Effects of Holliday junction position on Xer-mediated recombination *in vitro*

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Site-specific recombination mediated by XerC and XerD functions in the segregation of circular replicons in *Escherichia coli*. A key feature of most models of recombination for the family of recombinases to which XerC and XerD belong is that a Holliday junction forms at the position of the first pair of recombinase-mediated strand exchanges and then branch migrates 6–8 bp to the position of the second pair of strand exchanges. We have tested this hypothesis for Xer recombination by studying the effects of junction position on XerC-mediated strand exchange *in vitro*. Recombination of synthetic Holliday junction substrates in which junction mobility was constrained to a region extending over or removed away from the normal cleavage and exchange point was analysed. All substrates undergo strand cleavage at the normal position. We infer that the Holliday junction need not be at this position during strand cleavage and exchange. With substrates in which the Holliday junction is constrained to a region away from the XerC-mediated cleavage point, strand exchange generates products with the predicted mispaired bases.

Key words: branch migration/Holliday junction/Int family recombinases/site-specific recombination/Xer

Introduction

Xer site-specific recombination functions in the normal segregation of multicopy plasmids and the bacterial chromosome in *Escherichia coli* (Summers and Sherratt, 1984; Blakely *et al.*, 1991; Leslie and Sherratt, 1995). This system utilizes two related recombinases, XerC and XerD (Colloms *et al.*, 1990; Blakely *et al.*, 1993), which belong to the phage lambda integrase family of site-specific recombinases. As in other integrase family systems, the two pairs of strand exchanges in Xer recombination appear to be temporally and spatially separated, with Holliday junction-containing molecules as intermediates in the recombination process (McCulloch *et al.*, 1994; Arciszewska and Sherratt, 1995; Sherratt *et al.*, 1995). Holliday junction-containing molecules also occur as intermediates in homologous recombination (reviewed in West, 1992; Kowalczykowski *et al.*, 1994), and a substantial body of information is accumulating on the structure of Holliday junctions, their branch migration and their

interactions with proteins (reviewed in West, 1992; Lilley and Clegg, 1993; Kowalczykowski *et al.*, 1994).

A synthetic Holliday junction-containing molecule that is free to branch migrate through the 28 bp recombination core is a substrate for Xer-mediated recombination *in vitro*, although only the XerC-catalysed pair of strand exchanges was observed (Arciszewska and Sherratt, 1995). In related site-specific recombination systems, a wealth of data has supported models in which the initial Holliday junction forms at the point of the first pair of strand exchanges. These models then propose that the second pair of strand exchanges, which occurs 6–8 bp 3' of the initial pair of exchanges, requires that the Holliday junction branch migrates to this new position of cleavage and strand exchange (Figure 1; reviewed in Craig, 1988; Stark *et al.*, 1989, 1992; Landy, 1989, 1993). A prediction of such models is that synthetic Holliday junction-containing DNA substrates in which the crossover position is constrained to one or the other exchange point, or to regions located away from the exchange points, should show predictable and characteristic recombination properties. Here we show that these predictions are not fulfilled for XerC-mediated exchanges. Experiments with FLP/FRT and lambda Int/*attB/attP* using synthetic Holliday junctions with constrained mobility also suggest that 'classical' branch migration models need to be re-evaluated for this family of recombinases (Dixon and Sadowski, 1994; Nunes-Düby *et al.*, 1995).

Results

Construction of artificial Holliday junction-containing molecules in which junction position is constrained to different regions

A synthetic Holliday junction molecule that contains the core region of the *cer6* recombination site (HJ1; Figure 2A) is a substrate for Xer recombination *in vitro* (Arciszewska and Sherratt, 1995). The junction in this molecule is free to branch migrate within the 28 bp of perfect homology of the core sequence. Although both XerC and XerD are required for the recombination, only the XerC-mediated pair of cleavages and strand exchanges occur on this substrate; cleavage occurs between the XerC binding site and the central region (Figure 2A; Arciszewska and Sherratt, 1995).

In order to examine the effects of the position of the junction on Xer-mediated recombination, six derivatives of *cer6* with constrained junction mobility were made by introducing base pair substitutions at the indicated positions in the core recombination site (Figure 2B and C). The junction is free to migrate within the regions flanked by the substitutions, but branch migration outside of these regions would require the formation of energetically less favourable molecules containing a mismatched base pair

Fig. 1. A 'classical' scheme of lambda integrase family site-specific recombination as applied to the Xer system (adapted from Stark *et al.*, 1992). Recombinase binding is followed by synapsis (here depicted as antiparallel). XerC mediates the first pair of strand exchanges (step a; McCulloch *et al.*, 1994; solid arrows indicate cleavage/exchange points; Y depicts the tyrosine nucleophile) to give a Holliday junction intermediate with the crossover at the exchange point. Branch migration, accompanied by isomerization (steps b and c), moves the crossover to the expected site of XerD exchanges, with the two XerD molecules now positioned to mediate such exchanges (step d; open arrows indicate predicted cleavage and exchange points). The central region is that of *cer6*, the substrate used here. XerC and XerD bind to 11 bp sequences that flank the central region, binding being cooperative (Blakely *et al.*, 1993). The reactions observed in the experiments here are equivalent to the reverse of step a; i.e. the conversion of a Holliday junction-containing molecule back to substrate. XerD-mediated exchanges have not been observed on this substrate.

in each of two of the arms. Two groups of substrates were made. In one, the mobility of the junction was limited to a region of 4, 3 or 2 bp, that contains the top strand XerC-mediated cleavage point (HJ3, HJ5 and HJ6, respectively). In the other group, the junction mobility was limited to a region that was away from the cleavage point: in HJ4 the junction could move over the four central base pairs of the central region, while in HJ7 the junction was able to move within a 7 bp region over the presumptive site of the bottom strand exchanges; mobility of HJ2 was fixed in the middle of the central region. The substitutions were chosen so that the substituted nucleotides should not affect recombination or recombinase binding based on the known recombination proficiencies of the wide range of the natural and artificial sites (e.g. see Sherratt, 1993).

Branch migration of substrates with constrained junction mobility

Branch migration of the substrates with constrained mobility of the junction was probed with the Holliday junction resolving enzyme, RuvC (Dunderdale *et al.*, 1991; Iwasaki *et al.*, 1991), and with osmium tetroxide which can detect unstacked thymine residues at a four-way junction (McClellan *et al.*, 1986; McClellan and Lilley, 1987; Lilley, 1992; Palacek, 1992). Three substrates labelled at the 5' end of the top strand I (HJ1, HJ4 and HJ7) and two substrates labelled at the 5' end of the bottom strand II (HJ1 and HJ4) were digested with RuvC and analysed on a denaturing gel alongside Maxam-Gilbert AG reaction-modified DNA (Figure 3A and B). The positions of RuvC cleavages are indicated in the sequence of the top or bottom strands of the core region in Figure 3D. RuvC cleaves the HJ1 substrate at several sites in the top and bottom strands, suggesting that the

junction position moves freely in this substrate along its 28 bp region of perfect homology. The cleavages are very inefficient as the core region of *cer6* does not carry the A/T TT G/C RuvC consensus sequence (Shah *et al.*, 1994). The positions of cleavage in the other substrates map to the regions of their allowed junction mobility, demonstrating that branch migration does not occur beyond the heterology barrier, at least at a frequency that would be relevant in the experiments here. Interestingly, the cleavages in HJ7 occur with quite different efficiency and specificity compared with HJ1; this may reflect some local structural or conformational differences and/or relate to the time that the junction spends at a particular position. The results also show that RuvC can cleave DNA right up to the position of the junction crossover point at the 3' end of the mobile regions (for example, see cleavage of the top strands in HJ1, HJ4 and HJ7). Other work has shown that RuvC cleaves symmetrically on two strands of the same polarity at Holliday junctions (for example see Dunderdale *et al.*, 1991). We have not tested whether RuvC cleavage is symmetrical on our substrates, but believe it likely that this is the case.

Results supporting limited branch migration in the constrained substrates were also obtained when mobility of the junction in HJ1 and HJ4 was probed with osmium tetroxide (Figure 3C). In HJ1 modification occurred at every thymine residue along the *cer6* core region, while in HJ4 only the one thymine located within the mobility region of this substrate was modified.

Recombination of substrates with constrained junction mobility

The effects of junction position on Xer recombination were examined on substrates labelled at the 5' end of an

Fig. 2. (A) The structure of Holliday junction-containing substrates. The substrates contain two XerC (shaded) and two XerD (hatched) binding sites and a 6 bp central region (thick line); they were assembled from four synthetic oligonucleotides: I, 92; II, 84; III, 84 and IV, 76 nucleotides long. By convention, strands I and IV are designated 'top' strands, while strands II and III are 'bottom' strands. Recombination through exchange between the top strands (T) would generate two linear duplex molecules, each of 84 bp; recombination through bottom strand exchanges (B) would produce two different size duplexes of 92 and 76 bp. Only XerC-catalysed strand exchanges between the top strands have been observed with the substrate containing a *cer6* core site sequence (Arciszewska and Sherratt, 1995). The positions of XerC-mediated cleavages are indicated by the solid arrowheads, while the predicted positions of XerD cleavages are indicated by open arrowheads. The bottom of the panel depicts the structures of XerC recombinant products: Holliday junction covalent complexes (HJCC); linear duplex covalent complexes (LDCC) and rejoined linear duplex (RLD). (B) Regions of mobility of Holliday junction-containing substrates. The sequence presents the top strand I of the 28 bp *cer6* core recombination site which includes XerC and XerD binding sites (11 bp each) and a 6 bp central region (bold type). The regions of mobility of the junction in various substrates are framed with open boxes. The site of XerC-mediated cleavage is indicated with the arrow (Arciszewska and Sherratt, 1995). (C) Nucleotide sequence of the core region of various Holliday junction-containing substrates. Substrates with junction mobility constrained to a region extending across the XerC-catalysed cleavage and strand exchange point are presented in diagram I; those with the mobility region extending away from the cleavage point in diagram II. Only the relevant portion of the core sequence of the *cer6* substrate is presented; the central region (bold case) is framed by a box. The points of XerC-catalysed cleavage and strand cleavage are indicated with arrows. Note that in diagram I the junction is positioned at the point of XerC-mediated strand exchange, while in diagram II it is positioned in the middle of the central region. The changes in *cer6* nucleotide sequence that are present in the various substrates are indicated. A single base pair substitution was introduced into each of two arms in each substrate. The majority of the substitutions were in the central region of *cer6* and should not affect recombination (Sherratt, 1993). The base substitutions in the recombinase binding sites are present in the functional Xer recombination site, *dif* (Blakely *et al.*, 1991).

invariable strand I. In initial experiments, recombinant DNA from reactions incubated with recombinases for 30 min, was analysed on polyacrylamide gels containing

SDS, and on native polyacrylamide gels after proteinase K treatment (Figure 4A and B). Rejoined linear duplex DNA (RLD), covalent complexes of the XerC recombinase

Fig. 3. Probing the mobility of Holliday junction-containing substrates. (A and B) Resolution of the substrates with RuvC. The substrates were 5' end-labelled in top strand I (A) or bottom strand II (B). DNA from reactions carried out in the presence (+) or absence (-) of RuvC was analysed on 10% denaturing gel alongside DNA derived from Maxam-Gilbert AG modification reactions performed on the strand I (A) and HJ4-derived strand II (B). The regions of the expected junction mobility in the substrates are indicated with the vertical lines to the left; the order of the lines is the same as the order of the samples in the gel. The reactions shown were carried out in the presence of magnesium; similar results were obtained when top strand-labelled substrates were analysed in the presence of manganese (data not shown). (C) Chemical modification of unstacked thymine residues with osmium tetroxide. DNA labelled at the 5' end of strand I was modified with OsO_4 in standard recombination buffer, digested with piperidine and analysed on a 12% denaturing gel alongside DNA derived from a Maxam-Gilbert AG sequencing reaction carried out with strand I. The regions of expected junction mobility are indicated with vertical lines to the left of the sample tracks. (D) Sites of RuvC cleavage in top (T) and bottom (B) strands. The positions of RuvC-mediated cleavages in the core region are indicated with arrows. The lengths of the arrows are proportional to the efficiency of cleavage. Regions of junction mobility are indicated with thin lines. The 6 bp central region is shown in bold case. In HJ1, no cleavages outside the core region were observed.

Fig. 4. Recombination of substrates with constrained junction mobility. Recombination reactions were performed in the presence of cell extracts enriched for XerC and XerD (HJ1, HJ2 and HJ4) or partially purified XerC and XerD proteins (HJ1, HJ3, HJ5, HJ6 and HJ7). Recombinant DNA was analysed on a 4% SDS-containing polyacrylamide gel (A), or after deproteinization on a 6% native gel (B). All the substrates were labelled at the 5' end of strand I (+), XerC and XerD present in reaction; (-), no addition of the proteins. HJS, Holliday junction substrate; HJCC, Holliday junction covalent complex; LDCC, linear duplex covalent complex; RLD, rejoined linear duplex; HJ, Holliday junction; LD, linear duplex. Note that HJ and LD species contain intact as well as cleaved DNA molecules.

with the linear duplex (LDCC) and Holliday junction (HJCC) were observed with each of the substrates, although they were generated in different relative amounts (Figure 4A; see also Figure 2A). The size of the linear duplex products is that expected for XerC-mediated recombination (Arciszewska and Sherratt, 1995). Quantitation of the products in 30 min reactions from a number of experiments revealed that recombination produces a distinctive and repeatable pattern of recombinant products (Table I). In order to characterize these reactions in more detail, analyses of recombinant products as a function of time were made (Figures 5 and 6). Comparison of the amounts of recombinant products at 3 and 30 min for HJ1, HJ3, HJ5 and HJ6, all of which are free to branch migrate through the site of XerC-mediated cleavage and strand exchange point, is presented in Figure 5. The patterns of recombination for each of these substrates are very similar: 40–60% of the total product at 30 min is obtained by 3 min, with more of the product at 30 min rejoined than at 3 min. HJ3 was consistently the best substrate, both in terms of total product at either time and with respect to the fraction of product that is rejoined. These results suggest that restricting the mobility of the junction to four bases that include the XerC cleavage

Table I. Recombination of substrates with constrained junction mobility

Substrate	Recombinant product			
	Substrate recombined (%)	Percentage of total product that is:		
		HJCC	LDCC	RLD
HJ1 (<i>cer6</i>)	48	10	25	66
[4]	(42–54)	(6–15)	(12–46)	(48–78)
HJ3	67	3	14	83
[2]	(65–69)	(2–4)	(6–22)	(76–90)
HJ5	49	8	23	70
[2]	(46–52)	(6–9)	(13–33)	(61–78)
HJ6	49	9	32	60
[2]	(48–50)	(6–11)	(18–46)	(48–71)
HJ4	13	55	38	8
[2]	(8–17)	(50–59)	(38)	(3–12)
HJ2	21	35	32	34
[2]	(19–22)	(32–37)	(31–33)	(30–37)
HJ7	43	15	37	48
[3]	(37–46)	(6–22)	(27–58)	(36–57)

Relative reactivity of the substrates assayed by the amount of total recombinant product in a 30 min reaction. RLD, rejoined linear duplex; HJCC, Holliday junction covalent complex and LDCC, linear duplex covalent complex. Bold figures are mean values from the number of experiments indicated in square parentheses; ranges of values from different experiments are presented in round parentheses.

Fig. 5. Recombination of substrates with mobility constrained to the region that contains the point of XerC-mediated strand cleavage. Frequency of recombination is expressed as the percentage of input substrate DNA that has been converted to total recombinant product (i.e. RLD + LDCC + HJCC, see Figure 4A) or to rejoined linear product (RLD) after 3 and 30 min of incubation with XerC and XerD.

point increases the probability of recombination initiation and completion. Restricting the mobility further to two or three bases that contain the XerC cleavage point, resulted in reduced recombination as compared with HJ3. We therefore conclude that either optimal XerC-mediated strand exchange requires at least 4 bp over which branch migration can occur, or the optimal position of the junction crossover for XerC-mediated strand exchange is at least two nucleotides 3' of the exchange point.

Figure 6A–H shows recombination of HJ1 compared with that of junctions HJ4, HJ2 and HJ7, whose mobility is constrained to a region that lies away from the point of normal XerC-catalysed cleavage; i.e. the junctions can approach to within one, three or four nucleotides, respectively, of the XerC-mediated cleavage point. The left hand panels (A, C, E and G) show the accumulation of the total and rejoined product as a function of time. These

Fig. 6. Kinetics of recombination of substrates with mobility constrained to a region away from the XerC-mediated strand exchange point. The plots on the left (A, C, E and G) present the percentage of input DNA converted to either total recombinant product (□), or to rejoined linear duplex (RLD) (◇). The panels on the right (B, D, F and H) show the proportions of the various recombinant products as a percentage of the total recombinant DNA: RLD (*), LDCC (△) and HJCC (○).

reactions were all characterized by an initial rapid rate, followed by a further slow or zero net reaction. The initial rates of the total product accumulation all fell within a 2-fold range as compared with HJ1, indicating that a similar proportion of substrate was able to initiate recombination for each junction. These results suggest that the junction does not need to position itself at or immediately adjacent to the normal strand cleavage and exchange point in order to initiate and complete that strand exchange. In fact recombination of HJ7, a substrate in which the junction position should be at least 4 bp away from the cleavage point, is not very different from recombination of HJ1, as judged by the amounts of the total product accumulated at later times and the amounts of the product of completed strand transfer (see also Table I).

It is important to note that the substrates in this group differ from HJ1, HJ3, HJ5 and HJ6, in that XerC recombination, if it occurs at the predicted position, will generate linear products that contain mismatches. In HJ4, the mismatch would be located at the 5' nucleotide immediately 3' of the XerC cleavage, while in HJ2 and HJ7 the mismatches would be at the third and fourth position 3' of the cleavage, respectively. A decrease in duplex religation as compared with HJ1 was observed with the substrates of this group. This decrease correlates well with the distance of the mismatch position with respect to the predicted point of strand cleavage and rejoining (Table I and Figure 6). Recombination of HJ4, whose products contain a mismatched base pair at the

Fig. 7. Recombination of HJ4 is mediated by XerC. **(A)** Recombination of HJ1 and HJ4 in the presence of the indicated mutants of XerC and XerD. 6% native polyacrylamide gel. HJ, Holliday junction; LD, linear duplex. **(B)** Recombination in the presence of the maltose binding protein recombinase derivatives, MBPXerC and MBPXerD. Four percent polyacrylamide gel containing SDS. The various forms of DNA and protein-DNA complexes are indicated.

break point produces the least amounts of RLD, while recombination of HJ7, whose mismatch is located at the fourth position from the break generates quite high levels of RLD.

The right-hand panels of Figure 6 show data from the same experiments plotted to indicate the proportion of the total product which is in a given form; HJCC, LDCC or RLD. For HJ1, this clearly supports a precursor-product relationship between both covalent complexes and the rejoined product. The same trend is seen with all the other substrates; religated product accumulates with time whereas covalent complexes disappear, presumably being converted to ligated product (in the case of LDCC and HJCC), or back to substrate (in the case of HJCC).

Substrates with constrained junction mobility undergo XerC-mediated recombination

Recombination of all the substrates with restricted junction mobility generated rejoined linear duplex product of the size expected for XerC-mediated strand exchange (Figure 4A). However, analysis of recombinant products after treatment with proteinase K revealed that recombination of HJ4, and to a lesser degree HJ2, produced in addition DNA species of different electrophoretic mobility (Figure 4B). To test whether the products of HJ4 recombination are generated by XerC-mediated catalysis, experiments using derivatives of XerC and XerD mutant in their presumptive catalytic residues and maltose binding fusion proteins of both recombinases were performed (Arciszewska and Sherratt, 1995). Two mutants of each protein were

employed: XerCY275F, XerDY279F, XerCR243Q and XerDR247Q. Recombination of the HJ4 substrate displayed the same catalytic requirements as those for HJ1 (*cer6*); specifically recombinant products were observed when XerD was substituted with XerD mutants but not when XerC mutants substituted for XerC (Figure 7A). Recombination of HJ4 was also examined in the presence of maltose binding fusion proteins of XerC (MBPXerC) and XerD (MBPXerD) to determine which of the recombinases constitutes the protein moiety of the observed covalent complexes. Again, the pattern of electrophoretic mobility of covalent complexes in the presence of different combinations of wild-type or fusion proteins was the same for HJ1 and HJ4, suggesting that XerC is covalently attached to DNA in these complexes (Figure 7B). These experiments confirm that HJ4 recombination is mediated by XerC and indicate that the other substrates are recombined in the same way.

The XerC-mediated cleavage in substrates with constrained junction mobility occurs at the normal position

To test whether XerC-mediated cleavage occurs at the same position in the substrates with constrained junction mobility as observed with HJ1 (Figure 2; Arciszewska and Sherratt, 1995), the position of cleavage was analysed. Recombination reactions using the substrates labelled at the 5' end of an invariable strand I were stopped, treated with proteinase K and DNA was analysed on a sequencing gel (Figure 8). All the substrates produced the same length

Fig. 8. Position of cleavage of Holliday junction substrates with constrained mobility. Substrates were 5' end-labelled on top strand I. Deproteinized DNA from recombinant reactions was analysed on 8% (left) or 10% (right) sequencing gels. (+), XerC and XerD present; (-), no recombinationase. PS, parental strands; RS, rejoined strands; CS, cleaved strands. A longer exposure of the bottom portion of the gel on the left is presented in the inset below to visualise the cleaved fragment generated with HJ2. The several species of cleaved strands in each sample probably differ with respect to the number of amino acid residues that remained attached to the 3' end of DNA after proteinase K treatment.

fragments as observed with HJ1, demonstrating that XerC-mediated cleavage had occurred at the same position in all cases, thus showing that cleavage position is determined by DNA sequence rather than by Holliday junction position.

The two rejoined linear duplex products of HJ7 recombination contain the predicted mismatches

If the XerC-mediated strand exchange occurs at the normal position in substrates in which Holliday junction mobility is constrained to a region located away from this strand exchange point (HJ4, HJ2 and HJ7), then the rejoined linear duplex products of recombination of these substrates should each contain a mismatched base pair (see Figure 2C). The kinetics of the recombination reactions with these substrates is consistent with the presence of the predicted mismatches in the linear duplex products; there is an increased accumulation of cleaved compared with rejoined recombinant product.

Another indication of the presence of mismatches comes from the analysis of proteinase K-treated recombinant DNA: a product of reduced electrophoretic mobility was observed in reactions with HJ4 in addition to the linear duplex product expected for XerC-mediated recombination (Figure 4B). A similarly retarded product was observed

Fig. 9. Identity of the reduced mobility linear duplex product of HJ4 recombination. Deproteinized recombinant Holliday junction and linear duplex DNA were excised and recovered from a native polyacrylamide gel (as in Figure 4B) and analysed on an 8% sequencing gel. HJ, Holliday junction; LD, linear duplex; LD*, reduced mobility linear duplex.

with HJ2, although in this case the band 'smeared' between the normal and retarded positions. We have determined (shown above) that these recombinant products are generated by XerC-mediated catalysis and have inferred that they might represent unligated linear duplex DNA whose mobility is altered due to DNA distortion caused by the presence of mismatched bases close to the cleavage point. To test this hypothesis, HJ4 and HJ1 substrates 5' end-labelled in top strand I, were recombined, deproteinized and fractionated on a preparative polyacrylamide gel. DNA from bands corresponding to 'Holliday junction' as well as 'linear duplex' DNA derived from HJ1 recombination and the reduced mobility 'linear duplex' derived from HJ4 recombination (see Figure 4B), were recovered and analysed on a sequencing gel (Figure 9). As expected, the HJ1 and HJ4 Holliday junction DNA contained mainly parental strands, 92 nucleotides long, with a small proportion of cleaved strands. Linear duplex derived from HJ1 consisted largely of recombinant strands, 84 nucleotides long, with a small amount of cleaved DNA. However, the HJ4-derived linear duplex of reduced electrophoretic mobility contained mainly cleaved strands. The small amount of full-length recombinant strands observed in this sample almost certainly derives from cross-contamination with the faster migrating rejoined linear duplex DNA.

The cleaved products are not completely uniform in size, probably because they each carry a different small number of amino acid residues that remained attached to the 3' end of DNA after proteinase K treatment. The DNA fragments derived from the Holliday junction bands run

Fig. 10. The rejoined linear duplex products of HJ7 recombination contain the predicted mismatches. Rejoined linear duplex products derived from recombination of HJ1 and HJ7 were modified using hydroxylamine (strand II 5' end-labelled) or osmium tetroxide (strand III 5' end-labelled). The products of modification were analysed on a 10% denaturing gel after piperidine treatment alongside a marker (M) of hydroxylamine-treated HJ1 strand II. The sequences of the relevant portions of HJ7-derived strands II and III are presented above and below the gel. The positions of the mismatched C in strand II and the mismatched T in strand III among the products of HJ7 recombination are indicated in bold case in the sequence and with arrows on the gel. Note the difference in sequence between strands II of HJ1 and HJ7; HJ1 carries T at the position of the mismatched C in HJ7. + denotes treatment with hydroxylamine or osmium tetroxide, – denotes no treatment; treated and untreated samples were incubated with piperidine. HJ1 and HJ7 substrates were treated with DNA polymerase Klenow fragment to fill in the cohesive ends of these molecules in order to prevent modification of the four-nucleotide single-strand tails.

slightly ahead of those derived from the linear duplex bands. This might be caused by non-uniform digestion and different accessibility to the protein moiety of the complexes by proteinase K. We have previously shown using 3' end-labelled substrates that DNA strands in Holliday junction covalent complex and linear duplex covalent complex are cleaved at the same position (Arciszewska and Sherratt, 1995).

To test directly if XerC-mediated strand exchange occurs at the normal point in a substrate whose junction is constrained away from that position, we analysed rejoined duplex products for the presence of the predicted mismatches. HJ7 was chosen because its recombination yields sufficient amounts of rejoined recombinant products for analysis. Two HJ7 substrates 5' end-labelled either in bottom strand II or III were used with identically labelled HJ1 as a control. Samples from the recombination reactions were fractionated on a preparative SDS–polyacrylamide gel (for example see Figure 4A) and rejoined linear duplex product DNA was recovered from the gel. Recombination of HJ7 should generate a mismatch in each of the two linear duplex products; an A–C mismatch contains a C in the labelled non-recombinant bottom strand II, while the T–G mismatch contains a T in the labelled non-recombinant bottom strand III. Specific cleavage of the labelled strands at the mismatched C and T was obtained after hydroxylamine and osmium tetroxide modification respectively, and the DNA was analysed on a denaturing polyacrylamide gel (Figure 10). The results demonstrate the presence of unpaired C and unpaired T at the expected positions in strands II and III respectively, in HJ7-derived products. No mismatches were detected in the products

of HJ1 recombination. Some low-level background modifications of C and T bases were observed in the DNA of both substrates. The higher level of hydroxylamine modification at the two C bases adjacent to the mispaired C in the linear duplex product of HJ7 recombination was probably due to some local melting of the duplex DNA near the mismatch. These results demonstrate that in HJ7, the XerC-mediated strand exchange occurs outside the region to which the Holliday junction is constrained (see Figure 2).

We believe that the above data, along with those presented earlier with HJ4, showing inefficient rejoining and indicating a mismatch in unrejoined molecules, support the idea that strand exchange in all of the substrates occurs at the point of observed XerC cleavage. In HJ7, this suggests that XerC strand exchange occurs 4–11 nucleotides away from the expected junction position. Note that mispairing in the recombinant products derived from XerC-mediated exchanges in substrates like HJ4 or HJ7 will occur irrespective of the actual position of the junction at the time of recombination. For example, if a junction had been 'forced' through the heterology barrier towards the position of XerC strand exchange, a mismatched product would still result.

Discussion

Earlier work with lambda integrase/*attP/attB* and FLP/*FRT* (Kitts and Nash, 1987; Nunes-Düby *et al.*, 1987; Bauer *et al.*, 1989; de Massy *et al.*, 1989; Cowart *et al.*, 1991; reviewed in Stark *et al.*, 1992; Landy, 1993) supported the idea that the Holliday junction crossover point is initially located at the site of the first pair of cleavages and strand exchanges. Branch migration through the overlap region, accompanied by isomerization, then relocates the crossover to the point of the second pair of cleavages and strand exchanges, so that these can now occur in the same symmetry/structure context as the first pair of exchanges (Figure 1; discussed in Stark *et al.*, 1989, 1992).

Our data do not support this model for Xer recombination. DNA substrates in which the Holliday junction crossover is constrained to different regions of the *cer6* core site all undergo XerC cleavage at the expected position (Arciszewska and Sherratt, 1995). We conclude that the Holliday junction need not be at this position during recombination, based on the premise that a given junction is in the predicted region. Our data on RuvC and osmium tetroxide cleavage, which show that the majority of the junctions are constrained to the expected regions most of the time, support this assumption. Panyutin and Hsieh (1993), have previously demonstrated that creation of a mismatch is a major barrier to branch migration under conditions in which migration is either relatively slow (for example, in the presence of Mg^{2+} , conditions similar to those used here for RuvC cleavage), or more rapid because of the unstacked square planar nature of the junction (for example, in the absence of Mg^{2+} , buffer conditions used here for the recombination and osmium tetroxide modification reactions). We expect that energetically unfavourable branch migrations that move the crossover outside of the constrained regions and therefore generate two separate mismatches will be sufficiently rare

that they would not contribute significantly to the pool of recombining substrates. The observation that the initial rate of product formation is within a 2-fold range for all the substrates, indicates that a similar fraction of molecules is available for recombination in each case. Nevertheless, we cannot exclude the possibility that on XerC/XerD binding, the junctions are effectively 'forced' to a new recombinogenic position, though we think this unlikely. For example, the free energy difference between the Holliday junction substrates in which the four duplex arms are fully base-paired to derivatives in which branch migration has generated mismatches in two of the duplex arms should be of the order of 50 kJ/mol as a consequence of losses in Watson-Crick base pairs and reduced helical stacking. Since XerC and XerD each bind their sites with a K_D in the μM to nM range (with a cooperativity index of ~ 100 ; Blakely *et al.*, 1993; G.Blakely and D.J.Sherratt, unpublished data), it is unlikely that sufficient energy would be available from protein binding to stabilize the mismatched structures. The effects of recombinase binding on Holliday junction position can only be tested experimentally if we can find reagents that can detect the junction position in the presence of the recombinases.

The kinetics experiments point strongly to a precursor-product relationship between cleaved and rejoined products for all of the substrates tested, supporting the view that cleavage and strand exchange occur at the same position irrespective of the Holliday junction location. With substrates that are free to branch migrate through the exchange point, rejoined product is the major product after the first few minutes. In contrast, substrates whose junction is constrained away from the cleavage point, show reduced rejoining, presumably because such joining generates products with mispaired bases. We demonstrate directly the presence of the predicted mispaired bases in the rejoined products of HJ7 recombination and show that the cleaved product of HJ4 recombination has an aberrant electrophoretic mobility, presumably because of an unpaired base at the cleavage point. Strand rejoining is least efficient when the mispaired base is immediately adjacent to the cleavage point, whereas ligation is almost normal when the mispaired base is four nucleotides away, reinforcing the conclusion that the observed cleavages are on the strand exchange pathway.

The ability of XerC to rejoin mismatches and the observation that cleavage and strand rejoining can be relatively uncoupled in this system, as judged by the substantial amounts of covalent complexes observed, could relate to the ability of Xer recombination to recombine sequences *in vivo* that are heterologous in their central region (McCulloch *et al.*, 1994). Among junctions that can branch migrate through the XerC cleavage position (HJ1, HJ3, HJ5, HJ6), the most efficient substrate in terms of overall recombination and ligation efficiency was HJ3, indicating that either the optimal junction position is at least two nucleotides 3' of the cleavage point or that branch migration of four nucleotides provides a more optimal substrate than one in which it is limited to three or less nucleotides. We prefer the former explanation, a conclusion similar to that of Nunes-Düby *et al.* (1995), based on *in vitro* experiments with lambda Int on Holliday junction substrates.

The analysis of reaction kinetics also showed that a

rapid initial recombination rate was followed by a much slower or zero rate. We believe that this may indicate that only a proportion of the substrate is initially available for XerC-catalysed recombination. This could arise as a consequence of helical stacking induced by cooperative XerC and XerD binding to the initially unstacked Holliday junction substrate. Two 'stacked X' isomers are likely to result from such binding, one with top strands crossed (discontinuous) and the other with bottom strands crossed (see Duckett *et al.*, 1988; Nunes-Düby *et al.*, 1995). Preliminary data (L.K.Arciszewska and D.J.Sherratt, unpublished) are consistent with such recombinase-induced helical stacking. We would expect that only one of the two isomers would be competent for XerC-mediated recombination. The other isomer would need to undergo isomerization, which would require at least partial complex dissociation and re-assembly, before it could undergo XerC strand exchange. This process may be slow and could be reflected in the second phase of slow or zero recombination. Kinetic evidence from experiments with FLP recombinase has suggested the presence of two distinct forms of the Holliday junction intermediate and the requirement for an isomerization step (Meyer-Leon *et al.*, 1990). Nunes-Düby *et al.* (1995), studying lambda Int-mediated recombination, have proposed that only one Holliday junction isomer is competent in each separate pair of strand exchanges, with the crossed strands being cleaved by Int. T4 exonuclease VII also cleaves crossed strands in a Holliday junction (Duckett *et al.*, 1988; Mueller *et al.*, 1988).

Our results do not readily support the pivotal role of branch migration implicit in earlier models of recombination for integrase family members. We are attracted by the principles of the 'strand swapping' model of Nunes-Düby *et al.* (1995), who have proposed that Int-mediated resolution of a Holliday junction substrate with a 7 bp overlap region occurs optimally when the junction cross-over is positioned three nucleotides 3' of a cleavage point. After cleavage, three nucleotides are transferred to the partner duplex in a single strand 'swap'. This avoids the extensive helical rotation associated with conventional branch migration over 7 bp and suggests that branch migration would need to occur over no more than the central base pair of the overlap region. Isomerization of the junction as it moves over the central base pair would position the scissile bonds correctly for either top or bottom strand exchanges.

Our data, which suggest that the optimum junction position is at least two nucleotides 3' of the XerC cleavage point, are consistent with this model. Nevertheless, we note that junctions positioned closer to or more distant from the cleavage point recombine relatively efficiently, indicating that if 'strand swapping' occurs in the Xer system, either a variable number of nucleotides can be displaced in a single 'swap', or bound XerC/D can direct the junction to the optimal 'swap' position. In FLP recombination, strand exchange can also occur when the junction is constrained to different regions (Dixon and Sadowski, 1994). We have no evidence to support the idea that junction position dictates whether top or bottom strand cleavage will occur, perhaps by determining the isomerization state of the junction, as proposed for Int. For example, positioning of the junction close to the

presumed bottom strand exchange point did not lead to loss of top strand XerC cleavage at the normal position and gain of bottom strand cleavage with this substrate. Our failure to observe bottom strand cleavage on these substrates is a mystery, though substrates of different sequence and structure have been shown to undergo XerD-mediated bottom strand cleavage *in vitro* at the predicted position, between the central region and the XerD binding site (see Figures 1 and 2B; G.Blakely and D.J.Sherratt, unpublished).

Materials and methods

Strains and plasmids

The strains and plasmids that encode the wild-type and mutant derivatives of XerC and XerD, and the fusion derivatives of both recombinases with maltose binding protein were described in Blakely *et al.* (1993) and in Arciszewska and Sherratt (1995).

Preparation of substrates with constrained junction mobility

The *cer6* (HJ1) Holliday junction substrate was constructed from four synthetic oligonucleotides (92, 84, 84 and 76 nucleotides), as described in Arciszewska and Sherratt (1995). Oligonucleotides for other substrates were of the same length and carried the same sequences as strands I–IV of HJ1, except that they contained base pair substitutions as shown in Figure 2C. They also contained different sets of the 4 bp sequence at their 5' termini: all the junctions carried 5' GATC in strand I, 5' ATCG in strand II, 5' TCGA in strand III and 5' CGAT in strand IV to allow differential labelling of the 3' ends. The strand I which was invariant in all the substrates was labelled at 5' end with T4 DNA polynucleotide kinase using [γ - 32 P]ATP. The junctions were assembled by re-annealing equimolar amounts (either 5 or 10 pmol) of 5' end-labelled strand I with the other three unlabelled strands. 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 electroelution and the volume of the resuspending solution was adjusted accordingly.

Cell extracts and purified protein fractions

The method for the preparation of cell extracts enriched for wild-type or mutant XerC and XerD has been described (Arciszewska and Sherratt, 1995). Extracts enriched for maltose binding fusion proteins of both recombinases were prepared according to the procedure suggested by New England Biolabs, and were supplemented with glycerol to 50% volume. Preparation of purified fractions of XerC and XerD was as described in Arciszewska and Sherratt (1995).

Recombination reactions

The reactions were carried out and terminated as described previously (Arciszewska and Sherratt, 1995). For time course experiments reactions were upscaled; the aliquots were removed at time points and stopped by addition of a 20% volume of SDS buffer (50 mM Tris–HCl, pH 7.5, 1% SDS). The zero-time reactions did not contain XerC and XerD. All incubations were at 37°C. Recombinant DNA was analysed through native TBE (89 mM Tris–borate, pH 8.3, 2 mM EDTA)–6% polyacrylamide gels or through 4% polyacrylamide–TBE gels containing SDS (0.1%). The bands in the gels were quantified on a PhosphorImager. For analysis on sequencing gels DNA was precipitated in the presence of tRNA (10 μ g) and resuspended in formamide loading buffer.

RuvC cleavage

RuvC cleavage reactions were performed in buffer containing 50 mM Tris–HCl, pH 8.0, 1 mM DTT, 10 mM MgCl₂ and 100 μ g/ml bovine serum albumin and ~16.7 mg/ml RuvC at 37°C for 30 min. Incubations were stopped by addition of 20% volume of stop solution (50 mM Tris–HCl, pH 7.5, 1% SDS) and deproteinized by addition of 10% volume of proteinase K stock solution (4 mg/ml). DNA was precipitated in the presence of tRNA (10 μ g), resuspended in formamide loading buffer and analysed on 10% sequencing gel.

Chemical modification of DNA

Osmium tetroxide modifications (Lilley and Palacek, 1984; Duckett *et al.*, 1988; Lilley, 1992; Palacek, 1992) were performed in reactions (100 μ l volume) containing 3% pyridine and 1 mM OsO₄ for 15 min at room temperature and were stopped by addition of 15 μ l of 3 M NaAc

(pH 5.5) and 10 μ g of tRNA, which was followed by ethanol precipitation of DNA. For modifications with hydroxylamine (Rubin and Schmid, 1980; Johnston and Rich, 1985; Johnston, 1992) 20 μ l of hydroxylamine hydrochloride stock solution (4 M, adjusted to pH 6.5 with diethylamine) was added to 7 μ l DNA in TE and incubated at 30°C for 20 min. Reactions were stopped by addition of 200 μ l of 0.3 M NaAc (pH 5.5) and 10 μ g tRNA and DNA was precipitated with ethanol, resuspended in the same volume of NaAc and re-precipitated. Chemical modifications for Maxam–Gilbert sequencing and piperidine digestions were performed according to the procedure recommended by Amersham.

Acknowledgements

We are grateful to K.Grant for providing partially purified XerC and XerD; to M.Braddock for his kind help in phosphorimager analysis; and to S.West for the generous gift of RuvC. We thank S.Nunes-Düby and A.Landy for communicating unpublished results and S.Nunes-Düby, A.Landy, M.Jayaram and H.Nash for helpful discussions. These experiments were inspired by the ideas of S.Nunes-Düby. We thank S.Colloms for valuable comments on the manuscript. The work was supported by the Wellcome Trust and the Medical Research Council.

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Received on January 16, 1995; revised on March 13, 1995