the adjacent thymines. Only one of the two possible monoadducts is shown. (**C**) Plasmid vectors bearing the *URA3* reporter genes were incubated and irradiated *in vitro* in the presence of TFOs. The site-specific lesions were analysed for quantitation of monoadducts and crosslinks. Yeast cells were then transformed with the vectors. Quantitation of transformation efficiency was performed by plating dilutions on an appropriate selection medium. Samples were plated in parallel on a medium allowing the detection of changes in the *URA3* reporter gene. (**D**) Nucleotide and amino acid sequence of the modified *URA3* alleles. The codons containing the psoralen intercalation site are in bold. Detectable mutations are shown below the sequences. The position of the mutations in the sequence is indicated by an arrow. fs, frameshift mutations.

enzyme (*Dra*I, 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*::*hiv1taa*). 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 (AAAAGAA**GG**GG**A**GG**A**) 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 pRS1TTA 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+UVA), 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 (29) 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 trancription-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+UVA). 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*::*hiv1taa* 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

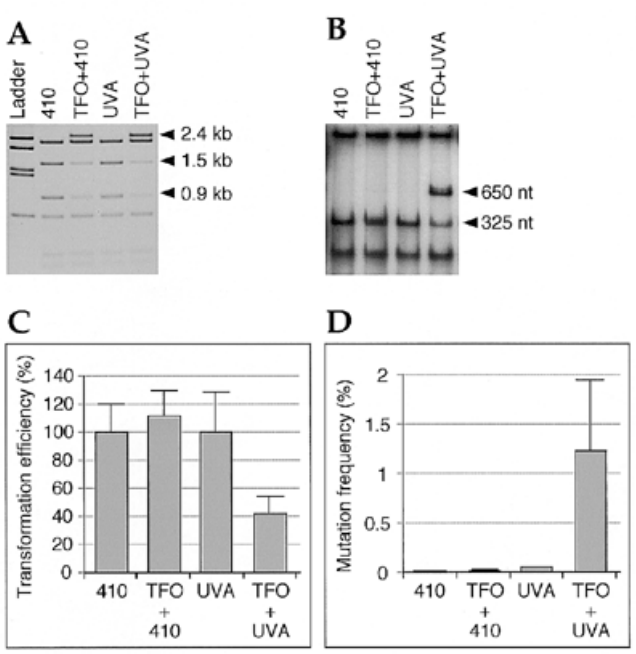

Figure 2. Comparison of the effects of monoadducts and interstrand crosslinks on repair and mutagenesis. Plasmid pRS1TTA was incubated with or without TFO and irradiated with visible light (TFO+410, 410) or UVA (TFO+UVA, UVA). *Dra*I restriction analysis (**A**) and renaturing gel electrophoresis (**B**) indicated the presence of ~69% of monoadducts in the case of TFO+410 with little or no crosslinks, whereas TFO+UVA treatment induced 8% of monoadducts and 69% of crosslinks. Three different transformations were performed for each sample, using FF18 733. (**C**) Transformation efficiency of the samples treated with TFO was measured and compared with the irradiated controls. (**D**) Mutation frequency was estimated from growth on 5-fluoroorotic acid.

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

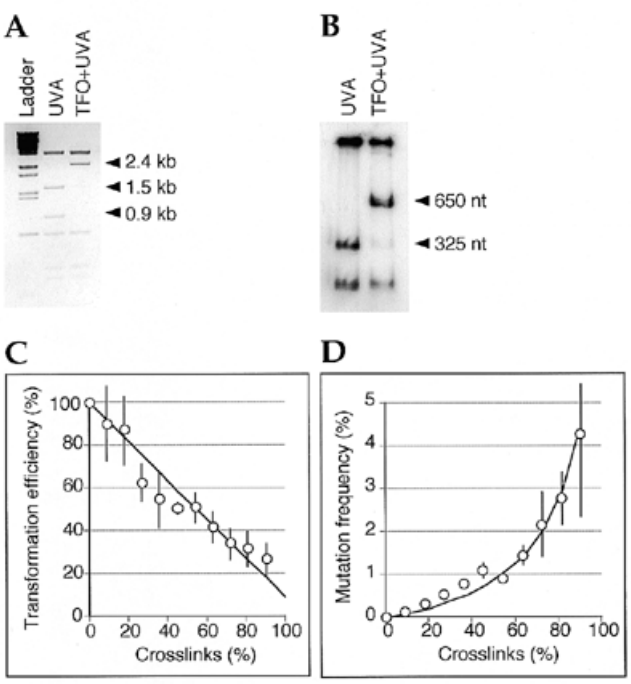

Figure 3. Repair and mutagenesis of plasmids treated with TFOs. Plasmid pRS2TTA was incubated with or without TFO prior to UVA irradiation (TFO+UVA, UVA). In the presence of TFO, ~95% of plasmids contained a lesion as assayed by *Dra*I restriction analysis (**A**) and 90% of plasmids were crosslinked, as revealed by the renaturing gel electrophoresis experiment (**B**). Samples containing intermediate levels of lesions were obtained by diluting the TFO+UVA-treated sample with the UVA-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.

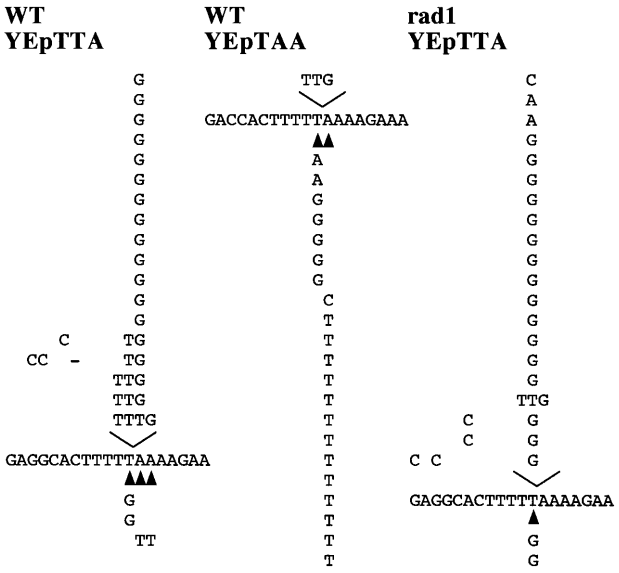

Figure 4. Mutations induced by the TFO. Plasmids YEpTTA and YEpTAA were treated with TFO and UVA. They were used to transform the yeast strain FF18 733 (WT). Two classes of mutations were observed, insertions and substitutions. Insertion events are shown above the *URA3* reporter gene sequence. All the insertion events occurred at the TpA site of the target (indicated by an open arrow). Substitution events are shown below the *URA3* sequence. Their position is indicated by a black arrow. Mutations obtained using the YEpTTA plamid sample and FF18 xxx (rad1) yeast strain are also reported.

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

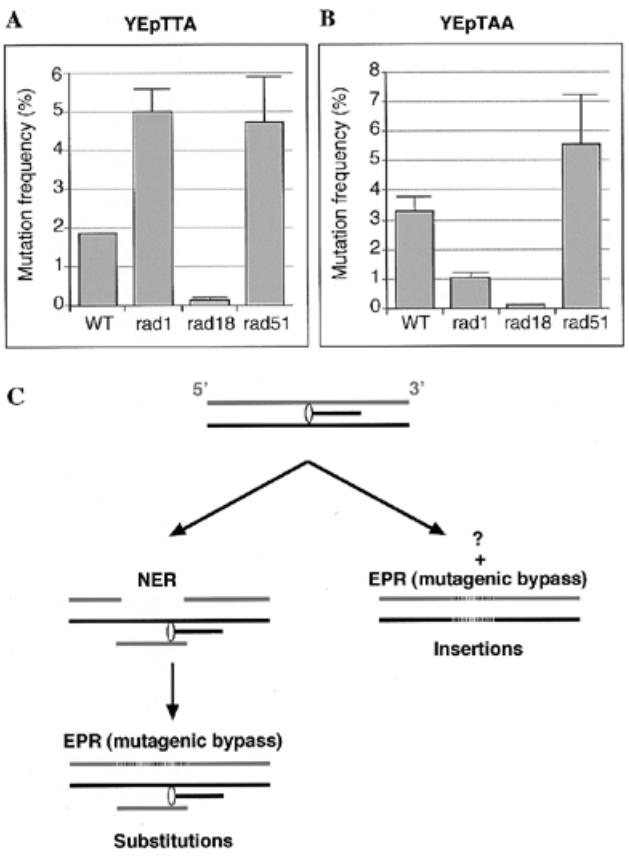

Figure 5. Effects of different mutations in the host repair process on the generation of TFO-induced mutants. Plasmids YEpTTA (**A**) and YEpTAA (**B**) were incubated with or without TFO, irradiated with UVA and introduced into yeast strains FF18 733 (WT), FF18 xxx (rad1), FF18 739 (rad18) and FF18 1079-2 (rad51). The YEpTTA sample contained 78% of adducts and the YEpTAA sample contained 95% of adducts as observed by *Dra*I restriction analysis. Three distinct transformation experiments were performed for each strain. (**C**) Proposed pathways involved in the mutagenic processing of TFO-targeted psoralen crosslinks.

not shown). This further points out the inefficiency of the repair of TFO-targeted crosslinks, the residual transformation efficiency of TFO-treated plasmid samples being mainly due to uncrosslinked molecules.

In contrast, mutations in the various repair pathways all had significant effects on the appearance of *URA3* mutants. Inactivation of the *rad18* gene, which encodes a protein that controls damage-avoidance repair and induced mutagenesis (39) with RAD6 (40), resulted in a complete disappearance of the mutants, no matter which plasmid was used as a target for the TFO, either YEpTTA (Fig. 5A, rad18) or YEpTAA (Fig. 5B, rad18), indicating that in both cases EPR played a central role in the appearance of mutants. The occurrence of multiple mutation events (Fig. 4, YEpTTA) further supported the involvement of the error-prone polymerase ζ of the EPR pathway (41,42) in the generation of mutations. Interestingly, in one mutant, the target sequence was found to have been replaced by the oligonucleotide sequence (data not shown) as if the polymerase had been misdirected on the oligonucleotide strand when encountering the lesion, a phenomenon that had been previously observed *in vitro* (43).

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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REFERENCES

1 Hélène,C. (1991) *Anticancer Drug Des.*, **6**, 569–584.
2 Giovannangeli,C., Thuong,N.T. and Helene,C. (1992) *Nucleic Acids Res.*, **20**, 4275–4281.
3 Grigoriev,M., Praseuth,D., Robin,P., Hemar,A., Saison-Behmoaras,T., Dautry-Varsat,A., Thuong,N.T., Helene,C. and Harel-Bellan,A. (1992) *J. Biol. Chem.*, **267**, 3389–3395.
4 Grigoriev,M., Praseuth,D., Guieysse,A.L., Robin,P., Thuong,N.T., Helene,C. and Harel-Bellan,A. (1993) *C.R. Acad. Sci. III*, **316**, 492–495.

5 Takasugi,M., Guendouz,A., Chassignol,M., Decout,J.L., Lhomme,J., Thuong,N.T. and Helene,C. (1991) *Proc. Natl Acad. Sci. USA*, **88**, 5602–5606.
6 Giovannangeli,C., Diviacco,S., Labrousse,V., Gryaznov,S., Charneau,P. and Helene,C. (1997) *Proc. Natl Acad. Sci. USA*, **94**, 79–84.
7 Friedberg,E.C., Walker,G.C. and Siede,W. (1995) *DNA Repair and Mutagenesis*. American Society for Microbiology Press, Washington, DC.
8 Grigoriev,M., Praseuth,D., Guieysse,A.L., Robin,P., Thuong,N.T., Hélène,C. and Harel-Bellan,A. (1993) *Proc. Natl Acad. Sci. USA*, **90**, 3501–3505.
9 Sandor,Z. and Bredberg,A. (1994) *Nucleic Acids Res.*, **22**, 2051–2056.
10 Havre,P.A., Gunther,E.J., Gasparro,F.P. and Glaser,P.M. (1993) *Proc. Natl Acad. Sci. USA*, **90**, 7879–7883.
11 Havre,P.A. and Glazer,P.M. (1993) *J. Virol.*, **67**, 7324–7331.
12 Degols,G., Clarenc,J.P., Lebleu,B. and Leonetti,J.P. (1994) *J. Biol. Chem.*, **269**, 16933–16937.
13 Sandor,Z. and Bredberg,A. (1995) *FEBS Lett.*, **374**, 287–291.
14 Sandor,Z. and Bredberg,A. (1995) *Biochim. Biophys. Acta*, **1263**, 235–240.
15 Wang,G., Levy,D.D., Seidman,M.M. and Glazer,P.M. (1995) *Mol. Cell. Biol.*, **15**, 1759–1768.
16 Wang,G. and Glazer,P.M. (1995) *J. Biol. Chem.*, **270**, 22595–22601.
17 Bredberg,A., Sandor,Z. and Brant,M. (1995) *Carcinogenesis*, **16**, 555–561.
18 Raha,M., Wang,G., Seidman,M.M. and Glazer,P.M. (1996) *Proc. Natl Acad. Sci. USA*, **93**, 2941–2946.
19 Glazer,P.M., Wang,G., Havre,P.A. and Gunther,E.J. (1996) *Methods Mol. Biol.*, **57**, 109–118.
20 Faruqi,A.F., Seidman,M.M., Segal,D.J., Carroll,D. and Glazer,P.M. (1996) *Mol. Cell. Biol.*, **16**, 6820–6828.
21 Musso,M., Wang,J.C. and Van Dyke,M.W. (1996) *Nucleic Acids Res.*, **24**, 4924–4932.
22 Prakash,S., Sung,P. and Prakash,L. (1993) *Annu. Rev. Genet.*, **27**, 33–70.
23 Gietz,R.D. and Sugino,A. (1988) *Gene*, **74**, 527–534.
24 Sikorski,R.S. and Hieter,P. (1989) *Genetics*, **122**, 19–27.
25 Zarzov,P., Mazzoni,C. and Mann,C. (1996) *EMBO J.*, **15**, 83–91.
26 Gietz,D., St. Jean,A., Woods,R.A. and Schiestl,R.H. (1992) *Nucleic Acids Res.*, **20**, 1425.
27 Boeke,J.D., Trueheart,J., Natsoulis,G. and Fink,G.R. (1991) *Methods Enzymol.*, **194**, 164–175.
28 Johnston,J.R. (1994) In Rickwood,D. and Hames,B.D. (eds), *Molecular Genetics of Yeast. A Practical Approach*. Oxford University Press, Oxford, UK.
29 Higgins,N.P., Kato,K. and Strauss,B. (1976) *J. Mol. Biol.*, **101**, 417–425.
30 Rupp,W.D. and Howard-Flanders,P. (1968) *J. Mol. Biol.*, **31**, 291–304.
31 Baynton,K., Bresson-Roy,A. and Fuchs,R.P. (1998) *Mol. Cell. Biol.*, **18**, 960–966.
32 Rose,M. and Winston,F. (1984) *Mol. Gen. Genet.*, **193**, 557–560.
33 Kent Moore,J.K. and Haber,J.E. (1996) *Mol. Cell. Biol.*, **16**, 2164–2173.
34 Segal,D.J., Faruqi,A.F., Glazer,P.M. and Carroll,D. (1997) *Mol. Cell. Biol.*, **17**, 6645–6652.
35 Brendel,M. and Ruhland,A. (1984) *Mutat. Res.*, **133**, 51–85.
36 Scott,B.R., Pathak,M.A. and Mohn,G.R. (1976) *Mutat. Res.*, **39**, 29–74.
37 Sage,E., Drobetsky,E.A. and Moustacchi,E. (1993) *EMBO J.*, **12**, 397–402.
38 Piette,J., Decuyper-Debergh,D. and Gamper,H. (1985) *Proc. Natl Acad. Sci. USA*, **82**, 7355–7359.
39 Cassier-Chauvat,C. and Fabre,F. (1991) *Mutat. Res.*, **254**, 247–253.
40 Bailly,V., Lauder,S., Prakash,S. and Prakash,L. (1997) *J. Biol. Chem.*, **272**, 23360–23365.
41 Nelson,J.R., Lawrence,C.W. and Hinkle,D.C. (1996) *Science*, **272**, 1646–1649.
42 Lawrence,C.W. and Hinkle,D.C. (1996) *Cancer Surv.*, **28**, 21–31.
43 Guieysse,A.L., Praseuth,D., Grigoriev,M., Harel-Bellan,A. and Helene,C. (1995) *Biochemistry*, **34**, 9193–9199.
44 Sladek,F.M., Munn,M.M., Rupp,W.D. and Howard-Flanders,P. (1989) *J. Biol. Chem.*, **264**, 6755–6765.
45 Van Houten,B., Gamper,H., Holbrook,S.R., Hearst,J.E. and Sancar,A. (1986) *Proc. Natl Acad. Sci. USA*, **83**, 8077–8881.
46 Bessho,T., Mu,D. and Sancar,A. (1997) *Mol. Cell. Biol.*, **17**, 6822–6830.
47 Coffin,J.M. (1995) *Science*, **267**, 483–489.