Xer-site-specific recombination functions in the stable maintenance of circular replicons in *Escherichia coli*. Each of two related recombinase proteins, XerC and XerD, cleaves a specific pair of DNA strands, exchanges them, and rejoins them to the partner DNA molecule during a complete recombination reaction. The rejoining activity of recombinase XerC has been analyzed using isolated covalent XerCDNA complexes resulting from DNA cleavage reactions upon Holliday junction substrates. These covalent protein-DNA complexes are competent in the rejoining reaction, demonstrating that covalently bound XerC can catalyze strand rejoining in the absence of other proteins. This contrasts with a recombinase-mediated cleavage reaction, which requires the presence of both recombinases, the recombinase mediating catalysis at any given time requiring activation by the partner recombinase. In a recombining nucleoprotein complex, both cleavage and rejoining can occur prior to dissociation of the complex.

Xer-site-specific recombination functions in the stable segregation of circular replicons in *Escherichia coli* by resolving circular multimers that can arise by homologous recombination or rolling circle replication (reviewed in Ref. 1). Recombination occurs at specific target sites and requires the catalytic action of two recombinase proteins, XerC and XerD (2). Homologues of XerC and XerD have been identified in many bacteria with circular chromosomes. The Xer proteins are members of the bacteriophage λ integrase family of recombinases. Members of this family contain two highly conserved motifs, containing four invariant residues: a tyrosine, which acts as the nucleophile during strand cleavage, and two arginines and a histidine (the RHR triad), which have been implicated in the activation of the scissile phosphodiester in both the cleavage and rejoining reactions. The two arginines are thought to stabilize the pentavalent phosphate transition state, whereas the histidine may act as a general base catalyst (3–6). Recent structural and biochemical studies have revealed two further residues that are directly implicated in catalysis. An invariant lysine is present in a conserved β turn, lying between motifs I and II, in the recombinases and the structurally and mechanistically related type 1B topoisomerases. A second histidine in motif II contacts the scissile phosphate and may participate in the general acid-base catalysis; this histidine, although well conserved, is not invariant, being a tryptophan in Cre recombinase (5, 6).

Integrate family-mediated recombination proceeds via two temporally and spatially separated pairs of strand exchanges, producing a Holliday junction as an intermediate (6–11). Each strand exchange involves two trans-esterification steps; the strand cleavage reaction occurs by a nucleophilic attack of the active site tyrosine upon the activated DNA phosphodiester producing a 3′-phosphotyrosyl bond and a 5′-OH. The second rejoining reaction occurs when nucleophilic attack of a 5′-OH on the 3′-phosphotyrosyl bond restores the continuity of the DNA strand and displaces the tyrosine. If the 5′-OH comes from the cleaved DNA partner, strand exchange has occurred.

Because site-specific recombinases break and rejoin DNA phosphodiester bonds, these enzymes have evolved mechanisms to ensure that efficient recombination occurs only on correctly aligned recombination sites that have been bound by the four recombinase molecules needed to catalyze the cleavage and rejoining of four DNA phosphodiester bonds. The structure of Cre bound to Holliday junction DNA (6) provides support for biochemically based models that suggest DNA conformation, and protein-protein interactions play a key role in determining which pair of recombinase molecules within a tetrameric recombination complex are to be active at any point in time (12–14). The failure of singly bound XerC or XerD molecules to catalyze efficient DNA cleavage suggests that XerC and XerD each activates catalysis of its partner recombinase when bound to DNA (12). The inappropriate positioning of the active site tyrosine for in-line attack on the scissile DNA phosphate in the crystal structure of a XerD provides a structural basis for both monomer inactivity and partner recombinase-mediated activation through correct repositioning of the tyrosine nucleophile (14). Flp recombinase uses a different strategy to control catalysis; a single active site contains contributions from two Flp monomers (15).

For two characterized Xer recombination sites, *psi* from the plasmid pSC101 (16) and *cer* from ColE1 (17), recombination occurs with a distinct order of strand exchange, with XerC catalyzing the first pair of exchanges to form the Holliday junction intermediate (18, 19). In *psi* site recombination, the junction is then resolved to products by XerD-mediated strand exchange (19, 20).

DNA molecules containing Holliday junctions and bearing recombination core sites have been shown to be substrates for *in vitro* strand exchange mediated by several different integrase family recombinases (8, 21–25). The use of a Holliday junction-containing substrate allows the analysis of the cleavage, rejoicing, and strand exchange steps, as well as their controlling factors, during a reaction on the same substrate. Neither XerC nor XerD on its own is active in DNA cleavage or strand exchange upon Holliday junctions (12, 25, 26), implying that interaction between the two recombinases is necessary to produce an active site configuration that is competent for cleav-
The rejoining reaction is much less well characterized than the cleavage step in integrase family-mediated recombination. Previous studies of rejoining have been carried out using the FLP recombinase with either activated substrates bearing a 3’-phosphotyrosyl bond or a half-site DNA substrate (29, 30). The homology dependence of rejoining was also studied using half-sites and full sites with mismatched bases (31, 32). In this study we have used isolated covalent protein-DNA complexes resulting from XerC-mediated cleavage of a Holliday junction to study the rejoining reaction and its requirements for interactions with partner recombinases. This fully paired linear full site substrate with a covalently attached wild-type protein should be equivalent to the true covalent intermediate produced in a wild-type reaction. As well as providing a wild-type substrate for the study of rejoining, it also extends the analysis of the rejoining step of recombination to a second member of the integrase family of recombinases and allows dissection of the protein interactions necessary for catalysis. A major conclusion is that a covalently bound XerC molecule can catalyze DNA rejoining in cis; the presence of XerD does not stimulate the reaction and therefore is not required to activate XerC-mediated rejoining.

EXPERIMENTAL PROCEDURES

Construction of Labeled Substrates—Holliday junction-containing substrates were constructed by annealing four synthetic oligonucleotides as described elsewhere (25, 26). The oligonucleotides used for the dif-cer6 hybrid site are identical to those used for HJ, except for the central region sequences, and are as follows: strand I, 5’-GATCGCTGATACGCGCTAACGCGTGATCACG-3’; strand II, 5’-ATCCTAGGGCCGCCGCTATAGCAATTCGAATATCGCTCCTAT-3’; strand III, 5’-CTCACCCGTGTAAATCTACATACACTCCCGCTGAGCATCGAAGCTTGATGGAATAGAT-3’; and strand IV, 5’-CGCCGCGCTTTAGGCGTTAGGCCATTTAACATAAAGGATTTGTTAAATGACACCCCGACCGTTGCCGGATCCG-3’.

DNA Rejoining in cis—Reactions of the Xer recombinases with synthetic Holliday junctions bearing various core recombination sequences produce both rejoined linear duplex strand exchange products and covalent recombinase-DNA complexes that have undergone cleavage at the expected position (12, 25, 26). The levels of unjoined cleavage product observed are higher than those reported for similar reactions mediated by either λ integrase or FLP recombinase, indicating that Xer rejoining reactions are rate-limiting under the assay conditions used. To test whether the covalent complexes obtained with XerC have the properties of reaction intermediates and can catalyze strand rejoicing, the covalent complexes were isolated and the requirements for strand rejoicing studied.

Untethered synthetic Holliday junction molecules based on either the chromosomal dif sequence (33) or the plasmid hybrid cer6 sequence (34) are substrates for XerC catalysis but do not undergo significant XerD catalysis. XerC catalysis is dependent on the presence of both recombinases (12, 25, 26) and is directed at a specific phosphodiester on a specific strand (by sequence (33) or plasmid hybrid cer6) (12, 25, 26).

Holliday Junction Reactions—Reaction conditions were as described previously (25). Standard reactions contained ~0.1 pmol of labeled DNA, 500 ng of poly(dI-dC)·poly(dI-dC), 1 µg of acetylated bovine serum albumin and ~10 pmol of XerC and ~5 pmol of XerD per 10 µl of reaction. Binding reactions were incubated on ice for 5 min followed by electrophoresis through 6% polyacrylamide in 1x TBE (89 mM Tris borate, pH 8.3, 2 mM EDTA). Cleavage reactions were incubated at 37 °C for 30 min and then stopped by the addition of 2 µl of SDS stop buffer (50 mM Tris-HCl, pH 7.5, 1% SDS). The DNA was then electrophoresed through 4% polyacrylamide-TBE containing 0.1% SDS at 200 V for 165 min. Gels were dried and exposed to x-ray film (Genetic Research Instrumentation) or analyzed quantitatively on a PhosphorImager (Molecular Dynamics). Where species were to be excised from a gel, ~0.5 pmol of DNA was used.

Isolation of XerC-DNA Covalent Complexes—Large scale Holliday junction cleavage reactions were electrophoresed in the presence of SDS as described above. The wet gel was exposed to x-ray film, and the bands corresponding to XerC-DNA covalent complexes were excised.

The complexes were electroeluted overnight at 4 °C in 0.1x TBE and 0.1% SDS at 16 V and were then ethanol-purified. Resuspension was in recombination buffer (25).

Proteinase K Treatment—A stock solution of proteinase K was made in aqueous solution to a concentration of 4 mg/ml and stored at ~20 °C. One µl of this stock was added per 10 µl of reaction. Proteinase K treatment was carried out at 37 °C for 5 min in the presence of 0.1% SDS.

XerC-DNA Covalent Complex Reactions—Isolated complexes were supplemented with bovine serum albumin and poly(dI-dC)·poly(dI-dC) to create identical conditions as in cleavage reactions. An aliquot of the resuspended covalent complex was transferred to SDS stop buffer before incubation to prevent any further reaction. Complexes were incubated on ice for 1 h to aid protein refolding, and then Xer proteins were added as stated and the reactions incubated for an additional hour at 37 °C. SDS stop buffer was added, and each reaction was divided into two aliquots; one of these was electrophoresed in the presence of SDS, and the other was treated with proteinase K, ethanol-purified, and electrophoresed through a 10% polyacrylamide-TBE gel containing 7 M urea. Size markers for denaturing gels were 5’ end-labeled oligonucleotides.

Proteinase K treatment was also carried out after resuspension in recombination buffer with SDS stop buffer in the experiments described. The DNA was phenol/chloroform-extracted and ethanol-purified. It was then resuspended in recombination buffer and treated in the same manner as the above described covalent complex reactions.

RESULTS AND DISCUSSION

XerC Covalently Attached to Linear Duplex DNA Catalyzes DNA Rejoining in cis—Reactions of the Xer recombinases with synthetic Holliday junctions bearing various core recombination sequences produce both rejoined linear duplex strand exchange products and covalent recombinase-DNA complexes that have undergone cleavage at the expected position (12, 25, 26). The levels of unjoined cleavage product observed are higher than those reported for similar reactions mediated by either λ integrase or FLP recombinase, indicating that Xer rejoining reactions are rate-limiting under the assay conditions used. To test whether the covalent complexes obtained with XerC have the properties of reaction intermediates and can catalyze strand rejoicing, the covalent complexes were isolated and the requirements for strand rejoicing studied.

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isolated complexes were then assayed for their ability to catalyze the strand rejoining reaction.

The purified HJ1 LDCCs migrated through polyacrylamide containing SDS as rather broad bands in which there was some smearing ahead of the main band, possibly because of some proteolytic degradation during processing of the sample (Fig. 1A). In the absence of renaturation, little or no rejoined linear duplex (RLD) was evident (Fig. 1A, lane SDS; less than 2% of the label was in RLD). When the covalently attached protein was allowed to renature, RLD was produced (Fig. 1A, lane “no protein”) or after the addition of the indicated proteins. The reactions labeled proteinase K were treated with proteinase before the addition of the indicated proteins. All reactions were proteinase K-treated prior to electrophoresis to reveal the cleaved species.

When isolated covalent complexes were treated with proteinase K, before incubation in recombination buffer, no increase in the level of rejoining was seen with or without SDS present (Fig. 1B). This observation confirms that the strand rejoining
reaction is XerC-catalyzed and occurs slowly, if at all, by spontaneous, noncatalyzed attack of a DNA 5'-OH on an adjacent 3'-phosphotyrosine. We therefore conclude that a XerC molecule covalently bound to its substrate DNA catalyzes rejoining by the displacement of its own phosphotyrosyl by the DNA 5'-OH. These results are consistent with the covalent complexes being trapped reaction intermediates and demonstrate that cleavage and rejoining can occur in a nonconcerted fashion.

Rejoining Is Stimulated by the Addition of Exogenous XerC, but Not XerD; This Effect Is Independent of the Active Site Tyrosine on the Exogenous XerC—The effect of adding exogenous recombinases to the covalent protein-DNA complex was also examined (Fig. 1). Addition of XerD had no significant effect on the amount of rejoined product formed (8% of label in RLD compared with 8.5% RLD when no exogenous protein was added), indicating that interactions between the covalently attached XerC and XerD are not required for the strand rejoining reaction. The addition of XerC led to an increase in the level of linear duplex observed (16% of strand IV label and 18% of strand I label as RLD). Addition of a mutant XerC lacking the active site tyrosine nucleophile, XerCY275F, also led to an

Fig. 2. Purified MBP-XerC covalent complexes show the same rejoining characteristics as wild-type XerC complexes. A, isolated LDCC complexes were reacted as indicated (lane descriptions as in Fig. 1) and electrophoresed in the presence of SDS. Note that the contaminating HJ does not decrease in intensity during reaction, indicating that it is not a substrate for XerC catalysis in the absence of XerD. B, the same samples electrophoresed on a denaturing gel.
increase in linear duplex production over the reaction with no added protein (15% from 6% of strand I label as RLD).

The addition of XerC and XerD together led to a further increase in the level of rejoining compared with the addition of XerC alone (Fig. 1B, lane XerC+XerD). This could be a consequence of XerC+XerD reaction on contaminating Holliday junction-related material and/or a direct stimulation of rejoining on the LDCCs (for example by stimulating XerC binding to DNA through cooperative XerC-XerD interactions on DNA). Similarly, the increase in 84-nt recombinant product strands in the proteinase K-treated LDCC reaction that was subsequently incubated with with XerC and XerD is likely to have arisen by stimulation of XerC-mediated rejoining on the proteinase K-treated LDCC. A similar reaction has been observed with Flp recombinase (29). The stimulatory effect of exogenous XerC may be the result of its binding directly to the left half-site and activating the phosphotyrosyl bond of the covalently attached XerC molecule. This process would require that the exogenous XerC molecule bind to the left half-site and correctly position its active site residues involved in phosphodiester activation, despite the presence of the existing protein–DNA phosphotyrosyl bond and the rest of the covalently attached protein. We would expect this to happen only on molecules in which the covalently bound DNA had failed to renature, because structural studies of both XerD and Cre suggest that a correctly folded recombinase molecule covalently bound to DNA is unlikely to allow access of the active site of a second molecule to the scissile phosphotyrosyl bond (6, 14, 35). An alternative explanation is that the action of the exogenous protein is to aid in the refolding of the covalently attached XerC molecule. This would be an unlikely result from a non-recombinase co-purified protein, because MBP-XerC, the maltose-binding protein fusion derivative of XerC, also stimulates rejoining, and MBP-XerC was purified by a different method than the wild-type XerC. Furthermore, if the possible refolding were a direct result of recombinase-recombinase interactions, then XerD would be expected to exhibit stimulation too.

The addition of exogenous XerC also led to the formation of a species migrating more slowly than the substrate covalent complex on the SDS-polyacrylamide gel. This species is probably

![Diagram](image-url)
of recombinase-bound Holliday junction and the lower band XerD were fractionated on a native polyacrylamide binding gel. Each actions of three different Holliday junction substrates with XerC and Re-

protein interactions before and following strand exchange.

DNA 5\'-OH must be able to position themselves correctly for

stimulate the attack of the DNA 5\'-OH upon this bond. Simi-

larly when XerC was added to covalent complexes formed by

MBP-XerC, no covalent complexes containing only wild-type XerC were produced (Fig. 2A). These observations are consistent with the idea that cleavage involves attack of the nucleo-

phile in line with the leaving group: to cleave the protein–DNA phosphotyrosyl bond, a nucleophile must approach from the direction opposite the phosphotyrosyl bond. Because the recombi-

nase proteins make contacts with the DNA of the central region (2, 36), steric hindrance may render the phosphotyrosyl bond unable to rotate sufficiently to allow in-line nucleophilic attack

by the tyrosine of the exogenous protein. Therefore, cleavage of an existing phosphotyrosyl by the tyrosine of an exogenous protein would not be expected.

DNA Rejoining in Holliday Junction Covalent Complexes Follows the Same Pattern as for Linear Duplex Covalent Complexes—Covalent complexes of XerC and MBP-XerC with HJs were isolated and treated in reactions similar to those of the linear duplex covalent complexes. These complexes exist as a mixture of two equivalent forms, with one or the other top strand having been cleaved. Because the core recombination site is identical on the two top strands of HJ1, it was expected that the two forms were equimolar.

Because the HJCC migrates in a polyacrylamide gel at almost the same position as the linear duplex covalent complex of MBP-XerC, the level of co-purified contaminating intact junction was similar to that observed upon extraction of the MBP-

XerC LDCCs. Again, only when both XerC and XerD were present (data not shown).

Exogenous XerC and MBP-XerC Do Not Cleave the Existing Protein–DNA Phosphotyrosyl Bond—When MBP-XerC was added to an XerC-DNA complex, no MBP-XerC-DNA covalent complex was produced (Fig. 1A). This result suggests that the exogenous MBP-XerC does not directly attack the existing phosphotyrosyl bond with its own catalytic tyrosine but can stimulate the attack of the DNA 5\'-OH upon this bond. Similarly when XerC was added to covalent complexes formed by MBP-XerC, no covalent complexes containing only wild-type XerC were produced (Fig. 2A). These observations are consistent with the idea that cleavage involves attack of the nucleo-

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XerC LDCCs. Again, only when both XerC and XerD are pres-

ent should the co-purified DNA produce any reaction.

Judging by the mobility of the isolated complexes, there does not appear to have been a great deal of proteolysis or DNA degradation during purification, although they are contami-

nated with some substrate Holliday junctions and Holliday junction degradation products (Fig. 3A; the three “SDS” lanes). Strand rejoining is demonstrated by an increase in the inten-
sity of the Holliday junction band in the non-SDS lanes (for example, with HJ7 the proportion of total labeled DNA as Holliday junction increases from 15% (+SDS lane) to 34% (no protein lane) and 32% (+XerD lane). Therefore, the covalently attached XerC is capable of catalyzing the rejoining reaction in cis, and the addition of XerD does not stimulate rejoining, paralleling the results with the linear duplex covalent complexes. Denaturing polyacrylamide gel analysis of the HJ7 reactions confirmed that rejoining occurred to give the parental strand size (Fig. 3B).

**Holliday Junction Covalent Complexes Are Substrates for XerC Cleavage Regardless of the Presence of XerD—Surprisingly, the incubation of renatured HJCCs in the absence of exogenous recombinase also produced RLD product (Fig. 3A). The denaturing gel of these reactions confirmed that these were completed strand exchange products of the correct size (Fig. 3B). We assume that these RLDs arise from attack of free XerC (released from HJCCs in rejoining reactions) on HJCCs; cleavage occurring on the second top strand of HJCC before rejoining of the initially cleaved strand occurs. Because XerC-mediated rejoining within a HJCC generates a Holliday junction that is not a substrate for XerC catalysis in the absence of XerD, the cleavage and rejoining reactions that generate the RLDs must occur directly on the HJCCs.

The appearance of RLDs is correlated by an increase in LDCCs, showing that the rejoining of the two duplexes derived from HJCC cleavage does not need to be concerted. Furthermore, these results show that XerC can cleave independently of XerD on a HJCC, whereas no products are seen when XerC alone is added to intact Holliday junction substrates; this could be because HJCCs are better cleavage substrates than intact Holliday junctions or because XerC can cleave in a nonconcerted fashion at a low frequency on both the intact junction and the covalent complex. On the HJCC the presence of the existing cleavage on the other top strand and the lack of protein–protein contacts to keep the junction together allows the complex to dissociate before rejoining occurs and yields two linear duplex covalent complexes, one or both of which are then rejoined to product. On the intact junction, two of these rare cleavage events would be required to occur concurrently for the junction to dissociate.

**Both DNA Cleavage and Rejoining Occur within a Protein-DNA Complex Derived from a Recombinase-bound Holliday Junction Substrate—RLD can be generated only from Holliday junction substrates that have undergone two recombinase-mediated cleavages. Although it seems likely that rejoining would occur within this recombinase-DNA complex under normal conditions, it is possible that the rejoining reaction can occur after dissociation of these complexes within single LDCCs. These possibilities are not mutually exclusive, and it is possible that the observed low level of LDCCs results from rare junction dissociation events prior to rejoining, whereas rejoined linear duplex strand exchange products result from cleavage and rejoining reactions that are completed within a nucleoprotein complex.

To test this hypothesis, it was necessary to ascertain whether LDCCs are associated with protein-DNA complexes that migrate during electrophoresis with the mobility of Holliday junction molecules bound with recombinase or with the mobility of recombinase-bound duplex. Reactions of three different Holliday junction substrates with XerC and XerD were electrophoresed on a binding gel. Each reaction yielded two species, the upper one corresponding to the recombinase-bound junction DNA and the lower to the bound linear duplex product (data not shown). DNA from these bands was extracted separately in the presence of SDS and treated with protease K. The DNA was then analyzed on a denaturing polyacrylamide gel (Fig. 4). The bound junction complex contains almost all of the detectable cleaved DNA and a small proportion of the RLD; most of the label is in the parental size strands as expected. Similar results were obtained for all three substrates, although the two containing the cerC central region gave a higher level of cleaved and rejoined products. The lower complex is made up almost exclusively of RLD, consistent with the idea that after rejoining, synaptic complexes dissociate. Because the majority of cleaved molecules are contained within LDCCs rather than HJCCs (e.g. see Fig. 1A), we conclude that most of the cleaved DNA is held together by protein-protein interactions prior to rejoining within the bound complex. However, we cannot exclude the possibility that SDS treatment of unreacted recombinase-bound Holliday junctions induces recombinase-mediated cleavage and some rejoining.

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