Processing of Targeted Psoralen Cross-Links in *Xenopus* Oocytes

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Psoralen cross-links have been shown to be both mutagenic and recombinogenic in bacterial, yeast, and mammalian cells. Double-strand breaks (DSBs) have been implicated as intermediates in the removal of psoralen cross-links. Recent work has suggested that site-specific mutagenesis and recombination might be achieved through the use of targeted psoralen adducts. The fate of plasmids containing psoralen adducts was evaluated in *Xenopus* oocytes, an experimental system that has well-characterized recombination capabilities and advantages in the analysis of intermediates in DNA metabolism. Psoralen adducts were delivered to a specific site by a triplex-forming oligonucleotide. These lesions are clearly recognized and processed in oocytes, since mutagenesis was observed at the target site. The spectrum of induced mutations was compared with that found in similar studies in mammalian cells. Plasmids carrying multiple random adducts were preferentially degraded, perhaps due to the introduction of DSBs. However, when DNAs carrying site-specific adducts were examined, no plasmid loss was observed and removal of cross-links was found to be very slow. Sensitive assays for DSB-dependent homologous recombination were performed with substrates with one or two cross-link sites. No adduct-stimulated recombination was observed with a single lesion, and only very low levels were observed with paired lesions, even when a large proportion of the cross-links was removed by the oocytes. We conclude that DSBs or other recombinogenic structures are not efficiently formed at psoralen adducts in *Xenopus* oocytes. While psoralen is not a promising reagent for stimulating site-specific recombination, it is effective in inducing targeted mutations.

Photoinduced interstrand psoralen cross-links in cellular DNA are detected as genomic damage, and cells of all types attempt to repair them. Cross-links present a particular challenge to the repair machinery, since both strands are damaged at a single site. Like some other types of lesion, psoralen cross-links induce recombination in bacterial (5, 22, 36), yeast (26, 29–31), and mammalian (8, 34, 39) cells. Double-strand breaks (DSBs) have been either inferred or directly demonstrated to be involved in this process. In yeast cells (7, 18, 23, 31), DSBs have been shown to be intermediates in the cellular repair of psoralen cross-links, and their production requires nucleotide excision repair (NER) machinery (*RAD3* epistasis group). Recombinational repair (*RAD52* epistasis group) is required to rejoin the breaks made during cross-link removal and may participate in generating the DSBs (7).

NER is the major mechanism by which all living systems repair DNA that has been damaged by helix-distorting modifications, such as pyrimidine dimers and psoralen adducts (10, 32, 42). Briefly, NER proteins recognize and bind to the damaged site. In eukaryotes, incisions are made on the damaged strand 22 to 24 nucleotides 5' and 5 nucleotides 3' to the damage. The 27- to 29-base oligonucleotide containing the damage is excised intact, and repair synthesis fills the resulting gap. In the case of a psoralen double-strand cross-link, two sequential rounds of excision and resynthesis must occur. After the first excision step, the resulting gap is filled either by repair synthesis which must bypass the damaged site, often mutagenically, or by recombinational repair with homologous sequences (5, 38).

The goal of the present study was to investigate the fate of plasmids containing psoralen cross-links in *Xenopus laevis* oocytes. Full-grown *Xenopus* oocytes have been shown to possess an active NER system with a capacity several orders of magnitude greater than that of mammalian cells (16, 21, 37). Therefore, it seemed reasonable to expect that psoralen cross-links would be repaired in oocytes and that DSBs would be produced during this process. Linear DNAs recombine efficiently in oocytes via the single-strand annealing (SSA) pathway (1, 20, 25, 35); thus, production of DSBs at an appropriately placed cross-link should stimulate recombination.

Psoralen adducts were introduced into plasmid DNAs that were subsequently injected into *Xenopus* oocytes. Several specific outcomes were anticipated and monitored: removal of cross-links, DSB formation during the attempt to repair, homologous recombination resulting from the DSBs, and mutagenesis resulting from bypass synthesis across the lesion. These analyses were facilitated by the use of site-specific adducts produced in vitro with a psoralen-conjugated triplex-forming oligonucleotide (pso-TFO) (8, 40, 41). This reagent and ones like it have been shown to induce site-directed mutations and a low level of homologous recombination in mammalian cells (8, 14, 33, 40, 41).

MATERIALS AND METHODS

Oligonucleotides and plasmids. The pso-TFO pso-AG30 (8, 40, 41) was synthesized by Oligos Etc., Inc. (Wilsonville, Oreg.) and has the sequence 5'-X-A GGAAGGGGGGGTGGTGGGGAGGGGAGC-Y-3' (see Fig. 2A). X is a 4'-hydroxymethyl-4,5',8-trimethylpsoralen with a six-carbon linker joining the 4' position of the furan ring with the oligonucleotide. Y is a 3' propylamine group included to minimize susceptibility to degradation by 3' exonucleases (17). Plasmids harboring tailless cross-links were produced with a TFO synthesized with a disulfide linker between the tethered psoralen and the nucleic acid. After triplex formation and UV irradiation, the TFO was detached by reduction with dithiothreitol (28, 40).

pRW4 (5,615 bp) is essentially pBR322 with a direct 1.25-kb duplication of the

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tetracycline resistance gene (24). pHSS6 (2,320 bp) was included in all oocyte injections as a recovery control (19). pZ189, pSupFG1, and pSupF2 have been described previously (8, 41). MpSupF2 is a modified version of pSupF2 containing a 3-bp deletion at the 3' end of the second SupF repeat, abolishing an *EagI* site. The sole remaining *EagI* site lies between the two SupF repeats.

pTD-O, -I, and -S (5,680 bp) were constructed in three stages. In stage one, complementary oligonucleotides containing cleavage sites for the restriction endonucleases *MluI* and *BssHII* and the meganuclease *I-SceI*, and appropriate sticky ends (5'-TCGACGCGTATTACCTGTTATCCCTAGCGTGCAGCG-3' and 5'-TCGAGCGCGCACGCTAGGGATAACAGGGTAATACGCG-3') were cloned into the *XhoI* site of pRW4. In stage two, a second set of complementary oligonucleotides containing the triplex binding site (TBS) for AG30 (5'-CGCG GATCCTTCCCCCCCCACACCCCTCCCCCT-3' and 5'-CGCGAGGGGG AGGGGGTGGTGGGGGGGAAGGATC-3') were cloned in both orientations into the *MluI* site or the *BssHII* site. In the final stage, all four clones were digested with *I-SceI* and *PstI*, and the appropriate fragments containing the inserted TBSs were ligated together.

Mutagenesis assay. pSupFG1 (90 nM) and pso-AG30 (9 μ M) were incubated in 55 μ l of binding buffer (10 mM Tris-Cl, pH8, 20 mM MgCl₂, 1 mM spermidine, 10% sucrose) for 2 h at 37°C, after which the samples were transferred to round-bottom microtiter plates, covered with Parafilm, and irradiated at room temperature on a 360-nm Blak-Ray C-50 UV transilluminator (Ultra-Violet Products, Inc., San Gabriel, Calif.) for 15 min, corresponding to a dose of approximately 2.7 J/cm². The samples were precipitated with isopropanol to remove un-cross-linked pso-AG30.

A 20-nl volume of injection mixture, consisting of 1 ng of treated or untreated pSupFG1, 1 ng of pHSS6, and 88 mM NaCl, was injected into the nuclei of 20 stage VI *Xenopus* oocytes by methods previously described (24), and the oocytes were incubated at room temperature for 48 h. The DNAs were extracted as previously described (24). An approximately 0.75 oocyte equivalent of injected or uninjected pSupFG1 mixture was electroporated into the *Escherichia coli* K-12 strain SY302 [*recA lacZ125*(Am) *trp-49*(Am) *hsdR2::Tn10, tet'*] (12). The transformants were plated on 1.5% Luria-Bertani agar containing either 25 μ g of kanamycin (for pHSS6) per ml or 200 μ g of 5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside (X-Gal) per ml, 3 μ M isopropyl- β -D-thiogalactopyranoside (IPTG), and 100 μ g of ampicillin (for pSupFG1) per ml.

Recombination assays. Plasmids with TFO-directed adducts were prepared by incubating pso-AG30 (9 μ M) with target plasmid (90 nM) in 55 μ l of binding buffer for 2 h at 37°C. The samples were irradiated as described above. A 20-nl volume of injection mixture, consisting of 1 ng of target plasmid and 1 ng of pHSS6, was injected into the nuclei of 60 to 100 stage VI *Xenopus* oocytes. For mixtures containing circular, un-cross-linked substrate plasmids, pso-AG30 was included at a 1:1 molar ratio with the substrate to address the possibility of TFO-dependent effects in the absence of a psoralen adduct. After incubation at room temperature for the indicated times, DNAs from 15 to 20 manually dissected nuclei were extracted as described previously (24).

For samples containing a single adduct, 2.5 oocyte equivalents were digested with *EcoRI* and *BamHI*, electrophoresed on a 5% polyacrylamide gel, electrotransferred to a nylon filter, and hybridized with whole plasmid probe. Samples containing two adducts were digested with *PstI* and electrophoresed on a 1.2% agarose gel. To determine the percentage of molecules that contained two cross-links, plasmids were digested with *BamHI* and processed as described above. *BamHI* cuts these plasmids at four sites: two flanking the TBS region and two precisely at the adduction sites. Therefore, molecules that were resistant to cleavage at both adduction sites were interpreted to contain two TFOs. Since this method does not distinguish monoadducts from double-strand cross-links, we made the assumption that two-thirds of the adducts at each site were cross-links (11).

Cross-linking and recovery assays. Injected and uninjected samples were digested with *NcoI*. To assay for cross-linking, 2.5 oocyte equivalents of digested DNA were loaded onto 0.75% alkaline agarose gels containing 33 mM NaOH and 2 mM EDTA (3) and the gels were run for 6 h at 20 V. To assay for recovery, 2.5 GV equivalents were loaded onto 1% agarose gels. Following electrophoresis and transfer, the DNA was hybridized with whole plasmid probe.

Substrates containing random cross-links. An 80- μ l volume containing 16 μ g (55 nM) of pRW4 and 8 μ g (438 μ M) of 4,5',8-trimethylpsoralen (TMP; Sigma, St. Louis, Mo.) was irradiated at room temperature with 360-nm-wavelength UV light. Aliquots (10 μ l) were removed after 0, 15, 30, and 60 s, corresponding to doses of approximately 0, 45, 90, and 180 mJ/cm², respectively. The samples were extracted with phenol-chloroform-isoamyl alcohol (25:24:1 [vol/vol]) and ethanol precipitated to remove unbound TMP.

To determine the average number of cross-links per molecules, 200 ng of each sample was digested with *PvuII* and then electrophoresed in a 0.75% alkaline agarose gel, as described above. Following electrophoresis, the gel was irradiated on a 250-nm-wavelength Chromato-Vue C-61 UV transilluminator (Ultra-Violet Products, Inc., San Gabriel, Calif.) for 15 min at room temperature to photo-reverse cross-links, and then the DNA was immobilized on positively charged nylon membranes (Boehringer Mannheim) by overnight capillary transfer in 0.4 M NaOH and hybridized by the method described by Church and Gilbert (4) with random-primed ³²P-labeled pBR322 probe. Quantitation was performed with ImageQuant software on a PhosphorImager (Molecular Dynamics, Sunnyvale, Calif.). The average number of cross-links per molecule was calculated from

FIG. 1. Preferential loss of randomly cross-linked plasmids. (A) Typical recovery assay by native gel electrophoresis and Southern blot hybridization. Samples containing pRW4 which had been irradiated in the presence of free TMP for the times indicated were incubated in oocytes for 24 h, along with un-cross-linked pHSS6. The average number of cross-links per molecule (mol) of pRW4 was calculated as described in Materials and Methods. Cross-linked and un-cross-linked pRW4 cannot be distinguished by this assay. Uninjected (U), mock-injected (M), and oocyte-injected (I) samples are shown. (B) Graphical representation of the normalized recovery of randomly cross-linked pRW4 after oocyte incubation. Error bars indicate the standard deviations of the values obtained by normalization to the U and M lanes, which gave slightly different numbers.

the experimentally measured fraction of uncross-linked molecules, assuming a Poisson distribution. The derived values of 3, 7, and 13 cross-links should be considered approximate, since they are based on rather long extrapolations of the primary data.

Cross-linked pRW4 and un-cross-linked pHSS6 (1 ng each) were coinjected into nuclei of 20 stage VI *Xenopus* oocytes in a 20-nl volume. DNAs were extracted following overnight incubation at 17°C, digested with *PvuII*, electrophoresed in a 1% agarose gel, and hybridized with probe pRW4 as described above.

RESULTS

Preferential loss of plasmids containing random adducts.

Initially we examined the fate in *Xenopus* oocytes of random adducts produced by irradiation of plasmid DNA in vitro in the presence of a psoralen monomer, TMP. pRW4 containing a variable number of cross-links was injected into oocyte nuclei, along with an un-cross-linked control plasmid, pHSS6. After overnight incubation, the DNA was recovered and analyzed. Recovery was normalized to uninjected controls and to uninjected samples that had been subjected to the extraction procedure (mock injected) and run on the same gel. This precaution addresses the possibility of preferential loss during extraction or inefficient hybridization of the cross-linked DNA.

Figure 1 clearly shows the preferential loss of the cross-linked plasmid compared to the un-cross-linked control. This effect became more dramatic as the initial number of cross-links increased, with less than 20% of injected molecules remaining after 24 h from plasmids containing an average of 13 cross-links (Fig. 1B). This indicated that the cross-links were being processed in the oocytes. Because DSBs have been shown to be intermediates in the repair of psoralen cross-links in yeast (7, 18, 23, 31), it is plausible to suggest that the

FIG. 2. Mutagenesis by TFO-directed adducts. (A) Diagram of pso-AG30 and its target plasmid, pSupFG1. The 5' psoralen and 3' propylamine of pso-AG30 are shown. The TBS is located at the 3' end of the *supF* tRNA gene (large arrow) as shown. Other features of the plasmid are also indicated. SV40, simian virus 40. (B) The spectrum of mutations in plasmids recovered from light-blue or white colonies. The TBS (partially shown) is underlined in the duplex sequence. The numbering scheme is according to the method described by Glazer and coworkers (41), and the nonsense strand is at the top for comparison with published results. The TFO-bound psoralen intercalates between and cross-links the T's of the base pairs at positions 166 and 167. Base substitutions are shown above the duplex; deletions and insertions are shown below. Each symbol represents a separate recovered plasmid. The eight A→T substitutions at position 167 led to light-blue colonies; all others were pure white.

preferential loss could be due to conversion of at least some of the cross-links to DSBs and subsequent degradation of the resulting linear molecules (2, 24). No recombination products were observed from these reactions, as expected for randomly broken DNA.

Mutagenesis at site-directed lesions. To get a clearer picture of the fate of individual psoralen adducts, we turned to specific, TFO-directed lesions. Mutations are produced at the adduct site when such plasmids are incubated in mammalian cells (8, 14, 33, 40, 41). To investigate this issue in oocytes, pso-AG30 was cross-linked to pSupFG1, which contains a single, functional copy of the *supF* tRNA gene (Fig. 2A). The irradiation treatment typically produced about 60% double-strand cross-links and 30% monoadducts to one of the target strands (11). These derivatized plasmids were injected into oocyte nuclei, incubated for 48 h, and then extracted and transformed into an *E. coli* strain which carries a *lacZ* gene with an amber mutation. Unmutagenized pSupFG1 allows suppression of the amber mutation, producing blue colonies on X-Gal-containing

media. pSupFG1 harboring mutations at the cross-link site leads to white or light blue colonies, depending on the nature of the mutation (8).

Cross-linked pSupFG1 that had been incubated in oocytes produced 37 white or light-blue colonies of 1,039 total colonies (3.6%). No nonblue colonies were observed from un-cross-linked, oocyte-incubated pSupFG1 (2,343 total colonies) or from cross-linked pSupFG1 which had not been incubated in oocytes (1,205 total colonies). DNAs from 13 white and 8 light-blue colonies were extracted and sequenced (Fig. 2B). All of the light-blue colonies contained plasmids with an A→T transversion at position 167 (by the numbering scheme previously described [41]). The resulting tRNA is apparently partly functional. White colonies harbored plasmids with a single-base-pair deletion on either side of the intercalation site (positions 166 and 167) or single-base-pair substitutions or insertions, as indicated in Fig. 2B. The tight clustering of mutations around the site of the lesion is typical of TFO-directed adducts, but the detailed pattern of mutations differs somewhat from that seen in mammalian cells (8, 40, 41).

Two mechanisms for the generation of DSBs during cross-link repair. We wanted to determine whether psoralen damage would lead to DSB formation in oocytes, in addition to mutagenesis. A priori there are at least two types of mechanisms that might generate DSBs during the attempt to repair inter-strand cross-links by NER. The first (Fig. 3A) requires only one cross-link and simply proposes that DSBs arise by initiating a second round of excision before repair on the first strand is completed. When synthesis has partially filled the initial gap, up to the position of the lesion, the second pair of incisions occurs in duplex DNA. In the second mechanism (Fig. 3B), repair of two separate lesions will, at some frequency, produce incisions on opposite strands such that nearby 5' termini are exposed. When two molecules of the endogenous 5'→3' exo-

FIG. 3. Two possible mechanisms for the generation of DSBs during cross-link repair. Mechanisms requiring one (A) or two (B) cross-links are diagrammed. Horizontal lines, DNA strands; vertical bars, psoralen cross-links; half arrowheads, 3' ends; the dashed line in panel A, newly synthesized DNA; Pacman symbols in panel B, exonucleases. See text for details.

FIG. 4. Homologous recombination assay for substrates containing one site-specific adduct. (A) The MpSupF2 substrate plasmid. pso-AG30 is shown aligned with its TBS. The large white and black arrows indicate the two SupF repeats, and the position of the unique *EagI* site is shown. (B) Autoradiogram of a typical recombination assay. All samples were digested with *EcoRI* and *BamHI* before electrophoresis, and DNA was detected after transfer with a radiolabeled MpSupF2 probe. *EagI*-linearized MpSupF2 (linear) is shown before (0) and after (48) incubation in oocytes for 48 h. Un-cross-linked MpSupF2 (uncrosslinked), MpSupF2 containing an AG30-directed adduct (TFO-crosslinks), and MpSupF2 containing a site-specific adduct without the oligonucleotide tail (tailless crosslinks) were incubated in oocytes for the times indicated. The locations of substrate bands containing two *supF* genes (S) and product bands containing one *supF* gene (P) are indicated at the right. Lane Uninj oo, a DNA fragment from uninjected oocytes which hybridizes with the MpSupF2 whole plasmid probe and fortuitously migrates with unrecombined substrate. The two bands in the linear 0-h lane that migrate just faster than the product are the terminal fragments of the pre-cut substrate. Stds, standards.

nuclease (24) initiate degradation at these ends, a DSB will result when they meet.

DSBs formed by either of the mechanisms described above might be observable directly, or the molecules might be degraded too rapidly after cleavage to be detectable. In fact, simple Southern blot analysis of recovered DNAs revealed no linear molecules with ends at the cross-linked site. We used a homologous recombination assay in an attempt to capture substrate molecules that might have received a DSB (35).

Homologous recombination assay—single adducts. Plasmid MpSupF2 contains an AG30 TBS between direct repeats of the *supF* gene (Fig. 4A). When this plasmid is linearized by cleavage between the repeats, nonconservative homologous recombination can occur in the oocytes by the SSA mechanism (20, 25, 27). An intermediate in this reaction would be an 88-bp heteroduplex containing four mismatches, with 13-base and 8-base nonhomologous 3' tails (8). Product plasmids would contain only one copy of the *supF* gene. These products can be detected readily by native polyacrylamide gel electrophoresis analysis, after digesting the plasmids at sites which flank the repeats. The lanes labeled linear in Fig. 4B show the formation of product from MpSupF2 that was cut with *EagI* prior to injection. In contrast, circular substrates did not generate recombination products.

Substrates containing a TFO-directed adduct were incubated in oocytes for various times and then analyzed. Substrates with a covalently attached AG30 can be seen running at a retarded mobility (Fig. 4B). No recombination products from these substrates could be detected even after 48 h of incubation in the oocytes, nor was any significant loss of the damaged

molecules observed, in contrast to the loss of randomly cross-linked substrates (Fig. 1).

These substrates contained not only a psoralen adduct on one or both target strands, but also the covalently attached 30-nucleotide TFO. This oligonucleotide tail has been shown to interfere with the processing of psoralen cross-links in COS cells (40). To determine if the presence of the oligonucleotide tail would influence recombination, substrates were prepared with a pso-AG30 analog with a disulfide linkage to the psoralen. After irradiation, the linkage was reduced with dithiothreitol and the TFO was removed to produce substrates containing a site-specific, tailless adduct between the repeats. Again, no recombination or preferential loss of these substrates could be detected even after 48 h of incubation in the oocytes (Fig. 4B). Thus, a single targeted psoralen adduct is not converted to a DSB in oocytes with measurable efficiency.

Homologous recombination assay—paired adducts. To investigate the second proposed mechanism for psoralen-stimulated DSB production, substrates which contained two AG30 TBSs between 1.2-kb direct repeats were constructed (Fig. 5A). The TBSs were arranged so that the two TFOs would bind with their 3' ends pointing outward (pTD-O), inward (pTD-I), or in the same direction (pTD-S). Substrates containing two TFO-directed adducts were prepared in vitro as described above. About 75% of the treated substrates carried adducts at both sites, and 20 to 30% had two interstrand cross-links (data not shown).

Figure 5B shows that pTD-O linearized prior to injection by *I-SceI* cleavage between the two TBSs yielded recombination products after incubation in oocytes demonstrating that these

FIG. 5. Homologous recombination assay for substrates containing two site-specific adducts. (A) The pTD series plasmids. Each has an I-SceI site between two AG30 TBSs, all of which are located between two 1.2-kb repeats (shaded arrows). The TBSs were oriented so that the 3' tail of TFOs bound to the sites would be pointing outward (pTD-O), inward (pTD-I), or in the same direction (pTD-S). (B) Typical recombination assay analyzed by native gel electrophoresis and Southern blot hybridization. Uncut, unadducted, undigested pTD-O. All other samples were digested with *Pst*I before electrophoresis. Material from uninjected oocytes (Uninj oo) is shown in the adjacent lane. I-SceI-linearized pTD-O (linear), unadducted pTD-O (unXlinked), pTD-O containing AG30-directed adducts (TFO), and pTD-O containing site-specific adducts without the oligonucleotide tail (tailless) are shown before (0) and after 48 h of incubation in oocytes. Similarly adducted samples of pTD-I and pTD-S are also shown. The locations of substrate bands containing two *supF* genes (S) and product bands containing one *supF* gene (P) are indicated at the right. A faint product band is visible in the TD-O tailless 48-h sample, and similar bands were observed in other 48-h samples upon longer exposure of the autoradiogram.

plasmids are capable of recombination. Only very faint product bands could be detected after 48 h of oocyte incubation of the adducted substrates. This trace level of recombination did not appear to be dependent on the orientations of the TBSs or the presence or absence of the oligonucleotide tail. No substantial loss of any of the doubly adducted plasmids was observed. If two neighboring lesions are capable of initiating DSB formation, the efficiency must be quite low.

Repair and recovery of plasmids containing a single, targeted cross-link. Having failed to observe dramatic psoralen-stimulated recombination, we investigated the fate of cross-linked plasmids in oocytes more directly. Substrates containing no cross-links or a single TFO-directed adduct were injected into oocyte nuclei, and the oocytes were incubated for various times before extraction. Remaining cross-links were assayed by alkaline agarose gel electrophoresis (Fig. 6A). Linearized cross-linked DNA migrates at a retarded mobility in an alkaline gel compared to un-cross-linked material. Approximately two-thirds of the original targets contained a cross-link, in agreement with earlier observations (11). The results of several such assays are shown graphically in Fig. 6B. Only about 20% of the TFO-directed cross-links were removed by the oocytes after 48 h of incubation. Tailless cross-links were processed somewhat more efficiently, with nearly 60% of these lesions removed after 48 h. However, a chi-square test revealed that the difference between the two data sets was of marginal significance.

Injected material was examined for preferential loss of the cross-linked plasmid compared to a coinjected un-cross-linked plasmid. The DNAs were linearized and analyzed on a native agarose gel. Figure 7A shows a typical recovery assay, with the cumulative results of several experiments shown graphically in Fig. 7B. In contrast to the results with plasmids containing

FIG. 6. Removal of cross-links upon incubation in oocytes. (A) Typical cross-linking assay analyzed by alkaline gel electrophoresis and Southern blot hybridization. All samples were linearized before electrophoresis. Un-cross-linked MpSupF2 (uncrosslinked), MpSupF2 containing an AG30-directed cross-link (TFO-crosslinks), and MpSupF2 containing a site-specific cross-link without the oligonucleotide tail (tailless crosslinks) were incubated in oocytes for the times indicated at the top. The locations of bands corresponding to cross-linked and un-cross-linked MpSupF2 are indicated at the right. Quantitation of the amount of cross-linked material as a percentage of the total amount of DNA is shown below the autoradiogram (% crosslinking). For purposes of comparison, these amounts were also expressed as a percentage of the amount of cross-linking in the uninjected samples (% of Original). (B) Graphical representation of the combined cross-linking data for several experiments, as indicated. Error bars show standard deviations.

FIG. 7. Recovery of plasmids containing a site-specific adduct after incubation in oocytes. (A) Typical recovery assay by native gel electrophoresis and Southern blot hybridization. All samples were linearized with *NcoI* before electrophoresis. *EagI*-linearized MpSupF2 (linear) is shown before (0) and after 48 h of incubation in oocytes. Other samples are designated as described in the legend to Fig. 6. The locations of bands corresponding to linear MpSupF2 and the unadducted control, pHSS6, are indicated at the left. Quantitation of the amounts of MpSupF2 after incubation, which were normalized to the amounts in the uninjected samples, are given below the autoradiogram (% Recovery). Adducted and nonadducted MpSupF2 cannot be distinguished in this assay. (B) A graphical representation of the combined recovery data for several experiments, as indicated. Error bars show standard deviations.

random cross-links (Fig. 1), no preferential loss was detected within the error of the measurement for any of the substrates containing a single cross-link. No molecules containing a site-specific DSB were detected.

DISCUSSION

The goal of this study was to characterize the processing of plasmids containing psoralen cross-links in *Xenopus* oocytes. Specifically, we asked whether psoralen adducts are recognized in oocytes, whether cross-links are removed, and whether DSBs or other recombinogenic intermediates are generated at the damaged site. Psoralen adducts are processed in oocytes, and, as in other cell types (14, 15, 41), mutations are often created at the site of the lesion. Psoralen cross-links are removed in oocytes, but at rates that are much slower than published rates for repair of UV-induced damage (16, 21, 37). The oocyte provides a particularly favorable setting in which to study DSB-induced recombination because of its high capacity for SSA events and the absolute dependence of homologous recombination on DSBs (1, 2, 35). In practice, essentially no recombination was observed, even in cases in which more than half of the original cross-links were removed by the oocytes. Thus, unlike yeast (7, 18), oocytes appear not to produce DSBs during the attempt to repair psoralen adducts.

Mutagenesis at the adduct site. With a cross-linking target in a bacterial *supF* gene and a bacterial assay for *supF* function (14, 15), psoralen-induced mutations were readily detected after incubation in oocytes. The absence of *supF* mutations in adducted samples transformed directly into bacteria gave us confidence that the mutations that we characterized were the result of oocyte activities, although it is possible that the mu-

tagenic events were initiated in oocytes and completed in the host bacteria. *psu*-TFO-dependent mutagenesis has been observed in *recA*⁺ *E. coli* in a cross-linked *supF* gene that had been inserted into the bacteriophage λ genome (15). Use of a *recA* host in our experiments limited its ability to repair the lesions and may also have minimized bacterial mutagenesis by suppressing the SOS response.

In comparable studies with mammalian (COS) cells, mutations at the site of the lesion were also recovered from plasmids containing TFO-directed adducts (8, 14, 40, 41). While the mutations in all cases were confined within a few base pairs of the damaged site, the spectrum of mutations recovered from oocytes was subtly different from that produced in COS cells. In the most analogous study (adduct formation in vitro with *psu*-AG30 prior to introduction into cells [41]), a variety of mutations was found: single-site substitutions, only a few of which correspond to those in the oocyte collection; a number of deletions in the range of tens of base pairs; and one single-base-pair deletion (Δ 167). The oocytes yielded a higher proportion (8 of 21) of single-base-pair deletions at the two sites immediately flanking psoralen intercalation (Δ 166 and Δ 167). The most common substitution mutation in COS cells was T166→A (in the nonsense strand) (8, 40); however, this was not found among the oocyte products. The most frequently observed substitution in oocytes, A167→T, was very rarely produced in COS cells. (It is possible that this is the result of an ascertainment difference, since the latter mutation yields a light-blue colony phenotype, and the difference may be exacerbated by the use of different bacterial host strains.)

As in most studies of this type, we cannot be certain whether the observed mutations arose from interstrand cross-links or from monoadducts in the target. In general, monoadducts are less mutagenic, and the predominance of mutations at position 167 suggests that they resulted from processing of cross-links (40).

The difference in mutation spectrum between oocytes and mammalian cells may reflect inherent properties of these cell types. Substitutions and small deletions are presumed to result from DNA synthesis that bypasses a remaining lesion. In COS cells, the target plasmid was replication competent, and pains were taken to ensure that only replicated DNAs were included in the analysis (8, 40, 41). *Xenopus* oocytes do not replicate injected DNA (9, 13), but they are capable of repair synthesis and NER (6, 16, 21). It is conceivable that different patterns of misincorporation would occur during replicative and repair-type synthesis.

Processing of cross-links. Randomly cross-linked plasmids were preferentially lost in oocytes in a dose-dependent manner. Because DSBs are generated during the repair of cross-links in yeast (7, 18, 23, 31), it seemed plausible that breaks were occurring in oocytes, followed by degradation of the resulting linear molecules (2, 24, 43). However, when TFO-directed, site-specific cross-links were introduced into oocytes, they were removed quite slowly, and no loss of DNA was observed. Removal was more rapid when the TFO was released before injection (tailless cross-links), corroborating an earlier finding that the presence of the oligonucleotide inhibited repair (40). Direct analysis showed no evidence of DSBs at the cross-link site. The difference between the targeted and random cross-links is not understood, but it is clear that a single cross-link, or even a pair of adducts, is not efficiently converted to a DSB in oocytes.

Adduct-stimulated recombination. Psoralen-stimulated recombination has been reported for bacterial (5, 22), yeast (26, 29–31), and mammalian (8, 34, 39) cells. With two exceptions (8, 34), the adducts in these studies were random in location

and variable in number. We investigated the potential stimulation of extrachromosomal recombination in oocytes by site-specific lesions, both from inherent interest and as a sensitive method for detecting DSBs.

No recombination was detected for substrates containing a single adduct, and only very low levels were observed with two adducts. The fact that the relative orientation of the paired sites had no effect on the recombination efficiency indicates that our simple model for DSB formation with two cross-links (Fig. 3B) was probably not applicable. The absence of psoralen-stimulated recombination is a strong indication that DSBs are not commonly formed at adducted sites in oocytes. This conclusion is limited by the sensitivity of the assays employed, but recombination of 1% of the original substrate would have been readily detected.

Our expectation that DSBs might be produced was based on the observation that cross-link-stimulated DSBs in yeast are dependent on NER activities (7, 18) and the demonstration of NER at UV lesions in oocytes (16, 21, 37). Our results make it likely that the processing of psoralen adducts in *Xenopus* differs from that in yeast. This could be due to differences in other repair components (e.g., elements of the *RAD52* pathway) or to cell cycle-related phenomena (7, 23). Regardless of the underlying mechanism, it is clear that more than 50% of the initial cross-links can be removed in oocytes (Fig. 6B) without being accompanied by detectable recombination (Fig. 4B).

During the course of this work, site-specific, psoralen-stimulated recombination was demonstrated in COS cells by using essentially the same TFO and plasmid (8). Multiple pathways were shown to act on the targeted damage producing mutations, gene conversions, nonconservative recombination events, and illegitimate deletions. Using a different experimental setup, Sandor and Bredberg also detected a low frequency of gene conversion induced by TFO-directed adducts in a human cell line (34). Recently, a redesigned substrate was used to demonstrate a higher level of nonconservative homologous recombination events in COS cells (7a). It is difficult to make direct comparisons between the product yields in mammalian cells (1 to 2% of recovered plasmids in the recent study) and the failure to observe significant stimulation of recombination in oocytes. In the case of the oocytes, all injected DNAs were recovered without amplification, while from COS cells only replicated DNAs were analyzed, and the effects of the nature of the adduct and the timing of repair on DNA recovery could not be evaluated directly (8).

The ultimate utility of TFO-directed, psoralen-stimulated recombination will depend on the ability to control the balance between desirable recombination events and undesirable mutagenesis. While the oocyte experiments failed to show the DSBs and recombination products predicted based on results from yeast, the observations with mammalian cells are more encouraging. In addition, it is conceivable that more direct cleavage reagents could be tethered to TFOs in an attempt to deliver site-specific, recombinagenic DNA damage.

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