RecA-dependent incision of psoralen-crosslinked DNA by (A)BC excinuclease

Suzanne Cheng⁺, Aziz Sancar¹ and John E. Hearst*

Department of Chemistry, University of California and Laboratory of Chemical Biodynamics, Lawrence Berkeley Laboratory, Berkeley, CA 94720 and ¹Department of Biochemistry, University of North Carolina, Chapel Hill, NC 27599–7260, USA

Received August 9, 1990; Revised and Accepted December 17, 1990

ABSTRACT

Previous work to elucidate the mechanism of crosslink repair by (A)BC excinuclease has shown that a psoralen-crosslinked duplex is selectively incised in the furan-side strand, while a three-stranded structure is incised in the pyrone-side strand of the crosslink. These observations support a sequential incision and recombination model for the complete error-free repair of a psoralen crosslink. The work presented here extends these findings by demonstrating that in the presence of RecA protein and a homologous DNA oligonucleotide, (A)BC excinuclease is induced to incise the pyrone-side strand of a crosslinked double-stranded plasmid molecule. This finding adds further support to the current model for error-free crosslink repair.

INTRODUCTION

Interstrand crosslink damage obstructs the critical processes of replication and transcription. In *Escherichia coli*, (A)BC excinuclease is responsible for repair through the nucleotide excision repair pathway (reviewed in 1, 2). The enzyme makes a specific incision on each side of a lesion, allowing the damaged nucleotide(s) to be removed. For single-stranded damage, the intact complementary strand enables DNA polymerase I to replace the damaged region. Crosslink damage, however, affects both strands and can thus result in mutations. The current model for error-free excision repair of crosslinks was developed from *in vivo* work of Cole and coworkers (3, 4) and *in vitro* work of Van Houten and coworkers (5, 6).

Cole and colleagues noted that survival of psoralen crosslink damage by *E. coli* cells depends upon intact *uvrA*, *uvrB*, *uvrC*, *recA*, and *polA* genes, and they found no evidence for a double-strand break mechanism (3, 4). A sequential incision and recombination mechanism was therefore proposed for error-free crosslink repair. Initially, only one strand of the crosslinked duplex is incised, and the resulting gap is filled through a *recA*-mediated homologous pairing event. The post-recombination intermediate, a three-stranded structure at the crosslink site, is

then incised a second time to fully remove the crosslink. Finally, DNA polymerase and ligase complete the repair process.

More recently, Van Houten and coworkers carried out detailed *in vitro* studies using uniquely psoralen-modified duplex substrates. Although both furan- and pyrone-side monoadducted substrates are incised along the damaged strand, a crosslinked duplex is selectively incised in the furan-side strand (5, 6). Three-stranded substrates in which the third strand is homologous to the furan-side strand are selectively incised on the pyrone side (7). These findings are all consistent with the sequential incision and recombination mechanism.

These *in vitro* studies examined the individual steps of the repair pathway. Reconstitution of the complete crosslink repair process *in vitro* by linking these steps is the next goal. Key intermediates as yet uncharacterized concern the interaction of recombination proteins such as RecA with (A)BC excinuclease and the damaged DNA. Sladek and associates (8) recently reported important findings regarding the requirements for efficient RecA-mediated strand exchange involving an incised crosslinked duplex. They observed that the presence of a psoralen-crosslinked 19-base oligonucleotide does not block RecA-mediated strand exchange between a single-stranded (ss) circle and a linear duplex. Secondly, they designed a crosslinked circular duplex containing one nick on each side of the adduct, to model the product of (A)BC excinuclease's first pair of incisions. This doubly-nicked substrate is inactive in strand exchange; initiation of RecA-mediated recombination requires extension of the nicked region to form a ssDNA gap. The 5'→3' exonuclease activity of polymerase I (pol I) is capable of generating the necessary gap, and since pol I would be present *in vivo*, this may be one of its roles. The proximity of the furan-side end of the psoralen (4-hydroxymethyl-4,5',8-trimethylpsoralen = HMT) to the nick on the 3' side apparently precludes synthesis off the 3'-OH terminus of the crosslinked oligomer. The precise nature of the post-incision and post-recombination nucleoprotein complexes is not known.

The work described here represents yet another step toward the goal of reconstituting crosslink repair *in vitro*. The RecA-mediated recombination step to enable complete repair of the

* To whom correspondence should be addressed

⁺ Present address: Eleanor Roosevelt Institute, Denver, CO 80206, USA

crosslinked site was studied using site-specifically HMT-adducted circular M13mp19 duplexes. In particular, a 'transiently' positioned third strand at the crosslinked site may be sufficient to induce (A)BC excinuclease to make its second pair of incisions. Sladek and coworkers observed a significant population of intermediates resulting from strand exchange reactions with gapped crosslinked duplexes and suggested that these intermediates might have a mechanistic role in the repair process (8). To investigate this possibility, a homologous oligonucleotide was tested as a 'transient' third strand to induce pyrone-side strand incisions of the crosslinked site.

MATERIALS AND METHODS

Proteins and DNA

Bacterial alkaline phosphatase (BAP), bovine serum albumin (BSA), and all restriction enzymes except KpnI were purchased from Bethesda Research Laboratories (BRL). KpnI and T4 polynucleotide kinase were from New England Biolabs. RecA protein was obtained from Pharmacia, and DNA ligases (T4 and *E. coli*) and *E. coli* DNA polymerase I were from Boehringer Mannheim Biochemicals (BMB). T4 gp43 was a gift of B. Alberts, University of California at San Francisco. *E. coli* DNA photolyase, UvrA, UvrB, and UvrC were purified as described elsewhere (9, 10), and helicase II (UvrD) was a gift of S. Matson, University of North Carolina at Chapel Hill. Rabbit muscle creatine phosphokinase (CPK) was purchased from Sigma.

DNA oligonucleotides (Figure 1) homologous to the minus strand of M13mp19 were prepared as described previously (7). Site-specifically HMT-monoadducted M13mp19, prepared using the primer extension method of Kodadek and Gamper (11), was a gift of I. Husain, Sancar lab. RF M13mp19 plasmid was purchased from BMB, and single-stranded (+) circular M13mp19 from BRL. Plasmids pUC19 and pBR322 were obtained from Pharmacia. [α - ^{32} P]dCTP (3000 or 7000 Ci/mmol) was from Amersham.

Preparation of crosslinked covalently closed M13mp19

Aliquots of site-specifically HMT-monoadducted M13mp19 (1 μ g in 40 μ L) were converted to crosslinked M13mp19 by a 3-minute irradiation at room temperature (23–25°C) with 320–380 nm light at approximately 125–150 mW/cm². Neither the monoadducted nor the crosslinked molecules were supercoiled.

Bacterial Strains

E. coli K-12 derivatives CSR603 (*uvrA*[–]*recA*[–]) and HB101 (*uvrA*⁺*recA*[–]) were used to test the transformation efficiency of psoralen-adducted DNA.

(A)BC excinuclease incisions and subsequent repair synthesis

(A)BC excinuclease reactions were carried out in 50 mM Tris·Cl (pH 7.4 at 25°C), 50 mM KCl, 10 mM MgCl₂, 2 mM ATP, 180 μ g/mL BSA, and 5 mM DTT. Each 40- μ L reaction contained approximately 200–400 ng of M13mp19, 0.6 μ g of UvrA, 1.4 μ g of UvrB, and 1.5 μ g of UvrC protein. DNA pol I (1 unit) and dNTP's were then added to the post-incision complex. Repair patches could be labeled internally with [α - ^{32} P]dCTP (typically 30 μ Ci, ~0.1–0.2 μ M). DNA ligase (either 0.5 unit *E. coli* enzyme with 1.5 mM NAD (from Sigma) or 1 unit T4 ligase) and helicase II (0.5 pmol) were also included in certain samples. Reaction mixtures were then deproteinized, ethanol-precipitated twice, and resuspended in REact4 buffer (BRL) and 100 μ g/mL

Figure 1. Oligonucleotide substrates, all homologous to the polylinker region of the minus strand of the M13mp19 plasmid. The arrows above the sequences of the 40- (a) and 50-mer (b) indicate the position of the HMT-adducted thymidylate within the unique KpnI recognition site. The sequence of the 107-mer overlaps that of the 40- and 50-mers as depicted in (c). The small vertical bars in (c) represent the HMT monoadduct.

BSA for digestion by HindIII and PvuII. To each completed digest, SDS to 0.4%, EDTA to 4 mM, and 5 $\times$ dyes in glycerol were added. The digests were loaded directly onto a 1.2-mm thick native 8% polyacrylamide gel, and the 143/139-mer HindIII/PvuII restriction fragment which contained the polylinker region from each sample was recovered. This fragment was then further analyzed on denaturing 8% polyacrylamide gels. The 139-mer could be separated from the 143-mer using 8 or 10% gels.

To test for complete repair, sets of samples were also incubated with KpnI at the time of the HindIII and PvuII digestions. Complete repair leading to restoration of the KpnI site was then detected by the appearance of 43/35-mer and 100/104-mer bands (Figure 2).

Initial isolation of the 139/143-mer restriction fragments for sequencing revealed band patterns that corresponded to the pattern of C's within each strand. This unexpected pausing by polymerase appeared to eliminate the need for that sequencing reaction. Abbotts and coworkers (12) reported that with 0.25 nM Klenow fragment and 10 nM template, a much higher frequency of termination occurred with 0.1 μ M levels of each dNTP than with 1.0 μ M. To raise the total concentration of dCTP during the post-incision repair synthesis here, two aliquots of 0.5 μ M unlabeled triphosphate each were added; the remaining dNTP's in each sample were at 25 μ M. These higher dCTP levels did enhance polymerization, but also reduced the total signal by diluting the labeled triphosphate. Total reaction times of 35–40 minutes were also used to maximize patch synthesis. This approach proved inadequate for detailed analyses, however, due to random termination events that interfered with data interpretation.

Preparation of gapped M13mp19

Uniquely gapped M13mp19 was prepared by previously published procedures (8, 13), with slight modifications. RF M13mp19 was first linearized using KpnI and HindIII, and the longer restriction fragment was isolated on a nondenaturing 1% agarose gel. KpnI/HindIII-linearized M13mp19 (5 μ g) and single-stranded (+) circular M13mp19 (10 μ g) were suspended in 115 μ L of 50% formamide and 10 mM EDTA. This mixture was divided across three wells of a Health Products Microdialyzer assembled with a membrane having a 12–14,000 dalton cutoff. The sample was first dialyzed against 98% formamide, 10 mM EDTA for 2.5 hours at room temperature (RT). After 10 minutes at 65–70°C, the sample was dialyzed against 50% formamide,

Figure 2. Schematic of the portion of the HindIII/PvuII restriction fragment that was used to analyze repair of the HMT crosslink site, before and after KpnI digestion. The 143/139-mer restriction fragment is shown with the (+) strand on top. Bases are numbered relative to the 5' end of each strand. Possible (A)BC excinuclease incision sites are marked on both the pyrone- (P) and furan-side (F) strands; the terms 'monoadduct incisions' and 'crosslink incisions' refer to the positions of the incisions relative to the adducted thymidine on that strand. The location of incorporated [α - ^{32} P]dCTP would indicate which strand was incised and where repair patch synthesis began. In practice, the 143/139-mer was not isolated until after repair patch synthesis. Samples were simultaneously digested with HindIII, PvuII, and KpnI. The susceptibility of the repaired DNA to KpnI digestion reflects the completion of repair and restoration of the KpnI recognition site.

10 mM EDTA and 0.2 M Tris·Cl (pH 8 at 25°C) for another 2.5 hours at RT. Next, the sample was left overnight at 4°C to equilibrate with 0.1 M Tris·Cl (pH 8 at 4°C), 10 mM EDTA and 0.1 M NaCl. Finally, the sample was dialyzed for 3 hours at RT against 10 mM Tris·Cl (pH 8 at 25°C), 1 mM EDTA. Following gel-purification, this procedure typically gave a 40–50% yield of the gapped (35 bases) M13mp19.

Preparation of crosslinked oligomer-M13 complexes

The KpnI/HindIII-gapped M13mp19 was crosslinked to HMT-monoadducted 40- or 50-mer (Figure 1) essentially as described previously for pUC19 (7). Although the 35-base gap permitted hybridization of the oligonucleotide at the crosslink site (KpnI recognition sequence) in the absence of RecA, the reaction was generally more efficient if RecA was present (data not shown). Each 20- μ L reaction was 5% glycerol, 25 mM Tris·acetate (pH 7.5 at 37°C), 50 mM Na·acetate, either 2.5 mM ATP γ S or ATP (with 5mM phosphocreatine and 0.75 units of CPK), 2 mM DTT, and initially 1 mM Mg·acetate for the presynaptic association of RecA with the oligonucleotide. Each oligonucleotide substrate was freshly 5'-end-labeled with kinase and gel-purified prior to use. RecA was at 2.5 μ M, and each oligonucleotide was at 6.25 nM (concentration in molecules). After a 10-minute presynaptic incubation at 37°C, 1.25 nM gapped M13 substrate was added and the Mg $^{2+}$ concentration was raised to 12.5 mM for synapsis. After 13–15 minutes, each sample was irradiated for 2 minutes with approximately 100 mW/cm ^{2} of 320–380 nm light. Plasmid and unreacted oligomer were separated on a

Figure 3. Complexes between KpnI/HindIII-gapped M13mp19 and furan-side HMT-monoadducted oligonucleotides. The 40- and 50-mer (Figure 1) are both complementary to the (+) strand of M13mp19. Each oligonucleotide was paired with the M13mp19 substrate through a RecA-mediated reaction. Each sample was then irradiated to convert these inserted monoadducts to interstrand crosslinks. The structures of the final M13/40 and M13/50 products have not been precisely characterized.

nondenaturing 1% agarose gel. As control reactions, three-stranded complexes between each oligonucleotide and FI pUC19 (ss:ds at 2:1) were also formed and crosslinked. The final products are referred to as M13/40 and M13/50 (Figure 3) or pUC/40 and pUC/50 for simplicity.

RecA-dependent (A)BC excinuclease incisions

RecA-mediated presynapsis with unmodified 107-mer (Figure 1c) and subsequent synapsis reactions with M13/40 or M13/50 were carried out under the ATP conditions described above for the preparation of crosslinked oligomer-M13 complexes. The 107-mer (12.4 ng), CPK (0.75 units) and RecA (5.2 μ g) were incubated in 20 μ L for 10 minutes. To initiate synapsis, 5- μ L aliquots were removed and added to prewarmed tubes containing 5 μ L (5–7000 Cerenkov cpm) of M13 substrate and 1 μ L of 0.115 M Mg $\cdot$ acetate. UvrA and UvrB were diluted in (A)BC excinuclease buffer, then mixed and prewarmed in (A)BC reaction tubes during the final minutes of the 15-minute synapsis period. The buffer in these (A)BC reaction tubes was prepared such that final concentrations of 50 mM Tris $\cdot$ Cl (pH 7.4 at 25°C), 50 mM KCl, 10 mM MgCl₂, 2 mM ATP, 150 μ g/mL BSA, and 5 mM DTT (excluding the contributions from the RecA reaction buffer) were achieved in the final 40 μ L. The 11- μ L synapsis reactions were then added to the UvrA (0.07 μ g) and UvrB (0.32 μ g) mixtures for UvrA-mediated formation of the UvrB $\cdot$ DNA complex (14). After 5 minutes, 0.4 μ g of freshly diluted UvrC was added. Incision reactions were incubated for 15 minutes at 37°C. Samples were deproteinized, ethanol-precipitated and analyzed on denaturing 10% polyacrylamide gels. In control reactions with the three-stranded pUC19 complexes, the RecA steps were omitted.

RESULTS

The first experimental goal was to characterize the pattern of incisions made by (A)BC excinuclease in the crosslinked circular M13mp19 duplex. Through characterization of the repair patches, the incised strand(s) could be identified, either furan- or pyrone-side, or both.

Properties of crosslinked M13mp19

The crosslinked substrate was prepared by irradiating site-specifically HMT-monoadducted M13mp19 (see 'Methods'). Transformation experiments were used to estimate the extent of photodamage introduced during the crosslinking process and the amount of monoadducted starting material remaining: (i) Unmodified pBR322 plasmid was irradiated and then transformed into HB101 (*recA*[−]) *E. coli* cells; damaged pBR322 would show a reduced frequency of transformation. Irradiated plasmid pBR322 showed no reduction in transformation efficiency, within experimental error, indicating that negligible photodamage was introduced in the crosslinking irradiations (data not shown). (ii) Transformation of CSR603 cells with crosslinked M13mp19 yielded 22 colonies on a 10^{−3} dilution plate while the unmodified control yielded 2050 colonies on a 10^{−4} dilution plate. Since CSR603 cells (*uvrA*[−]*recA*[−]) are deficient in nucleotide excision repair and recombination, the approximately 0.1% survival observed is attributable either to contaminating uncrosslinked substrate or to replicative bypass of crosslinks. Monoadduct lesions would not have been lethal because genetic information could be recovered through use of the undamaged strand as a template for replication (15). (iii) In HB101 cells (*uvrA*⁺*recA*[−]), a later preparation of the crosslinked M13mp19 yielded 18 colonies per ng M13 as compared to 700 per ng unmodified M13, equivalent to a 2.6% survival. This value is comparable to the 1.7–1.8% survival reported by Piette and coworkers (16), estimated to represent a maximum of 0.5% monoadduct contamination. Their crosslinked sample was

prepared at the same time as our own; therefore, this result suggests that our crosslinked M13mp19 was also approximately 0.5% monoadduct. Piette and colleagues noted that the location of the crosslinked site within the *lacZ* gene meant that only frameshift mutations or large deletions would result in inactive β -galactosidase (16). We did not observe any white or pale colonies indicative of impaired β -galactosidase. These results imply that in *recA*[−] cells, crosslinks are repaired by (A)BC excinuclease in an unknown manner with a low, but measurable, efficiency.

Incision of crosslinked M13mp19

Against this background, repair patches could be analyzed to determine which strand had been incised and to estimate the location of the 5' incision. Following incubation with (A)BC excinuclease and pol I under conditions for repair synthesis, the crosslinked region was isolated from the rest of the M13mp19 plasmid as a HindIII/PvuII fragment and analyzed by denaturing polyacrylamide gel electrophoresis. As Figure 4 indicates, post-incision repair synthesis by pol I yielded a family of [α -³²P]dCTP-labeled products. Closer examination of this pattern of pauses and terminations revealed that the bands

Figure 4. Example of data showing pattern of bands obtained following (A)BC excinuclease incision of HMT-adducted M13mp19 substrates and post-incision repair synthesis by pol I in the presence of [α -³²P]dCTP. A HindIII/PvuII restriction digest was used to focus on the region of interest; shown here is the denaturing polyacrylamide gel analysis of the isolated HindIII/PvuII fragment (Figure 2). The patterns of bands from the monoadducted and crosslinked M13mp19 were compared to identify the strand preference for incision of the crosslinked substrate. To seal any nicks or gaps, the sample in lane 2 was treated with pol I, dNTP's and ligase prior to the incision reaction. The full-length band is a 139-mer; termination after the last C yields a band at $n = 138$ bases. The band assignments were based upon the known sequence of the 139-mer (Figure 2) and adjacent sequencing lanes (not shown) of a 107-mer standard.

correspond to the pattern of C's in the sequence of the minus strand of the HindIII/PvuII fragment. The fact that the pattern of bands in lanes 2–6 (crosslink) is the same as in lane 8 (furan-side monoadduct) indicates that the furan-side strand of the crosslinked M13mp19 had been incised by (A)BC excinuclease. As is shown in Figure 2, the expected 5' incision site follows C₉₃. This deoxycytidylate marks the 3' end of the lowest molecular weight band in the crosslink lanes (2–6) in Figure 4. The presence of this 93-base product suggests that in a fraction of the sample population, pol I removed at least one additional base at the 5' incision site before initiating strand synthesis. In the monoadducted sample (lane 8), incorporation of [α -³²P]dCTP began at base 97 or 99, suggesting that these repair patches were initiated from the 5' (A)BC excinuclease incision site (between the 94th and 95th bases of the restriction fragment).

The disappearance of the 93-mer band (compare lanes 3 and 4) in the presence of UvrD (helicase II) suggests that this protein interfered with the 3'→5' exonuclease activity of pol I at the 5' incision site. RecA did not have the same effect (lane 5). Although helicase II has only a minor effect on the footprints of the UvrA and UvrB proteins and on polymerase activity, the protein appears to influence polymerase processivity and may interact with the DNA substrate at the 5' incision site (17).

The strong band at approximately $n = 126$ bases in lanes 1–6 of Figure 4 was later identified as undenatured crosslinked duplex (data not shown). Pretreatment of the crosslinked substrate with pol I and ligase to seal random nicks and gaps (lane 2) reduced the intensity of this band somewhat. Both lane 1, a sample that had been incubated with polymerase without prior digestion by (A)BC excinuclease, and the gel (not shown) used to initially separate the HindIII/PvuII restriction fragments did indicate that there was strand synthesis initiated at random nicks and leading to [α -³²P]dCTP-incorporation outside the crosslinked region. The approximately 104-mer band in lane 1 may reflect synthesis terminated because of the crosslinked template ahead. The absence of such a band in (A)BC excinuclease-treated samples (lanes 2–6) may reflect the behavior of polymerase that is initiating synthesis at the 5'-incision site rather than at a nick further away from the psoralen.

Another result seen in Figure 4 was that labeled 139-mer was obtained from crosslinked M13mp19. Radiolabeled full-length strands of the HindIII/PvuII restriction fragment (139 and 143 bases) were produced at more than either the 0.5% level of residual monoadducted M13mp19 estimated from the transformation data and could reflect a low level of bypass synthesis. Bypass of psoralen monoadducts has been previously reported (reviewed in 18). Although a <1% *in vivo* bypass of an HMT crosslink may have been observed (16), no other data for crosslink bypass has been reported.

Interaction between RecA protein and (A)BC excinuclease

Data such as that in Figure 4 demonstrated that the crosslinked M13mp19 duplex is incised on the furan-side strand. The absence of evidence of pyrone-side incision is consistent with the sequential incision and recombination model (6). Previous *in vitro* studies have demonstrated that (A)BC excinuclease requires a three-stranded intermediate before it will incise the pyrone-side strand of a crosslinked substrate (6, 7). The next goal was to duplicate conditions for RecA-mediated strand exchange with the incised crosslinked duplex.

Sladek and coworkers had demonstrated that RecA requires extension of the 11–12-base gap between (A)BC excinuclease's

incision sites. The necessary gap can be generated from the 3' incision site by the 5'→3' exonuclease activity of pol I (8). In addition to using pol I to create a gap following an (A)BC excinuclease reaction, we designed a uniquely gapped substrate to model the product. Strand exchange reactions using these substrates and M13mp19 FIII (linearized by AvaII, EcoRI, HindIII, Kpn I, PvuI, or XbaI) were not successful (data not shown).

The simpler system of an oligonucleotide and a circular duplex plasmid, however, did yield promising results. In this system, the crosslinked oligonucleotide mimics the fragment excised from the furan-side strand during the first incision step in (A)BC excinuclease-mediated repair of a crosslinked duplex, as in earlier studies (7). The KpnI/HindIII-gapped circular M13mp19 substrate was chosen as the duplex component because the KpnI restriction site corresponds to the 3' incision that (A)BC excinuclease would make on the furan-side strand, at the third phosphodiester bond 3' to the crosslink. The 35-base distance between the KpnI and HindIII restriction sites on the gapped strand was expected to be large enough to activate the substrate for RecA-mediated homologous pairing. For the oligomeric component, two furan-side monoadducted substrates were chosen from among those prepared for earlier studies (7). The 5' end of the 40-mer (Figure 1a) lies very near the KpnI recognition site and resembles the end of the fragment created by (A)BC excinuclease's furan-side strand incisions. The 50-mer (Figure 1b) was selected as a more efficient substrate for RecA than the 40-mer (7). The length of the oligomer between 30 and 107 nucleotides has little or no effect on the ability of (A)BC excinuclease to recognize and incise the pyrone-side strand of the crosslink (7). The resulting crosslinked M13/40 and M13/50 complexes are pictured in Figure 3. The three-stranded regions have *not* been precisely characterized.

The experiment was designed to test the ability of a three-stranded intermediate at the crosslink site to induce (A)BC excinuclease to make pyrone-side incisions. RecA and a 107-mer (Figure 1c) homologous to the furan-side strand (40- or 50-mer) were used to form three-stranded intermediates with the crosslinked M13mp19 complexes. As controls, analogously crosslinked three-stranded pUC19 complexes were also digested with (A)BC excinuclease. These pUC19 complexes were identical to those used in earlier work (7). The reaction products were analyzed on denaturing polyacrylamide gels. Only the 5' ends of the 40- and 50-mers were ³²P-labeled and therefore detectable. Furan-side incisions would be indicated by the appearance of shortened oligomers while pyrone-side (plasmid strand) incisions would leave the labeled oligomer intact and crosslinked to the small fragment excised from the plasmid.

As Figure 5 illustrates, 'transient' formation of a complex between the homologous 107-mer and the crosslinked M13/40 and M13/50 substrates did result in preferential incision of the pyrone-side strand by (A)BC excinuclease. Under these conditions, digestion by (A)BC excinuclease released fragments which migrated more slowly than full-length 40- and 50-mer, respectively. More specifically, these excision products comigrated with those from the three-stranded pUC19 complexes (pUC/40 and pUC/50), products previously shown to consist of an 11–12-base fragment of plasmid crosslinked to the ³²P-labeled oligonucleotide (7). Lanes 3 and 10 of Figure 5 indicate that the 107-mer alone was able to 'displace' the 50-mer, and to a lesser extent the 40-mer (apparent in a darker film exposure), to form the three-stranded intermediate which induced pyrone-side incision. However, the presence of RecA enhanced this interaction (compare lanes 3 with 5 and 10 with 12). In addition

Figure 5. Autoradiogram showing third strand-induced pyrone-side incisions. Gapped M13/40 and M13/50 complexes (Figure 3) were digested with (A)BC excinuclease in the presence of 107-mer complementary to the gap (lanes 3 and 10), RecA (lanes 4 and 11), or both (lanes 5 and 12). As positive controls for pyrone-side incision, the corresponding three-stranded pUC19 complexes were also digested with (A)BC excinuclease (lanes 7 and 14). In each complex, only the 40- or 50-mer was ^{32}P -labeled. Samples were analyzed on a denaturing 10% polyacrylamide gel. The crosslinked (XL) pyrone-side excision product is marked with an arrow. Uncrosslinked (either UM = unmodified or MA = monoadducted) oligonucleotide that had been retained by either the M13mp19 or pUC19 plasmid was released during the heat-denaturation step prior to loading of the gel.

Figure 6. Schematic illustrating the (A)BC excinuclease incisions induced by a three-stranded structure at the crosslink site in M13/40 and pUC/40. The pUC/40 complex which is comprised of a supercoiled duplex circle and an oligonucleotide insert yields only pyrone-side (plasmid) strand incision products. For the M13/40 complex, the single-stranded region activates the substrate for RecA-mediated homologous pairing. (A)BC excinuclease incision of the three-stranded intermediate that results from synapsis between the M13/40 and a RecA-coated homologous 107-mer releases the 40-mer crosslinked to an excision fragment (Figure 5). This product co-migrates with that from the three-stranded pUC/40, indicating that the pyrone-side (plasmid) strand was incised. Similar sketches can be drawn for the M13/50 and pUC/50 complexes. Note that the actual structures of these complexes have not been determined. (F = furan side, P = pyrone side)

to the crosslinked excision fragment, unmodified and monoadducted oligonucleotide that had been retained by the plasmid was released during the heat-denaturation prior to gel electrophoresis. The lower molecular weight bands in lanes 12 and 14 are suggestive of furan-side incisions: monoadducted oligomer (5' incision occurring at the eighth phosphodiester bond to the adduct) would yield a labeled 36-mer, while the crosslinked duplex substrate would yield a 35-mer (5' incision at the ninth bond). Lanes 9–11 contain a 37-mer that may be due to variation in the incision pattern such that the 5' incision occurred at the seventh phosphodiester bond from the psoralen; such variation has been noted by others (reviewed in 1). Other minor bands are attributable to random exonuclease activity. The steps leading to the results shown in Figure 5 are diagrammed in Figure 6. The absence of pyrone-side incision of the M13/40 and M13/50 complexes in the absence of the 107-mer indicated that the overlap of the M13mp19 strand and the homologous 40- or 50-mer was insufficient to produce the necessary three-stranded structure. A nonhomologous 107-mer was not tested for its ability to induce pyrone-side incision through formation of a three-stranded structure at the crosslink.

DISCUSSION

Previous work with linear HMT-crosslinked duplexes indicated that (A)BC excinuclease preferentially incises the furan-side strand (6). All of our results indicated that this was also true of the circular HMT-crosslinked M13mp19 substrate; the repair patch patterns were identical to those generated by the furan-side monoadducted substrate.

Jones and Yeung (19) recently postulated that the GC content of sequences flanking a psoralen crosslink determines the binding and subsequent strand specificity of incisions by (A)BC excinuclease. In their study of various trimethylpsoralen (TMP)-crosslinked duplexes, Jones and Yeung observed that a GC-rich sequence in the furan-side strand 6–12 bases to the 5' side of TMP-crosslinked thymines led to preferential incision of the

Figure 7. Model for the error-free repair of psoralen crosslinks mediated by (A)BC excinuclease (6). (A)BC excinuclease initially makes dual incisions adjacent to the crosslink on one strand of the helix. Displacement of the resulting fragment leaves a gap which is filled through a recombination event mediated by the RecA protein. (A)BC excinuclease then makes a second set of incisions, isolating the crosslink in the three-stranded post-recombination intermediate. Finally, the enzyme and the crosslinked fragment dissociate, and the gap is filled by the combined action of DNA polymerase I (pol I), ligase and helicase II, in the presence of all four deoxyribonucleoside triphosphates (dNTP's: dATP, dCTP, dGTP, and dTTP).

furan-side strand. If a GC-rich segment was on the 3' side or absent entirely, then incision of both strands was observed. Jones and Yeung proposed that the UvrA and UvrB subunits may exhibit sequence-dependent modes of binding.

In our own work, we have examined (A)BC excinuclease incision of HMT-crosslinked molecules. The furan-side sequences flanking the crosslink site (underlined) are given below for a 137-basepair duplex (i) and the M13mp19 substrate (ii).

5'-TA CGAATTC GAAGCTACGAGC TGAGGCC (i)

5'-GA ATTCGAG CTCGGTACCCGG GGATCCT (ii)

Sequence (ii) was also contained within the 40-mer oligonucleotide crosslinked into KpnI/HindIII-gapped M13mp19 to form M13/40; the 50-mer oligonucleotide in M13/50 began with the 3rd G in (ii). Both of the above sequences are more GC-rich 6–12 bases 3' to the crosslink than 5'; yet in both cases, only furan-side incisions were detected (data for sequence (i) not shown). With the M13/40 and M13/50 substrates, pyrone-side incisions were observed only in the presence of a third strand homologous to the furan-side strand.

We attribute the strand specificities noted by Jones and Yeung to their use of TMP. Unlike HMT, TMP lacks a 4' substituent which is known to contribute to the asymmetry between the furan and pyrone ends of the psoralen diadduct (20). Thus although sequence context may affect the incision of TMP crosslinks, our results with HMT-adducted substrates argue against this dependence for all psoralen crosslinks. As Jones and Yeung note, initial incision of a crosslinked duplex on the pyrone side could result in primarily error-prone repair through pol I-mediated strand synthesis. The 4' substituent in HMT may block furan-side strand synthesis (8) in favor of error-free recombinational repair.

The work described here represents our efforts to extend previous *in vitro* findings. We had observed that a crosslinked three-stranded complex in which the furan-side strand is an oligonucleotide will be preferentially incised by (A)BC excinuclease along the pyrone-side strand. Complete repair of the resulting excision gap could be carried out by DNA pol I and ligase (7). These observations were consistent with the sequential incision and recombination mechanism (Figure 7) proposed for crosslink repair (6). To reconstitute crosslink repair *in vitro*, we needed to further understand the interaction of the RecA protein with (A)BC excinuclease. Experiments were therefore designed to investigate (i) whether RecA would recognize an incised crosslinked duplex in the presence of (A)BC excinuclease and specifically catalyze homologous recombination at the incision gap, and (ii) whether (A)BC excinuclease would recognize the three-stranded post-recombination complex in the presence of RecA and specifically incise the remaining strand of the crosslink.

The most promising results came from study of a model system comprised of a uniquely gapped M13mp19 circular duplex with a complementary oligonucleotide crosslinked to the plasmid near the 5' end of the gap. The need for a gap to initiate RecA-mediated homologous pairing had been noted by Sladek and coworkers; the doubly-nicked post-incision duplex is not itself an active substrate for the pairing reaction (8). The data presented here demonstrate that in the presence of a presynaptic complex between RecA and an oligonucleotide homologous to the furan-side strand, (A)BC excinuclease can be induced to incise the pyrone-side strand of a gapped crosslinked duplex. The incoming oligonucleotide does not need to be covalently attached to the

plasmid, suggesting that a final recombination product may not be necessary for the second (A)BC excinuclease step in the crosslink repair process. The final product is, however, most likely necessary for completion of the repair process by polymerase and ligase. These observations add further support to the sequential incision and recombination mechanism for complete crosslink repair. Furthermore, these results are the first to demonstrate that the strand specificity of (A)BC excinuclease incisions may be affected by the presence of RecA protein.

Several possible lines of research are suggested by this work. The most immediate extension would require optimization of *in vitro* conditions for starting with a crosslinked duplex, creating a gapped post-furan-side-incision intermediate to serve as a substrate for RecA, and then forming a three-stranded intermediate which induces pyrone-side incisions. We have thus far been unable to detect the final excision fragment by beginning with the crosslinked M13mp19 and carrying out the steps of sequential incision and recombination. Incision of a psoralen-crosslinked plasmid may be significantly enhanced by supercoiling (cited in 21). A next step would be to determine whether addition of pol I, dNTP's and ligase can complete the repair process from that point or if a final recombination product is necessary. These would certainly be significant steps towards *in vitro* crosslink repair.

ACKNOWLEDGEMENTS

This work was supported by the Director, Office of Energy Research, Office of General Life Sciences, Molecular Biology Division, of the U.S. Department of Energy under Contract No. DE-AC03-76SF00098 and by NIH Grant GM32833.

REFERENCES

1. Sancar, A. and Sancar, G.B. (1988) *Ann. Rev. Biochem.*, **57**, 29–67.
2. Myles, G.M. and Sancar, A. (1989) *Chem. Res. Toxicol.*, **2**, 197–226.
3. Cole, R.S. (1973) *Proc. Natl. Acad. Sci. USA*, **70**, 1064–1068.
4. Cole, R.S., Levitan, D. and Sinden, R.R. (1976) *J. Mol. Biol.*, **103**, 39–59.
5. Van Houten, B., Gamper, H., Hearst, J.E., and Sancar, A. (1986) *J. Biol. Chem.*, **261**, 14135–14141.
6. Van Houten, B., Gamper, H., Holbrook, S.R., Hearst, J.E., and Sancar, A. (1986) *Proc. Natl. Acad. Sci. USA*, **83**, 8077–8081.
7. Cheng, S., Van Houten, B., Gamper, H.B., Sancar, A., and Hearst, J.E. (1988) *J. Biol. Chem.*, **263**, 15110–15117.
8. Sladek, F.M., Munn, M.M., Rupp, W.D., and Howard-Flanders, P. (1989) *J. Biol. Chem.*, **264**, 6755–6765.
9. Sancar, A., Smith, F.W. and Sancar, G.B. (1984) *J. Biol. Chem.*, **259**, 6028–6032.
10. Thomas, D.C., Levy, M. and Sancar, A. (1985) *J. Biol. Chem.*, **260**, 9875–9883.
11. Kodadek, T. and Gamper, H. (1988) *Biochemistry*, **27**, 3210–3215.
12. Abbotts, J., SenGupta, D.N., Zon, G., and Wilson, S.H. (1988) *J. Biol. Chem.*, **263**, 15094–15103.
13. West, S.C., Cassuto, E., and Howard-Flanders, P. (1982) *Mol. Gen. Genet.*, **187**, 209–217.
14. Orren, D.K. and Sancar, A. (1989) *Proc. Natl. Acad. Sci. USA*, **86**, 5237–5241.
15. Koffel-Schwartz, N., Maenhaut-Michel, G. and Fuchs, R.P.P. (1987) *J. Mol. Biol.*, **193**, 651–659.
16. Piette, J., Gamper, H.B., van de Vorst, A., and Hearst, J.E. (1988) *Nucl. Acids Res.*, **16**, 9961–9977.
17. Van Houten, B., Gamper, H., Hearst, J.E., and Sancar, A. (1988) *J. Biol. Chem.*, **263**, 16553–16560.
18. Moustacchi, E. (1988) *Arch. Toxicol. Suppl.*, **12**, 26–34.
19. Jones, B.K. and Yeung, A.T. (1990) *J. Biol. Chem.*, **265**, 3489–3496.
20. Tomic, M.T., Wemmer, D.E. and Kim, S.-H. (1987) *Science*, **238**, 1722–1725.
21. Pu, W.T., Kahn, R., Munn, M.M., and Rupp, W.D. (1989) *J. Biol. Chem.*, **264**, 20697–20704.