Deficient DNA repair of triple helix-directed double psoralen damage in human cells

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Abstract Damage induced by a single psoralen-modified triple helix-forming oligonucleotide has been reported to be efficiently repaired in human cells. In this study we investigated a set of psoralen coupled oligonucleotides introducing multiple lesions into the target DNA. A simian virus 40 (SV40) shuttle vector was in vitro treated with different triple helix-forming oligonucleotides and UVA radiation, leading to double psoralen adducts at the supF mutational target gene of the plasmid. After passage in the Raji human cell line the recovered vector was analysed in an indicator bacterial strain. The results show that double psoralen adducts, located at both ends of a long triple helix, cannot be repaired efficiently in human cells.

Key words: Triple helix DNA; Psoralen; Mutagenesis; DNA repair; SV40 shuttle vector

1. Introduction

Triple helix formation between a double-helical DNA and a single-stranded oligonucleotide is a novel approach to specifically inhibit the biological function of the target DNA [1,2]. Two levels where the inhibition can be performed are the double-helical structural and the base coded informational level of the DNA.

In antigene strategy the double helical structure of the target is altered by triplex formation, preventing various proteins from working on the target DNA [3–6]. With a simple unmodified triplex-forming oligonucleotide, the inhibition of gene function is inherently transient, due to the reversible nature of the binding of the oligonucleotide to the DNA. In vitro permanent repression can be achieved by covalent modification of the target duplex, e.g. crosslinking of the two strands of the DNA by triple helix-directed psoralen [7]. However, in vivo the cellular DNA repair process has been shown to be able to overcome this inhibition [8]. Therefore, oligonucleotides capable of forming repair-resistant DNA damage would be useful in antigene strategy.

To inhibit the function of the DNA on the coded informational level the nucleotide sequence of the target has to be modified. Although psoralen-modified triple helix-forming oligonucleotides have been used for targeted mutagenesis [9,10], the mutagenesis process has been inefficient, in the sense that only a small fraction of the psoralen damage can be converted into mutations [11]. Therefore, there is a great demand for triple helix directed mutagens capable of inducing damage that is more efficiently transformed into mutations.

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With either of these two levels of inhibition, the normal cellular repair of triple helix-directed damage needs to be disturbed for an efficient inactivation of the target to be achieved. Consequently, we have tried to devise psoralen-modified oligonucleotides capable of inducing more complex types of damage. Here we report that DNA damage induced by oligonucleotides forming long triplex regions with double psoralen modifications cannot be repaired efficiently in human cells.

2. Materials and methods

2.1. Plasmid and oligonucleotides

pSP2Tx (Fig. 1) is a derivate of the SV40-based shuttle vector pSPTx [11]. The plasmid contains the early transcription unit of SV40 which allows replication in human cells. The mutational target amber suppressor tRNA gene supF is placed between the bacterial replication origin and the β-lactamase gene. During the construction of pSP2Tx another 11 bp long purine rich region was placed immediately after the triple helix target site of pSPTx, providing a second triplex forming region.

Triplex-forming oligonucleotides (TFOs) were obtained from Appligene (Illkirch Cedex, France). TFO1 is a previously described [11], 22-mer guanine rich triple helix-forming oligonucleotide with psoralen linked to the 5′ end through a hexamethylene linker (Fig. 1). TFO2 is a 33-mer with a 5′ end psoralen modification similar to TFO1. TFO3 is an 11-mer oligonucleotide with a psoralen group attached to the 3′ end via a $C_9H_{17}O_2N$ linker [12]. TFO4 denotes the simultaneous use of TFO1 and TFO3. TFO5 is a 33-mer guanine rich oligonucleotide with psoralen groups at both ends.

2.2. Triple helix and psoralen adduct formation

For triple helix formation the target DNA was incubated in the presence of TFO in a 10 mM Tris-HCl, pH 7.4, 20 mM $MgCl_2$ buffer at 37°C for 1 h. For covalent psoralen adduct formation the samples were kept in a microtiter plate on ice and irradiated with 0.8 mW/cm^2 UVA of a hand held long-wave UV lamp. For detection of psoralen adduct formation a 180 bp long *Eco*RI-*Sac*I fragment of the supF gene was labelled by Klenow polymerase and [α-^{35}S]dATP at the *Eco*RI site and purified from an agarose gel. ~5 ng of the fragment was incubated with 10 pmol TFO in 5 μl buffer and UVA-irradiated as described. 2 μl was run on a denaturing polyacrylamide gel and autoradiographed. Radioactivity corresponding to autoradiogram bands was determined in gel slices by an LKB-Wallac 1209 liquid scintillation counter.

2.3. Cell transfection and mutant analysis

5 μg plasmid pSP2Tx was incubated with 150 pmol TFO in 30 μl buffer, UVA-irradiated for 32 min and electroporated into Raji human B lymphoblast cells. The cells were grown for 48 h to allow for repair and replication of the plasmid, then the cells were harvested, low molecular weight DNA was prepared, unreplicated vectors were eliminated by *Dpn*I digestion and the sample was transformed into the *MBM7070 E. coli* indicator strain by electroporation. Mutants were counted and their plasmid DNA was sequenced [11].

3. Results

3.1. Design of triplex-forming psoralen-modified oligonucleotides

Previously, we investigated the repair and mutagenic proper-

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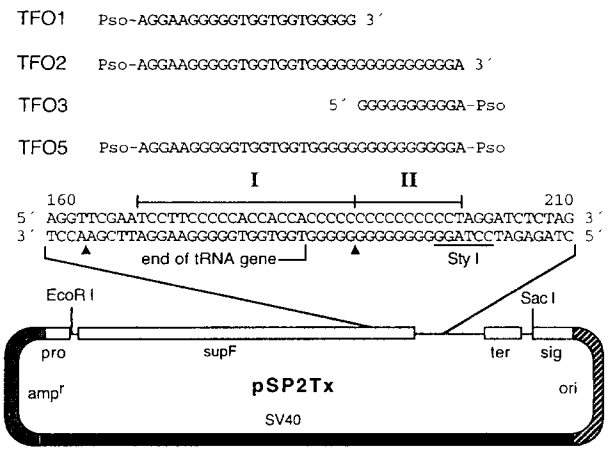

Fig. 1. Schematic presentation of the structures of the triple helix-forming oligonucleotides and plasmid pSP2Tx. The target sequence of the tRNA gene is detailed and triplex regions I and II are indicated. Black triangles mark the estimated repair patch of TFO1-directed damage. SV40 = simian virus 40 T antigen and replication origin; amp^r = ampicillin resistance gene; ori = replication origin of pBR327; supF = suppressor tRNA coding sequence; pro = transcription promoter; ter = transcription terminator; sig = signature sequence.

ties of a 22-mer triplex-forming psoralen-modified oligonucleotide (TFO1) which could target psoralen adducts to the thymine residues 166–167 of the supF gene of plasmid pSPTx [11]. We could observe induction of base changes mainly at position 167, but most of the damage was repaired correctly.

Since triple helix formation is known to prevent binding of proteins to and modification of the target DNA [1,2], it was somewhat surprising to observe an efficient repair of triplex psoralen adducts. The human excision repair complex is known to cut the DNA strand at the 4th-5th phosphodiester bond in 3′ direction and the 5′ 22nd-24th phosphodiester bond around a psoralen adduct, and to remove the damaged region as a 26–29 bp long oligonucleotide [13]. To explain the correct removal of TFO1-directed psoralen damage it can be assumed that the 22 bp long triplex region fits within the 26–29 bp excision repair patch and therefore does not interfere with the repair process.

To obtain triplex-directed psoralen damage which cannot be repaired, we tried to devise oligonucleotides which might interfere with the correct excision repair. Therefore, we inserted a 11 bp purine run (forming triplex site II) in plasmid pSP2Tx immediately after the first triplex site (Fig. 1). In this plasmid TFO1 can bind to triplex site I and upon UVA irradiation direct psoralen adduct formation to position 166–167. The 33-mer triplex forming oligonucleotide called TFO2 with 5′-psoralen modification can direct psoralen adducts to the same site as TFO1, but the longer triplex region might block the normal excision repair. The 11-mer oligonucleotide with 3′-psoralen modification (TFO3) can bind to triplex site II and direct psoralen damage to the thymine residues at position 199–200. This oligonucleotide applied together with TFO1 (this mixture is called TFO4) may give a 33 bp long triplex region with psoralen damage at both ends. Finally, the 33-mer oligonucleotide with psoralen groups at both ends (TFO5) was devised which can bind to both triplex sites and therefore can direct psoralen damage to both positions 166–167 and 199–200. Binding of TFOs to the expected target sites was confirmed by a DNase I footprint assay (data not shown).

3.2. *Analysis of triplex-directed psoralen adduct formation*

A labelled 180 bp fragment of the supF gene was incubated with TFOs, UVA-irradiated and analysed on a denaturing gel (Fig. 2).

In the control sample without TFO only one band can be seen corresponding to the labelled purine-rich strand of the target. With TFO1 and 2 min UVA dose a strong second slowly migrating band appears indicating psoralen monoadduct formation. At 32 min UVA a third band at the top of the gel indicates psoralen crosslink formation between the two strands of the target sequence. Quantitation of psoralen adduct formation showed that with TFO1 plus 32 min UVA approximately 20% of the target molecules have an interstrand crosslink, 60% have a monoadduct on the purine-rich strand, 15% have a monoadduct on the pyrimidine-rich strand and 5% of the target molecules are unmodified (results not shown). These data are similar to the data we previously obtained with the pSPTx plasmid and TFO1 [11].

Similar results were obtained with TFO2. Here the band containing monoadducts appears above the corresponding band of TFO1, since the attached oligonucleotide is 11 bp longer. Quantitation showed 20% with crosslink, 55% with monoadduct on the purine rich strand, 15% with monoadduct on the pyrimidine-rich strand and 10% of the target to be unmodified. This result shows that psoralen adduct formation is somewhat affected by the length of the triplex-forming oligonucleotide.

In the sample treated with the relatively short TFO3 both the monoadduct and the crosslink bands migrate faster than the corresponding TFO1 bands. In the 32 min UVA irradiated sample 80% crosslink, 5% purine-rich strand monoadduct, 5% pyrimidine-rich strand monoadduct and 10% unmodified plasmid were seen. This means that triplex-directed psoralen adduct formation at position 199–200 is different from that at position 166–167. Crosslink formation is more efficient and both strands of the target react similarly. These variations can be due to the

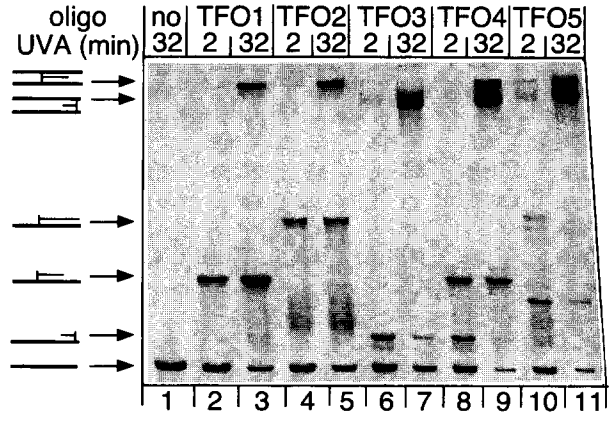

Fig. 2. Detection of triplex-directed psoralen adduct formation in the supF gene fragment. On the top of the gel the applied TFOs and the time of the UVA-irradiation are indicated. The structure of the reaction products in some bands are shown on the left side. Lane 1 = control sample without TFO, but with UVA radiation. lanes 2–11 = samples treated with different TFOs plus a low and a high dose of UVA radiation.

facts that TFO3 targets a 5'TpA3' sequence, instead of a 5'ApT3' sequence like TFO1 and TFO2, and that the psoralens are coupled differently to the oligonucleotides.

With two psoralen molecules directed against the target (in the cases of TFO4 and TFO5) 16 different reaction products can be expected. On a gel all these products cannot be distinguished, since some of them migrate similarly. However, the analysis indicates that with TFO4, 75% of the plasmids contained at least one crosslink, 1% is unmodified, and the rest (24%) contain at least one monoadduct.

When TFO5 was applied, 86% crosslinked vector (including when the two psoralens reacted as monoadducts on the opposite strands of the plasmid), 13% vector with at least one monoadduct and 1% unmodified vector was obtained. These results show that psoralen adducts can be simultaneously and efficiently formed at both ends of a triple helix region.

3.3. Analysis of shuttle vector recovered from cell transfections

Shuttle vector pSP2Tx was treated with different TFOs and UVA radiation and then transfected into Raji cells. Two days later the plasmid was recovered and transformed into *E. coli* indicator bacteria and mutant white colonies were scored. The results are presented in Table 1.

The control samples (no treatment, TFO1 alone, UVA alone) gave 0.05–0.08% mutation frequencies, similar to those seen previously with the pSPTx vector [11]. The plasmid survival of the TFO1 (without UVA) treated sample was chosen as 100%, since the presence of a high concentration of oligonucleotide in the sample can have some effect on the efficiency of transfection [14].

The sample treated with TFO1 gave a 0.91% mutation frequency which is somewhat lower than the 1.3% observed by us previously [11]. This difference can originate from the variations in cell type (Raji or Jurkat) or vector (in the case of pSP2Tx we have less crosslinks, and some of the frequent mutations give lightblue colonies and may therefore be able to escape detection). The plasmid survival decreased to 75%, but this value is within the range of the experimental variability.

With TFO2 we got 0.82% mutants which is very similar to the value we obtained with TFO1, and the survival is 118% indicating no plasmid elimination. These data show that TFO2 is well-repaired and has no more mutagenic effect than TFO1.

Interestingly, TFO3 caused a high (0.75%) mutation frequency and a reduction of the survival (55%) although it directs the psoralen adducts outside the supF gene. A part of this high value can be explained by the fact that TFO3 treatment can induce deletions (results shown below) reaching into the end of the tRNA gene and in some cases also into essential parts of the plasmid.

When both TFO1 and TFO3 were used together the mutation frequency was raised to 2.86%. Even more striking, the very low (14%) plasmid survival indicates that the triplex-directed double psoralen damage results in plasmid elimination. With TFO5 we obtained a lower (1.65%) mutation frequency. The plasmid survival data was only 10%, reflecting that TFO5-directed psoralen damage are poorly repaired, too.

Table 1
Effect of TFO treatments on mutation frequency and plasmid survival

Type of treatment[a]	Mutants/ scored colonies	Mutation frequency (%)	Plasmid survival (%)[b]
No treatment	12/18400	0.07	(123)
UVA	9/17800	0.05	(139)
TFO1	11/14100	0.08	100
TFO1 + UVA	172/18900	0.91	75
TFO2 + UVA	176/21400	0.82	118
TFO3 + UVA	138/18400	0.75	55
TFO4 + UVA	480/16800	2.86	14
TFO5 + UVA	316/19100	1.65	10

[a] UVA stands for 32 min UVA irradiation.
[b] Based on number of colonies among corresponding aliquots of plated bacteria.

3.4. Analysis of mutant plasmids

Plasmid DNA of 15 mutants from transfections with each of the TFOs + UVA was sequenced. The data are presented in Fig. 3.

The mutations from the TFO1-treated samples were mainly T→A and T→G transversions targeted to position 167. We could find one mutant with a 79 bp deletion, too. This pattern is the same as that previously observed with TFO1 and the pSPTx plasmid [11]. TFO2 mutants showed essentially the same picture indicating that a longer triple helix region downstream of the psoralen adduct site does not alter the mutation pattern.

The most frequent mutation type in the case of TFO3 treatment presumably is a non-detectable base substitution outside the supF region (at position 199–200). As expected, the pattern shows no mutations at position 167, but some deletions involving the end of the tRNA gene. Some mutations can be observed throughout the two triplex forming regions which can either originate from the repair of the TFO3-directed psoralen damage or be of spontaneous origin.

Mutants from sample treated with TFO4 and TFO5 show a very similar pattern compared to TFO1 treatment. This probably reflects the fact that plasmids with double psoralen damage become eliminated and do not induce detectable mutations, leaving the relatively few plasmids having adducts only at position 166–167 bp to be responsible for the observed mutations. This also means that the unreacted psoralen group at the 3' end of TFO5 does not alter the mutagenesis process.

4. Discussion

Upon 32 min UVA-irradiation TFO1 and TFO2 introduced psoralen damage into the great majority of the target molecules. However, this heavy load of damage induced no more than 0.91% and 0.82% mutants, respectively, and only a slight decrease in plasmid survival. This is in good agreement with previous observations of efficient repair of TFO1-directed psoralen damage [11] and indicates that the longer TFO2 triplex region downstream of the psoralen damage site has no negative effect on the repair process.

TFO3 treatment of the vector caused a decreased (55%) survival indicating that this type of damage is not repaired as efficiently as those of TFO1 and TFO2. Unfortunately, it is difficult to evaluate the mutation data of TFO3, since the adducts are located outside the supF gene and some of the mutations therefore probably remain undetected.

Each of TFO4 and TFO5 can form a continuous 33 bp long triplex region with covalent psoralen damage at both ends. Upon 32 min UVA-irradiation ~81% of the plasmids have psor-

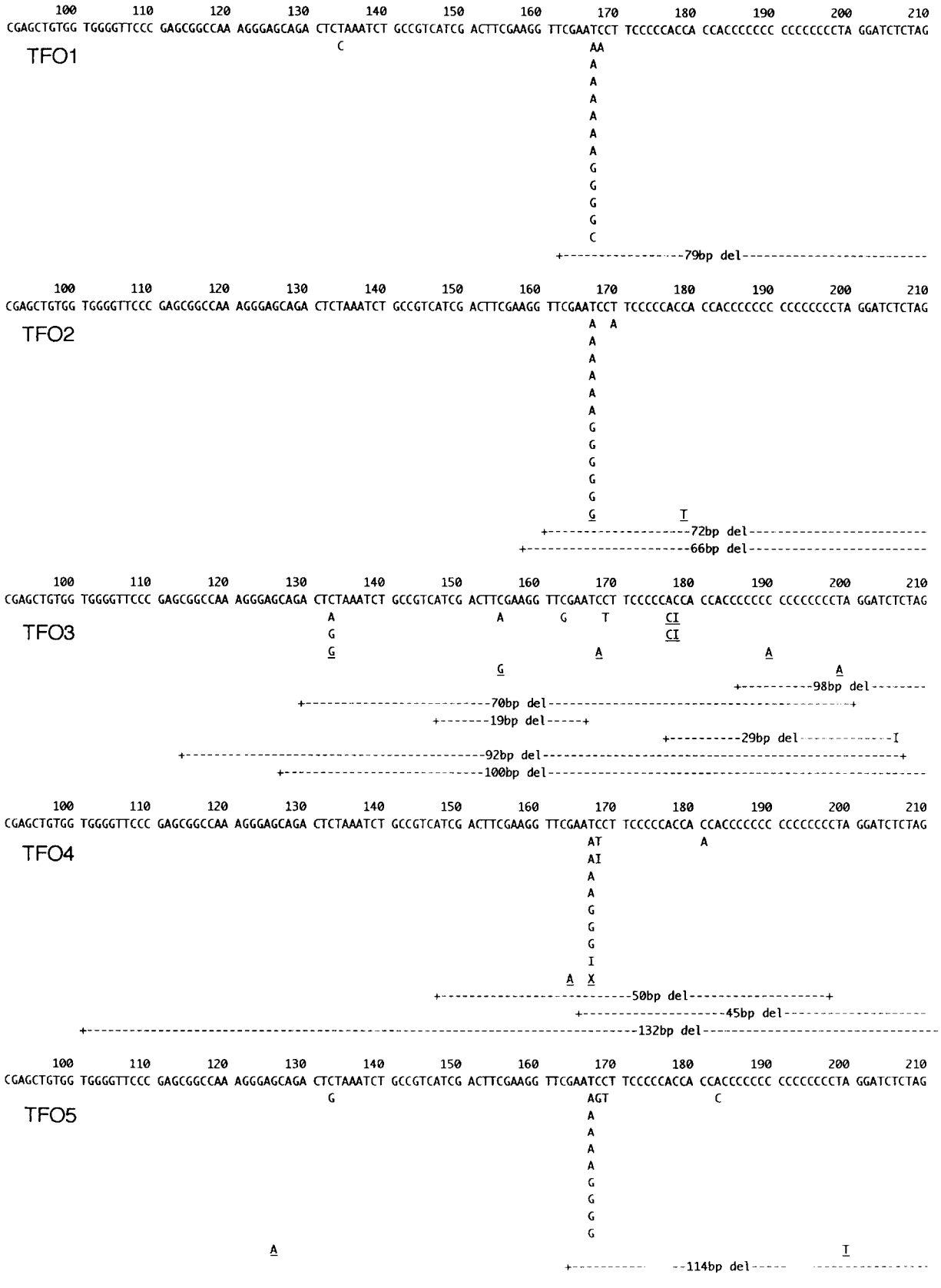

Fig. 3. Distribution of mutations in the supF gene region after TFO treatments. Mutations obtained with different TFOs are indicated separately under the wild type sequence. 'X' indicates one base pair deletion, 'I' means one base pair insertion. Multiple mutations in one plasmid are underlined.

alen adduct formation at both ends of the triplex, ~18% have psoralen damage at one end and ~1% is unmodified. After transfection we observed a higher (2.86% and 1.65%) mutation frequency and a very low (14% and 10%) plasmid survival. This low survival shows that most of the plasmids are eliminated and indicates that the cells are not able to repair the double psoralen damage. The mutation pattern resembles the TFO1 and TFO2 mutation patterns, suggesting that the mutations are induced when only the position 166–167 psoralen has reacted. These data also mean that the relative mutation frequency per survival is higher, whereas the absolute number of mutants per transfection is lower compared to the TFO1 and TFO2 treated samples.

These data indicate that plasmids having psoralen adducts at both ends of a long triplex region are not repaired in human cells, but eliminated. This result is in good agreement with observations showing that vicinal lesions are particularly problematic in DNA repair [15,16]. Unfortunately, we could not get information about the exact mechanism of this elimination. There are at least two possible explanations. The first explanation is that the damage is not repaired at all, therefore the plasmid cannot replicate and the *Dpn*I digestion removes it. Another explanation is that some kind of misrepair occurs resulting in long deletions. In our plasmid the supF gene is situated between two essential parts of the vector (the ampicillin resistance gene and the bacterial replication origin), therefore, plasmids having long (>250 bp) deletions inactivating one of these regions are not viable in bacteria.

Currently, there is some ambiguity in the literature as to whether triplex-forming oligonucleotide-directed single psoralen damage is repaired or not in mammalian cells. One group reported efficient repair in 48 h [8], while others observed no repair during 72 h (M.W. Van Dyke, personal communications). In our case, while damage from TFO1 and TFO2 seems to be repaired efficiently, the decreased survival observed with TFO3 indicates that some of the TFO3-directed damage is not repaired. Probably slight differences, e.g. in the sequence context around the damage, in the exact structure of the psoralen adducts and in the length and orientation of the triplex region will determine the efficiency of the repair process.

In conclusion, our results show that triplex-targeted double psoralen damage is unrepairable, even in the case when the corresponding triplex-directed single psoralen adducts are efficiently repaired.

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References

[1] Hélène, C. (1991) Anti-Cancer Drug Des. 6, 569–584.
[2] Maher, L.J. (1992) BioEssays 14, 807–815.
[3] Cooney, M., Czernuszewicz, G., Postel, E.H., Flint, S.J. and Hogan, M.E. (1988) Science 241, 456–459.
[4] Duval-Valentin, G., Thuong, N.T. and Hélène, C. (1992) Proc. Natl. Acad. Sci. USA 89, 504–504.
[5] Birg, F., Praseuth, D., Zerial, A., Thuong, N.T., Asseline, U., Le Doan, T. and Hélène, C. (1990) Nucleic Acids Res. 18, 2901–2908.
[6] Mouscadet, J-F., Carteau, S., Goulaouic, H., Subra, F. and Auclair, C. (1994) J. Biol. Chem. 269, 21635–21638.
[7] Takasugi, M., Guendouz, A., Chassignol, M., Decout, J.L., Lhomme, J., Thuong, N.T. and Hélène, C. (1991) Proc. Natl. Acad. Sci. USA 88, 5602–5606.
[8] Degols, G., Clarenc, J., Lebleu. B. and Léonetti, J. (1994) J. Biol. Chem. 269, 16933–16937.
[9] Havre, P.A., Gunther, E.J., Gasparro, F.P. and Glazer, P.M. (1993) Proc. Natl. Acad. Sci. USA 90, 7879–7883.
[10] Havre, P.A. and Glazer, P.M. (1993) J. Virol. 67, 7324–7331.
[11] Sandor, Z. and Bredberg, A. (1994) Nucleic Acids Res. 22, 2051–2056.
[12] Kurfurst, R., Roig, V., Chassignol, M., Asseline, U. and Thuong, T.N. (1993) Tetrahedron 49, 6975–6990.
[13] Svoboda, D.L., Taylor, J., Hearst, J.E. and Sancar, A. (1993) J. Biol. Chem. 268, 1931–1936.
[14] Hobbs, C.A. and Yoon, K. (1994) Antisense Res. Dev. 4, 1–8.
[15] Svoboda, D.L., Smith, C.A., Taylor, J.-S.A. and Sancar, A. (1993) J. Biol. Chem. 268, 10694–10700.
[16] Chaudhry, M.A. and Weinfeld, M. (1995) J. Mol. Biol. 249, 914–922.