Xer site-specific recombination *in vitro*

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Two related recombinases, XerC and XerD, belonging to the lambda integrase family of enzymes, are required for Xer site-specific recombination *in vivo*. In order to understand the roles of these proteins in the overall reaction mechanism, an *in vitro* recombination system using a synthetic Holliday junction-containing substrate has been developed. Recombination of this substrate is efficient and requires both XerC and XerD. However, only exchange of one pair of strands, the one corresponding to the conversion of the Holliday junction intermediate back to the substrate, has been observed. Recombination reactions using XerC and XerD derivatives that are mutant in their presumptive catalytic residues, or are maltose-binding fusion recombinase derivatives, have demonstrated that this pair of strand exchanges is catalysed by XerC. The site of XerC-mediated cleavage has been located to between the last nucleotide of the XerC binding site and the first nucleotide of the central region. Cleavage at this site generates a free 5'-OH and a covalent complex between XerC and the 3' end of the DNA.

Key words: Holliday junction/*in vitro*/site-specific recombination/Xer

Introduction

The Xer site-specific recombination system functions in *Escherichia coli* in the normal segregation of multicopy plasmids and the bacterial chromosome. We have provided evidence that this system converts circular multimers (that arise by homologous recombination) to monomers, which can be segregated efficiently at cell division (Summers and Sherratt, 1984; Blakely *et al.*, 1991). This recombination system is novel in that it utilizes two related recombinases of the phage lambda integrase family (Blakely *et al.*, 1993). Moreover, the requirements and outcomes of recombination are different depending on whether the recombination sites are derived from natural plasmids (e.g. *cer*, in plasmid ColE1) or from the bacterial chromosome (*dif*, located in the replication terminus of the *E.coli* chromosome; Blakely *et al.*, 1991, 1993; Kuempel *et al.*, 1991). Recombination at *cer* is exclusively intramolecular and requires the accessory proteins ArgR and PepA, and 190 bp of accessory DNA sequences adjacent to the 30 bp core recombination site that binds XerC and XerD (Stirling *et al.*, 1988, 1989; Summers and Sherratt, 1988; Colloms

et al., 1990; Blakely *et al.*, 1993). The accessory proteins and accessory sequences have been implicated in the process of synapse formation and are essential for ensuring that recombination is exclusively intramolecular (Summers, 1989; Blakely *et al.*, 1993). In contrast, recombination at *dif* occurs both inter- and intramolecularly, and requires only XerC and XerD, and a core recombination site of 28 bp (Blakely *et al.*, 1991, 1993).

Several site-specific recombination systems of the integrase family have been studied in detail (reviewed in Sadowski, 1986; Cox, 1988; Craig, 1988; Landy, 1989, 1993; Stark *et al.*, 1992). The characterization of the lambda integrase/*attP*/*attB*, the Cre/*loxP* and the FLP/*fRT* systems, which are the best understood, has benefitted enormously from the development and exploitation of *in vitro* recombination systems that assay partial or complete recombination reactions (Mizuuchi *et al.*, 1980; Abremski *et al.*, 1983; Vetter *et al.*, 1983; Hsu and Landy, 1984; Meyer-Leon *et al.*, 1984; Sadowski *et al.*, 1984; Kitts and Nash, 1987; Nunes-Düby *et al.*, 1987, 1994; Burgin and Nash, 1992; Chen *et al.*, 1992; Pan and Sadowski, 1992). Xer site-specific recombination shows features different to these systems: e.g. its use of two recombinases; its ability to show selectivity for intramolecular resolution when recombination is between identical sites present on natural plasmids; its exploitation of unexpected accessory proteins; its apparent ability to recombine sites containing substantial heterology (McCulloch *et al.*, 1994); and its novel role in chromosome segregation (Blakely *et al.*, 1991). We were therefore prompted to develop and exploit an *in vitro* Xer recombination system. A potential bonus of such studies is that the use of two recombinases, rather than a single recombinase, provides a valuable tool for understanding the individual roles of each of the recombinase molecules during the strand-exchange reactions mediated by the lambda integrase family of enzymes (e.g. see Chen *et al.*, 1992, 1993; Han *et al.*, 1993; Landy, 1993; Jayaram, 1994; Nunes-Düby *et al.*, 1994).

Results

A synthetic Holliday junction-containing molecule is a substrate for Xer site-specific recombination *in vitro*

We reasoned that a Holliday junction-containing substrate would be a good candidate for *in vitro* recombination mediated by XerC and XerD, since such molecules are intermediates in recombination mediated by other members of the lambda integrase family of recombinases (Hoess *et al.*, 1987; Kitts and Nash, 1987; Nunes-Düby *et al.*, 1987; Jayaram *et al.*, 1988; Meyer-Leon *et al.*, 1988), and have been shown to serve as *in vitro* DNA recombination substrates (Hsu and Landy, 1984; Hoess

Fig. 1. The Holliday junction-containing substrate. The substrate was assembled from four synthetic oligonucleotides: I, 92; II, 84; III, 84; and IV, 76 nt long. It contains two XerC and two XerD binding sites. The 28 bp core sequence 5'GGTGCCTACAAGGGATGTTATG-GTAAAT3' derives from the *cer6* recombination site in which the XerC binding site (the 11 5' nucleotides) and the XerD binding site (the 11 3' nucleotides) are separated by the 6 bp central region (thick line). The arm containing the 5' end of strand I carries *cer* site sequences upstream of the core region, other arms carry unrelated, non-homologous sequences. All four arms have cohesive ends with 5'GATC protruding. Diagnostic restriction sites and the relevant sequence distances are indicated. By convention, strands I and IV are designated 'top strands', while II and III are 'bottom strands'. Resolution of the substrate through exchange between the top strands would generate two linear 84 bp duplex molecules, while resolution through exchange between the bottom strands would produce 92 and 76 bp duplexes. The directions of top (T) and bottom (B) strand resolution are indicated by arrows.

et al., 1987; Jayaram *et al.*, 1988; Meyer-Leon *et al.*, 1990; Dixon and Sadowski, 1993). The Xer recombination-mediated accumulation of Holliday junction-containing molecules has been observed under certain experimental conditions *in vivo* (McCulloch *et al.*, 1994), suggesting that this molecule is an intermediate in Xer recombination as well. The use of a recombination substrate containing a Holliday junction alleviates the requirement for the potentially rate-limiting step of synapsis of two duplex segments, and seemed to provide the best available means to assess the relative roles of XerC and XerD in the recombination mechanism.

The structure of the Holliday junction-containing substrate is diagrammed in Figure 1; it contains the XerC and XerD binding sites of *cer* separated by a 6 bp central region (also see Figure 3C). This *cer6* core recombination site is identical to the core site of the 'type II hybrid' that was generated *in vivo* by recombination between *cer* and the plasmid CloDF13 homologue of *cer* (Summers, 1989). This core site recombines inter- and intramolecularly *in vivo* without accessory sequences and accessory proteins, but can respond to their presence, when recombination is predominantly intramolecular (Summers, 1989;

Fig. 2. Recombination of the Holliday junction-containing substrate requires the presence of both XerC and XerD. (A, B) Recombination in the presence of cell extracts enriched for XerC and/or XerD; (C) recombination in the presence of the purified protein fractions. Products of recombination reactions were analysed on 6% native (A) or 4% SDS-containing (B and C) polyacrylamide gels. Samples analysed on the gel in (A) were proteinase K-treated before electrophoresis. (A, B) The substrate was labelled at the 5' end of the top strands; lanes: 1, no extract added; 2, extract from cells lacking XerC and XerD; 3, XerC extract; 4, XerD extract; 5, XerC and XerD extracts. (C) The substrate was labelled at the 5' end of the bottom strands; lane 1, no extract; 2, extract lacking XerC and XerD; 3, XerC and XerD extracts; 4, XerC and XerD purified proteins. The various recombinant products are indicated. The symbols are HJ (Holliday junction), LD (linear duplex), HJCC (Holliday junction covalent complex), HJS (Holliday junction substrate), LDCC (linear duplex covalent complex) and RLD (rejoined linear duplex). The DNA species designated HJ and LD in (A) contain intact as well as cleaved DNA molecules which carry a small residual polypeptide at the 3' end.

J.Roberts and D.Sherratt, unpublished data). These properties, intermediate between *cer* and *dif*, made *cer6* a good candidate for initial *in vitro* studies. The junction was assembled from four synthetic oligonucleotides of 92, 84, 84 and 76 nucleotides; the arm that contains the 5' end of strand I carries 30 bp of *cer* accessory sequences, while the other arms contain unrelated sequences. Resolution of the junction by a pair of 'top' (I and IV) or 'bottom' (II and III) strand exchanges can easily be distinguished: the top strand exchanges would generate two linear duplex molecules of 84 bp, while the bottom strand exchanges would produce two linear fragments of 92 and 76 bp. The DNA substrate should be free to branch migrate throughout the whole 28 bp of the core region. This has been confirmed experimentally by analysing the products of digestion with the Holliday junction resolving enzyme RuvC (Dunderdale *et al.*, 1991) which cuts the junction at several positions throughout the core region (data not shown).

In order to determine the requirements for Xer recombination *in vitro*, the Holliday junction substrate was incubated with cell extracts enriched for XerC or XerD, or with the purified fractions of both proteins (Figure 2). Figure 2A shows a native polyacrylamide gel analysis of proteinase K-treated reactions in which a substrate 5' end-labelled on both top strands (I and IV) was incubated with

cell extracts. A single band of recombinant product was observed in reactions with extracts containing both XerC and XerD, but not with either recombinase alone. Comparison of the mobility of this product with the mobility of DNA size markers suggested that it was ~84 bp long. Digestion of DNA from recombination reaction with restriction enzymes *Bgl*III or *Not*I revealed that the recombinant 'band' consists of the two expected DNA fragments of the same size and in the same amounts (data not shown).

In order to visualize the presence of possible covalent protein–DNA complexes produced during the reaction, DNA from reactions which had not been proteinase treated was analysed on polyacrylamide gels containing sodium dodecyl sulphate (SDS; Figure 2B). Three recombinant product bands were observed. The fastest mobility band contains the 84 bp linear duplex DNA that has resulted from complete strand exchange (RLD; Rejoined Linear Duplex); two other bands represent covalent complexes of the protein with the linear duplex DNA (LDCC; Linear Duplex Covalent Complex) and with the Holliday junction DNA (HJCC; Holliday Junction Covalent Complex). Quantitative analyses of several experiments revealed that 42–56% of the substrate DNA was converted into the product during 30 min incubation (data not shown). The RLD constituted the majority of the converted DNA (48–78%); among the DNA–protein complexes, the LDCC was always more abundant (12–46%) than HJCC (5–15%).

Analysis of deproteinized DNA from the recombination reaction (as in Figure 2A, lane 5) on a sequencing gel confirmed the presence of the new 84 bp recombinant strands (data not shown). In addition, two other species, of much higher mobility were observed. Judging from their relative sizes, they were most likely fragments of parental strands I and IV that were cleaved but not religated. This result suggests that the covalent protein–DNA complexes observed on the SDS gel in Figure 2B resulted from cleavage of the top strands. The two cleaved strands were present in similar amounts and their proportion to religated recombinant strands was similar to the proportion of the covalent complexes to religated duplex analysed on the SDS gel shown in Figure 2B. To test whether covalent complexes resulting from cleavage of the bottom strands were present in the recombination reaction, experiments were performed with the Holliday junction substrate labelled at the 5' ends of strands II and III (Figure 2C, lane 3). Although both covalent complexes were present on the SDS gel in amounts similar to those observed in Figure 2B, cleaved bottom strands were not found on the sequencing gel (data not shown). The same recombinant products were generated when highly purified fractions of XerC and XerD were used instead of the cell lysates (Figure 2C, lane 4), indicating that XerC and XerD were sufficient for recombination on this substrate.

These experiments demonstrate that the *cer6* Holliday junction substrate can be recombined *in vitro* in the presence of XerC and XerD. The generation of two linear duplex molecules of the same size indicates that the junction has undergone one of the two possible pairs of strand exchanges: that involving the top strands. Neither cleavage nor exchange at the bottom strands was observed in these experiments.

Cleavages at the top strands occur at the junction of the XerC binding site and the central region

The recovery of recombinant products covalently attached to protein provided the opportunity to determine the sites of cleavage in the top strands. Since proteinase K treatment of the covalent complexes leaves behind a few amino acid residues attached to the 3' end of the DNA, the precise localization of the cleavage point required measuring the length of the cleaved derivatives of strands I and IV which contained the recombinase-generated 5' end. To do this, Holliday junction-containing substrates were 3' end-labelled either in all four strands or only in strand I or IV. DNA was incubated with XerC and XerD cell extracts under standard reaction conditions; reactions were stopped with SDS, deproteinized and resolved on a native polyacrylamide gel (not shown). The Holliday junction and linear duplex DNA bands (similar to those in Figure 2A, lane 5) were recovered and analysed on a sequencing gel (Figure 3A). Two bands that could represent the cleaved single-strand DNA fragments were visible when linear duplex DNA resulting from the recombination of four strand-labelled substrate was analysed (lane 4), but only one or the other was present when substrate was labelled at strand I or IV, respectively (lanes 5 and 6), thus allowing the identification of the two cleavage products. Comparison of their mobility with that of phosphorylated and dephosphorylated markers reveals that the cleavage products are 51 and 43 nt for strands I and IV, respectively, and likely contain a 5'-OH end. This result locates the sites of the first transesterification at the top strands to between the last nucleotide of the XerC binding site and the first nucleotide of the central region (Figure 3C). To confirm this, recombination was carried out on a substrate 3' end-labelled on top strand IV with terminal deoxynucleotidyl transferase. The electrophoretic mobility of the cleaved single-stranded DNA fragment was compared with the mobility of a 3' end-labelled oligonucleotide of the same length and sequence as that expected of the cleaved fragment (Figure 3B). The mobility of the two fragments was the same. Identical cleavage positions were observed on analysis of the Holliday junction DNA (Figure 3A, lanes 9–11).

XerC mediates the top strand cleavage and exchanges

Two independent approaches have been used to determine the catalytic requirements for the pair of top strand exchanges. One has used XerC and XerD proteins mutant at the amino acid residues which are believed to be involved in catalysis (Argos *et al.*, 1986; Abremski and Hoess, 1992; Lee *et al.*, 1992), while the other has used fusion derivatives of both recombinases with maltose-binding protein.

Three mutants of each protein were used. They carried substitutions in the putative active site tyrosine nucleophile (XerCY275F, XerDY279F) or in the conserved arginine of domain II (XerCR243Q, XerDR247Q), or both (XerCY275FR243Q, XerDY279FR247Q). It has been proposed that the domain II conserved arginine is involved in phosphodiester activation (Parsons *et al.*, 1988; Chen *et al.*, 1992; Friesen and Sadowski, 1992). The mutants were able to bind their respective DNA binding sites in *in vitro* band shift assays (data not shown). Recombination

Fig. 3. Localization of the site of first transesterification in the top strands. (A) The position of XerC-mediated cleavage. Recombinant DNA derived from 3' end-labelled substrates was fractionated on a preparative native polyacrylamide gel, and linear duplex and Holliday junction 'bands' (e.g. Figure 2A, lane 5) were extracted and analysed on a 10% denaturing polyacrylamide gel (lanes 4, 5, 6, 9, 10 and 11). Lanes 1, 2, 8 and 12 are size markers (phosphorylated and unphosphorylated) resulting from *RsaI* digestion of the substrate DNA labelled on all four strands. The length of the markers (nt) is indicated on the left side of the gel. Lanes 3 and 7 are Maxam–Gilbert AG modification-derived DNA from strands I and IV, respectively; the inset shows an autoradiograph overexposure that visualizes these markers clearly. Lane 4, linear duplex product from the substrate labelled in all four strands (A); lane 5, linear duplex from substrate labelled in strand I (I); lane 6, linear duplex from substrate labelled in strand IV (IV); lane 9, Holliday junction DNA from substrate labelled in all four strands (A); lane 10, Holliday junction from substrate labelled in strand I (I); lane 11, Holliday junction from substrate labelled in strand IV (IV). The lengths of the cleaved products are indicated on the right side of the gel. The cleaved fragments run slightly behind the Maxam–Gilbert generated fragments, suggesting that they contain 5'-OH ends. More than one species of either recombinant or marker DNA is observed in all the lanes; this is most likely due to non-uniform end-labelling of the Holliday junction substrate with the Klenow fragment of polymerase I. (B) Confirmation of the position of XerC-mediated cleavage on the top strand. Recombinant products derived from a substrate in which strand IV was 3' end-labelled with [α - 32 P]ddATP. The reaction was analysed on a 10% denaturing polyacrylamide gel (lane 2) alongside an [α - 32 P]ddATP-labelled oligonucleotide of the same length and sequence as the expected cleaved fragment (lane 3). Lane 1 shows unreacted substrate. The arrow indicates the cleaved product and marker. (C) Core sequence of the *cer6* recombination site. The arrow indicates the position of the XerC-mediated cleavage on the top strand.

reactions were performed with cell extracts enriched for each mutant XerC protein in combination with wild-type XerD or for each mutant XerD protein in combination with wild-type XerC (Figure 4A and B). No recombinant products were observed when any of the mutant XerC substituted for the wild-type XerC protein (Figure 4A and B, lanes 6–8); however, products similar to those observed in the presence of both wild-type proteins (lane 2) were present when XerD mutants were added instead of the wild-type XerD (lanes 3–5). These results show that XerC catalyses the top strand exchanges in the Holliday junction substrate and that although XerD needs to be present, it does not contribute the presumptive active site tyrosine or the active site arginine of domain II. We therefore

believe that XerD is not catalytically involved in the strand exchanges that occur in this reaction.

The result that catalytic activity of XerC alone was sufficient to mediate one pair of strand exchanges suggests that both the activation and the nucleophilic attack at the scissile phosphodiester bond during the first transesterification reaction come from XerC. Are both of these activities donated by the same XerC molecule, or is cooperation of two protomers of the protein, one contributing the activation function and the other the nucleophile, necessary for this reaction, as has been proposed for FLP (Chen *et al.*, 1992, 1993)? If two protomers are involved in the reaction, it should be possible to complement an activation-proficient mutant that lacks the nucleophile

Fig. 4. Recombination of the Holliday junction-containing substrate in the presence of mutant XerC and XerD. (A) 4% SDS-containing polyacrylamide gel; (B) 6% native polyacrylamide gel. (A) and (B) Lane 1, no extract; lane 2, XerC and XerD; lane 3, XerC and XerDY279F; lane 4, XerC and XerDR247Q; lane 5, XerC and XerDY279FR247Q; lane 6, XerCY275F and XerD; lane 7, XerCR243Q and XerD; lane 8, XerCY275FR243Q and XerD. The DNA-binding activity in extracts containing XerC or XerD mutants was compared with the binding activity in the wild-type protein extracts by gel retardation assay using a linear DNA fragment carrying the *cer6* core site sequence (not shown). The recombinant products are as indicated (see the legend to Figure 2).

tyrosine, XerCY275F, with an activation-deficient mutant carrying the active site tyrosine, XerCR243Q. Such complementation was attempted in the presence of various relative concentrations of cell extracts of both XerC mutants and wild-type XerD extract. Note that if such complementation occurred, only one catalytic unit would be present per substrate molecule; therefore, only cleaved covalent complexes of the protein with Holliday junction DNA would be expected. DNA from the reactions was analysed on a polyacrylamide gel containing SDS to visualize the products of first transesterification trapped in the covalent protein–DNA complexes (Figure 5).

Fig. 5. Lack of recombination with XerCY275F and XerCR243Q in the presence of XerD. DNA was analysed on a 4% SDS-containing polyacrylamide gel. Lane 1, no extract; lane 2, XerD and XerC; lane 3, XerD and XerCY275F; lane 4, XerD and XerCR243Q; lanes 5–8, XerD and XerCY275F/XerCR243Q in the following ratios of binding activity (XerCY275F:XerCR243Q): lane 5, 6:1; lane 6, 2:1; lane 7, 1:1.5; lane 8, 1:2.5. The DNA-binding activity in extracts containing XerCY275F and XerCR243Q mutants was compared by gel retardation assay using a DNA fragment carrying the *cer6* core site sequence (not shown).

However, no recombinant products were observed (lanes 5–8), even after long exposures of the gel (data not shown), indicating that XerCR243Q could not complement XerCY275F under these experimental conditions. One possible interpretation of this result is that the nucleophilic attack in the Xer system may not be delivered *in trans*. This would be consistent with the failure to observe cleavage of the Holliday junction substrate by the external nucleophile, tyramine, in the presence of XerCY275F (data not shown; e.g. see Lee and Jayaram, 1993). However, lack of complementation could be also explained by insufficient protein levels in the reactions, the inability of XerCY275F to activate the scissile bond, or the inability of XerCR243Q mutant to present the nucleophile tyrosine, or because the interactions between the two mutant protomers are different than those between the two wild-type protomers of XerC. We have observed that the XerCR243Q protein gives complexes with *cer6* and XerD that have reduced mobility compared with the complexes containing wild-type XerC or XerCY275F (data not shown). The significance of this fact for the interpretation here is not clear.

The results presented above suggest that XerC catalyses the exchange of the top strands in the *cer6* Holliday junction substrate. It would therefore be expected that XerC is a protein component of the observed covalent DNA–protein complexes that arise upon the first transesterification of the top strands. To examine which of the

Fig. 6. Recombination of Holliday junction-containing substrate in the presence of maltose-binding XerC (MBPXerC) and XerD (MBPXerD) fusion proteins. Recombinant DNA was analysed on a 4% polyacrylamide gel containing SDS. Lane 1, no extract; lane 2, extract lacking XerC and XerD; lane 3, XerC; lane 4, XerD; lane 5, MBPXerC; lane 6, MBPXerD; lane 7, XerC and MBPXerD; lane 8, MBPXerC and XerD; lane 9, MBPXerC and MBPXerD; lane 10, XerC and XerD. The covalent DNA–protein complexes are indicated.

two recombinases is present in these complexes, fusion proteins of XerC and XerD with maltose-binding protein (MBPXerC and MBPXerD) were used in recombination experiments. Complexes with MBP fusion proteins contain an additional 43 kDa per protein molecule and would, therefore, be expected to have reduced mobility during PAGE compared with the complexes carrying wild-type protein. The results of this analysis are presented in Figure 6. The cell extracts enriched in MBPXerC and MBPXerD could support a complete recombination between the top strands, as evidenced by the presence of a religated linear duplex product (Figure 6, lane 9). In addition, covalent complexes (HJCC and LDCC) of reduced mobility, as compared with those produced in the presence of wild-type XerC and XerD, were observed in this reaction (compare lanes 9 and 10). Similar mobility complexes were observed in the presence of MBPXerC and XerD, but not with XerC and MBPXerD (lanes 8 and 7, respectively), suggesting that XerC or MBPXerC are attached to DNA in the covalent complexes. These results show that XerC provides the nucleophile in the first transesterification of the top strand exchanges. Taken together with the previous results, we conclude that the top strand cleavages and exchanges are catalysed by XerC.

Discussion

Xer site-specific recombination functions in the normal segregation of the *E. coli* chromosome and in the stable inheritance of its multicopy plasmids. We believe that homologues of this system are broadly distributed in bacteria and have the same function. It seems reasonable that this type of system appeared early during the evolution of bacteria with circular replicons, and may have provided the genetic material from which integrase family recombinases with other functions have evolved. In order to understand how Xer recombination functions in circular replicon segregation, we need to characterize the system structurally and mechanistically *in vitro*, to confirm that the *in vitro* structures and mechanisms are relevant *in vivo*, and to show how the operation of this system relates temporally and spatially to cellular metabolism.

Earlier studies of Xer recombination *in vivo* revealed the need for two recombinases, XerC and XerD, each encoded by a distinct segment of the *E. coli* chromosome (Blakely *et al.*, 1993). Here we show that these two proteins can mediate recombination on a Holliday junction-containing substrate. The use of two recombinases by this system provides a powerful tool to understand the precise function of each of the recombinase molecules in reactions mediated by a member of the lambda integrase family of enzymes. The fact that Holliday junction molecules are recombination substrates lends support to the view that such structures are intermediates in the normal *in vivo* recombination reaction (McCulloch *et al.*, 1994). As with the other characterized integrase family systems, supercoiling is not necessary to promote *in vitro* strand exchange in such substrates (Hsu and Landy, 1984; Hoess *et al.*, 1987; Jayaram *et al.*, 1988; Meyer-Leon *et al.*, 1990; Dixon and Sadowski, 1993). In the substrate used here, we have only observed one of the two pairs of strand exchanges. This pair of top strand exchanges is catalysed by XerC, and the XerC-mediated cleavage that we believe leads to this strand exchange occurs adjacent to the XerC binding site on each of the top strands. The position and chemistry of this cleavage corresponds to that determined for other lambda integrase recombinases (Craig and Nash, 1983; Andrews *et al.*, 1985; Hoess and Abremski, 1985; Senecoff *et al.*, 1985). The strand exchanges described here involve the same strands as those whose exchange *in vivo* generated supercoiled Holliday junction-containing molecules from a substrate containing two directly repeated *cer* sites (McCulloch *et al.*, 1994). The *in vitro* reaction, therefore, is equivalent to the conversion of a Holliday junction intermediate back to duplex substrate.

The failure to see the bottom pair of strand exchanges or bottom strand cleavages on this substrate is perplexing. We would expect XerD to be catalytically involved in this pair of strand exchanges and have inferred from *in vivo* experiments that such exchanges occur during normal recombination in the cell (Blakely *et al.*, 1993). This failure could be explained by inactivity of our XerD preparations, the requirement for specific cofactor(s) or conditions for XerD exchange, or the requirement for a specific substrate structure or conformation. For example, the Holliday junction substrate, which should be in the square planar configuration in recombination buffer before the addition of recombinase (Lilley and Clegg, 1993),

might adopt either of two stacked helical isoforms upon addition of recombinase; if only one of these is produced or is active in recombination, then only one of the two pairs of strand exchanges would be observed. The requirement for specific substrate conformation or structure appears to be the most likely explanation, since we have recently observed (S.Colloms, N.Ganguly, G.Blakely and D.Sherratt, unpublished) that the same XerD preparations as used here will mediate bottom strand cleavage or strand exchanges (when in the presence of XerC) on substrates whose recombination cores have different sequences and structures. The recombination cores of these substrates, like those used here, are flanked by sequences different to those in natural sites, indicating that all sequences necessary for both pairs of strand exchanges are contained in the recombination core site. Since it appears likely that each pair of strand exchanges requires the catalytic activity of a different recombinase, differential biochemical control of the two pairs of strand exchanges is possible and may be important in the *in vivo* role of this recombination system. Differential control may also be reflected by our observations here of a single pair of strand exchanges, and by results *in vivo* which infer that XerC exchanges can occur in the absence of XerD exchanges (McCulloch *et al.*, 1994).

In addition to the linear duplex recombinant products in which the strand exchanges have been completed, we observed in our reactions a substantial proportion of covalent recombinase–DNA complexes (ranging from ~10% to almost 50% of the total recombinant product; Figure 2B and data not shown). One type of complex contained Holliday junction molecules in which top strand esterification had occurred, while the other contained linear duplex DNA that had resulted from esterification at both top strands of the substrate. The majority of the recombinase-bound DNA was in linear duplex complexes. We believe that the complexes are intermediates in the top strand exchange reaction because they are not present in reactions with XerC mutants; their mobility is changed in the predicted way when maltose-binding protein derivatives of XerC are substituted for XerC; and because the cleavages occur at the predicted positions by comparison with other systems. The ready observation of the XerC recombinase–DNA complexes contrasts with the situation in other lambda integrase systems in which the capture of these intermediates has proved to be difficult unless special steps are taken to trap such molecules (Craig and Nash, 1983; Hoess and Abremski, 1985; Nunes-Düby *et al.*, 1987; Kitts and Nash, 1988; Pargellis *et al.*, 1988). Our ability to detect complete and partial recombination products provides a useful analytical tool; it could reflect a relative uncoupling of cleavage and religation that is intrinsic to the Xer system, or that arises as a consequence of our reaction conditions.

The experiments with mutants of XerC and XerD show that two presumptive active site residues of XerD are not required for the XerC-mediated cleavage or exchange of the top strands. Therefore, XerC and XerD do not cooperate to form a single active site in these reactions. This excludes mechanisms equivalent to the 'trans-diagonal' and 'trans-horizontal' models proposed by Jayaram and co-workers for FLP based on the observations that at least two recombinase molecules are involved catalytically in a

single cleavage and exchange (Chen *et al.*, 1992, 1993; Jayaram, 1994). Our results do not exclude a mechanism equivalent to the 'trans-vertical' model of these workers; this would require the active site cooperation of two XerC molecules. Nevertheless, because we have failed to observe complementation between XerCY275F, which lacks the tyrosine nucleophile and the presumptive activation-deficient XerCR243Q, a mechanism in which a single recombinase molecule acts *in cis* seems most likely. Action *in cis* has recently been shown for lambda integrase acting on a Holliday junction substrate *in vitro* (Nunes-Düby *et al.*, 1994), although earlier data using linear 'suicide' substrates had demonstrated *trans*-cleavage (Han *et al.*, 1993). *Cis* or *trans* cleavage do not necessarily imply a fundamental difference in catalytic mechanism [see Nunes-Düby *et al.* (1994) for discussion]. It may be that FLP, on the substrates used, has the active site tyrosine of a given molecule positioned incorrectly for *cis* attack of the phosphodiester activation 'pocket' of that protein–DNA complex. This allows an exogenous nucleophile or the tyrosine from another protomer to attack the activated phosphodiester. With XerC and lambda Int, a given active site tyrosine may be positioned so that it can act on its own phosphodiester activation pocket, thus preventing an external nucleophile from attacking. Tyrosine nucleophile positioning could be an invariant product of the primary recombinase structure or could vary depending on the particular nucleoprotein complex that contains it. We note that with gamma-delta resolvase, the serine nucleophiles of a dimer bound to the *res* substrate appear not to be correctly positioned to attack the scissile phosphodiester bonds and presumably conformational changes that occur during productive synapse formation lead to a correct positioning of the nucleophiles (Rice and Steitz, 1994). Such processes may help ensure that strand cleavages are not normally initiated until the appropriate complex is formed, and may be a general feature of recombinase/transposase-mediated rearrangement systems.

Materials and methods

Strains and plasmids

The host used to harbour wild-type and mutant XerC- and XerD-overproducing plasmids was *E.coli* DS9009, a *xerC*[−], *xerD*[−] derivative of DS941 (Blakely *et al.*, 1993). The maltose-binding protein fusion derivatives of XerC and XerD were produced from plasmids in TB1 [*araΔ(lacproAB) rpsL(φ80lacZΔM15) hsdR*]. The plasmids overproducing various XerC and XerD variants are listed in Table 1.

For the construction of pGB500, the *xerC* gene was amplified by the polymerase chain reaction (PCR) using pSDC104 as a template (Blakely *et al.*, 1993). The primers were designed in such a way that an *EcoRI* restriction site was introduced at the 5' end of the coding region of *xerC* (the first ATG codon of *xerC* has been replaced) and a *HindIII* restriction site was introduced at the 3' end of the gene. The amplified DNA fragment was digested with *EcoRI* and *HindIII* and, after gel purification, cloned directly into pMAL-c2 vector (New England Biolabs). For the construction of pGB501, an *EcoRI* restriction site was introduced in front of the 5' end of the coding sequence of the *xerD* gene. This involved PCR amplification of the entire plasmid pRM132 (Blakely *et al.*, 1993) using primers that introduced three extra base pairs at each end of the newly generated linear fragment. These six base pairs produced upon plasmid religation an *EcoRI* site in front of the ATG codon of the *xerD* gene. The amplified *xerD* gene in this plasmid supported Xer recombination *in vivo* (not shown). The *EcoRI*–*BamHI* fragment from this plasmid that contained the *xerD* gene was subsequently subcloned into the pMAL-c2 vector.

pLA130, pLA137, pLA138 and pLA134 were constructed following

Table I. Plasmids

Plasmid	Recombinase produced	Reference
pSDC104	XerC	Blakely <i>et al.</i> (1993)
pLA121	XerCY275F	Blakely <i>et al.</i> (1993)
pLA130	XerCR243Q	this work
pLA138	XerCY275FR243Q	this work
pRM130	XerD	Blakely <i>et al.</i> (1993)
pLA137	XerDY279F	this work
pLA127	XerDR247Q	Blakely <i>et al.</i> (1993)
pLA134	XerDY279FR247Q	this work
pGB500	MBPXerC	this work
pGB501	MBPXerD	this work

All plasmids (except pGB500 and pGB501) are derived from pTZ18R vectors (Pharmacia) in which recombination is expressed from the *plac* promoter. The pGB500 and pGB501 are derived from pMal-c2 vector (New England Biolabs); the fusion proteins are under the control of *ptac* promoter.

exactly the procedure described in Blakely *et al.* (1993). The plasmids pLA138 and pLA134, which carry double mutations in the *xerC* and *xerD* genes, were generated using template plasmids pLA130 and pLA127, respectively, that already contain arginine to glutamine mutation. The amplified fragments of the *xerC* and *xerD* gene used in the constructions were sequenced to ensure the absence of any additional substitutions. PCR amplifications were carried out using Vent polymerase (New England Biolabs). Reaction conditions and the sequences of the primers used are available upon request.

Preparation of a Holliday junction-containing substrate

The four oligonucleotides used to assemble Holliday junction DNA were: I, 92 nt (5'-GATCCTGCCGTGATCAGCTGAACGCGTTTACGCGTGCGTACAAGGGATGTTATGGTAAATGGCCTAACGCCTAACGCGCCGCTAGTCC); II, 84 nt (5'-GATCGGACTAGGCGGCGCTTTAGGCGTTAGGCCATTTACCATAACATCCCTGTACGCACCCGACCGTTGCCGGATCCGCTGC); III, 84 nt (5'-GATCGGATTCGAGATCTCAGGATGTCATTTACCATAACATCCCTGTACGCACCGCTAAACGCGTTACGCGTATCAGCGCAG); IV, 76 nt (5'-GATCGCAGCGGATCCGGCAACGGTCCGGTGCGTACAAGGGATGTTATGGTAAATGACATCCTGAGATCTCGAATCC). The oligonucleotides were synthesized on an Applied Biosystems DNA synthesizer and purified on 10% denaturing polyacrylamide gels. The oligonucleotides (10 pmol) were 5' end-labelled with [γ - 32 P]ATP using T4 DNA polynucleotide kinase and mixed with the other unlabelled oligonucleotides (10 pmol each). The reannealing was conducted in 10 mM Tris-HCl (pH 8.0), 1 mM EDTA buffer by heating to 65°C and slow cooling. The Holliday junction-containing molecules were purified on a 6% polyacrylamide gel, recovered from excised gel slices by electroelution, precipitated and resuspended in 10 mM Tris-HCl (pH 8.0), 1 mM EDTA buffer. The Holliday junction substrate used in the strand cleavage mapping experiment was assembled using cold oligonucleotides (10 pmol each); all four strands were then labelled at the 3' ends with [α - 32 P]dATP using the DNA polymerase I Klenow fragment. In order to obtain the substrate labelled only at the 3' end of strand I or IV, the four strand-labelled substrate was digested with the restriction enzymes *Sau3AI* (removes 3' end of strands II, III and IV) or with *BclI*, *NorI* and *BamHI* (remove 3' end of strands I, II and III), respectively. The Holliday junction-containing molecules were purified on a 6% polyacrylamide gel and resuspended in TE buffer. The 3' end-labelled strands I and IV used in Maxam-Gilbert sequencing reactions were obtained after fractionation of a substrate labelled on all four strands on a denaturing polyacrylamide gel. The Holliday junction substrate used in the experiment shown in Figure 3B was labelled at the 3' end of strand IV with [α - 32 P]dATP and terminal deoxynucleotidyl transferase; the 39 nt oligonucleotide 5'-GGGATGTTATGGTAAATGACATCCTGAGATCTCGAATCC used as a size marker in this experiment was end-labelled in the same way.

Cell extracts and purified protein fractions

Cell extracts enriched for wild-type or mutant XerC or XerD were prepared from DS9009-derived strains carrying overproducing plasmids listed in Table I. A cell extract depleted for XerC and XerD was prepared from DS9009. Aliquots (10 ml) of LB medium supplemented with 1% glucose and appropriate antibiotics (50 µg/ml ampicillin and 50 µg/ml

kanamycin for strains carrying plasmids or 50 µg/ml kanamycin and 25 µg/ml chloramphenicol for DS9009) were inoculated and incubated overnight at 37°C. Cultures were diluted (50- to 100-fold) in fresh medium as above, grown to early mid-log phase at 37°C, spun, resuspended in the same medium lacking glucose and induced with isopropylthio- β -galactoside (IPTG) at 1 mM overnight, at either room temperature or 30°C. Cells were spun, washed and resuspended in cell lysis buffer (3 ml buffer/1 g cells) supplemented with phenylmethylsulphonyl fluoride (PMSF, 1 mM) and lysozyme (400 µg/ml), and incubated on ice for 30 min. After the incubation, cells were disrupted by three cycles of freezing and thawing, and cell debris were removed by centrifugation at 48 000 g. Glycerol was added to collected lysate to 25% v/v. The final composition of the cell lysis buffer was 50 mM Tris-HCl (pH 7.5), 100 mM NaCl, 1 mM EDTA and 25% glycerol. The total protein content in the extracts was ~7 mg/ml. Extracts enriched for XerC and XerD fusion proteins with maltose-binding protein were prepared according to the procedure for crude extracts, as suggested by New England Biolabs. The extracts were supplemented with glycerol to 50% volume. The total protein content in these extracts was 0.35–0.40 mg/ml, of which a high proportion was XerC or XerD fusion protein (G.Blakely and D.Sherratt, unpublished). The preparation of purified fractions of XerC and XerD will be described in detail elsewhere (K.Grant and D.Sherratt, unpublished). In outline, nucleic acids were removed from centrifuged lysates (above) by DEAE-Sephacell chromatography. The resulting recombination-containing fractions were then applied either to MonoS or FastS column (Pharmacia). Purified recombination was obtained after elution by a 0–1 M NaCl linear gradient [in 50 mM Tris-HCl (pH 7.5), 10 mM NaCl, 1 mM EDTA], made 50% in glycerol and stored at -70°C. The purified fractions contained ~25% of XerC and 60% XerD.

Recombination reactions

Recombination reactions were run in 20 mM Tris-HCl (pH 7.5), 50 mM NaCl, 1 mM EDTA and 0–10% glycerol, and contained ~2.5 ng of labelled DNA, 500 ng of poly(dI-dC):poly(dI-dC) and 2 µl of protein cell extracts per 10 µl reaction. Reactions that contained purified protein fractions were supplemented with bovine serum albumin (BSA) at 100 µg/ml. Following 30 min incubation at 37°C (or room temperature), the reactions were stopped by addition of 2 µl of SDS buffer [50 mM Tris-HCl (pH 7.5), 1% SDS and 0–10% glycerol], and when necessary deproteinized by the addition of 1 µl of proteinase K stock solution (4 mg/ml). Reactions were scaled up when required. The recombinant DNA was analysed on native TBE [89 mM Tris-borate (pH 8.3), 2 mM EDTA] polyacrylamide gels or on TBE gels containing SDS (0.1%) at 200 V for 2 h. The gels were dried and exposed to X-ray film or analysed on a phosphorimager.

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