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Biochemical and Biophysical Research Communications 336 (2005) 69–75
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Repair of psoralen interstrand cross-links in *Xenopus laevis* egg extracts is highly mutagenic

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Received 2 August 2005
Available online 18 August 2005

Abstract

The recognition and removal of interstrand cross-links is perhaps the least understood of all repair pathways in eukaryotic cells. We have shown previously that uncoupling of cross-links occurs in mammalian cell extracts and have identified a number of factors that mediate this process. However, we have not observed complete repair of the substrate in this system. Here, we show that uncoupling of interstrand cross-links also occurs in Xenopus laevis egg extracts, and that the initial products of this reaction are identical to the products observed in mammalian cell extracts suggesting a common mechanism. However in contrast to mammalian cell extracts, we observe repair of the cross-linked substrate in the Xenopus extracts presumably by a translesion bypass mechanism that allows replication past the uncoupled monoadduct, and its likely subsequent removal by nucleotide excision repair. This repair process is shown to be highly mutagenic consistent with bypass synthesis.

Keywords: Interstrand cross-links; DNA repair; *Xenopus laevis* extract; Mutagenesis

The repair of interstrand cross-links (ICLs) in eukaryotic cells, particularly the early stages of lesion recognition and incision, has remained surprisingly refractory to elucidation. Subsequent to uncoupling of the ICL, the completion of repair is known to occur either by homologous recombination [1,2] or possibly by a pathway of translesion bypass [3]. The latter pathway would be highly mutagenic compared to a recombination mechanism. To overcome the lack of understanding of the uncoupling steps, we have developed an in vitro assay to monitor the repair processing of interstrand cross-links (ICLs) in mammalian cell extracts [4–8]. This assay uses a plasmid DNA substrate containing a site-specific psoralen ICL that allows for the detection of specific products produced by the uncoupling of the lesion. Using this assay we have previously identified a number of proteins required for this reaction. These proteins include replication protein A (RPA), the nucleotide excision repair (NER) heterodimer Ercc1-Xpf, and the mismatch repair (MMR) factor MutSβ. In addition, we have shown that PCNA enhances the uncoupling reaction by a stimulation of MutSβ binding to the psoralen ICL. To a large extent, our in vitro findings have been validated by results of genetic studies in somatic cells, as both Ercc1-Xpf and Msh2 have been shown to be involved in a common pathway that mediates resistance to ICLs in human cells [9]. In addition, it has been shown that MMR is involved in a nonmutagenic pathway of repair of ICLs in mammalian cells [10]. Although we have been able to demonstrate the uncoupling of ICLs in mammalian cell

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doi:10.1016/j.bbrc.2005.08.042

extracts [6], we have not observed bypass and repair on the resulting substrate containing a monoadduct.

Extracts prepared from *Xenopus laevis* eggs have proven extremely useful for biochemical studies on any number of pathways including DNA replication, DNA repair, and cell cycle checkpoint signaling (reviewed in [11,12]). These extracts are particularly useful for studies involving replication, since upon introduction of DNA substrates, replication initiates in an extremely synchronous manner. In eukaryotic cells, the major pathways of ICL repair are thought to initiate during S phase when the replication fork encounters the blocking lesion. Thus, the *X. laevis* extracts may provide a very unique system in which to study the processing of ICLs in vitro. Here, we show with our defined cross-linked substrate that ICLs are processed in these extracts, and that the initial products of this reaction are identical to the products observed in mammalian cell extracts. In addition, unlike in the mammalian extracts, we observe repair of the cross-linked substrate in the *X. laevis* extracts, and this repair was found to be highly mutagenic.

Materials and methods

Plasmid substrates. Psoralen interstrand cross-linked substrates for the in vitro ICL assay were prepared as previously described [4].

Preparation of Xenopus egg extracts. A clarified, crude extract of unactivated eggs of *X. laevis* was prepared as described [13,14] with some modifications. Briefly, unactivated eggs are laid over a 12–16 h time period after priming several female *X. laevis* with human chorionic gonadotropin (Sigma). The eggs are collected and rinsed several times in 1× MMR buffer (100 mM NaCl, 2 mM KCl, 1 mM $MgSO_4$, 2 mM $CaCl_2$, 5 mM Hepes, pH 7.8, and 0.1 mM EDTA). Rinsed eggs are dejellied in excess 2% cysteine (pH 7.8), followed by multiple rinses in XBII buffer (100 mM KCl, 4 mM $MgCl_2$, 10 mM K–Hepes, pH 7.2, 100 mM sucrose, and 0.1 mM EGTA). The eggs are transferred in a minimal volume of this buffer, supplemented with 1 mM DTT and 100 μg/ml cycloheximide, to SW41 (Beckman) ultracentrifuge tubes. Low-speed centrifugation at 920*g* for 15 s is used to pack the eggs and excess buffer is removed. Preparation of a highly concentrated low-speed supernatant (LSS) extract is achieved by overlaying a 5 mm layer of the lubricant Nyosil M-25 (Nye Lubricants, Fairhaven, MA) over the packed eggs and repeating the spin at 920*g* for 30 s. After the lubricant and buffer layers are removed, the packed eggs are lysed by spinning at 49,000*g*, 2 °C, for 20 min in a SW41 rotor (Beckman). The cytoplasmic fraction (LSS) is removed carefully by syringe and needle from the side of the tube, above the dark pigmented layer, and collected on ice. A cocktail of protease inhibitors (Sigma) and cytochalasin B (10 μg/ml, Sigma) is added and the extract is flash-frozen in small aliquots in liquid nitrogen.

ICL repair assay. Approximately 20 ng of CLT or DT substrate was incubated with *Xenopus* egg extract (50 μg of total protein) supplemented with an ATP regenerating system (150 mM creatine phosphate, 1 mM ATP, 0.1 mM EGTA, 1 mM $MgCl_2$, and creatine kinase at 0.15 μg/μl). For labeling, [α-^{32}P]dATP (10 mCi/ml, 6000 Ci/mmol, Amersham) was added to the reaction mixture. All reactions were incubated at 25 °C for the times indicated, and EDTA was added to 10 mM to terminate the reaction. Reaction mixtures were treated with RNase at 37 °C for 10 min and then with proteinase K at 37 °C for 30 min in buffer A (20 mM Hepes, pH 7.6, 50 mM NaCl, 5 mM EDTA, and 2% SDS). DNA was extracted with phenol/chloroform and precipitated with ethanol.

Plasmid rescue and PCR. Plasmid DNAs were recovered at 0 and 6 h after incubation with *Xenopus* egg extract, and after extraction were electroporated into AB2480 (*recA uvrA*) mutant *Escherichia coli* strain. PCR was performed on rescued plasmid clones using T3 and M13 primers.

Results

Repair of psoralen ICLs in X. laevis egg extracts

The substrates used in the in vitro assay are shown in Fig. 1A. The CLT contains a site-specific psoralen cross-link, and the DT is a control plasmid that is identical to the CLT, except for the region encompassed by the two *Ssp*I sites and the absence of the ICL [4]. Incubation of these two substrates independently in *Xenopus* egg ex-

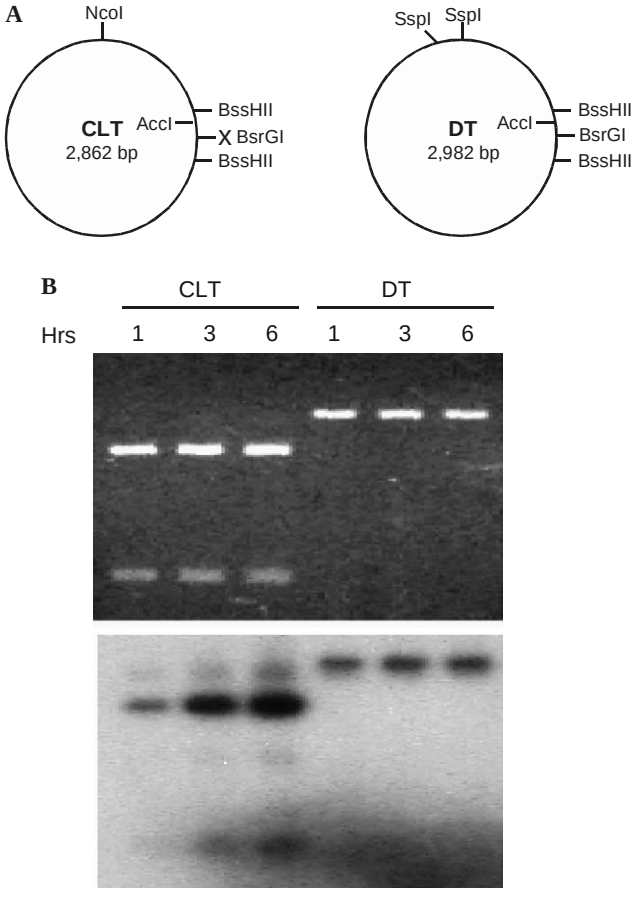

Fig. 1. Psoralen ICL stimulates DNA synthesis in *Xenopus* egg extracts. (A) Substrates used for in vitro DNA repair assay. CLT (cross-linked template); DT (donor template). "X" indicates the site of psoralen interstrand cross-link in the CLT. Restriction enzyme sites are as indicated. (B) Autoradiogram (lower panel) shows an increase in nucleotide incorporation in the CLT compared to the control DT plasmid. Ethidium bromide staining of DNA in same gel is shown in upper panel. DNAs were digested with a combination of *Acc*I and *Nco*I restriction enzymes.

tracts showed that incorporation into the CLT was significantly higher than incorporation into the DT plasmid, suggesting that the psoralen ICL was stimulating DNA synthesis presumably due to repair processing (Fig. 1B).

We have previously shown that incubation of the CLT in mammalian cell extracts followed by subsequent cleavage with the *Bss*HII restriction enzyme results in the production of two fragments of 86 and 113 nt upon denaturing gel electrophoresis [6]. An analysis of these fragments indicated that they resulted from an uncoupling of the ICL followed by a gap-filling step that terminates at the remaining monoadduct (Fig. 2). No detectable bypass of the remaining monoadduct was observed in the mammalian cell extracts. Incubation of the CLT, but not the DT, in the *Xenopus* egg extracts resulted in the production of the same 86 and 113 nt fragments (Fig. 3A). However, in addition we also

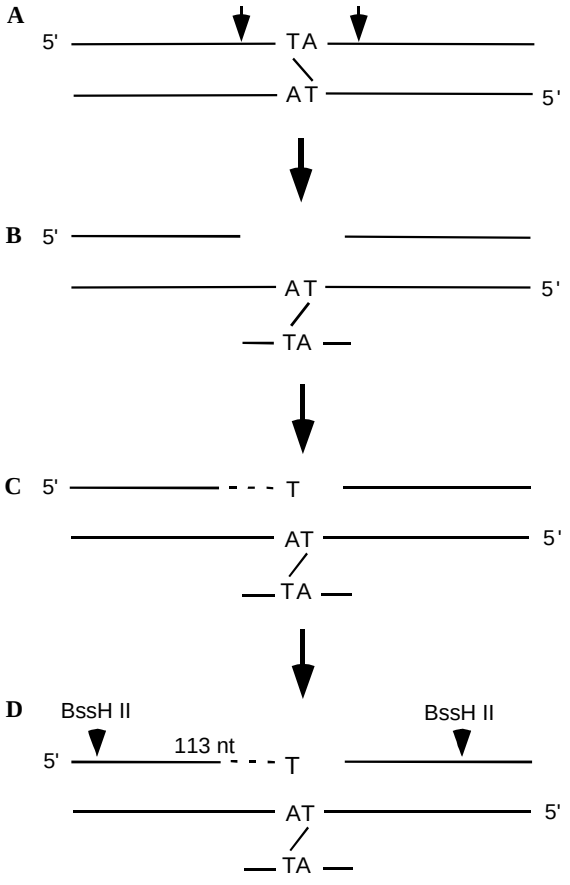

Fig. 2. Schematic model of the CRS assay indicating the processing of a psoralen ICL in cell extracts. An ICL in duplex DNA stimulates cleavage (indicated by arrows) on both sides of the cross-link in one strand (A), which creates a gapped intermediate (B). DNA synthesis (dashed line) fills in the gap but is blocked at the adducted thymine in the template strand from further progression (C). Subsequent cleavage of the substrate with the restriction enzyme *Bss*HII creates a fragment of 113 nucleotides (nt) (D). Symmetrical processing of the cross-link on the bottom strand yields a fragment of 86 nt.

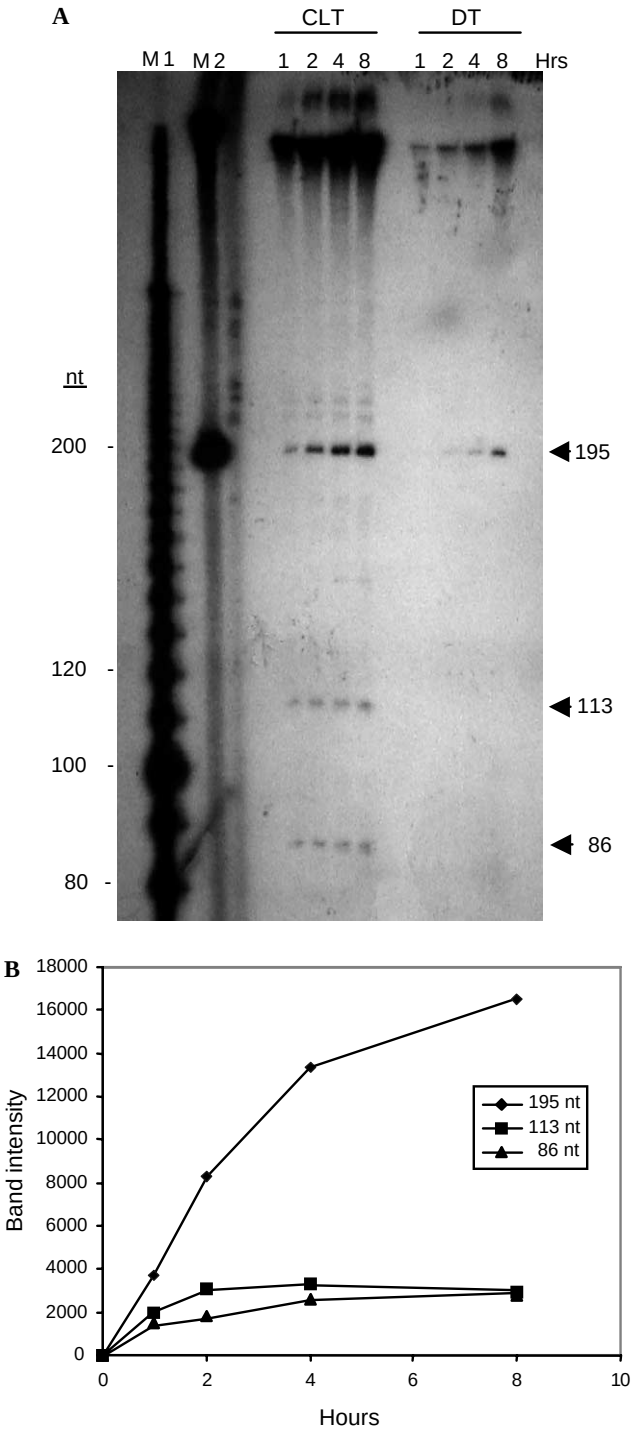

Fig. 3. Processing of psoralen ICLs in *Xenopus* egg extracts. (A) Autoradiogram showing the products of derived from the CLT and DT plasmids after varying times of incubation in the *Xenopus* egg extract. The 195 nt fragment indicates repair of the ICL. The 86 and 113 nt fragments are intermediates in the reaction. (B) Quantitation of the products of the repair reaction shown in (A) as a function of time.

observed the production of a 195 nt fragment, resulting from the *Bss*HII digestion, that would only occur if the remaining monoadduct was bypassed by a translesion polymerase. A cross-linked 195 bp fragment would

migrate higher in the gel due to the fact that both strands are coupled together; thus, the occurrence of the single-stranded 195 nt fragment indicates that repair of the ICL occurred in the *Xenopus* egg extracts. We were not able to determine directly whether the remaining monoadduct was removed, however, we have shown previously that there is a very potent nucleotide excision repair pathway in these extracts which would likely remove this lesion [15]. In addition, after incubation in the *Xenopus* egg extracts the CLT can be rescued in an *E. coli* strain deficient in both NER and homologous recombination (see below). The other interesting deduction gained from this experiment is that the levels of the 86 and 113 nt fragments initially rise, but then reach a steady state level while the level of the 195 nt fragment continues to increase (Fig. 3B). These observations indicate that the 86 and 113 nt fragments represent intermediates in the ICL processing, which suggests that there is a pause at the remaining monoadduct followed by bypass and complete gap filling.

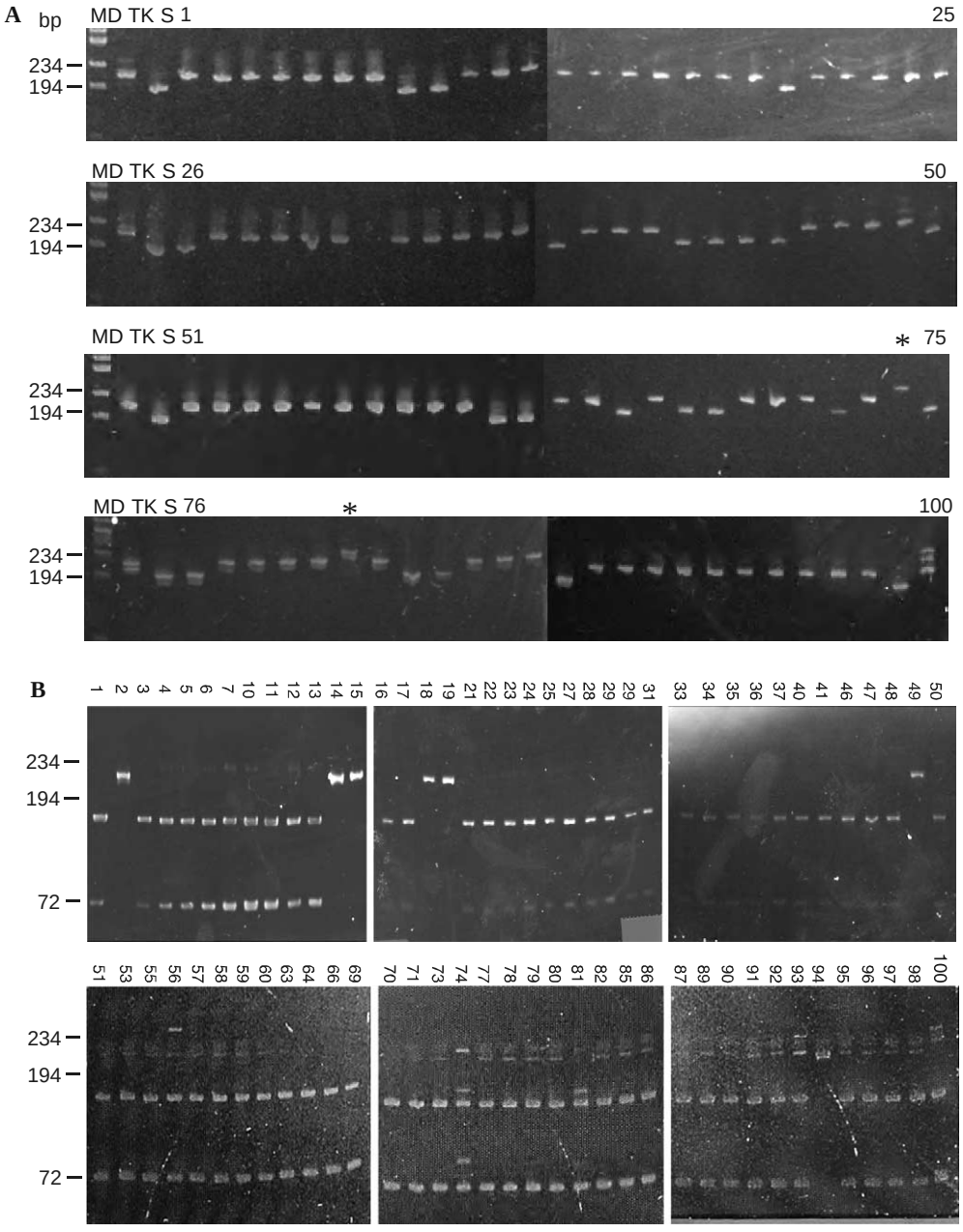

Fig. 4. Analysis of clones rescued into *E. coli* shows ICL-induced mutagenesis. (A) Ethidium bromide gel showing PCR products obtained from clones rescued into *E. coli* strain AB2480 after incubation in *Xenopus* egg extract. Faster migrating bands indicate clones that do not contain the insert. Slower migrating bands contain the insert. Clones #74 and #81 indicated by asterisks exhibited abnormally slow migration. ''M'' indicates marker bands; ''DT'' indicates the PCR product from the DT plasmid; and ''KS'' indicates the PCR product from a plasmid without the insert. (B) Digestion of clones containing inserts shown in (A) with *Bsr*GI. Clones not digestable with *Bsr*GI indicate mutation of this site.

To determine the extent and nature of the repair of the CLT in the *Xenopus* egg extracts, we first determined the fraction of our substrate that contained a cross-link. The CLT is prepared by insertion through ligation of a small cross-linked oligonucleotide [4]. This procedure also results in molecules that do not contain the oligonucleotide or contain the oligonucleotide without a cross-link. To determine the proportions of these species in our CLT substrate, we compared undigested CLT with that digested with a combination of *Hin*dIII and *Bsr*GI restriction enzymes. *Hin*dIII will linearize plasmids without an insert and *Bsr*GI will linearize plasmids with an insert not containing a cross-link (the cross-links blocks digestion by this enzyme). This analysis showed that approximately 6.5% of the CLT substrate was digestible with these two enzymes (data not shown).

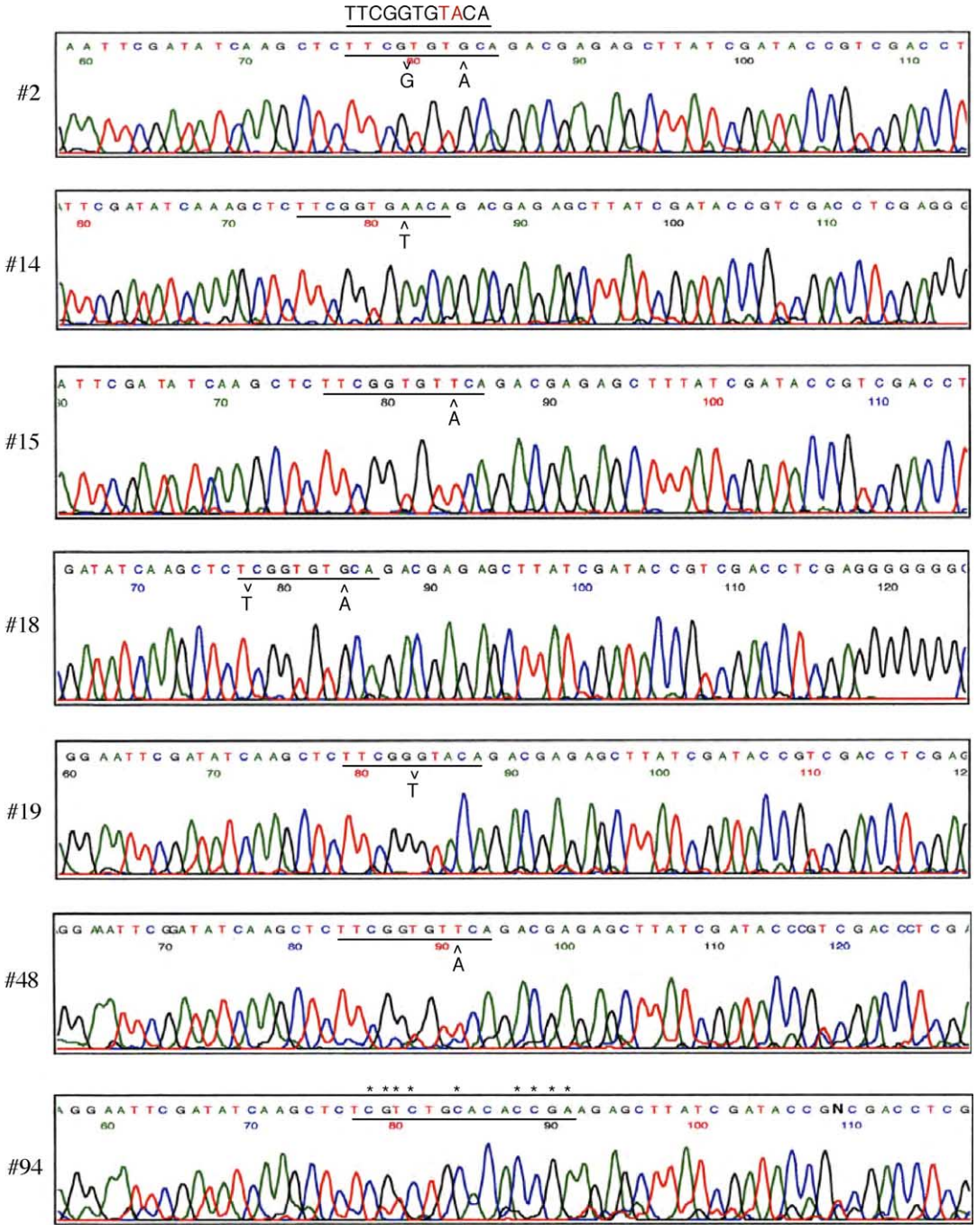

Fig. 5. Analysis of mutations by DNA sequencing. Clones that were not subjected to *Bsr*GI digestion as shown in Fig. 4 were analyzed by DNA sequencing to determine the nature of the mutation. The wild-type sequence is shown at the top. Clone numbers are indicated at the left of each sequencing histogram. Arrowheads pointing up indicate a base change and arrowheads pointing down a deletion.

The remainder of the substrate thus contained a cross-link. To determine the extent of repair in the *Xenopus* extracts, we first recovered CLT plasmids from the extracts without incubation (0 h samples) and transfected them into the *recA uvrA* mutant *E. coli* strain AB2480 [16]. This strain is incapable of repairing cross-links [17]. Sixty-five clones were examined by PCR and subsequent restriction enzyme digestion. Of the 65 clones 29 (44.6%) did not contain an insert and 36 (55.4%) contained an insert without a cross-link (data not shown). Thus, the proportion of these species in the original CLT substrate is 2.9 ($0.445 \times 6.5\%$) and 3.6% ($0.554 \times 6.5\%$), respectively. This same experiment was performed after 6 h of incubation of the CLT in the *Xenopus* egg extract. One hundred PCRs were performed with one failure (Fig. 4A). Twenty-two of the clones did not contain an insert and 77 did. This indicates that the percentage of noncross-linked plasmid increased from 55.4% at 0 h to 77.8% after 6 h of incubation. A simple calculation $(3.6 + r)/(3.6 + 2.9 + r) = 77.8\%$, where r is the extent of repair, indicates that approximately 6.6% of the CLT underwent repair in the *Xenopus* extracts and were able to replicate in the *E. coli* AB2480 strain.

Repair of psoralen ICLs in Xenopus egg extracts is highly mutagenic

We next examined the 77 recovered clones that contained an insert for mutations at or near the psoralen ICL by digestion of the PCR product with *Bsr*GI. Of the 77 clones examined 7 were *Bsr*GI resistant, indicating that they had undergone a mutation that altered this restriction enzyme site (Fig. 4B). In the 0 h sample, all 36 clones that contained an insert were found to be digestible by *Bsr*GI, indicating a lack of mutations in this control group. To verify the mutations in the experimental group, all seven clones were sequenced. This analysis indicated that four clones had single base mutations, one contained a single base deletion, two contained a single base mutation and a deletion, and one contained multiple base mutations (Fig. 5). In addition, two clones (#74 and #81, Fig. 4A) were digestible with *Bsr*GI, but contained complex insertion/deletion mutations that resulted in an overall increase in the size of the region just upstream from the cross-link site. Interestingly, both of these clones contained a second *Bsr*GI site in the insertion region. To determine the frequency of mutagenesis, we used the following calculation: $[(6.5)/(6.5 + 3.6)] \times 77 = 50$, which represents the approximate number of recovered clones that were repaired in the *Xenopus* egg extract. Nine of these 50 clones were mutated, yielding a frequency of mutagenesis of approximately 18%. This value might be an underestimate since mutations that did not alter the *Bsr*GI site or did not result in detectable size alterations would have been missed by our analysis.

Discussion

We have shown here that repair of psoralen ICLs occurs in *Xenopus* egg extracts and that the pathway of repair results in a high rate of mutagenesis. To our knowledge this is the first demonstration of complete repair of ICLs in vitro. The most likely scenario for this process is that the ICL is first uncoupled by a mechanism involving Ercc1-Xpf, MutSβ, and RPA, as the initial products of the reaction are identical to the products that we have observed in mammalian cell extracts [5]. Production of these products is dependent upon these three factors in mammalian cell extracts. In contrast to the mammalian system, however, bypass of the remaining monoadduct occurs in the *Xenopus* extract. This conclusion is based on the occurrence of the 195 nt band which is the product of digestion of the plasmid with *Bss*HII. We have not observed this band after incubation in mammalian cell extracts, rather, only the 86 and 113 nt bands resulting from blockage of the polymerase at the remaining monoadduct are observed. In the *Xenopus* extracts, these bands appear to be intermediates in the repair reaction indicating a pause at the monoadduct and possibly a switch from the replicative polymerase to a translesion bypass polymerase. Homologous recombination is unlikely to play a role in this repair since there is only a small amount of plasmid that would be able to act as a homologous donor (the 3.6% of uncross-linked plasmid). Once translesion bypass and gap-filling have occurred, the remaining monoadduct would likely be repaired by the potent NER activity contained in the *Xenopus* extracts [15]. The high frequency of mutagenesis found in the recovered plasmids is consistent with a translesion bypass mechanism. These results thus confirm our earlier conclusion that ICLs are uncoupled in mammalian cell extracts since the intermediate products are the same in both systems and can be clearly extended in the *Xenopus* extracts. The ability of *Xenopus* egg extracts to carry out bypass synthesis of the monoadduct may be due to the fact that replication of the plasmids occurs in this system and/or due to the much higher concentration of replicative and bypass polymerases that exist in these extracts [11,12]. The demonstration of repair of ICLs in vitro indicates that the *Xenopus* egg extract system has great potential for further studies of the repair processing of this highly deleterious lesion.

Acknowledgments

This work was supported by NCI Grants CA075160 and CA097175. DNA sequencing resources were supported by the Cancer Center Support (Core) Grant CA16672.

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