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Triplex targeted genomic crosslinks enter separable deletion and base substitution pathways

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ABSTRACT

We have synthesized triple helix forming oligonucleotides (TFOs) that target a psoralen (pso) interstrand crosslink to a specific chromosomal site in mammalian cells. Mutagenesis of the targeted crosslinks results in base substitutions and deletions. Identification of the gene products involved in mutation formation is important for developing practical applications of pso-TFOs, and may be informative about the metabolism of other interstrand crosslinks. We have studied mutagenesis of a pso-TFO genomic crosslink in repair proficient and deficient cells. Deficiencies in non homologous end joining and mismatch repair do not influence mutation patterns. In contrast, the frequency of base substitutions is dependent on the activity of ERCC1/XPF and polymerase ζ, but independent of other nucleotide excision repair (NER) or transcription coupled repair (TCR) genes. In NER/TCR deficient cells the frequency of deletions rises, indicating that in wild-type cells NER/TCR functions divert pso-TFO crosslinks from processes that result in deletions. We conclude that targeted pso-TFO crosslinks can enter genetically distinct mutational routes that resolve to base substitutions or deletions.

INTRODUCTION

Triple helix forming oligonucleotides (TFOs) (1) have been under development for many years as gene targeting reagents (2–4). Triplexes form most readily on intact duplexes containing uninterrupted polypurine:polypyrimidine elements. They are stabilized, in sequence-specific fashion, by hydrogen bonds between the bases of the third strand and purine strand of the duplex. Third strands may be composed of either purines or pyrimidines, depending on the nature of the target sequence. One goal of triplex research is bioactive TFOs that could bind specific chromosomal sequences in living cells, and provoke events that yield a desired outcome, such as gene knockout by mutagenesis, targeted recombination/gene conversion, etc. With certain purine motif third strands, it appears that the triplex structure is sufficiently distorting to provoke a response by cellular repair functions resulting in detectable mutagenesis of the target site (5,6). However, linkage of TFOs to a DNA reactive compound, such as the interstrand crosslinking agent psoralen (7,8), results in much higher mutation frequencies (9,10). We have described pyrimidine motif pso-TFOs, with modified sugar residues, that have strong biological activity in living cells as measured in an assay of gene knockout at a specific chromosomal site (11,12). These TFOs did not have any inherent mutagenic activity, and the targeted mutagenesis was dependent on photoactivation of the psoralen. Pso-TFOs can be viewed as dual component reagents, (oligonucleotide and psoralen) and further development and exploitation will require an understanding of both components. Thus, in addition to continued studies on the chemistry and activity of TFOs, it is also important to understand the factors that influence the ultimate fate of the target sequence crosslinked by the pso-TFO. In turn, the information gained from these studies might reveal new insights into the processing of crosslinks formed by well characterized compounds such as psoralens, Mitomycin C, nitrogen mustards, etc.

Early studies in *Escherichia coli* on the repair of crosslinks concluded that incision, by the NER apparatus, of one strand on either side of the crosslink, produced a gapped, 'unhooked', substrate with the excised fragment still attached to the non-incised strand by the crosslinking agent. The gap is repaired by homologous recombinational repair mediated (HRR) by *recA*, using information from an undamaged homologous

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chromosome (13–17). The 'other side' of the crosslink can then be repaired by conventional NER. In the absence of recombination, the gap may be filled by lesion bypass by *polB* polymerase (18,19). These studies define the essentials of a major crosslink repair pathway-recognition followed by incision and gap formation (unhooking), then gap repair by recombination or lesion bypass synthesis.

In yeast multiple pathways for crosslink metabolism have been defined, engaging NER, error prone polymerases, recombinational functions and post replication lesion avoidance (20–22). In mammalian cells the situation is also complex and there are many unresolved questions. Based on biochemical studies, it is widely accepted that the ERCC1/XPF complex (23,24) is essential for 'unhooking' crosslinks (25,26), although this has not been shown directly *in vivo*. However, experiments employing the alkaline comet assay demonstrate that incision of genomic DNA in cells following exposure to crosslinking agents is markedly reduced in cells deficient in ERCC1/XPF (27,28). Attention has also been called to the activity of this complex at replication forks stalled or broken at crosslinks (27–32). While cells with deficiencies in ERCC1 or XPF are quite sensitive to crosslinking agents, cells with defects in other NER genes are only moderately sensitive, suggesting that conventional NER does not make an essential contribution to crosslink repair (27,33,34). However, the literature on the role of NER is contradictory, with different conclusions reached in different experimental systems. For example, repair synthesis on crosslinked plasmids in cell extracts was dependent on ERCC1 and XPF, but not other NER genes (35). Similarly, incision in cells treated with crosslinking agents was unaffected by NER deficiency other than ERCC1/XPF (27,28).

In contrast, a dependence on NER genes other than ERCC1/XPF was demonstrated in another plasmid system (36). Transcription coupled repair (TCR) was also implicated (37), consistent with an earlier report (38). Psoralen crosslink repair was defective in CHO cells deficient in the XPD helicase (39). It has also been proposed that the NER apparatus introduces gaps on one side of a crosslink without actually unhooking the crosslinked strands, leading to a 'futile cycle' of incision and gap filling (40,41).

Although most recent work has addressed aspects of crosslink repair such as incision or resolution of stalled or broken replication forks, there have been a few studies of sequence alterations induced by crosslinking agents. Psoralen induced base substitutions were recovered in the *HPRT* gene (42,43) presumably due to error prone lesion bypass during gap filling across templates still carrying the unhooked crosslink and associated oligonucleotide. It has been proposed that polymerase η contributes to this process (37,44), while the role of other lesion bypass polymerases has yet to be established. Unrepaired crosslinks can provoke the formation of breaks in one of the daughter arms at a replication fork, resulting in chromosome rearrangements (32). Deletions and insertions are also recovered although how these occur is not understood (45,46). The relationship between different repair pathways and different sequence alterations for chromosomal crosslinks has not been established.

In the experiments reported here we have determined the mutational consequences of targeted crosslinks in cells with different repair deficiencies. We show that the frequency of base substitutions reflects the activities of the ERCC1/XPF complex and pol ζ. NER/TCR functions are involved in crosslink metabolism, although they are not necessary for the formation of base substitutions. The generation of deletions is independent for all NER/TCR activity, including ERCC1/XPF.

METHODS

Cell lines

The Chinese hamster ovary (CHO) wild-type cell lines AA8 and V79, and various CHO repair deficient cell lines (UV5/XPD, UV41/XPF, UV24/XPB, UV61/CSB, XR-V15B/Ku86) were obtained from Dr Larry Thompson (Lawrence Livermore Laboratory) and grown in α-MEM supplemented with penicillin, streptomycin and 10% fetal bovine serum (FBS). Mismatch repair deficient D35 and the parental CHO-pro3- cell lines were obtained from Dr Lawrence Chasin (47,48). ERCC1 deficient (knockout) CHO727 cells (49) were transfected with an expression vector containing hamster ERCC1 cDNA under the control of a CMV promoter and stable integrants were selected by growth in medium containing 200 μg/ml hygromycin B (Invitrogen) to generate ERCC1 complemented cell lines. XR-V15B (Ku86 deficient) cells were obtained from the ATCC. The 9N human fibroblasts and the 6I derivative expressing antisense message against the REV3 subunit of pol ζ have been described (50,51). The Artemis deficient fibroblast cell line (52) was the kind gift of Dr Jean Pierre Villartay. Werner Syndrome skin fibroblasts supplemented with the tert gene were obtained from Dr J. Shay (53).

Triplex forming oligonucleotides and *HPRT* deletion assay

The 17 nt triplex forming oligonucleotide, AE-07, against the hamster *hprt* target, and the 16 nt human version were synthesized and purified as described previously (54). Prior to experiments, cells were cultured in medium containing HAT (10^{-4} M hypoxanthine, 5×10^{-6} M aminopterin, 10^{-5} M thymidine) to remove pre-existing *HPRT*-deficient cells. Chinese hamster cells were synchronized in G_0/G_1 by a variation of the method described by Sawai et al. (55,56). Briefly, cells were plated at subconfluent levels and the next day the medium changed to DMEM with 2% FBS and 2% dimethyl sulfoxide (DMSO). After 48 h the cells were washed with (85–88% G_0/G_1 cells by FACS analysis) and either electroporated or fed with complete medium (for G_1 phase experiments), or incubated with complete medium containing 100 μM mimosine for 16 h to block them in early S phase (~90% early S phase cells) (57). After 16 h the cells were released from the mimosine block by feeding with DMEM/10% FBS. Pso-TFOs were introduced by electroporation using an Amaxa nucleoporator. Conditions were developed for each cell line such that transfection efficiencies were on the order of 60–80%, as monitored by expression of GFP following electroporation with a GFP encoding plasmid. Transfection was followed by incubation and exposure in the Rayonet chamber to UVA light for 3 min at 1.8 J/cm^2. The cells were passaged twice over an 8 day period and then plated in culture medium containing 20 μM 6-thioguanine (6-TG). Cells were also

plated in medium without 6-TG to determine plating efficiency. After 7–10 days, colonies were counted and the deletion frequencies calculated as the ratio of 6-TG resistant colonies/total colony forming cells. The relationship between dose of TFO and the frequency of 6-TG resistant colonies is given in the Supplementary Data.

Small pool PCR

TFO treated cells were harvested at the time of 6-TG selection at which time the cells had undergone ~8–10 population doublings. Crosslinks cannot persist in proliferating cells, and so the targeted crosslinks would be cleared from the cell population by the time of harvest. DNA was extracted and PCR performed on 50 pg aliquots of DNA (five diploid genome equivalents). Typical reactions contained 50 pg DNA, 0.2μM primers (E5Fb: CTAGTTTGAGGCCAGCTTTGGC; E5Rb: GGGATTCCAGGCATGCCTTACTG), 1.5 mM $MgCl_2$, 0.2 mM dNTP and 0.5 U *Taq* polymerase in a final volume of 20 μl. A total of 10 μl aliquots of the 750 bp PCR product were digested completely with XbaI in 0.5× NEB buffer 2 containing BSA in a total volume of 30 μl, electrophoresed on a 2.5% agarose gel and visualized by ethidium bromide staining. Fragments with mutations were identified by resistance to cleavage by XbaI. A nested PCR amplification was performed on 1 μl of the XbaI digested small pool PCR products diluted 1/100–1/1000 using previously published Exon 5 PCR primers (E5R: GGCTTACCTATAGTATACACTAAGCTG; E5F: AACATATGGGTCAAATATTCTTTCTAATAG). The resultant PCR products were digested with XbaI to check for resistance to cleavage and sequenced using the Sequitherm cycle sequencing kit (Epicentre) according to the manufacturer's instructions using the forward PCR reactions primer as the sequencing primer. Base substitution frequencies were calculated as the number of mutations detected over the total number of successful PCR multiplied by five (since each reaction consisted of five genome equivalents). Typical analyses were based on 960 or, in some cases, 1440 genome equivalents. The validation of this approach is given in the Supplementary Data.

Characterization of deletions

Colonies from 6-TG selected plates were picked and grown up on 96-well plates in medium containing 6-TG. DNA was extracted and the Exon 5 region amplified and the PCR products were sequenced.

Determination of ICL induced incision by alkaline comet assay

The comet assay is based on the differences in electrophoretic mobility of supercoiled and relaxed (by breaks) loops of genomic DNA from single cells. The DNA in relaxed loops migrates in an electrophoretic field forming a tail relative to the 'head' of non-migrating unbroken DNA. DNA from cells treated with crosslinking agents failed to migrate but incision of the crosslinked genomic DNA during repair restores the comet tail (28,58). Cells treated with 8-methoxypsoralen (8-MOP) and irradiated with UVA were washed with phosphate-buffered saline (PBS) and scraped immediately or 6 h after the UVA treatment, and suspended in PBS. Approximately 1.5×10^4 cells were mixed with 1.5% low-melting-point agarose, and spread on a microscope slide that had been pre-coated with 1% agarose. A total of 100 ml of low-melting-point agarose was applied on top of the sample layer as the last step of slide preparation. Slides were placed in cold lysis buffer for at least 1h at 4°C (2.5 M NaCl, 100 mM EDTA, 10 mM Tris, 1% Triton X-100 and 10% DMSO), followed by rinsing three times in PBS for 5 min. After lysis, slides were incubated in DMSO free lysis solution containing 1 mg/ml of proteinase K for 2 h at 37°C. Slides were washed in PBS three times before incubation in cold unwinding solution [300 mM NaOH and 1 mM EDTA (pH 13)] in the dark at 4°C for 45 min. Electrophoresis was carried out under the same condition at 25 V for 45 min. Slides were then rinsed in neutralizing solution [0.4 M Tris (pH 7.5)] three times for 5 min and fixed in 100% ethanol before staining with ethidium bromide. Images of 50–100 cells per sample were obtained by using a fluorescence microscope (Axiovert 200 M, Carl Zeiss) and Axiovision 4.2 software. Individual comet images were evaluated by using Komet 5 image software. The relative tail intensity was used as a measure of DNA ICL damage. The removal of ICL was analyzed by comparing the tail intensity of the UVA irradiated 8-MOP treated cells with that of the UVA irradiated control cells.

RESULTS

hprt target sequence

The CHO *hprt* triplex target sequence is next to the splice acceptor sequence adjacent to Exon 5 (Figure 1). At the end of the sequence is a TA step which is a preferred site for psoralen crosslinking. The crosslink is between the T at the end of the triplex target (proximal T) and the T on the complementary strand one base removed (distal T). The site also includes the recognition sequence for the XbaI restriction enzyme. The schematic of the pso-TFO, AE-07, described previously (12), is shown. This TFO has 4 clustered 2′-O-(2-aminoethyl)-ribose (2′-AE) residues, which are important for bioactivity (11,12), with the remainder 2′-O-methylribose (2′-OMe) (Figure 1). The corresponding human target has the same sequence except for a run of 5 Ts instead of the 6 Ts in the hamster.

Sequence alterations are similar in wild-type, MMR and NHEJ deficient cells

We have determined the frequency and character of deletions and base substitutions in cells with several DNA repair deficiencies. These were chosen because prior studies argued for a direct role of the relevant genes in crosslink repair, or because they might be expected to influence the processing of intermediates formed during pso-TFO crosslink metabolism. For example, based on experiments in cell free extracts it has been suggested that MutSβ (the heterodimer of MSH2-MSH3) is involved in crosslink recognition (59). We found a slight difference (reproducible, but not statistically significant) between wild-type and MutSβ deficient hamster cells in their deletion frequencies (Figure 2a), but no difference in base substitution frequencies (Figure 2b). We repeated the analysis in the human MMR deficient lines HHUAchr2 (*MSH3*$^-$) and HHUAchr5 (*MSH6*$^-$), and found similar results with both

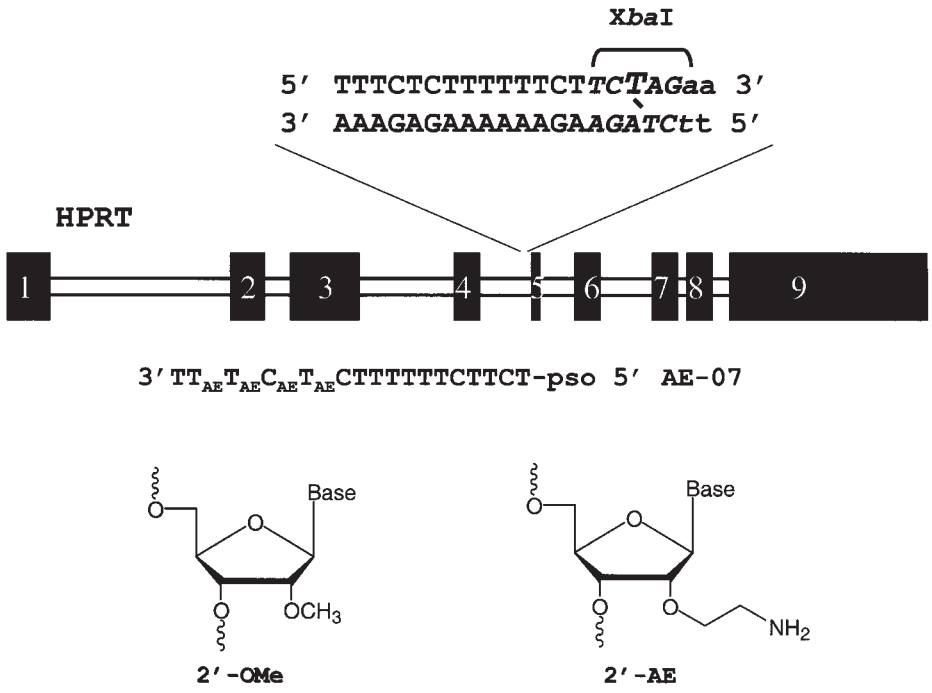

Figure 1. Schematic of the CHO *hprt* target sequence. The psoralen crosslink site, 5′ TA, lies between the triplex target sequence and the start of Exon 5 (lower case letters). The crosslink site is also included in an XbaI recognition site. The human target sequence differs from the hamster in that there is a run of 5 rather than 6 Ts. The AE-07 pso-TFO contains a patch of four 2′-AE substitutions at the 3′ end, and 2′OMe residues in the remainder of the molecule. The structures of the 2′-AE and 2′-OMe ribose nucleosides are shown.

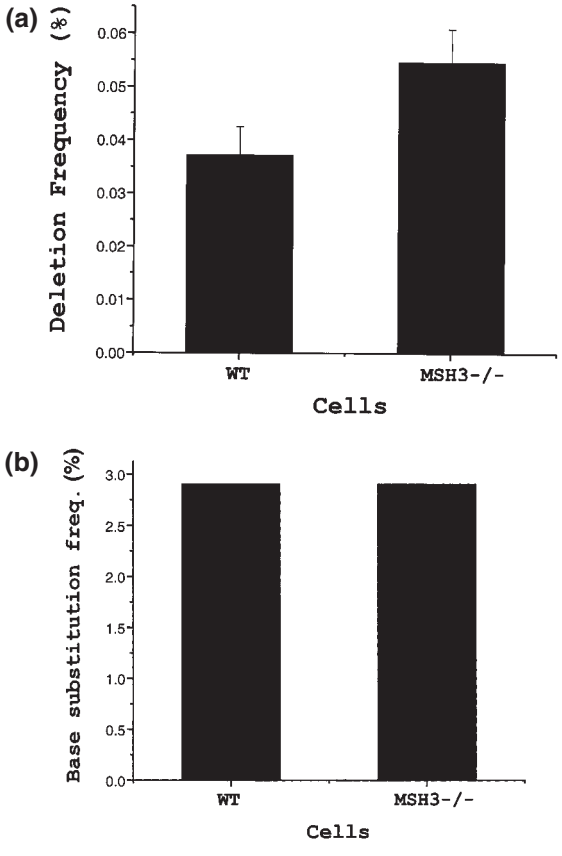

Figure 2. Cells with wild-type mutation frequencies. (**a**) Deletion frequencies in mismatch repair deficient cells; (**b**) Base substitution frequencies in MMR deficient and wild-type cells.

(data not shown). The base substitutions in all the lines were largely T->C transitions as well as some T->A transversions, and were located at the enlarged T in the target sequence as shown in Figure 1 (proximal T). These data do not contradict a role for MMR in the recognition of pso-TFO crosslinks, but they do indicate MMR is not a major influence on the mutagenesis of targeted genomic crosslinks.

We performed the experiments in cells deficient in two components of the NHEJ pathway, Artemis (52) and Ku86, found wild-type deletion and base substitution frequencies, and the standard pattern of base substitutions (data not shown). The protein defective in Werner Syndrome (WS) interacts with the Ku proteins (60), and cells derived from patients with WS have been shown to have a mild sensitivity to crosslinking agents (61). However, the frequencies and patterns of base substitutions and deletions in cells derived from a WS patient were similar to wild-type (data not shown).

Role of ERCC1/XPF

Previous studies indicated that the pso-TFO targeted deletions were consistent with the processing of double-strand breaks (45,54). A dependence on ERCC1/XPF in the formation of double-strand breaks at crosslinks has been reported (59). Rothfuss and Grompe concluded that efficient DSB formation during ICL repair was dependent on preceding ERCC1-dependent incision events (28). However, DSB formation, independent of ERCC1/XPF, has been linked to breakage of replication forks stalled at crosslinks (27,32). We treated wild-type AA8, CHO727 (ERCC1 deficient) and UV41 (XPF deficient) cells with the pso-TFO in mid S phase and found that the frequency of deletions was much greater in the CHO727

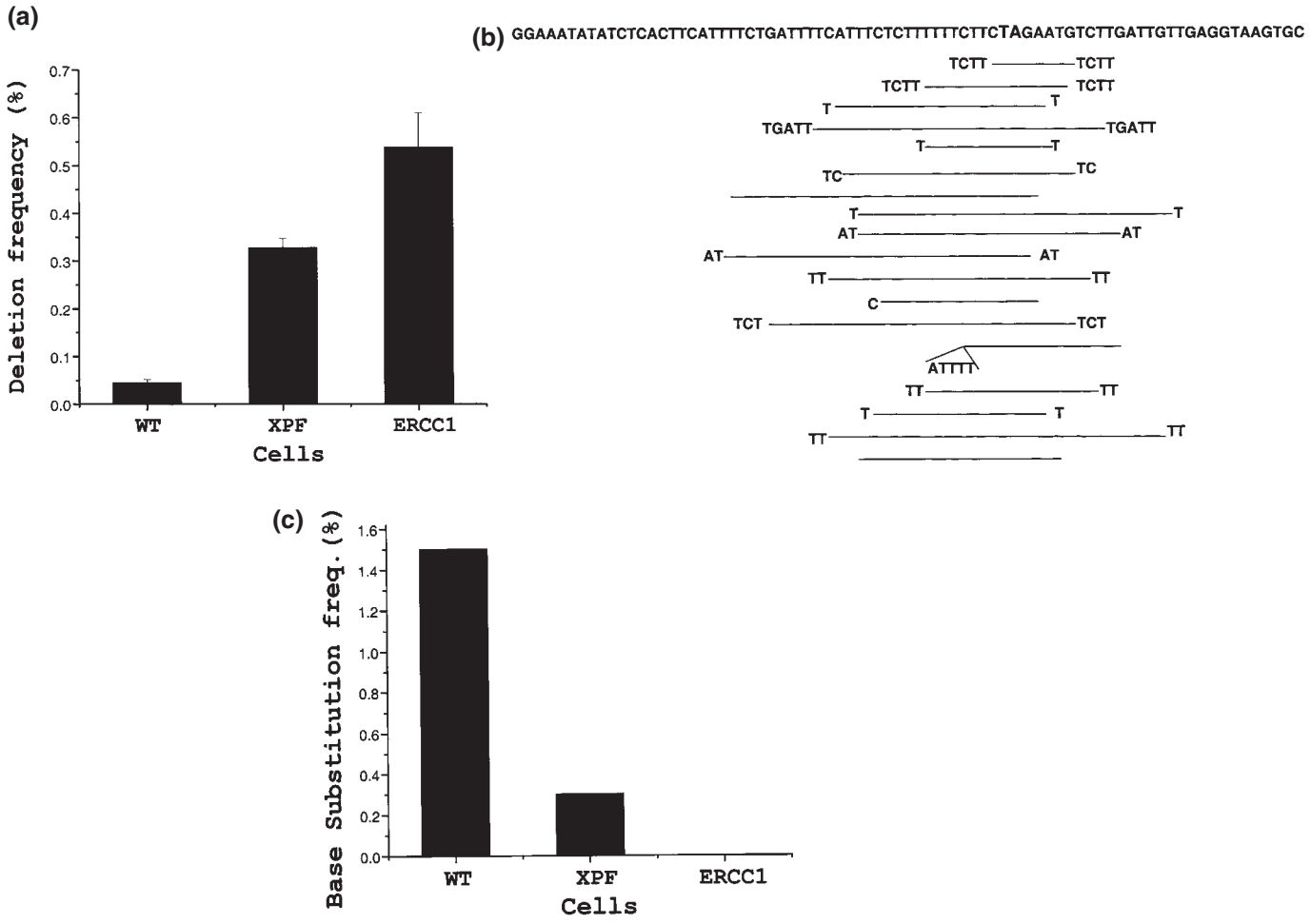

Figure 3. Analysis in ERCC1/XPF deficient cells. (**a**) Frequency of deletion mutations in wild-type, or ERCC1, or XPF deficient cells treated with AE-07, (**b**) Pattern of deletion mutations in cells deficient in XPF; (**c**) Frequency of base substitutions.

and UV41 cells than in the wild-type cells (Figure 3a). We considered the possibility that these deletions might be different from those generated in wild-type cells, since they were derived from a pathway necessarily independent of ERCC1/XPF. We analyzed the sequence of 26 deletions from UV41 cells from cells treated in mid S phase. The deletion size ranged from a few nucleotides to 40. We found that 85% were bounded by microhomologies of 1–4 nt (Figure 3b). These results were similar to those obtained with wild-type cells (54,56).

The *hprt* gene replicates early in S phase (62,63). It has been proposed that crosslinks provoke double-strand breaks as a result of collision with replication forks (30,32,64). Thus, in cells treated in mid S phase there would be many hours before a pso-TFO crosslink would have the opportunity to block a fork in the *hprt* gene. During that time other repair pathways might be operative, and the frequency and kinds of events might differ from those arising from fork encounters. In order to consider this possibility we treated AA8 and CHO727 cells in late G_1 phase with the pso-TFO. We found that the frequency of deletions in the CHO727 cells treated in late G_1 phase was the same as that from the S phase experiments. Furthermore, the size distribution was similar.

In contrast to the rise in deletions, there was a dramatic decline in the frequency of base substitutions in the XPF and ERCC1 deficient cells, with a complete loss of these events in the CHO727 cells (Figure 3c). To verify the requirement for the ERCC1/XPF complex for base substitutions we complemented the CHO727 cells by transfection with wild-type hamster ERCC1 cDNA. Stable clones were isolated and shown to be as resistant to psoralen and MMC as wild-type cells. Treatment of the complemented cells with the pso-TFO yielded targeted base substitution and deletion frequencies similar to those in wild-type cells (data not shown).

These results demonstrated that the ERCC1/XPF complex was necessary for the base substitutions. However, the formation of deletions was independent of the activity of the complex, and the absence of the complex did not influence their properties.

Lesion bypass polymerases and base substitution mutagenesis of pso-TFO crosslinks

Base substitutions at sites of DNA damage are a result of lesion bypass by specialized error prone polymerases (65). Polymerase η has been implicated in this process for crosslinks, based on a plasmid assay (37). We asked if the frequency

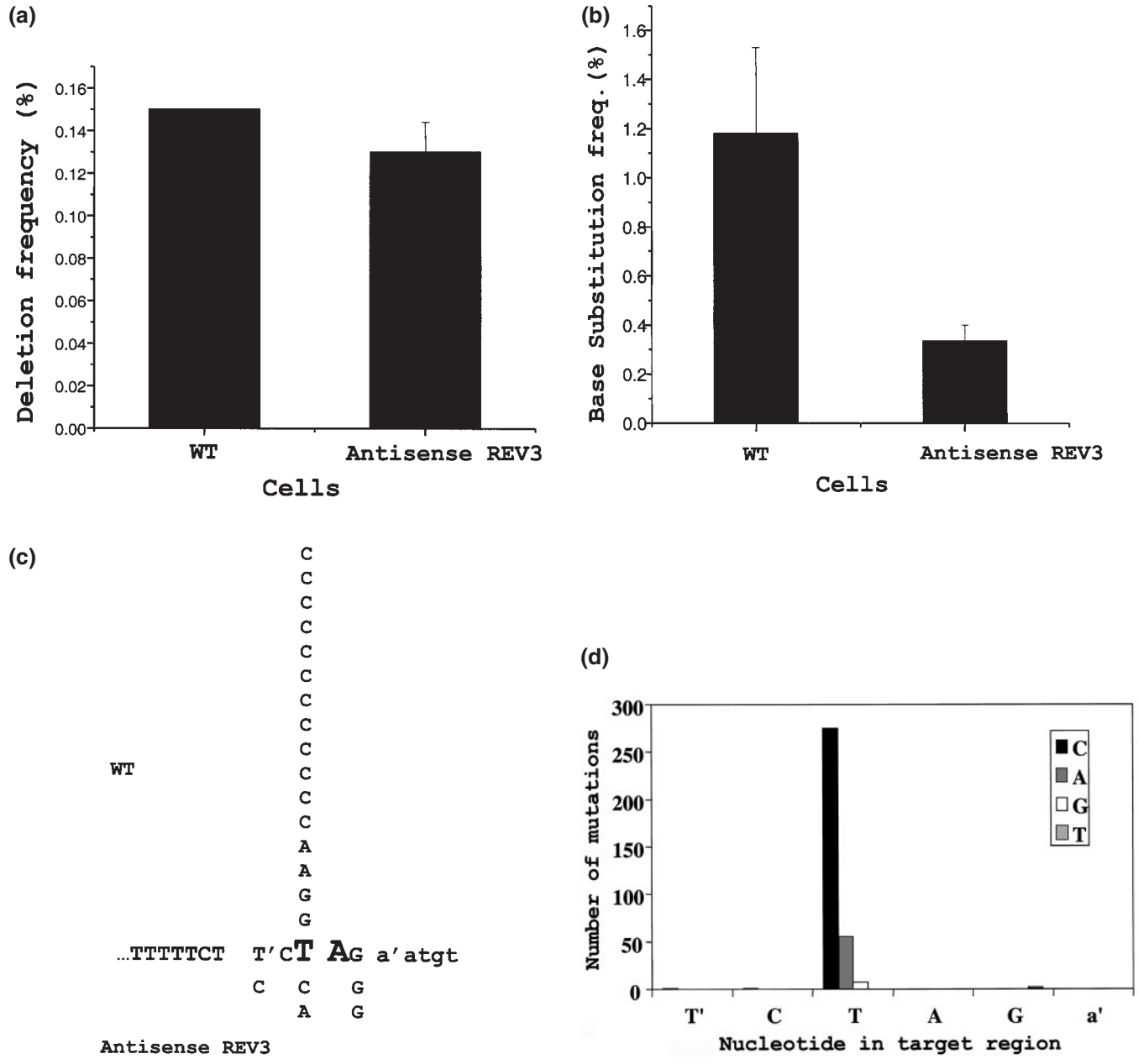

Figure 4. Mutation analysis in cells expressing antisense message against polymerase ζ. (**a**) Deletion frequencies, (**b**) Base substitution frequencies, (**c**) Mutation spectra, control cells (above) and antisense cells (below). The TA crosslink site is enlarged. The difference in the frequency of base substitutions at the proximal T position in the two cells is statistically significant, $P < 0.008$. (**d**) The compilation pattern from cell lines with wild-type base substitution patterns. The difference between pol ζ antisense cells and the compilation pattern in the frequency of events at the proximal T position is statistically significant, $P < 0.0001$. The mutations at the individual nucleotides in the target region were: T→C (1); C→A (1); T→C (275), T→A (55), T→G (7); G→T (2).

or pattern of base substitutions would be influenced by a deficiency in polymerase η. The Xeroderma pigmentosum variant cells (polymerase η-null) were not amenable to colony forming assays, and so a measurement of deletion frequency was not possible. However, we were able to recover and characterize base substitutions, and these proved to be identical to those recovered from wild-type human cells, T→C and T→A at the proximal T.

We then examined the targeted mutagenesis in cells engineered by cDNA antisense expression to be deficient in polymerase ζ (50,51). Survival assays with these cells and the parental counterparts demonstrated no enhanced sensitivity to psoralen (data not shown). The analysis showed no effect on deletion frequency by pol ζ suppression (Figure 4a). However we saw a 3-fold decline in base substitution frequency (Figure 4b). Sequence analysis indicated a shift in the nature and location of base substitutions, as compared to the wild-type control (Figure 4c). Those from the control cells were located exclusively at the site of the proximal T in Figure 1. Only a few were recovered from the pol ζ antisense cells. These were located at positions outside the crosslink site, as well as at the proximal T. The results contrasted with the wild-type control and with the compilation of 341 events, derived from experiments with cell lines that showed the

wild-type base substitution pattern (Figure 4d). Those patterns were dominated by T to C and, to a lesser extent, T to A, at the site of the proximal T. A few substitutions (4/341) in the compilation were located outside the crosslink site. These were similar to those in the pattern from the pol ζ antisense cells, although they were proportionately much less common. The simplest interpretation of these results was that the polymerase ζ was required for base substitutions at the crosslinked proximal T, while other polymerases introduced mutations at the sites away from the targeted crosslink. The two events at the wild-type site in the spectrum from the antisense cells most likely reflected the incomplete suppression of pol ζ in those cells.

Role of NER and TCR in incision of crosslinked genomic DNA in G_1 cells

As discussed above, the role of NER/TCR functions in crosslink repair in mammalian cells is unresolved. It has been argued that replication forks blocked or broken by crosslinks would present a substrate to the ERCC1/XPF complex (32). In proliferating cells the activity of ERCC1/XPF at crosslink stalled forks could obscure contributions by the larger ensemble of NER/TCR activities, which might function independent of replication (22). Thus it was important to ask if components of NER/TCR were involved in the metabolism of crosslinked genomic DNA, in the absence of replication. To address this question we chose an assay that could report a cellular response to treatment with a crosslinking agent in a phase of the cell cycle and time frame that would preclude replication. We treated wild-type and XPD deficient cells with psoralen/UVA in early G_1 phase, and then measured the introduction of DNA breaks into genomic DNA by comet assay. We found that the tail intensity had nearly recovered to control levels after 6 hr in the wild-type cells, before entry into S phase, in good agreement with a recent report (28). However, in XPD deficient cells there was essentially no recovery in this time (Figure 5a and b). Transcription coupled repair of psoralen crosslinks was demonstrated by Hanawalt *et al*. (38) and is consistent with the strand asymmetry of mutations described by several groups (37,42,43,66) and our own data (56). The experiment was repeated in CSB deficient UV61 cells in G_1 phase. The results

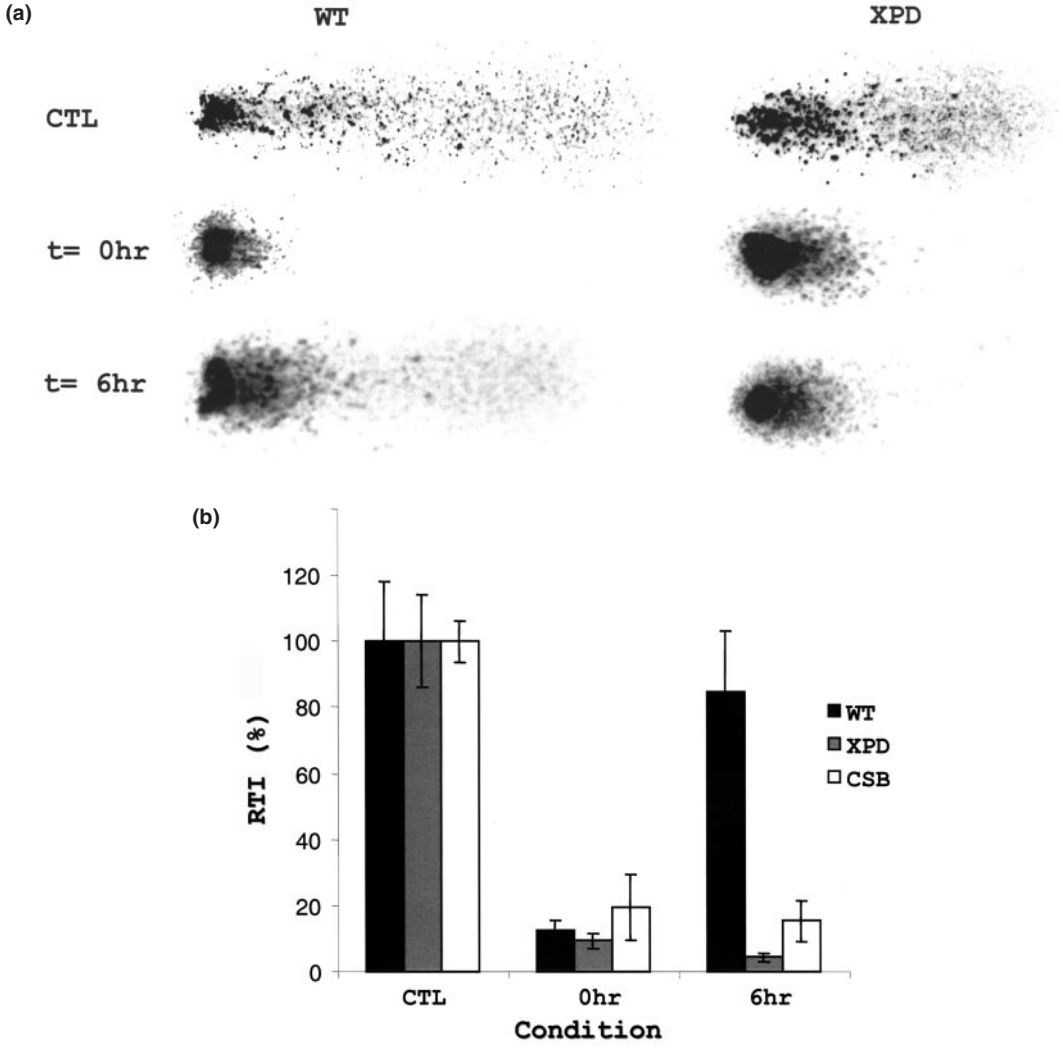

Figure 5. DNA breaks introduced into genomic DNA in repair proficient and deficient cells treated with psoralen in G_1 phase. (**a**) Representative DNA comet assay, (**b**) Quantitative analysis of breakage in wild-type, XDP or CSB deficient cells 6 h after psoralen treatment. RTI = relative tail intensity.

were quite similar to those obtained with the XPD deficient cells-relative to the 0 time sample there was little recovery in the 6 h after treatment (Figure 5b). Thus it appeared that NER/TCR functions played a role in the response to treatment with the crosslinking agent.

Mutagenesis of pso-TFO crosslinks in NER and TCR deficient cells

In the light of these results and the pronounced effect of ERCC1/XPF deficiency shown in Figure 3 we were curious to examine the effect of NER/TCR deficiency on the mutation pattern. Treatment of the XPD deficient cells with the pso-TFO resulted in a 4.5-fold increase in deletions relative to wild-type cells (Figure 6a) with no change in the frequency of base substitutions (Figure 6b). Similar results were obtained with XPB deficient CHO UV24 cells (data not shown). We found that the frequency of deletions was elevated ~5-fold in CSB deficient UV61 cells, while the base substitution frequency was unchanged (Figure 6a and b). Sequence analysis showed that the pronounced strand asymmetry of base substitutions was the same as in wild-type cells, indicating that the CSB function was not critical to the strand asymmetry.

These data in these two series of experiments indicated that NER/TCR functions were involved in the processing of DNA crosslinked by both psoralen and the pso-TFO. They demonstrated that in wild-type cells NER/TCR activities were unnecessary for entry into pathways that resulted in base substitutions, but drew targeted crosslinks away from pathways that resulted in deletions.

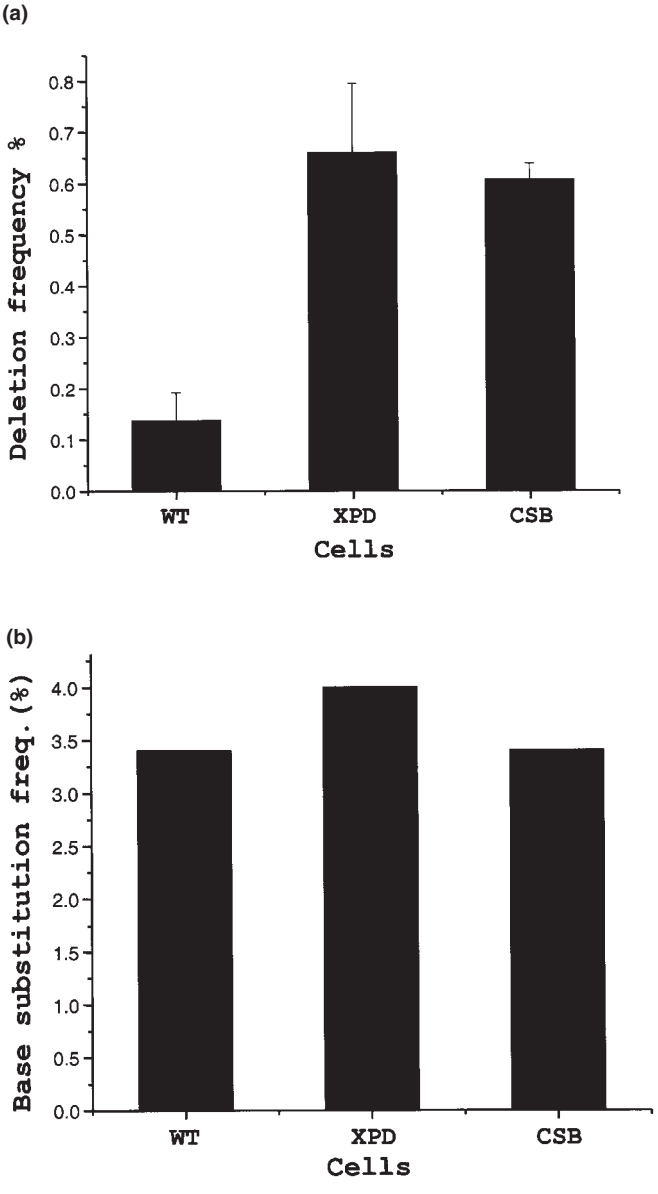

Figure 6. Mutation analysis in NER and TCR deficient cells. (**a**) Frequency of deletions in wild-type, XPD deficient, or CSB deficient cells following treatment with AE-07, (**b**) Frequency of base substitutions in wild-type, XPD, and CSB cells.

DISCUSSION

Targeted DNA damage by TFO conjugates

Previous studies on the metabolism of site specific chromosomal damage have been limited to double-strand breaks introduced by rare site restriction endonucleases (67). TFOs, under development for many years (2), have been conjugated to a variety of DNA damaging agents. These TFO conjugates would appear to offer an approach to covalent modification of specific chromosomal sites in mammalian cells. However TFOs with genomic targeting activity have become available only recently. We have described a pattern of 2′-AE substitution (68) in TFOs that confers bioactivity (11,12), and have used these modified TFOs to place a psoralen crosslink at a defined genomic site in living cells (12,56). The experiments described here are the first analysis of some of the genetic factors involved in the metabolism of targeted DNA damage.

NER-triplex interactions

Based on experiments with plasmids and cell extracts it has been suggested that a triplex linked to a psoralen crosslink can be an inhibitor of NER mediated incision activity (69,70), or a target of repair activities (5). It is not technically possible at this time to compare the metabolism of the targeted psoralen-TFO crosslink with an unconjugated psoralen crosslink at the same chromosomal target site. However it should be noted that the sequence alterations described in this report required psoralen photoactivation. Thus they were dependent on psoralen crosslink formation, rather than on attack by cellular functions on the triplex structure itself. This is probably the consequence of our use of an RNA analogue pyrimidine TFO, which imposes very little distortion on the underlying duplex (71), in contrast to the greater deformation that results from the formation of non isomorphous triplets by the deoxy purine TFOs (2) employed in earlier studies (6,9). Furthermore, if the TFO used in our experiments were a potent inhibitor of NER, we would have expected to see little difference in results between wild-type cells and NER deficient cells. As indicated in Figure 6, however, there are clear quantitative differences between wild-type and NER deficient cells, implying that NER must contribute to the processing of the chromosomal pso-TFO crosslink in wild-type cells.

Base substitution mutagenesis of pso-TFO crosslinks

Recent work in yeast (20–22) identifies multiple pathways available for crosslink metabolism, some of which are likely to be operative in mammalian cells and applicable to the pso-TFO crosslinks. From the perspective of this report, those pathways that proceed through intermediates generated by the activity of ERCC1/XPF on the targeted crosslink would have some probability of terminating in base substitutions. An increase in the activity of the ERCC1/XPF incision complex (drawing more lesions into the pathway) and/or increased levels of polymerase ζ would result in enhanced base substitutions. It is likely that there is more than one entry into this pathway, perhaps dependent on cell cycle status (22) (see Figure 7).

The results with CSB deficient cells are consistent with a role for CSB in processing the pso-TFO crosslink, perhaps through its association with RNA polymerase II and function in TCR. However CSB has several additional activities, including chromatin remodeling and DNA wrapping (72), and could influence pso-TFO crosslink metabolism via a mechanism independent of TCR. Our data are in accord with a role for TCR/NER genes in the processing of the pso-TFO crosslinks, although it should be noted that the structure(s) of intermediates generated by these activities is/are not known. At the same time our results also indicate that these functions (other than ERCC1/XPF) are not essential for the formation of base substitutions. Deficiencies in XPD, XPB and CSB had no effect on base substitution frequencies. This conclusion contrasts with that from studies of error prone repair of psoralen crosslinks in a transcribed gene in a non-replicating plasmid. Repair, which was accompanied by base substitutions, was reduced when the reporter plasmid was transfected into NER deficient cells (36). A resolution of this apparent contradiction would be that in proliferating cells deficiencies in NER/TCR would be compensated for by the eventual encounter of a crosslink with a replication fork. There is an origin of replication near the transcriptional promoter of the *HPRT* gene (73), and the replication fork and transcription bubbles would move through the gene in the same direction. This would explain the same mutational strand bias observed in both wild-type and TCR deficient cells in our experiments.

Deletion events

The deletions in all of our experiments are typical of those resulting from resolution of a double-strand break by NHEJ. An invariant property of all the deletions is the loss of the nucleotides engaged in the pso-TFO crosslink. A key intermediate would be a target region with double-strand breaks on both sides of the crosslink. Our experiments indicate quite clearly that deletion formation can occur in the absence of NER/TCR functions, including ERCC1/XPF. Thus, models which invoke successive rounds of incision by the

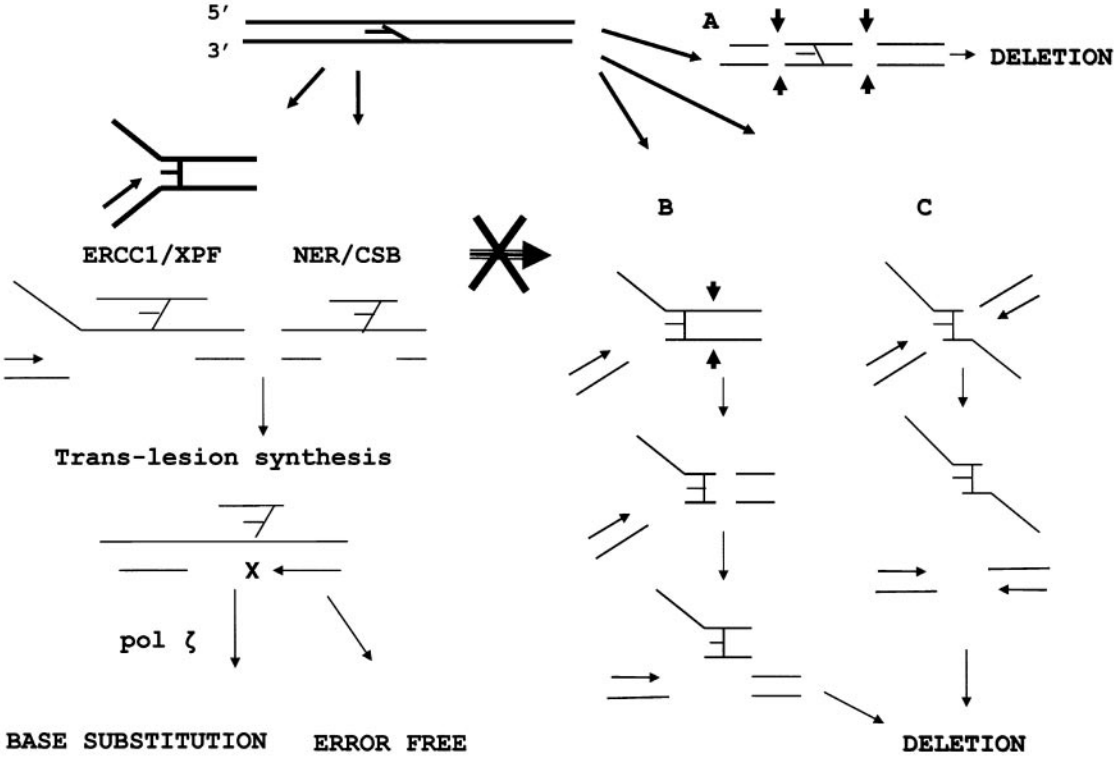

Figure 7. Schematic of mutation pathways for pso-TFO crosslinks. The pso-TFO crosslinks enter an ERCC1/XPF dependent base substitution pathway as a result of a block to duplex unwinding incurred during transcription, replication, etc. An intermediate may also be formed in the absence of replication through the action of NER/CSB, although this is speculative. Base substitutions are introduced during translesion synthesis by pol ζ. Translesion synthesis could be by error free polymerase(s) without mutation. Error free recombination pathways may also resolve the intermediates generated by ERCC1/XPF. Deletion formation is not dependent on NER/TCR/ or ERCC1/XPF and but does require cleavage of strands on either side of the crosslink (heavy arrows). These may arise in duplex DNA (**A**) or at a blocked replication fork through a series of breakage and cleavage reactions (**B**) or as a result of the collision of two replication forks on either side of the crosslink, followed by breakage of one arm of each fork (**C**). Unresolved crosslinks, or unrepaired ends may be the precursors for large rearrangements.

ERCC1/XPF or the NER apparatus on either side of the pso-TFO crosslink cannot explain our results. Indeed, in wild-type cells these activities draw crosslinks away from entry into pathways that result in deletions. There are multiple, speculative, routes to deletion formation at the pso-TFO crosslink. One would derive from a series of cleavage events on either side of the crosslink in intact duplex DNA, likely involving more than one activity (Figure 7A). A replication fork, broken on one side of the crosslink, could be a precursor to the deletions, but only if there were additional cleavage events on the other side of the crosslink in the unreplicated DNA (Figure 7B). Otherwise there would be no partner for the end joining reaction [see (74) for a detailed discussion of the repair of broken replication forks]. Another possibility would be the collision of two replication forks, traveling towards each other, with the crosslink (Figure 7C). Breakage of one arm of each fork would release two broken ends that could be joined by NHEJ or similar pathway. In the two replication models unjoined ends would be candidates for large scale rearrangements or broken chromosomes which might be lost if cells carrying them failed to survive expansion of the culture before selection. Double-strand breaks enhance the frequency of recombination, and an understanding of their generation and repair will have implications for protocols for pso-TFO targeted gene conversion and recombination.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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