Psoralens produce DNA interstrand cross-links which are thought to be repaired via a sequential excision and recombination mechanism in *Escherichia coli*. The first round of incision by UvrABC has been characterized: it results in an 11-base oligonucleotide cross-linked to an intact DNA strand (Van Houten, B., Gamper, B., Holbrook, S. R., Hearst, J. E., and Sancar, A. (1986) Proc. Natl. Acad. Sci. U. S. A. 83, 8077–8081). In the present work, DNA substrates containing 4′-hydroxymethyl-4,5′,8-trimethylpsoralen (HMT) cross-links in defined positions are constructed and used to analyze the other steps in repair. It is shown that RecA protein mediates strand transfer past an oligonucleotide cross-linked to a single-stranded DNA circle and that the resulting heteroduplex is a substrate for the UvrABC complex: it excises a double-stranded oligonucleotide which contains the HMT cross-link. It is also found that the first round of UvrABC incision does not lead directly to strand exchange but that an intervening step is needed. That step is carried out in vitro by the 5′-exonuclease activity of DNA polymerase I (pol I) which creates a single-stranded DNA region (a gap) at an incised cross-link such that RecA can initiate strand exchange. *Studies using cross-linked oligonucleotides showed that the gap produced by pol I results from the inability of the polymerase to add nucleotides to a 3′-OH end two to three nucleotides away from the furan side of an HMT cross-link. Pol I can, however, extend a 3′-OH end next to the pyrone side of the cross-link. Since UvrABC incises predominantly the furan side of psoralen cross-links in duplex DNA, this discrepancy has important consequences for repair.*

Psoralens (furocoumarins) are one of many carcinogenic and chemotherapeutic agents that produce interstrand cross-links—covalent linkages between complementary strands of DNA which block replication and transcription. They are naturally occurring heterocyclic compounds which act by intercalating into DNA at alternating pyrimidine-purine sequences and forming cross-links (and monoadducts) with pyrimidines when irradiated with near-UV light (320–400 nm) (reviewed in Cimino et al., 1985). Cross-links can be toxic and mutagenic in a wide variety of organisms, but they can also be repaired (reviewed in Scott et al., 1976; Hanawalt et al., 1979; Ben-Hur and Song, 1984). The mechanism of repair of psoralen cross-links has been studied most in the bacterium *Escherichia coli*.

A model for the repair of cross-links in *E. coli* was proposed a number of years ago which consisted of the sequential action of excision (UvrABC) and recombination (RecA) enzymes (Cole, 1973). It was suggested that cross-linked DNA was incised by Uvr proteins and that this incision led to RecA-mediated strand exchange past the lesion. A second round of Uvr-mediated excision was then thought to remove the psoralen moiety from the DNA.

From *in vitro* studies (Van Houten et al., 1986a, 1986b) with purified proteins and defined DNA substrates, it is now known that the UvrABC enzyme complex makes two incisions in one DNA strand, one on each side of the cross-link but several bases away from it. The result is an 11-base oligonucleotide covalently attached via the psoralen molecule to an intact strand. From *in vivo* studies on λ-phage-prophage crosses (Lin et al., 1977), it is also known that under non-replicating conditions, UvrABC incision of psoralen cross-links is required for RecA-mediated recombination.

While the first round of Uvr-mediated incision of cross-linked DNA and RecA-mediated strand exchange of undamaged DNA has been well characterized (reviewed in Sancar and Sancar, 1988; Radding, 1982; Cox and Lehman, 1987), several critical steps in the repair of psoralen cross-links remain obscure. One question is whether RecA can mediate strand exchange past the cross-linked oligonucleotide left by UvrABC incision. Strand exchange reactions are thought to take place close to the axis of RecA spiral filaments where space must be limited (Stasiak et al., 1984). Therefore, it is possible that a cross-linked oligonucleotide could present an obstacle to strand exchange.

A second question is if strand exchange can proceed past the incised cross-link, can the UvrABC complex excise the other side of the cross-link so that the psoralen moiety is released. Psoralen is an asymmetric molecule containing a furan ring on one end and a pyrone ring on the other (see Fig. 1). In double-stranded DNA (dsDNA), the UvrABC complex incises predominantly the DNA strand covalently bound to the furan side of a cross-link (Van Houten et al., 1986b). In order to remove the psoralen molecule from the DNA after strand exchange, however, UvrABC would have to incise the DNA strand bound to the pyrone side of the cross-link. Can

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*Indicates references to specific research papers or studies related to the repair mechanism of psoralen cross-links in *E. coli*.

1. The abbreviations used are: dsDNA, double-stranded DNA; ssDNA, single-stranded DNA; pol I, *E. coli* DNA polymerase I; SSB, *E. coli* single-stranded DNA-binding protein; HMT, 4′-hydroxymethyl-4,5′,8-trimethylpsoralen; cccDNA, covalently closed circular DNA; dNTPs, deoxyribonucleotide triphosphates.
UvrABC carry out such an incision with a great enough efficiency to play a significant role in the repair of psoralen cross-links in vitro?

The third and final question addressed was that of the step between incision and recombination. In vitro and most likely in vivo, a region of single-stranded DNA (ssDNA) is needed to initiate RecA-mediated strand exchange (Radding, 1982; Cox and Lehman, 1987). UvrABC incision of a cross-link, however, does not produce ssDNA per se; it produces a double-stranded structure containing two nicks (incisions). How then do UvrABC-incised cross-links induce strand exchanges in vivo? One possibility is that the DNA at an incised cross-link is sufficiently distorted to allow RecA protein to bind and initiate strand exchange. Another possibility is that there is an exonuclease step in the repair pathway which creates a single-stranded region (a gap) in the DNA. Previous studies have shown that E. coli strains deficient in the 5'-exonuclease activity of DNA polymerase I exhibit decreased removal of psoralen cross-links from DNA (Cole et al., 1976; Yacoub and Cole, 1977) as well as decreased cross-link induced recombination (Howard-Flanders et al., 1975). These findings prompted us to ask if pol I played a role in the step between incision and recombination.

In the present paper these questions are addressed and answered by constructing DNA substrates with psoralen cross-links in defined positions and reacting them in vitro with purified proteins. The photochemical properties of psoralens made the construction of the substrates possible. Far-UV light (254 nm) preferentially photoreverts the pyrone side of the cross-link to produce a furan-side monoadduct (Cimino et al., 1986a; Shi and Hearst, 1987).

**EXPERIMENTAL PROCEDURES**

**Enzymes and Reagents**—The E. coli RecA and single-stranded DNA binding (SSB) proteins and wheat germ topoisomerase I were the generous gifts of S. West (Imperial Cancer Research Fund, Hammersmith, Great Britain). J. Chase (Case Western Reserve University, Cleveland, OH), and N. Cozzarelli (University of California, Berkeley), respectively. UvrA and UvrC proteins were prepared by the method of Yeung et al. (1986) and Sancar and Rupp (1983), respectively. UvrB protein was prepared as in Thomas et al. (1985) except that a single Affi-Gel Blue (Bio-Rad) column was substituted for the two Blue Sepharose (Sigma) columns and run before the DEAE-agarose column.

4′-Hydroxymethyl-4,5′,8-trimethylpsoralen (HMT) was obtained from HRi Associates, Emeryville CA. Oligonucleotides FS1 (5′-CTCGAGCTACA-3′) and FS2 (5′-GATCTGTAGCTGACTGC-3′) (referred to as the 11- and the 19-mer, respectively) were synthesized by the Department of Chemistry, Yale University. Labeling refers to 32P. TE was 10 mM Tris-HCl, pH 8.0, 1 mM EDTA.

**Preparation of M13mp19 and M13mp19FS1 DNA**—M13mp19FS1 DNA was constructed by ligating the annealed oligonucleotides FS1 + FS2 into M13mp19 replicative form DNA (prepared as in Godson and Boyer, 1974) cut at the PstI and BamHI sites of the polynucleotide. M13mp19 (Norrander et al., 1983) and M13mp19FS1 phage DNA (single-stranded circle) were prepared from E. coli K12 strain JM107 (Yanisch-Perron et al., 1985) as in Messing (1983). 32P-Labeled phage DNA was similarly prepared except that the host was grown in a low phosphate medium as in Cunningham et al. (1980), and the DNA was purified on an equilibrium cesium chloride gradient.

**Preparation of Cross-linked Oligonucleotides**—The cross-linked oligonucleotides (FS1 + 2, A and B) (see Fig. 1 for orientation) were prepared by labeling the 5′ end of the 19-mer with T4 polynucleotide kinase, annealing it to equimolar amounts of the 11-mer in TE, adding HMT in a 1:03 M ratio of HMT to DNA (all DNA concentrations are expressed in mol of nucleotides), and irradiating it with 50 kJ/m² of near-UV light (predominantly 360 nm) from a 15-watt black light. A plastic Petri dish lid was used to absorb fluences at 254 nm. The orientational isomers were separated by electrophoresis through a 20% polyacrylamide, 7 m urea, 40-cm gel. Bands were visualized by autoradiography, cut out and the DNA was eluted from them and precipitated with Nenzo columns (Du Pont). The isolated fragments were routinely 97% pure.

Irradiation of cross-linked DNA was by irradiation with a Sylvania model G15T8 germicidal UV lamp, henceforth referred to as far-UV light (254 nm).

**Preparation of DNA Substrates Containing Cross-linked Oligonucleotides in Defined Positions**—The construction of the cross-linked ssDNA and dsDNA substrates are outlined in Fig. 1, paths A and B, respectively. In path A, FS1 + 2 cross-link isomer A was dephosphorylated and irradiated with 1.2 kJ/m² of far-UV light (254 nm) so that about 50% of the HMT cross-links were photoreverted on the ssDNA only, leaving a furan-side monoadduct on the 19-mer. After labeling the 5′ end, the monoadducted 19-mer (0.6 μM) was annealed to viral strand M13mp19FS1 DNA (0.33 mM) in TE containing 0.1 M NaCl and irradiated with 50 kJ/m² of near-UV light. The phage DNA carrying the labeled cross-linked oligonucleotide was purified on an alkaline sucrose gradient or by passage through a Sephacrose CL-4B (Pharmacia LKB Biotechnology Inc.) column in 0.1 N NaOH, 0.5 mM EDTA. In path B, M13mp19 replicative form DNA was cut with BarnHI, dephosphorylated, and then cut with PstI. Isomer B (25–50 nM) was ligated into the double cut DNA (5 μM, 1:4 insert ratio), and the unlabeled circular dsDNA containing a cross-linked 11-mer was purified by agarose gel electrophoresis. The presence of the nicks and the cross-linked oligonucleotide was verified by restriction analysis and photoreversal (not shown).

**Preparation of Circular dsDNA Markers Containing or Lack the Cross-linked Oligonucleotide**—Aacl-cut M13mp19FS1 DNA (0.16 μM) was dissolved in 98% formamide plus 10 μM EDTA and incubated at 55 °C. 32P-Labeled M13mp19FS1 phage DNA or the cross-linked ssDNA substrate of Fig. 1, path A, was added to 0.18 mM and NaCl to 0.1 M. The final solution (63% formamide) was dialyzed against 1 liter of TE containing 0.1 M NaCl overnight such that the temperature were dropped from 4 °C and then against TE at 4 °C. Roughly 30–40% of the labeled circular ssDNA was converted to circular dsDNA by this procedure.

**RecA Reaction**—RecA reactions with the cross-linked ssDNA substrate were carried out in 40 μl and contained 4.0 μM circular ssDNA, 2 units of creatine phosphokinase (Sigma), 1 μg/ml phosphocreatine, 0.18 μM SSB, 0.1 μg/ml bovine serum albumin, and 2.5 μM RecA protein in 2 mM ATP, 10 mM MgCl₂, 25 mM Tris-HCl, pH 7.5, and 1 mM diethiothreitol. After 10 min at 37 °C, the reaction was supplemented with 8.0 μM homologous linear duplex DNA, and the incubation was continued for another 40 min. The control reaction was carried out using M13mp19 replicative form DNA (+ strand) in the absence of linear duplex DNA. Reactions with the cross-linked dsDNA substrates were as above except that the volume was 10 μl, and they contained 3.8–8.0 μM cross-linked dsDNA substrate, either unmodified or after pol I treatment, 2–8 μM labeled linear duplex, 2.6–5.5 μM RecA protein, and 0.0–0.18 μM SSB as indicated. (The linear duplexes were labeled either at the 5′ end with kinase in Fig. 6A, Control, and Fig. 7 or at the 3′ end with T4 DNA polymerase in Fig. 8.) The reaction was scaled up 20-fold, and the DNA, RecA, and SSB concentrations were doubled to prepare product for the UvrABC and XhoI reactions. Reactions were stopped by incubating at 37 °C (10–30 min) in the presence of EDTA (20–25 μM), sodium deoxycholate (0.2–1%), and protease K (0–400 μg/ml). The DNA was extracted with phenol/chloroform and precipitated prior to subsequent reactions.

Samples from the RecA and restriction enzyme reactions were brought to 1–2% sodium dodecyl sulfate and analyzed by agarose gel electrophoresis at 150 volts for 2–3 h using either 1% agarose in TAE (system I) or 0.8% agarose plus 0.5 μg/ml ethidium bromide in TBE (system II). (TAE and TBE were made as described in Maniatis et al., 1982.) After staining and photographing the gels, they were dried and visualized by autoradiography using Kodak XAR film.

**In vitro Topoisomerase Assays**—Topoisomerase I was assayed using recA reaction (96 μM total DNA) was incubated in 0.8 μl with 50 units of ligase at room temperature for 2–3 h. The product—covalently closed circular DNA (cccDNA)—was purified on a cesium chloride gradient. Supercircular were introduced into the cccDNA as judged by agarose gel electrophoresis at 100 volts for 4–5 h in 1× TBE. Supercircular DNA was defined as the 15–20 units of topoisomerase I in the presence of 0.3 μg/ml ethidium bromide in 10 μM Tris-HCl, pH 7.9, 50 mM NaCl, 10 mM MgCl₂ for 30 min at 37 °C.
*UvrABC Reaction*—The incision reaction was carried out in 25 μl and contained 50 mM Tris- HCl, pH 7.5, 150 mM NaCl, 10 mM MgCl₂, 5 mM dithiothreitol, 2 mM ATP, and 0.8 μM cccDNA. The reaction mix was incubated at 37 °C prior to the addition of the UvrA and UvrB proteins at 100 nM each. After 1 min at 37 °C, the UvrC protein was added at 50 nM, and the incubation was continued for 20 min. The reaction was stopped by adding EDTA to 15 mM, sodium dodecyl sulfate to 0.1%, proteinase K to 250 μg/ml, and glycogen to 20 μg/ml and incubating at 37 °C for 10 min. The DNA was extracted and precipitated.

*Pol I Reactions*—Pol I reactions were performed in 30 μl at 37 °C for 10 or 30 min and contained 21 μM of one of the cross-linked dDNA substrate and 4.5 units of *E. coli* pol I (New England BioLabs) in pol I reaction buffer: 25 mM Tris-HCl, pH 7.8, 10 mM MgCl₂, 30 mM NaCl, 4 mM dithiothreitol, 100 μg/ml bovine serum albumin, and 50 μM of each of the four dNTPs (dATP, dCTP, dGTP, and dTTP) were indicated. The reactions were stopped by adding EDTA to 20 mM, sodium dodecyl sulfate to 0.2%, and proteinase K to 180 μg/ml and incubating at 37 °C for 15–30 min. The DNA was extracted and passed through a 1-ml Sepharose CL-4B column in 50% formamide, 20 mM Tris-HCl, pH 7.8, 1 mM EDTA. Nicked circular dDNA was recovered by ethanol precipitation in the presence of 50 μg/ml glycogen.

Control reactions with ³H-labeled plasmid DNA containing approximately 1 nick per plasmid molecule (made by DNase I treatment and verified by sedimentation through an alkaline sucrose gradient) showed that the reactions described above were with saturating amounts of pol I (±2.5 units/21 μM DNA) and created gaps of 290 ± 30 or 1200 ± 200 bases after 10 or 30 min at 37 °C, respectively.

**RESULTS**

The Strand Exchange Step: RecA Promotes Strand Transfer Past a Cross-linked Oligonucleotide—The first step in psoralen cross-link repair is UvrABC-mediated incision of duplex DNA on the furan side of the cross-link. The result is an oligonucleotide 11 bases long covalently attached via psoralen to an oligonucleotide. As in the structure formed by UvrABC incision, the final product of each path. a control substrate which did not contain the cross-linked oligonucleotide (Fig. 3, lane 6). The labeled DNA species (Fig. 3, lane 9) which contained the labeled cross-linked oligonucleotide in a three-stranded region.

Since the exchange of base pairs in a RecA reaction begins at the 3' end complementary to a region of ssDNA (Radding, 1982; Cox and Lehman, 1987), the cross-linked ssDNA substrate was reacted with an unlabeled homologous linear duplex containing a 3' end 345 bases away from the cross-link (AvuII-generated) as shown in Fig. 2A. RecA-mediated strand transfer is expected to start at the AvuII site and proceed clockwise (Cox and Lehman, 1981; Kahn et al., 1981; West et al., 1981). The anticipated product is a nicked circular heteroduplex containing the labeled cross-linked oligonucleotide in a three-stranded region.

The results, shown in Fig. 3, indicate that RecA protein converted the cross-linked ssDNA substrate into a labeled DNA species (Fig. 3, lane 9) which migrated exactly as a nicked circular dDNA marker which contained the cross-linked oligonucleotide (Fig. 3, lane 6). The labeled DNA species also migrated as the product of a RecA reaction with a control substrate which did not contain the cross-linked oligonucleotide (Fig. 3, lane 4). A comparison of the time course of the reaction with the cross-linked ssDNA substrate to that with the control substrate revealed that the presence of the cross-linked oligonucleotide only slightly delayed the...
Fig. 2. Substrates and expected products of RecA reactions. A, the cross-linked ssDNA substrate from Fig. 1, path A, was reacted with AvcII-cut M13mp19FS1 DNA. Strand transfer starts at the vertical dotted line and proceeds clockwise. The products are linear ssDNA and nicked circular dsDNA containing the 5'-32P-labeled (*), cross-linked 19-mer. Two possible structures are shown for the latter: in one (on top), the 19-mer is base paired to its complement, and in the other the 19-mer is excluded from the double helix. A triple helical structure involving base pairing between all three strands is also possible. The presence of the cross-linked oligonucleotide was detected by release of the 32P-labeled 19-mer upon irradiation with far-UV light (254 nm) (Fig. 3). Strand transfer past the cross-linked oligonucleotide was verified by cutting with Bal I (Fig. 4). The nucleotide positions of restriction sites and the cross-link are given in parentheses. B, the cross-linked dsDNA substrate from Fig. 1, path B, was treated with pol I to create a gap with an undefined 5' end. It was reacted with a Pvu I-generated linear duplex (M13mp19FS1) containing a 32P-labeled (*) 3' end complementary to the region of the gap and 144 bases from the 3' end of the cross-linked oligonucleotide. Strand exchange starts at the vertical dotted line and proceeds counterclockwise. The products are labeled linear dsDNA and nicked circular dsDNA containing the cross-linked 11-mer (Fig. 8).

appearance of the final product (by 5 out of 60 min, gels not shown).

To demonstrate that the labeled oligonucleotide was attached to the circle via a psoralen cross-link and to show that it remained attached during the RecA reaction, the DNA substrates and the RecA reaction products were exposed to far-UV light (254 nm). Far-UV light photoreverts the cyclobutane addition between the psoralen molecule and the pyrimidine so that the covalent linkage between the two DNA strands is destroyed (Cimino et al., 1986). Therefore, with the cross-linked substrates used, far-UV irradiation should release a labeled 19-mer, as shown in Fig. 2A. In Fig. 3, it can be seen that far-UV irradiation reduced the amount of label at the ssDNA and nicked circle positions of the cross-linked substrate (Fig. 3, lanes 8 and 10, respectively), but as expected it did not affect the label in the control (Fig. 3, lane 5; the decreased amount of label in lane 3 was due to loading less DNA inadvertently; another gel, not shown, confirmed that the irradiation did not affect the control substrate). Ethidium bromide staining of the gel in Fig. 3 confirmed that the dsDNA was otherwise unaffected (not shown). The labeled species running at the solvent front in lane 8 of Fig. 3 was identified as the 19-base oligonucleotide by denaturing polyacrylamide gel electrophoresis (not shown). Less of this species was produced from the RecA reaction product (Fig. 3, lane 10), suggesting that a higher dose of 254-nm light is needed to photorevert the cross-link in that structure.

To verify that the RecA reaction product from the cross-linked ssDNA substrate was indeed a full length heteroduplex circle and not some intermediate of the reaction perhaps blocked at the cross-link, it was incubated with Bal I restriction enzyme. As depicted in Fig. 2A, there is a unique Bal I.

Fig. 3. RecA mediates strand transfer past a cross-linked oligonucleotide. Structures of the DNA substrates used in the strand transfer (RecA) and photoreversal (254 nm) reactions are given above the grid. Lanes 1-5 correspond to M13mp19FS1 phage DNA labeled with 32P in the backbone (asterisks and arrow) and lanes 6-10 to the cross-linked ssDNA substrate of Fig. 1, path A. The linear dsDNA in each case is M13mp19FS1 replicative form DNA cut with AvcII. The position of the AvcII site on the circle is indicated by the tick mark. Preparation of DNA markers from the DNA substrates (M, lanes 1 and 6) and the conditions of the RecA reactions (lanes 4, 5, 9, and 10) are described under "Experimental Procedures." The photoreversal reactions were carried out by irradiating an aliquot of the DNA (45 μM in TE) that went into lanes 2, 4, 7, and 9 with 5 kJ/m2 of far-UV light (254 nm) and adding formamide to 40% to produce the samples in lanes 3, 5, 8, and 10, respectively. The samples were resolved on an agarose gel, system I. Nicked circle refers to circular dsDNA.
site in M13mp19FS1 which is located on the other side of the cross-linked oligonucleotide from the AvaII site where strand transfer is initiated. The results of the BalI reaction are shown in Fig. 4. All of the RecA reaction product from the cross-linked ssDNA substrate was converted to linear dsDNA (Fig. 4, lane 6). This result as well as the fact that the RecA reaction product could be converted to covalently closed circles by T4 DNA ligase (see below) confirmed that RecA mediated strand transfer completely around the ssDNA circle containing the cross-linked oligonucleotide.

The Second Incision Step: UvrABC Excises a Cross-linked Oligonucleotide from the Strand Transfer Product—It has been proposed that recombination past psoralen cross-links is followed by a second round of UvrABC cutting which releases the psoralen from the DNA. To test whether UvrABC endonuclease is capable of such activity, we used the oligonucleotide-adducted heteroduplex produced by strand transfer in an excision reaction. This structure is similar to that which would be produced after RecA-mediated strand exchange of a UvrABC-incised cross-link, the main difference being the length of the cross-linked oligonucleotide—19 versus 11 nucleotides, respectively.

The heteroduplex containing the labeled cross-linked oligonucleotide was ligated to produce cccDNA. This cccDNA was purified, reacted with UvrABC proteins, and analyzed by gel electrophoresis. Approximately 90% of the label was released from this DNA by UvrABC and appeared as a series of bands on a denaturing polyacrylamide gel (Fig. 5, 3rd lane). (No label was released when the reaction was performed with UvrA, UvrB, or UvrC alone; gel not shown.) Irradiation with far-UV light (254 nm) showed that these bands contained the cross-linked 19-mer (Fig. 5, 4th lane). The most prominent bands (the upper two) in the 3rd lane of Fig. 5 ran close to the cross-linked oligonucleotide FS1 + 2 A (an 11-mer cross-linked to a 19-mer); this suggests that UvrABC made incisions approximately 11 bases apart on the side of the cross-link attached to the circle, i.e., the pyrene side. The appearance of multiple bands in the 3rd lane of Fig. 5 could be due to minor cutting by UvrABC on both the furan and the pyrone sides (others have reported a similar effect, i.e., Jones and Yeung, 1988), but they could also be accounted for by a trace exonuclease activity present in our UvrC protein preparation.

The cross-linked oligonucleotide from unligated and supercoiled products of the RecA reaction was also excised by UvrABC (gels not shown).

The UvrABC complex binds to adducted DNA presumably by recognizing distortions in the DNA helix introduced by the adducts (Sancar and Rupp, 1983; Sancar et al., 1985). Therefore, to investigate why the pyrene side of the cross-link is a substrate for UvrABC in the strand transfer product but not in cross-linked duplex DNA, we examined the susceptibility of the three-stranded region of the heteroduplex to cutting by a restriction enzyme. M13mp19FS1 contains a single XhoI site which lies within the 19-mer on the opposite side of the cross-link from the 5' label (see Fig. 1, lower portion, A). Susceptibilities to XhoI of the covalently closed and supercoiled forms of the oligonucleotide-adducted heteroduplex product (shown in Fig. 2A) were compared with each other and with controls lacking the cross-linked oligonucleotide. The results show that the cccDNA substrate carrying the cross-linked oligonucleotide was completely cleaved by XhoI but that approximately 5 times more enzyme was needed compared with the control substrate (Fig. 6A, top half).
percoiling increased the susceptibility of the site by approximately 5-fold, but the effect was the same with the control substrate (Fig. 6A, bottom half).

In the reactions in Fig. 6A, XhoI cutting of the oligonucleotide-adducted heteroduplex produced linear duplex DNA but little if any nicked circular DNA. This indicates that the two full length strands of the circle are paired in the three-stranded region and suggests that the oligonucleotide is not paired to its circular complement; if it were paired and cut by XhoI, then nicked circles would be formed (see Fig. 2A for a schematic representation of the two possible base pairings). To determine if the cross-linked oligonucleotide could indeed pair to its circular complement and be cut by XhoI, the cross-linked ssDNA substrate was reacted with XhoI. The results are presented in Fig. 6B and show that almost complete linearization of the ssDNA circle was achieved and that 5 times less enzyme per number of DNA molecules was needed compared with the heteroduplex structure (5 units cut 60 pmol of ssDNA in Fig. 6B versus 6 pmol of cccDNA in Fig. 6A, top right).

These results indicate that the cross-linked oligonucleotide paired with its complement in the ssDNA substrate but not in the heteroduplex. Therefore, it appears that the DNA strand brought in via strand exchange disrupted the base pairing of the cross-linked oligonucleotide to its complement. This change in the conformation of the DNA in the region of the cross-link could be the reason that the UvrABC complex now incises the DNA strand bound to the pyrone side of the cross-link. In Fig. 2A, the three-stranded region is shown as an equilibrium between the two possible base pairings. The XhoI results, however, suggest that the cross-linked oligonucleotide probably does not participate in base pairing to a significant degree so that the structure shown on the bottom is probably the more frequent one.

The Step between Incision and Strand Exchange: A Role for DNA Polymerase I—Having shown that RecA can promote strand transfer past a cross-linked oligonucleotide such that a structure susceptible to UvrABC excision is produced, we focused our attention on the initiation of strand exchange. To do this, we constructed a substrate similar to what would be found after the first round of incision by UvrABC. As shown in Fig. 1, path B, it is a dsDNA molecule containing two nicks in one DNA strand, one on either side of a single psoralen cross-link. This cross-linked dsDNA substrate is similar to an UvrABC-incised cross-link as defined by Van Houten et al. (1986b) for oligonucleotides and by M. M. Munn2 for M13 DNA in that: 1) the incisions or nicks in the DNA are on the furan side of the cross-link; 2) the oligonucleotide defined by the nicks is 11 bases long; and 3) the psoralen moiety is near the 3' end of the oligonucleotide. The only differences are that the substrate in Fig. 1, path B, lacks 5'-terminal phosphates and that it contains three nucleotides 3' to the cross-link, while Van Houten et al. (1986b) (and M. M. Munn) found two nucleotides in that position.

Initiation of RecA-mediated strand exchange normally requires a region of ssDNA. However, it is possible that the nicks and a possible helical distortion at the cross-link (Tomic et al., 1987) could be sufficient to serve as a focus for RecA binding and eventually strand exchange. Therefore, to determine if UvrABC incision of cross-linked DNA is sufficient to initiate RecA-mediated strand exchange, the cross-linked dsDNA substrate was incubated in the presence of RecA with several different 32P-labeled homologous linear duplexes containing 3' ends near the cross-link. None of these reactions changed the mobility of the labeled linear DNA, including a reaction with a linear duplex containing a 3' end at the position of the nick closest to the cross-link (Fig. 7, lane 4). Therefore, we conclude that by itself UvrABC incision of cross-linked DNA is not sufficient to allow the initiation of RecA-mediated recombination.

The above result indicates that a step between incision and strand exchange is needed in cross-link repair. Due to the requirement of ssDNA for the initiation of RecA-mediated strand exchange and to the polarity of strand exchange—5' to 3' with respect to the ssDNA—the missing step is most likely to be the formation of a ssDNA region (a gap) by a 5'-exonuclease (see Fig. 2B). A likely candidate for the formation of a gap is the 5'-exonuclease activity of UvrABC.
was prepared from M13mplSF1 replicative form DNA by restriction with Sau3A1, an isoschizomer of BamHI. The 4042-base pair Sau3A1 strand exchange. Structures of the DNA substrates used in the strand exchange reactions are shown at the top. The linear dsDNA substrate of the cross-linked dsDNA substrate, was gel purified and labeled product of the control reaction, a full length M13mplSF1 ssDNA dsDNA substrate of Fig. 1, path B. The starting point of homology between the linear duplex and the circle is shown by the tick mark. Strand exchange would proceed counterclockwise. The circle on the left is the control, M13mplSF1 (+) strand DNA. Lanes 1 and 2, control reaction with 4 μM ssDNA incubated with 0 or 2.6 μM RecA protein, 0.18 μM SSB, and 2 μM linear dsDNA as described under “Experimental Procedures.” Lanes 3 and 4, reaction with the cross-linked dsDNA substrate performed as in lanes 1 and 2. The samples were resolved on an agarose gel, system I. Gapped circle refers to the product of the control reaction, a full length M13mplSF1 ssDNA circle (7250 bases) containing the 4042-base fragment. If strand exchange occurred with the cross-linked dsDNA substrate, a labeled DNA species migrating behind the gapped circle would be visible in lane 4.

In contrast to the untreated cross-linked dsDNA substrate which showed no evidence of interacting with the labeled linear duplex in the presence of RecA protein (Fig. 7, lane 4), the pol I-treated substrate yielded numerous labeled intermediates indicating that it is a substrate for RecA (Fig. 8, lanes 2–5). A small but significant amount of full length heteroduplex product (nicked circle) was also produced (as much as 5%). This result held true not only in the absence of deoxyribonucleoside triphosphates (dNTPs) (Fig. 8, lanes 2 and 3), but also in the presence of dNTPs (Fig. 8, lanes 4 and 5).

Even though the initial goal was to produce full length heteroduplex product, the appearance of the intermediates is significant; it may be that in vivo it is on “intermediates” such as these that UvrABC acts. The reasons for not obtaining a higher yield of full length product, on the other hand, may

of such a gap is pol I: it contains a 5′-exonuclease activity which digests dsDNA by acting at nicks or incisions (Cozzarelli et al., 1969) and which can do so independently of the polymerase activity (Klett et al., 1968). Furthermore, the 5′-exonuclease activity of pol I has been previously implicated in recombinational repair of cross-links (Howard-Flanders et al., 1975; Cole et al., 1976; Yoakum and Cole, 1977).

The cross-linked dsDNA substrate of Fig. 1, path B, was treated with pol I and then reacted with RecA protein in the presence of labeled homologous DNA, as outlined in Fig. 2B. Pilot studies indicated that gaps having an average length of 300 nucleotides were made by the pol I treatment (10 min at 37 °C) (see “Experimental Procedures”), and restriction studies (not shown) indicated that the gap was to the right of the cross-link as shown in Fig. 2B. Control studies also suggested that oligonucleotides produced by the pol I treatment were inhibitory to the strand exchange reaction, so they were removed from the DNA by passing the pol I-treated substrate over a Sepharose CL-4B column in a buffer containing 50% formamide.

In order to assess the effect of the gap on strand exchange, the cross-linked dsDNA substrate of Fig. 1, path B, which showed no evidence of interacting with RecA, was treated with pol I for 10 min at 37 °C without dNTPs (−) or for 0.5 min at 37 °C without and then 9.5 min at 37 °C with dNTPs (−, +) and then passed over a Sepharose CL-4B column in a 50% formamide buffer. This pol I-treated DNA (3.8 μM) was incubated with 5.2 μM RecA, 0.18 μM SSB, and 2.4 or 4.8 μM Pol-generated linear duplex (0.6 or 1.2 molecules linear/circle, respectively) as described under “Experimental Procedures.” The duplex was labeled at the 3′ termini with [α-32P]dCTP by T4 DNA polymerase in the presence of dATP and TTP, and then dGTP. Lane 1, linear duplex DNA as marker; lanes 2 and 3, RecA reaction with the cross-linked dsDNA substrate treated with pol I in the absence of dNTPs, lanes 4 and 5, the same but treated with pol I in the presence of dNTPs; lanes 6 and 7, RecA reaction with M13mplSF1 phage DNA passed over the Sepharose CL-4B column. The samples were resolved on an agarose gel, system I, but run for 14.5 h at 40 volts.
not be related to cross-link repair in vivo and may only reflect the difficulties of achieving complete strand exchange with gapped substrates in vitro. RecA reactions even with substrates with defined gaps are seldom as efficient as with completely single-stranded circles (usually 25–60%, West et al., 1982; Hahn et al., 1988). They also tend to yield more intermediates and less product if the end of the homologous linear duplex is in the middle of the gap (West et al., 1982) as was the case in our experiments; the average gap size was 300 bases, but the 3' end of the linear duplex extended only 144 bases into the gap (see Fig. 2B). A RecA reaction between the Psul-generated linear duplex and a pol I-treated substrate with a gap of approximately 1200 nucleotides yielded significantly more intermediates and less nicked circle product (gel not shown).

While it is evident that the pol I and RecA reaction conditions need to be optimized, it is also clear that in the presence of dNTPs, pol I converts a structure analogous to a UvrABC-incised cross-linked duplex from one that is refractory to strand exchange to one that participates in RecA-mediated homologous pairing and strand exchange. The most likely explanation for this conversion is that pol I is creating a region of ssDNA in which RecA polymerization can be initiated. This therefore suggests that pol I is unable to add nucleotides to the 3'-OH end of the cross-linked oligonucleotide.

To analyze this hypothesis directly, a polymerase (Klenow) extension assay was employed using the cross-linked oligonucleotides FS1 + 2, A and B. The 11-mer (FS1), labeled at its 5' end prior to cross-link formation, served as the primer and the 19-mer (FS2) as the template. Extension of the 11-mer was monitored by photo-reverting the cross-link after treatment with Klenow and running the products on a denaturing polyacrylamide gel.

The results are presented in Fig. 9. The 11-mer of the non-cross-linked control was extended readily to a 15-mer (Fig. 9, 2nd lane). A small amount of 16-mer was also produced and is most likely a +1 addition product such as that described in Clark and Beardsley (1987). Roughly 80% of the 11-mer in cross-link isomer A was also extended to a 15-mer (Fig. 9, 7th lane): the band immediately above the 15-mer is a 15-mer with monoadduct. In contrast, less than 10% of the 11-mer in isomer B was extended (Fig. 9, 13th lane). Most of the label migrated as a 10-mer or a 10-mer with monoadduct, indicating that even though the polymerase activity of Klenow could not act efficiently on isomer B, the 3'-exonuclease activity could. The increased mobility of both cross-linked isomers after the Klenow treatment was due to 3'-exonuclease activity on the 19-mer.

In isomer B, the 3'-OH end to be extended is next to the furan side of the cross-link, which is the situation expected in vivo. However, UvrABC incision leaves two nucleotides on the 3' side of an HMT cross-link (Van Houten et al., 1986b), while isomers FS1 + 2, A and B, contain three nucleotides in that position. The extra nucleotide was therefore removed from the cross-link isomers by preincubating with Klenow in the absence of dNTPs. The results show that while cross-link isomer A could still be extended (Fig. 9, 9th lane), isomer B was now completely inert to polymerase action (Fig. 9, 15th lane). These results demonstrate that under the conditions used, pol I cannot extend the 3' end next to the furan side of an HMT cross-link. They therefore suggest that in vivo pol I might fail to extend the 3' end produced by UvrABC incision.

The extension by Klenow of the pyrone side raises the intriguing possibility that minor cutting by UvrABC on the pyrone side of cross-links in duplex DNA observed by some researchers (Jones and Yeung, 1988) may not lead to recombination repair in the presence of pol I or any other polymerase. It could also reflect the preference of the UvrABC complex for the furan side of the cross-link. Greater distortion on the furan side could prevent extension by pol I, which requires a 3' end base paired to a template in order to synthesize DNA (Kornberg, 1981).

**FIG. 9.** Klenow is unable to extend a 3'-OH end which is two or three bases from the furan side of an HMT cross-link.

The three DNA substrates depicted at the top (p, pyrone side; f, furan side of the HMT cross-link) were incubated with the large fragment of pol I (Klenow) as indicated. The cross-linked oligonucleotides FS1 + 2, XL - A and B were prepared as under "Experimental Procedures" except that the 11-mer was labeled at the 5' end with 32P prior to the formation of the cross-link. The oligonucleotides (0.6 μM) were incubated in 10 μl with 0.8 units of Klenow (International Biotechnologies, Inc.) in the pol I reaction buffer plus 8 mM dithiothreitol for 30 min at 19 °C. Reactions were performed either in the absence of dNTPs (-), in the presence of 15 μM each of the four dNTPs (dATP, dCTP, dGTP, and TTP) (+), or without dNTPs for 15 min then with dNTPs for 15 min (+). EDTA was added to 25 mM, and half of the reaction was irradiated with 1.8 kJ/m2 far-UV light (254 nm) to photorevert the cross-link. Samples were resolved on a denaturing 20% polyacrylamide gel electrophoresed for 11 h at 450 volts. The band immediately above the 11-mer in the lanes containing the photoreverted cross-link is an 11-mer with a monoadduct.

**DISCUSSION**

Using defined DNA substrates in reactions with purified proteins, we found that: 1) *E. coli* RecA protein mediates strand transfer past a 19-base oligonucleotide cross-linked to an ssDNA circle; 2) UvrABC excises the cross-linked oligonucleotide from the heteroduplex product of that reaction; 3) a dsDNA substrate with nicks on either side of a cross-link, similar to those that UvrABC would make, does not initiate RecA-mediated strand exchange; 4) preparations of the same substrate containing a gap made by the 5'-exonuclease of pol
I in the presence of dNTPs, however, do undergo strand exchange and pol I cannot extend the 3’ end next to a cross-link similar to that which UvrABC incision would leave. These results shed light on several critical steps in the sequential excision-recombination mechanism of cross-link repair in E. coli and define a role for the 5’-exonuclease activity of pol I in that repair. Diagrammed in Fig. 10 is our current understanding of the mechanism. The first step is incision of the DNA by UvrABC on the furan side of the psoralen cross-link (Van Houten et al., 1986b; Jones and Yeung, 1988). In vitro data suggest that the UvrABC protein complex remains bound to the DNA after incision and that UvrD and pol I are needed to release it (Caron et al., 1985; Husain et al., 1985). Whereas with UV dimers, the removal of the protein complex appears to be accompanied by the release of the incised oligonucleotide (Caron et al., 1985), it is not likely that the oligonucleotide would be released in the case of psoralen cross-links; that would require breaking the covalent bond between the psoralen molecule and the thymine residue. Therefore, the double-stranded structure containing the incised cross-link shown in Fig. 10B is most probably the structure left after incision by and removal of the UvrABC complex. This structure, however, did not initiate RecA-mediated strand exchange (Fig. 7), indicating that an extra step is needed.

The concerted action of pol I and UvrD and a possible physical association between them (discussed in Rothman, 1980; Caron et al., 1985; Sancar and Sancar, 1988) suggest that in vivo pol I would be at the site of the UvrABC-incised lesion. In vitro pol I uses nicks in DNA as starting points of concomitant synthesis and digestion—nick translation (Kelly et al., 1970; Kornberg, 1981). DNA synthesis starting from the nick that is to the left of the cross-link in Fig. 10B, however, will most likely be blocked by the cross-link (Ou et al., 1978; Piette and Heerst, 1983), while synthesis from the nick that is to the right will probably not be initiated at all (Fig. 9). This inhibition of repair synthesis could be the reason why psoralen cross-links are recombinogenic under nonreplicating conditions (Lin et al., 1977; Cassuto et al., 1977): it allows a gap to be made in the DNA.

In contrast to the polymerase activity, the 5’-exonuclease of pol I can act at the UvrABC incisions and was found to create a rather large gap (potentially thousands of bases) to the right of the cross-link as shown Fig. 10C. Due to the polarity of RecA-mediated branch migration (to the left in Fig. 10, see legend to C), a gap to the right of the cross-link is what is needed in order for strand exchange to go past the cross-link. Strand exchange could be initiated to the left of the cross-link, e.g. at a gap made by a 3’-exonuclease activity, such as exonuclease III, but it would not go past the cross-link. This could explain why strains deficient in exonuclease III (xth-) do not show a decreased capacity for cross-link-induced recombination (Lin et al., 1977), while strains deficient in the 5’-exonuclease activity of pol I do (Howard-Flanders et al., 1975).

The gap produced by pol I provides a region of ssDNA to which RecA protein can bind and initiate strand exchange (Fig. 10, D and E). Once initiated, RecA-mediated strand exchange has little difficulty proceeding past the incised cross-link (Fig. 10, F and G), indicating that there is sufficient space available within the RecA protein filament for the cross-linked oligonucleotide. Previously, RecA has been shown to carry out strand exchange reactions involving four DNA strands (reviewed in Howard-Flanders et al., 1984; West and Howard-Flanders, 1984) as well as reactions with DNA containing other types of adducts such as pyrimidine dimers (Livneh and Lehman, 1982; Hahn et al., 1988).

After RecA has converted the gapped structure to a heteroduplex containing a cross-linked oligonucleotide, the other side of the cross-link, the pyrene side, becomes susceptible to cutting by UvrABC (Fig. 10H). Since UvrABC does not incise the pyrene side of cross-links in duplex DNA very efficiently (Van Houten et al., 1986b; Jones and Yeung, 1988), the creation of a structure with a three-stranded region appears to be critical to repair. The results from our Xhol reactions (Fig. 6) indicate that the strand brought in by recombinination disrupts the base pairing of the cross-linked oligonucleotide to its complement. We therefore propose that this disruption is responsible for the increased activity by UvrABC on the pyrene side of the cross-link. Others have also found that a cross-linked oligonucleotide is excised from a three-stranded structure by UvrABC incision on the pyrene side (Cheng et al., 1988). Since the oligonucleotides in those experiments

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**Fig. 10. Proposed mechanism of excision-recombination repair of DNA containing psoralen cross-links in E. coli.** A and B, the UvrABC complex acts on cross-linked DNA, making two incisions in the DNA strand adducted to the furan side of the cross-link, one on either side of the cross-link (Van Houten et al., 1986b). C, the 5’-exonuclease of pol I acts on the nicks to form a gap 3’ to the incisions (5’→3’). D, RecA protein binds to the ssDNA in the gap and polymerizes in the 3’ direction, extending the head end of the polymer into the dsDNA region (Register and Griffith, 1985; Cassuto and Howard-Flanders, 1986) to the left). E and F, deep grooves in the RecA protein allow contacts between the DNA molecules which lead to homologous alignment or pairing of the two DNA molecules (Howard-Flanders et al., 1984; Cox and Lehman, 1987). Strand exchange starts in the gap (West et al., 1982) and proceeds in the same direction as RecA polymerization to the left), continuing past the incised cross-link. G, RecA monomers dissociate from the tail end of the polymer (Cox et al., 1983; Soltis and Lehman, 1983). Once free of RecA protein, the newly paired 3’ end is extended by DNA polymerase (dotted line) (West et al., 1982, 1983). H, spontaneous branch migration at the crossover (on the right) allows completion of repair synthesis and ligation. The two cross-overs are resolved, either vertically (as shown on the left) or horizontally (as shown on the right). The strand carrying the cross-linked oligonucleotide, now paired with an intact complementary strand, is incised by UvrABC. I, repair synthesis and ligation at the site of the excised cross-link complete the repair (Cheng et al., 1988).
were as long as 85 bases, the length of the cross-linked oligonucleotide is apparently not critical to the UvrABC reaction.

The second round of UvrABC incision removes the psoralen moiety from the DNA (Fig. 10F). A gap of 11–13 bases is produced (Fig. 5 and Cheng et al., 1988) and is subsequently filled in by pol I-mediated repair synthesis (Cheng et al., 1988). Pol I may also fill in the gaps left by strand exchange (Fig. 10G); mutants defective in the polymerase activity of pol I (polA1) show decreased cross-link-induced recombination and strand rejoining (Howard-Flanders et al., 1975; Lin et al., 1977; Sinden and Cole, 1978).

While the model presented above contains the essential elements for psoralen cross-link repair, the situation in vivo will undoubtedly be more complex. Protein-protein interactions and competition with other cellular processes can be expected to influence repair. For example, the recombinagenic gap that we propose as being made by pol I could be produced by the separation of the DNA strands for replication as appears to be the case of pyrimidine dimers and psoralen monoadducts (Casu et al., 1977; Howard-Flanders et al., 1976; Lin and Howard-Flanders, 1976). The size of the gap is also likely to be affected by other enzymes such as exonuclease VII (5’-3’ exo-nuclease), RecBCD (5’-exo-nuclease and helicase), RecF (unknown), and UvrD (helicase) (for all but exonuclease VII, see Yookum and Cole, 1977; Sinden and Cole, 1978). However, at least two of those, RecBCD and RecF, do not appear to play as critical a role in cross-link-induced recombination (Lin et al., 1977; Howard-Flanders et al., 1978; Howard-Flanders and Bardwell, 1981) or in cross-link removal (RecBCD only, Yookum and Cole, 1977) as does the 5’-exo-nuclease of pol I. UvrD, on the other hand, appears to be important both in recombination (Lin et al., 1977) and cross-link removal (Yookum and Cole, 1977), but it apparently needs a single-stranded region at least 12 nucleotides long for its helicase activity to act (Kuhn et al., 1979). Therefore, its role in repair is more likely to be that of turnover of the UvrABC proteins and in widening rather than in initiating the gap. Finally, while exonuclease VII is the only one of the above mentioned enzymes that has as a substrate nicked double-stranded DNA (Chase and Richardson, 1974; for RecBCD, see Cox and Lehman, 1987), its role in psoralen cross-link repair has as yet to be established.

Therefore, these data in combination with the following observations have led us to conclude that in addition to UvrABC and RecA, DNA polymerase I plays a critical role in psoralen cross-link repair: 1) pol I is likely to be at the site of damage during cross-link repair (Sancar and Sancar, 1988); 2) the 5’-exo-nuclease activity of pol I plays a role in cross-link removal (Cole et al., 1976; Yookum and Cole, 1977) and cross-link-induced recombination (Howard-Flanders et al., 1975); and 3) in vitro pol I converts a dsDNA substrate containing an incised cross-link into a gapped structure which is a substrate for RecA-mediated strand exchange (this work). While others (Cole et al., 1976; Yookum and Cole, 1977) had previously concluded that pol I played a critical role in repair, they misinterpreted the role as being one of producing a second incision at the cross-link. However, since we now know that UvrABC makes both incisions, it seems more probable that the role of pol I is to produce a gap so that the next step in repair—recombination—can take place.

In conclusion, we have reproduced in vitro several critical steps in the mechanism of excision-recombination repair of psoralen-DNA cross-links in E. coli; and for one previously ill defined step, that between incision and recombination, we provide biochemical evidence showing a role for the 5’-exo-nuclease activity of DNA polymerase I. Genetic recombination has been implicated in the repair of psoralen cross-links in mammalian cells as well as in bacteria (Hall, 1982; Hanawalt et al., 1979; Ben-Hur and Song, 1984). It will be interesting to see if the excision-recombination mechanism found in E. coli is conserved in higher organisms and if it is applicable to other cross-linking agents.

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