Action of site-specific recombinases XerC and XerD on tethered Holliday junctions

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In Xer site-specific recombination, two related recombinases, XerC and XerD, mediate the formation of recombinant products using Holliday junction-containing DNA molecules as reaction intermediates. Each recombinase catalyses the exchange of one pair of specific strands. By using synthetic Holliday junction-containing recombination substrates in which two of the four arms are tethered in an antiparallel configuration by a nine thymine oligonucleotide, we show that XerD catalyses efficient strand exchange only when its substrate strands are 'crossed'. XerC also catalyses very efficient strand exchange when its substrate strands are 'crossed', though it also appears to be able to mediate strand exchange when its substrate strands are 'continuous'. By using chemical probes of Holliday junction structure in the presence and absence of bound recombinases, we show that recombinase binding induces unstacking of the bases in the centre of the recombination site, indicating that the junction branch point is positioned there and is distorted as a consequence of recombinase binding.

Keywords: Holliday junctions/XerC/XerD/Xer site-specific recombination

Introduction

Holliday junction-containing DNA molecules (Holliday, 1964) are intermediates in homologous recombination (reviewed in West, 1992; Kowalczykowski *et al.*, 1994; Eggleston and West, 1996) and in site-specific recombination mediated by the λ integrase family of site-specific recombinases (reviewed in Landy 1989, 1993; Stark *et al.*, 1992). In homologous recombination, a range of different proteins recognize Holliday junctions with little or no sequence specificity, and either cleave the junctions to generate 'recombinant' or 'non-recombinant products', or promote branch migration of the junction (Duckett *et al.*, 1988; Mueller *et al.*, 1988; Lu *et al.*, 1991b; Bennett and West 1995a; West, 1996; White and Lilley, 1996). In contrast, integrase recombinases are sequence-specific DNA-binding proteins that recognize recombination site DNA whether it is in linear duplex or a Holliday junction-containing molecule, and mediate the strand exchange reactions at precisely defined positions.

A substantial body of work has led to insight into

Holliday junction structure and branch migration (Lilley and Clegg 1993a,b; Panyutin and Hsieh, 1994; Seeman and Kallenbach, 1994; Panyutin *et al.*, 1995). In the absence of metal ions, Holliday junctions adopt an unstacked square configuration that shows 4-fold symmetry and readily allows branch migration. In this configuration, the bases at the branch point of the junction are sensitive to chemical probes, such as osmium tetroxide or potassium permanganate, that react with unpaired or unstacked thymine residues (Duckett *et al.*, 1988; Bennett and West, 1995b). In the presence of metal ions, the junction folds into a 2-fold symmetric antiparallel right-handed X structure in which the helices of the arms are coaxially stacked pairwise onto each other and the helical stacks are rotated at an angle of $\sim 60^\circ$ to each other (Figure 1A). Branch migration of these structures is slow. Parallel isoforms of Holliday junctions are also possible, but they are energetically less favourable (von Kitzing *et al.*, 1990; Lu *et al.*, 1991a).

In the stacked antiparallel right-handed X structure, the two 'continuous' strands run in antiparallel orientation to each other along the axes of the stacked X arms (Figure 1A). The other two strands exchange between the two helical stacks at the branch point and have been called 'discontinuous', 'exchanging' or 'crossed'. Here, we refer to them as 'crossed' strands in order to avoid confusion with strands that contain nicks (discontinuous) or strands that undergo exchange during recombination (exchanging). Depending on the choice of helical partners during stacking, the junction can form two different isoforms in which a specific pair of strands is either continuous or crossed (Figure 1A).

Xer site-specific recombination, which functions in the stable inheritance of circular replicons, is an atypical member of the integrase family because, instead of using a single recombinase, it uses two related recombinases, XerC and XerD, each of which catalyses the exchange of one specific pair of strands (Blakely *et al.*, 1993, 1997; Colloms *et al.*, 1996; reviewed in Sherratt, 1993; Sherratt *et al.*, 1995). Xer recombinases act on a variety of natural sites present in plasmids (e.g. *psi* present in plasmid pSC101; Cornet *et al.*, 1994; Colloms *et al.*, 1996) and at the site, *dif*, present in the replication terminus region of the *Escherichia coli* chromosome (Blakely *et al.*, 1991; Kuempel *et al.*, 1991).

Our current view of the Xer recombination mechanism is shown schematically in Figure 1B, which shows recombination sites having a 6 bp *dif* central region. On supercoiled substrates containing two directly repeated copies of *psi*, strand exchange is sequential, with XerC mediating the first pair of 'top' strand exchanges to generate a Holliday junction-containing intermediate. Then XerD catalyses exchanges between the 'bottom' strands to generate recombinant products. By convention, we term

Fig. 1. (A) The two isoforms of the right-handed antiparallel stacked X Holliday junction. In the isoform on the left, top strands are crossed (dark blue and red), while in the other isoform the bottom strands are crossed (light blue and yellow). The junction crossover is positioned in the middle of the 6 bp central region. The positions of cleavages of XerC and XerD (open and filled circles, respectively) are mapped onto the phosphate backbone. The arms bound by XerC and XerD are indicated. Note the proximity of XerC cleavage sites in the isoform in which top strands are crossed and the identical proximity of XerD cleavage sites in the isoform in which the bottom strands are crossed. The colour code is as follows: strand I, dark blue; II, light blue; III, yellow; and IV, red. (B) A scheme for Xer recombination adapted from the model of Nunes-Düby *et al.* (1995) for λ Int. Binding of recombinases is followed by synapsis, shown with the *dif* recombination sites arbitrarily in antiparallel orientation. XerC mediates the first pair of strand exchanges between the top strands (step a, expanded in the box; McCulloch *et al.*, 1994; Colloms *et al.*, 1996). As with other λ family recombinases, XerC catalyses cleavage and rejoining in a two-step transesterification reaction in which the conserved tyrosine acts as the initial nucleophile generating a 3' phosphorylated protein-DNA complex. Three nucleotides of each of the top strands are exchanged between the partners in a single swap and then the terminal 5' OH of the partner strand acts as the nucleophile in the second transesterification step. The branch point of the generated Holliday junction is located in the middle of the central region and the top strands are crossed. Resolution of the junction to products by XerD-mediated exchange between the bottom strands (step d) requires isomerization (steps b + c) to adopt a configuration in which bottom strands are crossed. During isomerization, any cross-core XerC-XerD contacts must be broken and contacts with the new partners need to be re-made. The arrows indicate positions of XerC and XerD cleavages and rejoining; Y depicts the tyrosine nucleophile.

the pair of strands exchanged by XerC as the 'top' strands and the pair of strands exchanged by XerD as the 'bottom' strands (McCulloch *et al.*, 1994; Arciszewska and Sherratt, 1995; Blakely and Sherratt, 1996; Colloms *et al.*, 1996). We do not know if there is a preferred order of strand exchange with *dif*, though, in the cartoon, we have shown the XerC-mediated exchange occurring first.

Key issues in understanding the mechanism of strand exchange during integrase family site-specific recombination are the roles of Holliday junction branch migration and isomerization in the recombination process. This and the accompanying paper by Azaro and Landy address the question of how Holliday junction structure and position determine which of the two pairs of strand exchanges is to occur.

Earlier work on recombination mediated by the λ integrase enzymes was interpreted as suggesting that after the first pair of strand exchanges the Holliday junction branch point was located where cleavage and rejoining

had occurred (reviewed in Stark *et al.*, 1992; Landy, 1993; Nunes-Düby *et al.*, 1995). Branch migration, accompanied by isomerization, would then move the junction 6–8 bp to the position of the second pair of cleavages, thereby allowing recombination to be completed.

Recent data from experiments using substrates in which branch migration is constrained led to the idea that the Holliday junction branch point need not be at the position of strand cleavage for efficient strand exchange to occur (Dixon and Sadowski, 1994; Arciszewska *et al.*, 1995; Nunes-Düby *et al.*, 1995). The 'strand swapping' model for λ integrase recombination (Nunes-Düby *et al.*, 1995) proposes that the Holliday branch point is located close to the middle of the 7 bp region between the two cleavage points. During Holliday junction formation and resolution, 2 or 3 bp of each partner duplex is swapped in a single step during each pair of strand exchanges, and branch migration is limited to 1–3 bp. This avoids the need for extensive branch migration and the associated helical

rotation, though isomerization is still expected since it provides an equivalent stereochemical environment for each pair of strand exchanges (Figure 1A; Stark *et al.*, 1989). In the model, strands that are crossed, or become crossed on Holliday junction formation, are exchanged by strand swapping. Figure 1B shows this model when applied to Xer recombination. In this case, branch migration may not be required since the Holliday junction branch point could be located in the middle of the 6 bp central region after swapping of three nucleotides, though isomerization might still be necessary to allow both pairs of strand exchanges.

Here, we use synthetic Holliday junction-containing substrates carrying the recombination site *dif*, derived from the *E. coli* chromosome. Two substrates contain a nine thymine oligonucleotide tether to constrain the junction into one or the other of two antiparallel isoforms. Our results suggest that XerD strand exchange occurs only if its substrate strands are initially in the crossed configuration. XerC also efficiently exchanges crossed strands, though apparently it is able to mediate less efficient recombination on strands that were initially continuous. Binding of the recombinases to the Holliday junctions distorts the branch point and localizes it to the centre of the core recombination site.

Results

Structure of tethered Holliday junction-containing substrates

Two antiparallel tethered *dif* Holliday junction-containing substrates were constructed. In TSX the top strands are crossed, while in BSX the bottom strands are crossed (Figures 1A and 2A and B). A nine thymine oligonucleotide tether ensures that these molecules adopt only one of the two possible antiparallel isoforms and cannot isomerize to a parallel configuration (Kimball *et al.*, 1990). The 28 bp core recombination site of TSX and BSX is identical to that of the untethered *dif* Holliday junction, and branch migration can occur throughout this region (see below). The heterologous arms outside of the core site are of similar size and sequence in the tethered and untethered substrates. The expected products of the recombinase-mediated strand exchanges in the tethered substrates are shown in Figure 2C.

Tethered molecules are expected to have properties similar to untethered Holliday junctions; for example it has been shown that in the presence of metal ions tethered junctions adopt a conformation close to that of the untethered right-handed stacked X form, since they exhibit similar sensitivities to osmium tetroxide and hydroxyl radical attack (Kimball *et al.*, 1990; Bhattacharyya *et al.*, 1991). The interactions of T4 endonuclease VII and RuvC with tethered and untethered Holliday junction substrates were shown to be similar (Bhattacharyya *et al.*, 1991; Bennett and West, 1995a). Below, we extend these observations by demonstrating that XerC and XerD interactions with tethered and untethered Holliday junctions are also very similar.

The products of recombinase-mediated catalysis on tethered Holliday junction substrates

Incubation of XerC and XerD with substrate TSX gives a single major product of recombination (Figure 3A),

Fig. 2. (A) The structure of the untethered Holliday junction-containing substrate. The substrate carries two 28 bp *dif* core recombination sites. The two XerC- and two XerD-binding sites (each 11 bp, filled and hatched ovals, respectively) flank 6 bp central regions. The lengths of the arms outside the core region are indicated. The sequences of these arms are unrelated to each other, allowing the junction to migrate only within the limits of the core region (Arciszewska *et al.*, 1995). Strands I and IV are designated 'top' strands (thick lines), while II and III are 'bottom' strands (thin lines); designation of arms follows the numbers of strands which contribute the 5' end to the arm's terminus, for example, arm I contains the 5' end of strand I. Exchange of the top (T) strands generates two linear duplex molecules, each of 76 bp; while exchange of bottom strands (B) produces linear duplexes of 84 and 68 bp. XerC cleavage positions are indicated by the solid arrowheads, while expected XerD cleavage positions are shown by open arrowheads. The junction molecule is presented in unstacked square conformation. (B) The structure of tethered Holliday junction substrates, TSX (top strands crossed) and BSX (bottom strands crossed). In TSX, arms III and IV, while in BSX arms I and III, are brought together. To allow assembly of these substrates from synthetic oligonucleotides, each of them contains a nick (indicated by an arrow) in one of the arms located 18 nucleotides away from the oligo(T) tether. It was anticipated that the nick would not have a significant effect on junction structure or recombination, since it is outside of the core recombination site. Filled circles indicate positions of the label in substrates used in recombination experiments presented below. (C) The products of exchanges between a pair of top and a pair of bottom strands in TSX and BSX substrates. The lengths (nucleotides) of recombinant strands are indicated.

whose electrophoretic mobility is consistent with it having resulted from a pair of top strand exchanges (Figure 2C). Reactions using catalytically inactive mutants XerCY275F or XerDY279F show, as expected, that the recombinant product is generated by XerC. The presence of the recombinant strand was confirmed on sequencing gels (data not shown). The equivalent recombinant product of XerC strand exchange was seen on the untethered *dif* substrate (HJ). All of the detectable product of XerC strand exchange on TSX had retained the tether. If a TSX substrate had become untethered prior to recombination by dissociation of the 3' end of the strand II tether from

strand IV, a linear product containing 54 nucleotides on one strand and 76 nucleotides on the other strand would have been generated. This product has not been observed in our experiments. Therefore, the great majority of TSX molecules remain tethered before and after strand exchange. No recombinant products of the size predicted for XerD-mediated bottom strand exchange were detected in reactions with either TSX or untethered *dif* in most of the experiments (Figure 3A, and data not shown). In a few experiments, we have detected what may be a very low level of XerD strand exchange product in reactions containing either TSX or an untethered Holliday junction substrate. It is not clear whether this product is derived from the intact substrate or from another DNA molecule present in small amounts in some recombination reactions.

Two major recombinant products were produced in similar amounts when XerC and XerD were incubated with the BSX substrate (Figure 3B). The electrophoretic mobility of these products indicates that one of them was generated through exchanges between a pair of top strands and the other between a pair of bottom strands (Figure 2C). Use of the catalytically inactive mutants demonstrated that the larger product was generated by XerD while the smaller was from XerC-mediated strand exchange (Figure 3B). Analysis of recombination reactions with BSX on a sequencing gel confirmed the presence of recombinant top and bottom strands (data not shown).

In addition to the strand exchange products, small amounts of covalent complexes of recombinase with DNA were seen running above the TSX and BSX substrates (Figure 3A and B). As expected, they disappeared when samples were treated with proteinase K before electrophoresis (data not shown). The amounts of covalent complexes generated in reactions with TSX and BSX were similar to those observed with the untethered *dif* Holliday junction, indicating that cleavage and rejoining in all these substrates occur in a similar way.

Taken together, these results suggest that XerD can mediate strand exchange on a *dif* Holliday junction only when its substrate strands are crossed. XerC appears to be able to mediate strand exchange when its substrate

Fig. 3. (A) Recombination of TSX substrate. Recombinases were XerC and XerD or their catalytically inactive derivatives XerCY275F or XerDY279F. The gel (4% polyacrylamide containing SDS) shows DNA from 20 min reactions at 20°C. The products of XerC recombination run with the mobilities expected from their structure (Figure 2C). The numbers shown in shadowed font represent the average amounts of strand exchange products generated in the 20°C incubations for 20 min in seven experiments [standard error of means (SEM) are: for TSX/XerC, 4.4; HJ/XerC, 4.3] or in 37°C incubations for 40 min in four experiments (SEM; TSX/XerC, 3.6; HJ/XerC, 1.6); other values represent the amount of recombinant products in the gel shown. nd; not detected ($\leq 0.2\%$ of total DNA). (B) Recombination of BSX substrate. The gel (4% polyacrylamide containing SDS) shows DNA from 30 min reactions at 37°C. The numbers in shadowed font represent average amounts of strand exchange products generated in five experiments in 30 min incubations at 37°C (SEM; BSX/XerC, 6.3; BSX/XerD, 2.8; HJ/XerC, 1.8); other values give the amounts of products generated in reactions incubated at 20°C for 20 min in one experiment. nd; as for (A). (C) Time course of XerC and XerD strand exchange in tethered and untethered *dif* substrates. Recombination reactions containing ligase-treated TSX, BSX or untethered *dif* substrates were incubated in the presence of XerC and XerD at 37°C. The plots present the percentage of substrate DNA converted to rejoined recombinant product.

strands are either crossed or continuous. In the above experiments, the 150 mM Na⁺ present in the recombination reaction partially stacks the arms of the substrates (see below). Similar results of recombination were observed in the presence of 1 mM Mg²⁺, which should fully stack the junctions prior to recombinase binding. It was not possible to analyse recombination in the absence of metal ions because of recombinase insolubility in these conditions. Recombination of substrates containing an 18 thymine oligonucleotide tether generated the same products as TSX and BSX (data not shown).

Relative rates of catalysis on tethered Holliday junction substrates

In the experiments shown in Figure 3A and B, the levels of strand exchange were dependent on the incubation temperature of the reaction. The levels of XerC and XerD strand exchange on BSX were low at 20°C when compared with XerC strand exchange on the untethered *dif* Holliday junction (Figure 3B). Shifting the incubation temperature to 37°C resulted in a several-fold increase in both strand exchanges on BSX when compared with exchange on untethered junction. The levels of XerC strand exchange on TSX and *dif* are similar at 20°C (Figure 3A). At 37°C, less XerC strand exchange occurred on TSX as compared with *dif*.

In order to gain more insight into the strand exchange reactions on TSX and BSX, we carried out kinetics experiments. Before doing this, we wished to confirm that the recombination behaviour of TSX and BSX was not perturbed by the presence of a nick outside of the core recombination site. Therefore, samples of TSX and BSX that had been treated with DNA ligase in order to close the nick were used as recombinant substrates. Typically 50–60% of the nicks could be rejoined. The level and pattern of products of BSX recombination were identical on ligated and unligated substrates. Ligated TSX gave higher levels of XerC recombinant product than unligated TSX (a mean of 48% as compared with 30% in three 30 min, 37°C incubation experiments; data not shown). No evidence for XerD strand exchange in ligated TSX was obtained. Therefore, the nick which is immediately 5' of the XerD-binding site does not change the nature of strand exchange products, though it does appear to reduce the level of recombination. We show later that this nick is not a 'sink' for branch migration, though it may be responsible for some local DNA distortion.

The time course experiments were performed on TSX and BSX that had been treated with ligase (Figure 3C). XerC-mediated recombination of untethered *dif* junction is fast; typically ~50% of strand exchange product generated in a 30 min reaction at 37°C was produced within the first minute, a result similar to those observed with Holliday junction-containing substrates carrying other core recombination sites (Arciszewska *et al.*, 1995). The initial rate of XerC-mediated strand exchange on TSX was very similar to that in untethered substrate, as was the level of recombinant product at 30 min.

On BSX, the initial rate of XerC-mediated strand exchange was at least 3-fold lower than on TSX. The level of product was >2-fold less at 30 min. The initial rate of XerD strand exchange on BSX was lower than that mediated by XerC, though by 30 min the level of

Fig. 4. Restriction endonuclease digestion of tethered junctions and their recombination products. Substrates were incubated with XerC and XerD for 20 min at 20°C, the reactions were stopped with SDS, treated with proteinase K, phenol extracted and ethanol precipitated. The recovered DNA was digested with *Bam*HI (BH) or *Bgl*III (Bg) and analysed on a 6% polyacrylamide gel. DNA not treated with either recombinases or restriction enzymes was included as a control. The sites of cleavage by both enzymes are indicated in the structures of substrates and products (only labelled products are shown). Top strands are drawn as thick lines; filled small circles indicate the positions of the label; the position of the nick in each substrate is indicated with an arrow.

XerD product was some 50% higher than that of XerC. A similar pattern of recombination rates was observed when reactions were analysed at 20°C, though in this case the initial rate of XerC strand exchange on BSX was at least eight times lower than on the untethered junction control (data not shown). The higher initial rates of XerC-mediated exchanges on crossed strands of TSX as compared with continuous strands in BSX indicate that XerC has a preference for exchanging crossed strands.

Restriction endonuclease analysis of substrates and products

Because of the apparent different substrate specificities for XerC and XerD catalysis, we felt it necessary to verify the structure of the recombination products and the integrity of the substrates using *Bam*HI and *Bgl*III restriction endonuclease digestion (Figure 4). In particular, we wished to test whether recombination had occurred on tethered molecules, or on a small sub-population of molecules that were untethered prior to recombination. Despite *Bam*HI digestion being inefficient, because its recognition site is located very close to the linear duplex ends, sufficient cleavage was obtained to allow verification of structure. The product of TSX strand exchange is cleaved with both restriction enzymes, giving DNA fragments of the predicted sizes for a linear molecule retaining a tether. Analysis of reacted BSX DNA revealed the expected pattern of *Bgl*III cleavage of the XerD-generated strand exchange product. As expected, *Bgl*III did not cleave the XerC recombinant product.

Cleavage of TSX by *Bgl*II and *Bam*HI, and BSX by *Bgl*II, should untether the arms of these substrates and therefore retard their electrophoretic mobility (compare the relative mobilities of the tethered and untethered Holliday junctions; Figure 3A and B). The predicted retarded DNA was observed (Figure 4), though digestion was incomplete. Cleavage of TSX with *Bgl*II also generated DNA that ran ahead of the undigested substrate; this most likely reflects the dissociation and loss of an oligonucleotide between the 3' end of strand IV and the *Bgl*II cleavage position (8 nucleotides), or between the strand III nick and the *Bgl*II cleavage position (14 nucleotides), or both.

These results confirm that the products of XerC strand exchange on TSX and XerD strand exchange on BSX retain their tethers and are therefore derived from recombination on substrates that were tethered. However, the product of XerC strand exchange on BSX is identical to that which would have resulted from recombination on an untethered BSX substrate (Figure 2). We believe that this product derives from XerC exchange on tethered BSX for the following reasons. First, we do not observe slower mobility Holliday junction DNA, indicative of untethered substrate, in recombination reactions containing tethered substrates (Figure 3A and B). Second, we did not detect a XerC strand exchange product derived from untethered molecules that might be present in the TSX substrate (see above). Third, XerC-mediated recombination on BSX is strongly inhibited in the presence of one particular XerD mutant; this inhibition is much less pronounced in the untethered *dif* substrate, indicating that XerC is acting on different molecules in the two cases (B.Hallet, I.Grainge and D.J.Sherratt, unpublished data). Fourth, any untethering of BSX by denaturation of the region from the tether up to the nick (Figure 2B) would have generated single-stranded regions that would have been hypersensitive to osmium tetroxide and KMnO_4 modification; these were not observed (see below). Finally, an experiment was carried out in which BSX (treated with and without ligase) was labelled on strand III (Figure 2). This allowed visualization of the other top strand exchange product, which was seen to have the mobility expected of the small gapped circle that should result from continuous strand exchange on the tethered junction (data not shown).

Chemical probing of tethered Holliday junction structure

Previous studies on Holliday junctions have shown that in the absence of metal ions, the junctions adopt a 4-fold symmetric square configuration and the base pairs located at the branch point are sensitive to osmium tetroxide and potassium permanganate, which react specifically with the C5–C6 double bond of unstacked or unpaired thymine bases (Lilley and Palacek, 1984; Duckett *et al.*, 1988; Sasse-Dwight and Gralla; 1989; Bennett and West, 1995b). Metal ion-induced coaxial stacking of the junction arms in the right-handed stacked X structure, reduces this sensitivity. Therefore, these chemicals provide a useful probe for determining the extent of coaxial stacking and the position of the junction branch point. Osmium tetroxide and potassium permanganate only modify the sensitive thymine residues; cleavage of the DNA backbone occurs during the following piperidine treatment. Therefore, the

Fig. 5. Chemical probing of the structure of tethered Holliday junctions. Substrate DNA, labelled at the 5' end of strand IV, was modified with OsO_4 in the absence of metal ions or in the presence of 1 mM Mg^{2+} or 150 mM Na^+ . Following modification, DNA was digested with piperidine and analysed on a 15% denaturing gel alongside DNA derived from the Maxam–Gilbert A+G sequencing reactions carried out on strand IV. The bars to the left of each set indicate the extents of XerC- (shaded) and XerD- (hatched) binding sites and the central region (open). The position of the thymine residue opposite the nick in TSX is indicated with a filled arrow, and the positions of sensitive thymine residues close to the tether are marked with open arrows.

integrity of the tethered Holliday junctions is retained during the course of the assay.

Osmium tetroxide was used to probe the structure of tethered substrates in the absence of metal ions or in the presence of 1 mM Mg^{2+} , a concentration expected to stack the junctions, and 150 mM Na^+ , the concentration used in our recombination reactions. The core recombination sites of TSX, BSX and the untethered Holliday junctions exhibit a similar pattern of increased cleavages at all thymine residues in the absence of metal ions (Figure 5). This hypersensitivity of thymines reflects branch migration of the junction throughout the core region and suggests that the extent of branch migration is similar in all the substrates and is not hindered by the tether. The hypersensitivity of thymine residues is suppressed in the presence of 1 mM Mg^{2+} and partially suppressed in the presence of 150 mM Na^+ due to coaxial stacking of the arms. These results indicate that the junction-containing substrates are partially stacked in our recombination reactions prior to recombinase binding.

Some thymine residues outside the core region of TSX and BSX were also hypersensitive to osmium tetroxide modification. As expected, the residues located in the nine

thymine oligonucleotide tether of BSX are very sensitive (Figure 5). We expect the tether of TSX, which is present in the unlabelled strand, to be equally sensitive to osmium tetroxide modification. The thymine residues of the tether are sensitive to modification in the absence or presence of metal ions.

In TSX and BSX, several thymine residues sensitive to osmium tetroxide were also found close to the tether. The sensitivity of these residues was attenuated by the presence of Mg^{2+} or Na^+ , indicating a distortion of DNA induced by the tether. This result provides further confirmation that TSX is tethered; in an untethered TSX, the portion of strand IV between its 3' end and the position of the nick would be single stranded and therefore susceptible to osmium tetroxide modification in the presence as well as the absence of metal ions. A similar experiment showed no evidence for the presence of an equivalent single-stranded region in BSX (data not shown).

The nick in TSX is located at the 5' end of the core recombination site, a position to which the junction branch point could migrate and at which it might 'stall'. This appears not to be the case, since the thymine residue opposite the nick is no more sensitive to osmium tetroxide than are other thymine residues within the region of branch migration (Figure 5). Moreover, the distribution of the branch points in TSX is not affected by the presence of the nick. In the presence of metal ions, the thymine residue opposite the nick remains sensitive to osmium modification, indicating some local distortion of DNA structure at the nick. This could account for the decreased level of recombination in unligated TSX.

Potassium permanganate cleavage of Holliday junctions in the presence of XerC and XerD

We used potassium permanganate as a modifying reagent to analyse the structure of XerC–XerD complexes with Holliday junction DNA, since the osmium tetroxide reaction conditions resulted in no detectable binding of recombinases to DNA, as observed for other complexes (Bennett and West, 1995b; White and Lilley, 1997). Preliminary experiments showed that XerC and XerD bind with comparable affinity to tethered and untethered junctions (data not shown). In order to minimize recombination of the substrates upon recombinase binding, the reactions were incubated on ice for a very short time. This resulted in <10% of the Holliday junction substrate being converted to recombinant product, a level of recombination that should not influence interpretation of the data.

Potassium permanganate treatment of tethered and untethered Holliday junction DNA revealed no hyper-sensitive thymine residues within the branch migrating core region in the absence of metal ions (data not shown), though tether-induced DNA distortions, similar to those detected by OsO_4 , were observed in the same experiment. This shows that there is less reactivity of unstacked thymine residues at the branch point to $KMnO_4$ than to OsO_4 under the experimental conditions used.

$KMnO_4$ modification of XerC–XerD complexes with linear *dif* DNA induced increased thymine cleavage in the XerC-binding site at position –7 on the bottom strand. Two central region top strand thymines at positions –1 and –3 also showed increased cleavage (Figure 6A; summarized in Figure 6B). These small increases in

Fig. 6. Potassium permanganate probing of Holliday junction structure in the presence of XerC and XerD. **(A)** Substrates were 5' end labelled on top strand IV or on bottom strand II. Following modification, DNA was digested with piperidine and analysed on 15% sequencing gels alongside DNA derived from the Maxam–Gilbert A + G reactions performed on the strands which were labelled in a given substrate. Reactions without protein or potassium permanganate have undergone all the same steps as the other reactions, including piperidine cleavage. The bars to the left of each set indicate the extents of XerC- (shaded) and XerD- (hatched) binding sites and the central region (open). **(B)** Summary of the positions of increased potassium permanganate-induced cleavages in the presence of the recombinases mapped onto the *dif* core sequence. The size of arrows is a semi-quantitative representation of the level of cleavage (quantitation used a PhosphorImager; the bigger the arrow the larger the increase in cleavage; relative increases of <1.5-fold are not shown). As expected, all the positions of increased cleavage correspond to thymine residues. The sequences of XerC- and XerD-binding sites and the central region are indicated. Sequence coordinates are according to Blakely *et al.* (1993).

cleavage may represent distortions arising from protein-induced DNA bending. Binding of XerC and XerD to the untethered *dif* Holliday junction led to greatly increased levels of cleavage, most notably at position -1 on the top strand. The thymine at position +1 on the bottom strand, adjacent to this hypersensitive site, also shows an increased level of cleavage. Similar results were observed when XerC and XerD bound to tethered TSX and BSX junctions; in BSX the two central region thymines show an even greater sensitivity to modification. These large increases in sensitivity in the middle of the central region almost certainly reflect the presence of the branch point at this position when the recombinase proteins are bound. The *dif* central region is very AT rich, but it is a single thymine on the top strand and the symmetrically placed thymine on the bottom strand which are hypersensitive, suggesting that the branch point is located precisely in the middle of the central region between positions -1 and +1. This result also suggests that the recombinase-bound Holliday junction has some unstacking or loss of base pairing at the branch point.

Several slightly increased cleavages within the XerC-binding site adjacent to the central region were observed in untethered *dif* and TSX, but not in BSX. Similar increases were also seen on the linear *dif* fragment, and may result from a protein-induced bend. The lack of sensitivity of these residues in BSX may reflect the absence of the presumptive protein-induced bend or some other change in protein-DNA interaction specific for BSX-protein complexes.

The results suggest that in the presence of recombinases, the branch point is localized to the same position in the tethered and untethered substrates. The different isoforms that the tethered junctions are forced to adopt do not alter the apparent position to which the recombinases localize the branch point.

Fe-EDTA hydroxyl radical footprinting of Holliday junctions

Hydroxyl radical footprinting, which can give high resolution information about the structure of the stacked Holliday junctions as well as protein-DNA complexes (Tullius and Dombroski 1985, 1986; Churchill *et al.*, 1988; Kimball *et al.*, 1990; Bennett and West, 1995a), was used to analyse the structure of tethered and untethered Holliday junctions in the presence and absence of recombinase proteins (Figure 7). The footprinting reactions were carried out in solution containing ~150 mM Na⁺, which leads to partial stacking of the Holliday junction arms (see above). Protein-DNA complexes were then separated from unbound DNA in a polyacrylamide gel lacking metal ions (TBE) prior to analysing the cleavage pattern on a sequencing gel. Unlike modification with osmium tetroxide or potassium permanganate, hydroxyl radical attack causes disruption of the DNA backbone during the assay. We believe that this does not interfere with the analysis of the tethered junctions because much less than one cleavage per DNA molecule was expected to take place under our experimental conditions. Previous experiments have also shown that Fe-EDTA cleaves single-stranded DNA with much lower efficiency than double-stranded DNA (Jezewska *et al.*, 1989; Kimball *et al.*, 1990). Indeed, less

Fig. 7. Fe-EDTA hydroxyl radical footprinting of Holliday junction complexes with XerC and XerD. **(A)** Complexes of XerC and XerD with substrates 5' end labelled on the top strand IV or bottom strand II analysed on 15% sequencing gels alongside DNA derived from the Maxam-Gilbert A+G reactions carried out on the 5' end labelled oligonucleotide used for the construction of the given substrate. 'u' and 'l' indicate the DNA and DNA-recombinase complex derived from the upper and lower band, respectively, obtained after separation through a native polyacrylamide gel. The bars to the left of each set indicate the extents of XerC- (shaded) and XerD- (hatched) binding sites and the central region (open). **(B)** Summary of the protection patterns against hydroxyl radical attack mapped onto a sequence of the *dif* core recombination site. The sequences of XerC- and XerD-binding sites and the central region are indicated. Black boxes represent protection and grey boxes represent reduced or partial protection.

cleavage was observed at the tether as compared with double-stranded DNA.

Binding of XerC and XerD to an untethered *dif* junction and to TSX results in two complexes which migrate close together. These complexes were isolated separately and are marked as 'upper' (u) and 'lower' (l) complexes. There is very little difference, if any, between the patterns of protection observed with the upper and lower complexes of the bound DNA, indicating that they are each bound by both XerC and XerD (Figure 7A). The molecular basis of the observed difference in mobility of the complexes is not clear.

Hydroxyl radical footprinting of the Xer recombinases bound to tethered and untethered Holliday junctions showed substantial protection of the core region; in particular, the central region is strongly protected (Figure 7A; summarized in Figure 7B). The patterns of protection of the XerC- and XerD-binding sites show only minor differences. Notably, the short regions of lack of protection within the XerC- and XerD-binding sites on the bottom and top strands, respectively, are present consistently in all junction substrates. The linear *dif* fragment used as a control showed an almost identical protection pattern (Blakely and Sherratt, 1996).

These results indicate similar interactions of XerC and XerD with DNA of all the junction-containing substrates. The close similarity of footprinting patterns of the junction-containing substrates to linear DNA suggests that the arms of the junctions must be coaxial so that each recombinase protein can interact with the cross-core partner as though on a linear piece of DNA.

Since the Fe-EDTA reaction is carried out in solution and the bound DNA is enriched subsequently by gel electrophoresis, there is the possibility that the strand interruptions in the junction DNA could alter the affinity of the proteins for the junction and so produce an 'interference' pattern as well as a footprint. We consider this unlikely.

Fe-EDTA footprinting of Holliday junctions in the absence of proteins

When untethered *dif* junction DNA is subjected to electrophoresis in TBE buffer after the treatment with Fe-EDTA, two bands of very similar mobility are observed. These bands, labelled 'upper' (u) and 'lower' (l), revealed complementary cleavage patterns (Figure 7A). The upper species was cleaved throughout the core region on both the top and bottom strands of the junction, whereas the lower species was cleaved well outside and poorly within the core. Since branch migration of the junction is limited to the region of core recombination site, the complementary pattern of cleavages of the two complexes must be related to branch migration. We explain these results as follows. The population of molecules in the lower band was cleaved outside the core recombination region by hydroxyl radicals; these molecules migrate faster through the gel because of their stacked structure. In contrast, molecules in the upper band, which were cleaved in the core region, migrate slower because they have adopted a 'stacked square planar' configuration as a consequence of the branch position being stalled at the cleavage point (Pöhler *et al.*, 1994). Consistent with this explanation is the observation that untethered junctions pre-incubated in

150 mM Na⁺ run as two closely spaced bands on a TBE gel. We assume that they represent populations of molecules in square unstacked and stacked X configurations. Hydroxyl radical treatment of DNA from each of these bands carried out in the gel showed an identical ladder of cleavages throughout the whole molecules as expected (data not shown). In the presence of 1 mM Mg²⁺, only a single band corresponding to stacked X molecules is observed.

Discussion

One important finding from the work presented here comes from the experiments in which chemical probes were used to investigate the structure of Holliday junctions bound by XerC and XerD. Binding of the recombinases leads to an enhanced sensitivity to modification and cleavage in the middle of the central region of both the tethered and untethered Holliday junction-containing molecules. We believe the only explanation for this is that the branch point becomes located there as a consequence of protein binding. This supports the idea that either top or bottom strand exchanges can be initiated on a molecule whose branch point is located in the centre of the central region, 3 bp away from either position of strand cleavage (Figure 1B; Nunes-Düby *et al.*, 1995). Consistent with this are our previous results showing that the junction crossover need not be at the site of strand cleavage to allow efficient strand exchange (Arciszewska *et al.*, 1995).

The chemical sensitivities of the bases at the Holliday junction branch points induced by recombinase binding reflect local DNA distortion. Holliday junction structure is also changed by the binding of other proteins that either resolve the junction or promote branch migration (Bennett and West, 1995b; Duckett *et al.*, 1995a; Parsons *et al.*, 1995; Pöhler *et al.*, 1996; White and Lilley, 1997). In the absence of recombinases, the tethered and untethered Holliday junction substrates have the expected properties, with branch migration occurring throughout the *dif* recombination site. XerC and XerD interactions with Holliday junction and linear substrates appear to be similar; in particular, the central region in these substrates is protected from hydroxyl radical attack, indicating that a given XerC molecule bound to the junction is able to interact with the cross-core XerD partner in the same manner as on a linear substrate. This suggests that the arms in the junctions must have a degree of coaxial stacking in the presence of the recombinases similar to those in complexes of the recombinases with linear DNA. The protection of the branch point from hydroxyl radical attack also demonstrates that the proteins must be close enough to the branch point to generate the distortion revealed by potassium permanganate modification.

The difference between the two isoforms of the right-handed antiparallel *dif* Holliday junction is striking when the branch point is placed in the middle of the central region and the relative positions of the cleavage points are compared (Figure 1A). In the isoform with top strands crossed (left panel), the XerC cleavage positions are close together (~25 Å apart) on the major groove face of the branch point, whereas the XerD cleavage positions are much further apart (~40 Å). The isoform with bottom strands crossed has exactly the opposite positioning, with

the XerD cleavage sites now close together and those of XerC further apart.

Our second major finding is that XerC and XerD respond differently to the two Holliday junction isoforms. XerD mediates efficient strand exchange only on molecules in which its substrate (bottom) strands are initially crossed. This is consistent with the strand-swapping model of Nunes-Duby *et al.* (1995), which proposes that the swapping of three nucleotides in a single step during exchange of crossed strands in a Holliday junction could occur readily with relatively little strand movement (see Figure 1A and B). During Holliday junction formation from two duplexes, swapping of the strands that are to become crossed would simply be the reverse process. The results from experiments on λ integrase in the accompanying paper by Azaro and Landy, using Holliday junctions that adopt a preferred isoform according to the nucleotide sequence near the branch point, also support the view that it is the crossed strands that are exchanged most efficiently by Int.

Exchange of strands that are continuous, or are to become continuous, would require a more extensive movement of the transferring strands and could lead to clashes with the crossed strands. It might also need the duplex substrates and products to be highly distorted before and after Holliday junction formation (see later).

XerC also catalyses very efficient strand exchange when its substrate strands are initially crossed, as in TSX, supporting the view that both of these recombinases act preferentially on molecules with crossed substrate strands. However, XerC also appears to be able to catalyse strand exchange when its tethered substrate strands are initially continuous, as in BSX. The kinetics experiments show that the initial rate of strand exchange on TSX crossed strands (or on the untethered Holliday junction) is at least three times higher than on BSX continuous strands. Does this relatively inefficient activity on continuous strands reflect a promiscuity of XerC or does it reflect the presence of a contaminating substrate in the BSX preparation which is not appropriately tethered? In experiments designed to test this latter point, we have failed to find evidence that the products are from anything other than tethered BSX molecules of the expected structure. Moreover, it is clear that all detectable XerC strand exchange on TSX generates products retaining the tether. However, we cannot eliminate the possibility that XerC strand exchange occurred on molecules that had become transiently untethered by local denaturation of the duplex between the tether and the nick, followed by re-annealing and re-tethering after strand exchange.

If XerC can catalyse strand exchange on two apparently different substrates, the recombinases must either be able to manipulate the local structure of a junction to one that is a XerC strand exchange substrate irrespective of the constraints of a tether, or XerC must show promiscuity in its cleavage. This might lead to cleavage of continuous strands in BSX, followed by dissociation of the substrate into two linear duplexes with covalently bound XerC at the cleavage positions. Rejoining might then occur on these linear molecules.

A stringent substrate requirement for XerD catalysis as well as probable promiscuity in XerC catalysis are also suggested by the recombination properties of other sub-

strates. Linear suicide substrates are cleaved efficiently *in vitro* by XerC, but not by XerD (Blakely and Sherratt, 1996; Blake *et al.*, 1997), while XerC strand exchange *in vivo* and *in vitro* on supercoiled substrates containing two directly repeated *cer* sites generates supercoiled Holliday junction-containing molecules which are not substrates for XerD strand exchange (McCulloch *et al.*, 1994; Colloms *et al.*, 1996). This could be because the product of the initial XerC strand exchange is a Holliday junction-containing molecule that is not in a suitable configuration for XerD strand exchange and is incapable of isomerization.

On untethered Holliday junction substrates containing a variety of recombination sites, we have detected either no or trace amounts of XerD strand exchange, using wild-type XerC and XerD (Arciszewska and Sherratt, 1995; our unpublished results). Moreover, we have not observed XerD cleavage as measured by the appearance of XerD–DNA covalent complexes. In all of these substrates, XerC strand exchange was efficient. Substantial XerD cleavage on untethered junctions *in vitro* can be observed by using either a particular XerC variant (in the presence of wild-type XerD), or by some XerD variants (G.Blakely, I.Grainge, B.Hallet, L.Neilson and D.J.Sherratt, unpublished data). This could reflect relaxed substrate specificity or more efficient manipulation of Holliday junction structure with the mutants. Intriguingly, one of these XerD mutants also appears able to cleave both crossed and continuous strands on TSX, indicating that its substrate specificity has become relaxed so that it is equivalent to that of XerC.

Our observations with wild-type recombinases could mean that all of the untethered junctions that we have used naturally adopt a conformation close to that in TSX. The results of the footprinting experiments are consistent with this, since TSX and the untethered junction are more similar to each other than to BSX when the recombinases are bound. Alternatively, binding of recombinases to an untethered junction in any isoform might generate a recombinase–DNA complex that is suitable for XerC, but not XerD, strand exchange.

How do the above results, along with those on the properties of the whole recombination reaction, relate to the model outlined in Figure 1B? On plasmid sites *psi* and *cer*, recombination between a pair of recombinase-bound duplexes is initiated after synapsis, by a pair of XerC-mediated strand exchanges (Colloms *et al.*, 1996, 1997). If recombinase catalysis is limited to strands that are crossed, or are to become crossed, as shown schematically in Figure 1B, the two duplexes involved in the recombination reaction would be represented by arms I/III and II/IV (Figure 1A, left panel). Then the strands exchanged by XerC will become crossed on Holliday junction formation (the structure being equivalent to that of TSX). Isomerization to a structure capable of XerD strand exchange would then occur. With *psi* site substrates, XerD strand exchange must be very efficient (as must isomerization be, if the model is correct), since complete recombinant products appear quickly and only very low levels of Holliday junction intermediate are observed. If recombination between two duplexes is initiated by XerD strand exchange (this may be the case for some recombination sites; B.Hallet and D.J.Sherratt, unpublished data),

synapsis would bring the sites together in a way that would leave the bottom strands crossed on Holliday junction formation. XerC strand exchange could then occur after isomerization.

In those integrase family systems that use a single recombinase, isomerization provides an equivalent stereochemical environment for both pairs of strand exchanges. In systems that use two recombinases, different recombinase catalytic specificities could obviate the need for isomerization, as described below in an alternative mechanism that depends on the apparent ability of XerC to catalyse strand exchange on continuous strands. In this case, the initial duplex partners would be arms I/III and arms II/IV (Figure 1A, right-hand panel; since recombinase binding to duplex is accomplished by substantial bending, synapsis might occur in this way, though recombinase-bound duplexes do not show permanganate sensitivity that would be indicative of major distortion). XerC exchange of the strands that are to become continuous in the Holliday junction would generate an intermediate equivalent to BSX and the isoform in the right-hand panel of Figure 1A. This molecule could then be a substrate for XerD strand exchange without isomerization. Similarly, if recombination between duplexes were initiated by a XerD exchange of crossed strands, the Holliday junction intermediate could undergo strand exchange of continuous strands by XerC, without isomerization. Even if XerC-mediated exchange of continuous strands could occur in *dif*, which has a 6 bp central region, it would seem less likely to occur in those plasmid sites that have 7 and 8 bp central regions (e.g. *cer*). This is because of the increased length of DNA that would need to be transferred during swapping, unless some significant branch migration now occurred. We need to continue to consider this model because of the apparent exchange by XerC of continuous strands on BSX.

How and when isomerization occurs and what determines which isomer predominates remains unclear. The normal cross-core interactions between XerC and XerD bound to duplex DNA must be broken and re-formed during isomerization, since each recombinase-binding site swaps its partner (Figure 1); XerC and XerD binding to *dif* is highly cooperative, having a cooperative index of several hundred (Blakely and Sherratt, 1996), suggesting that the cross-core interactions are rather strong. It will also almost certainly be necessary to break and make other recombinase-recombinase interactions during isomerization. Because the two Xer recombinases bind to non-identical sequences and induce different DNA bending (Blakely and Sherratt, 1996), the disposition of the proteins and any protein-induced bends with respect to a given reference point will be different in the two isoforms, as should the DNA structure at the branch points. Any one of these differences could help determine the preferred isoform and provide recombinase cleavage specificity. It could be that the Xer system uses two recombinases in order that the recombination outcome can be controlled precisely, perhaps by some unknown signalling mechanism.

cer and several other natural plasmid sites (but not *psi* and the chromosomal site, *dif*) have purine-rich top strand sequences in the middle of their central regions (Sherratt, 1993). Such sequences preferentially adopt a conformation

in which the top strands are crossed (von Kitzing *et al.*, 1990; Duckett *et al.*, 1995b; Azaro *et al.*, 1997) and, therefore, should favour Holliday junction configurations that would lead to XerC, but not XerD strand exchange, as observed experimentally for several Xer recombination sites (McCulloch *et al.*, 1994; Arciszewska and Sherratt, 1995; Arciszewska *et al.*, 1995; Colloms *et al.*, 1996; our unpublished data).

Comparison of the properties of XerC and XerD with other Holliday junction-resolving enzymes shows that all are able to discriminate between different Holliday junction isoforms, with different proteins being able to resolve a given isoform in opposite ways. This could provide the basis for determining which type of recombinant product is to be formed. In homologous recombination, 'splice' recombinants arise when there has been an exchange of outside markers, while 'patch' recombinants retain their parental configuration of outside markers. There are many situations in which there is a bias for one or the other of the recombinant types. This could be determined either by the original configuration of the Holliday junction, or by the substrate specificity of the enzyme. For example, RuvC, CCE1 and T7 endonuclease I bind as dimers *in vitro* to either of the right-handed crossed X isoforms and cleave the strands that initially were continuous (Lu *et al.*, 1991b; Duckett *et al.*, 1995a; Bennett and West, 1995a,b; White and Lilley, 1996, 1997). Binding opens the DNA into a form approaching square planar, and the mechanism that leads these enzymes to cleave continuous strands may be determined by the substrate isoform prior to enzyme binding rather than by the structure after binding. If a junction is constrained to a particular isoform by DNA sequence, by a tether, DNA topology, the presence of a second neighbouring junction or by chromosome structure, a given enzyme may resolve the molecule preferentially in one direction (Bhattacharyya *et al.*, 1991; Bennett and West, 1995a; White and Lilley, 1996; Azaro *et al.*, 1997; Zerbib *et al.*, 1997; this work). An alternative way to generate recombination bias is to target enzymes that show different resolution specificity to a given Holliday junction; for example, whereas RuvC, CCE1 and T7 endonuclease I cleave continuous strands, T4 endonuclease VII, like the integrase family recombinases described here and in the accompanying paper, prefers to cleave strands that initially are crossed in the substrate (Duckett *et al.*, 1988; Mueller *et al.*, 1988).

An illustration of how DNA topology can determine recombination outcome is provided by the properties of supercoiled molecules containing a Holliday junction (Colloms *et al.*, 1996; Zerbib *et al.*, 1997). During inter-molecular recombination between two supercoiled circular molecules, a Holliday junction-containing figure-of-eight intermediate will be converted back to two monomer circles by enzymes that preferentially cleave continuous strands, and to dimer product by enzymes that resolve crossed strands. Intra-molecular recombination in a supercoiled dimer molecule will normally generate a catenated figure-of-eight Holliday junction intermediate as a consequence of entrapping supercoiling between the synapsed recombination sites. Again, this intermediate will be restored to substrate configuration by continuous-strand cleaving enzymes and to product monomer circle product by enzymes that cleave crossed strands (Colloms

et al., 1996; Zerbib *et al.*, 1997). DNA topology could be one 'signal' that determines the outcome of a Xer recombination reaction.

Materials and methods

Bacterial strains and plasmids

The bacterial strains and plasmids that encode the wild-type and mutant XerC and XerD are described in Blakely *et al.* (1993) and in Arciszewska and Sherratt (1995).

Preparation of Holliday junction substrates

The untethered *dif* Holliday junction substrate was constructed from four synthetic oligonucleotides: I (84 nucleotides), 5'-GATCCGTGAT-CACGCTGAACGCGTTTATAGCGGTGCGCATAATGATATATTATGT-TAAATGGCCTAACGCTAAAGCGGCCGCTA; II (76 nucleotides), 5'-ATCGTAGGCGGCCGCTTTAGGCGTTAGGCCATTTAACATAATATACATTATGCGCACCCGACCGTTGCCGGATCCG; III (76 nucleotides), 5'-TCGATCGAGATCTCAGGATGTCATTTAACATAATATACATTATGCGCACCCGCTAAAGCGCGTTTCAGCGTGATCACG; and IV (68 nucleotides), 5'-CGATCGGATCCGGCAACGGTCCGGTGGCG-ATAATGATATATTATGTAAATGACATCCTGAGATCTCGA (central regions are shown in bold).

The 'ideal' construction of tethered substrates from synthetic oligonucleotides would require oligonucleotides ~150 nucleotides long carrying both continuous strands joined by a tether. Because of the length limitations in synthetic DNA synthesis, two shorter oligonucleotides were used to substitute for the long oligonucleotide. After reannealing, this generated a nick in one arm of each substrate located 18 nucleotides away from the tether in the 3' direction (Figure 2B). The oligonucleotides used to construct the tethered substrates contained the same sequences as the oligonucleotides used for the untethered substrate, except for the presence of nine thymine tether residues. Also, nucleotides constituting three out of four cohesive ends present in untethered *dif* were absent in tethered substrates. The only remaining cohesive ends were in arm I of TSX and in arm II of BSX. The sequence of the oligonucleotides used for construction of the tethered substrates is available upon request.

For annealing of each substrate, 10 pmol of one strand, 5' end labelled with T4 DNA polynucleotide kinase, was mixed with a 2- to 3-fold excess of unlabelled strands. Annealing was carried out as previously described (Arciszewska and Sherratt, 1995). To obtain substrates with a sealed nick, phosphorylated oligonucleotides of strands that contributed the 5' terminus at the nick were used, and annealing was followed by ligation carried out at 20°C for 1 h. To ensure that similar amounts of different substrates were used in recombination reactions, the amounts of radioactivity in the substrates were measured after gel purification and DNA recovery, and the volume of resuspending solution was adjusted accordingly.

Proteins

XerD was purified from a 3 l culture of *E.coli* DS9009 containing plasmid pRM132 carrying *xerD* under control of the *plac* promoter (Blakely *et al.*, 1993). Cells were grown at 37°C in LB supplemented with ampicillin (200 µg/ml) and 2% glucose. At A_{600} 0.4 the culture was spun, resuspended in the same medium lacking glucose and induced with isopropyl-β-D-thiogalactopyranoside (IPTG; 1 mM) for 4 h. The cells were harvested, washed and frozen. For fractionation, cells were resuspended in buffer A [50 mM Tris-HCl (pH 8.0), 100 mM NaCl], incubated on ice in the presence of lysozyme (400 µg/ml) for 30 min, sonicated and spun. The cell lysate was loaded onto a 5 ml NiNTA column (Qiagen) which was washed with buffer B [20 mM NaOAc (pH 5.0), 10 mM NaCl]; XerD was eluted with a 15 ml imidazole gradient (0–0.5 M). Collected pooled fractions were dialysed into buffer C [50 mM Tris-HCl (pH 8.0), 50 mM NaCl, 1 mM EDTA] and loaded on a 1 ml MonoS column (Pharmacia), which was eluted with a 20 ml NaCl gradient (0.05–1 M). Eluted pooled XerD fractions were supplemented with glycerol to 50% volume and stored at –70°C. Protein concentration in the final preparation was ~1.0 mg/ml; XerD constituted ~90% of the total protein, with the majority of the contaminants being proteolytic fragments of XerD.

XerC was purified as described in Colloms *et al.* (1996), except that the Mono S column was omitted; the protein concentration of fractions used was 0.75 mg/ml; XerC was ~30% pure in this preparation. Purification of XerCY275F and XerDY279F has been described in Colloms *et al.* (1996). The protein concentration in purified fractions of

XerCY275F was ~0.2 mg/ml and was estimated to be 75% pure. XerDY279F contained ~0.2 mg/ml and was ~60% pure.

Recombination reactions

The reactions (10 µl) were carried out in 20 mM Tris-HCl (pH 7.5), 50 mM NaCl, 1 mM EDTA buffer and contained ~2.5 ng of labelled DNA, 500 ng of poly(dI-dC)-poly(dI-dC), 100 µg/ml bovine serum albumin (BSA) and 1 µl of each XerC [in 25 mM Tris-HCl (pH 8.0), 500 mM NaCl, 1 M urea, 0.5 mM EDTA and 50% glycerol] and XerD [in 50 mM Tris-HCl (pH 7.5), 500 mM NaCl, 50% glycerol] or their derivatives. Following incubation at the temperature and time specified in particular experiments, reactions were terminated by addition of 2 µl of stop buffer [50 mM Tris-HCl (pH 7.5), 1% SDS] and DNA was analysed on native 6% polyacrylamide-TBE gels or through 4% polyacrylamide-TBE gels containing SDS (0.1%). The bands were quantified on a PhosphorImager.

Binding reactions

The binding reactions (20 µl) were carried out in buffer containing 50 mM Tris-HCl (pH 7.5), 50 mM NaCl, 1 mM EDTA, in the presence of 2 µg of poly(dI-dC)-poly(dI-dC) and BSA (100 µg/ml) and contained ~25 ng of labelled substrate DNA and 2 µl each of XerC [in buffer containing 37.5 mM Tris-HCl (pH 7.5), 750 mM NaCl, 0.625 mM EDTA, 500 mM urea, 125 µg/ml BSA and 50% glycerol] and XerD [in buffer containing Tris-HCl (pH 7.5), 250 mM NaCl, 50% glycerol]. The reactions were incubated on ice for 2 min. The protein fractions used in binding reactions for hydroxyl radical footprinting were in buffer containing no glycerol: 50 mM Tris-HCl (pH 7.5), 300 mM NaCl and 1 mM EDTA. The total protein concentration was ~0.4 mg/ml and 0.2 mg/ml, for the XerC and XerD preparations, respectively. The binding reactions for hydroxyl radical footprinting contained no BSA.

Chemical probing of DNA structure

OsO₄ modifications were performed as described in Arciszewska *et al.* (1995).

For KMnO₄ modifications, DNA in binding reactions was treated as described in Bennett and West (1995b), except that SDS at a final concentration of 0.1% was also added to stop the reaction. DNA was then digested with piperidine, and analysed on 15% sequencing gels. The bands were visualized by autoradiography, and quantitated on a PhosphorImager. Chemical modifications for Maxam-Gilbert sequencing and piperidine digestions were performed as described in Sambrook *et al.* (1989).

Fe-EDTA hydroxyl radical footprinting

Reaction conditions were adapted from those outlined in Dixon *et al.* (1991). DNA in binding reactions was cleaved by addition of the following reagents at final concentrations: 250 µM [(NH₄)₂Fe(SO₄)₆·6H₂O], 500 µM EDTA, 5 mM sodium ascorbate and 0.03% hydrogen peroxide. Reactions were incubated for 2 min at 20°C and stopped by addition of 5 µl of a solution containing 50% glycerol and 125 mM EDTA. Protein-DNA complexes were separated from free DNA by electrophoresis through a 6% polyacrylamide-TBE gel at 4°C. The wet gel was exposed to film at 4°C for 30 min, and the bands were excised. DNA was eluted from the gel slices, ethanol precipitated and analysed on 15% sequencing gels.

Other methods

Restriction digestions and other standard procedures were as described in Sambrook *et al.* (1989).

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