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Site-specific recombination and circular chromosome segregation

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SUMMARY

The Xer site-specific recombination system functions in *Escherichia coli* to ensure that circular plasmids and chromosomes are in the monomeric state prior to segregation at cell division. Two recombinases, XerC and XerD, bind cooperatively to a recombination site present in the *E. coli* chromosome and to sites present in natural multicopy plasmids. In addition, recombination at the natural plasmid site *cer*, present in ColE1, requires the function of two additional accessory proteins, ArgR and PepA. These accessory proteins, along with accessory DNA sequences present in the recombination sites of plasmids are used to ensure that recombination is exclusively intramolecular, converting circular multimers to monomers. Wild-type and mutant recombination proteins have been used to analyse the formation of recombinational synapses and the catalysis of strand exchange *in vitro*. These experiments demonstrate how the same two recombination proteins can act with different outcomes, depending on the organization of DNA sites at which they act. Moreover, insight into the separate roles of the two recombinases is emerging.

1. INTRODUCTION

In site-specific recombination, recombinase proteins catalyse conservative break–join reactions at specific sites in DNA. Site-specific recombination is involved in a range of programmed DNA rearrangements in microbes. These include the integration and excision of viruses into and out of the bacterial chromosome, inversion gene switches that control the expression of bacterial cell-surface proteins and phage tail proteins, the processing of transposition intermediates and the copy-number control and stable segregation of plasmids (reviewed in Stark *et al.* 1992).

The physical state of circular chromosomes, unlike linear chromosomes, can be changed by homologous recombination. Any number of homologous recombinational exchanges between sister, or homologous, linear chromosomes will generate linear chromosomes of the same size as the parents (assuming the parents are of the same length and have the same gene organization). In contrast, odd numbers of homologous recombinational exchanges between sister, or homologous, circular chromosomes will lead to circles in which two chromosomes-worth of DNA are present in a single dimeric DNA molecule (Figure 1).

The Xer site-specific recombination system appears to function in *Escherichia coli* to convert circular dimers of the *E. coli* chromosome and of multicopy plasmids to monomers, thereby helping chromosome and plasmid segregation at cell division. This system requires two related recombinase proteins, XerC and XerD, en-

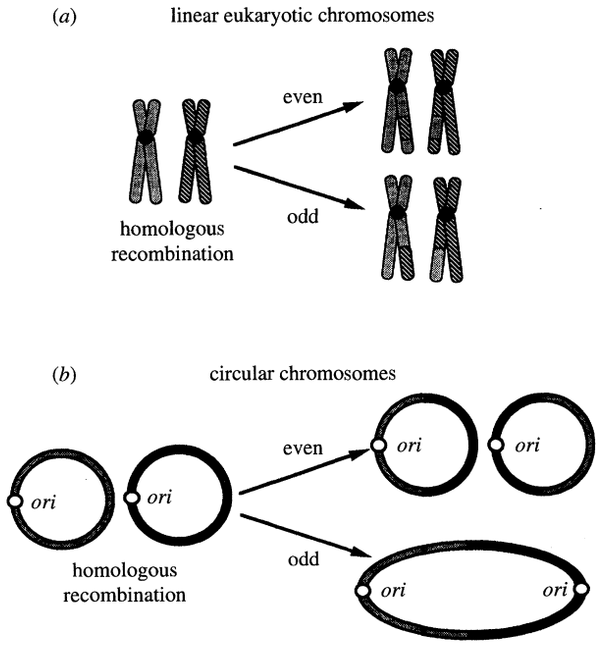

Figure 1. Cartoon illustrating the consequences of homologous recombination on (*a*) linear and (*b*) circular chromosomes. Note that on linear chromosomes, homologous exchanges can occur between homologues (shown) or between sister chromosomes.

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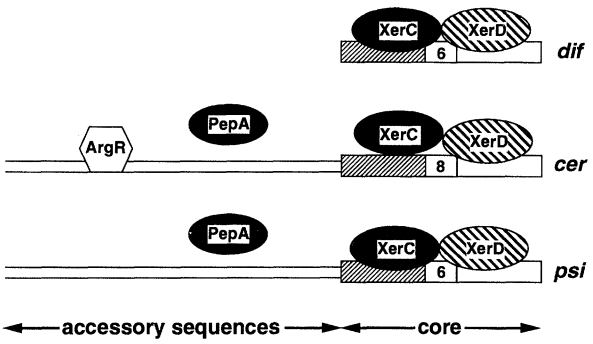

	XerC	central region	XerD
cer	GGTGCGTACAA	TTAAGGGA	TTATGGTAAAT
psi	GGTGCGCGCAA	GATCCA	TTATGTTAAAC
clf	GGTACCGATAA	GGGATG	TTATGGTAAAT
cer 8-1	GGTGCGTACAA	TTGGGATG	TTATGGTAAAT
cer 6-1	GGTGCGTACAA	GGGATG	TTATGGTAAAT
dif	GGTGCGCATAA	TGTATA	TTATGTTAAAT
dif 8	GGTGCGCATAA	TTTGTATA	TTATGTTAAAT
dif 1-1	GGTGCGCATAA	TCTAGA	TTATGTTAAAT
consensus	GG[TG/GA]C[GTAT/CGGC/C]AA		TTATG[G/T]TAAA[T/C]

Figure 2. Xer recombination sites. The top panel provides a cartoon of the *dif*, *cer* and *psi* recombination sites while the bottom panel shows the sequence of a number of core recombination sites. A functional *dif* site consists of just a recombination core of XerC and XerD binding sites separated by 6 b.p. A functional *cer* site is about 220 b.p. ArgR binds about 100 b.p. upstream of the core site. We do not yet know if PepA binds DNA and/or interacts with one of the other recombination proteins. *psi* (present in plasmid pSC101; Cornet & Louarn 1994) has accessory sequences that do not interact with ArgR. PepA is required for its recombination, which shows resolution selectivity. Note that in *cer* the XerC binding site is 2 b.p. removed from the XerD binding site when compared to *dif*. This will result in increased spacing and approximate 72° rotation in relative position between the two binding sites.

coded at 4024 kb.p. and 3050 kb.p. on the *E. coli* chromosome respectively. Each recombinase is co-expressed with at least two other proteins that appear not to have a role in Xer recombination (Colloms *et al.* 1990; Blakely *et al.* 1993). XerC and XerD contain amino acid residues conserved in the integrase family of site-specific recombinases. Recombination at sites present in natural multicopy plasmids (for example, the 220 b.p. *cer* site in plasmid ColE1) requires two additional proteins, ArgR and PepA (Stirling *et al.* 1988, 1989), and is exclusively intramolecular (i.e. it shows resolution selectivity). In contrast a 32 b.p. sequence, *dif*, normally present in the replication terminus region of the *E. coli* chromosome and required for normal chromosomal segregation, recombines both inter- and intramolecularly when inserted into a plasmid recombination substrate (Blakely *et al.* 1991). Recombination at *dif* requires XerC and XerD but not ArgR and PepA. These and other results indicate that ArgR and PepA act as accessory proteins and are involved in determining resolution selectivity (Summers 1989; Blakely *et al.* 1993). Similarly 190 b.p. of the 220 b.p. *cer* site are accessory sequences that function in resolution selectivity, whilst the remaining 30–32 b.p. constitute the recombination core, that is similar in sequence to the 32 b.p. *dif* site (see figure 2; Blakely *et al.* 1993).

Current experiments are aimed at understanding: (i) how the interactions of the recombination proteins with recombination sites generate a functional recombination synapse that may (*cer*) or may not (*dif*) show resolution selectivity during recombination; (ii) the catalytic mechanism of recombination; and (iii) how, precisely, recombination at *cer* and *dif* leads to stable circular replicon segregation at cell division.

2. RESULTS AND DISCUSSION

(*a*) *Interaction of XerC and XerD with* cer *and* dif *recombination core sites*

In figure 2 the overall structure of functional *cer* and *dif* sites are cartooned, and the DNA sequences of the core recombination sites of *cer*, *dif* and some related derivatives are shown. XerC binds to the left half of the core sites and XerD to the right half of the core sites (Blakely *et al.* 1993). The affinity of XerC for its half site is lower than that of XerD for its half site. Binding of both XerC and XerD is highly cooperative, with the affinity of each protein for its half site being 40–160-fold higher when the other recombinase is already bound (Blakely *et al.* 1993). Note that the *cer* core site and *dif* have 8 b.p. and 6 b.p. respectively in their central regions that separate the XerC and XerD binding sites. This difference results in reduced overall binding of XerC/XerD to *cer* when compared to *dif*, and a different overall geometry to the protein/DNA complex for the complexes with *cer* and *dif* (Blakely *et al.* 1993), so that the XerC/XerD complex with *cer* migrates more slowly than the equivalent complex with *dif* (Blakely *et al.* 1993).

Because the core *cer* site differs from *dif* not only in the length of the overlap region but in residues involved in XerC and XerD binding, a *dif* derivative containing 8 b.p. in its central region was constructed by inserting the dinucleotide TT to the 5′ end of the central region (see figure 2, which shows there is a one nucleotide difference in the XerD-binding site and a two nucleotide difference in the XerC-binding site). *dif8* has similar properties to *cer*; it is inactive as a core recombination site but when supplied with accessory sequences and the accessory proteins ArgR and PepA, as well as XerC and XerD, intramolecular resolution occurs *in vivo* with a plasmid containing two directly repeated sites.

The affinities of XerC/XerD to *dif8* and the overall geometry of the protein/DNA complex are more similar to that of the complex with *cer* than to the complex between wild-type *dif* and XerC/XerD. Similarly, a *cer* derivative containing only 6 b.p. in its central region (*cer6-1*) has the recombination properties of *dif*, recombining inter- and intramolecularly without accessory sequences and accessory proteins. Taken together, these observations show that the different recombination properties of *cer* and *dif* (i.e. exclusive

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intramolecular recombination compared to both inter- and intramolecular recombination respectively) and their different recombination requirements can be determined simply by changing the relative spacing of the XerC and XerD binding sites. Note, however, that recombination properties and requirements can also be changed by alterations to the XerC binding site. For example, *psi* and *clf* sites (see figure 2) have only a 6 b.p. central region. Nevertheless, they require accessory proteins and accessory sequences for recombination, which is exclusively intramolecular. *dif* differs from the *cfl* core site only in the XerC-binding site and central region and therefore these differences must determine the different recombination requirements and outcomes for these sites.

We have proposed (Blakely *et al.* 1993) that resolution selectivity can arise when recombinase–core site interactions and recombinase–recombinase interactions are too weak to allow stable recombination synapse formation. In systems where such interactions are strong enough to allow functional synapse formation, recombination can occur without additional proteins and DNA sequences and will show no selectivity for a particular configuration of recombination sites (e.g. recombination at *dif*, *cer6-1*; with FLP at *frt* sites and with Cre at *loxP* sites). We propose that sites with weaker recombinase interactions need additional DNA sequences and accessory proteins that interact with these sequences to form a functional recombinational synapse (e.g. *cer*, *dif8-1*, *psi*, *clf*; *res* and *gix* (the sites at which Tn3 resolvase and Gin invertase interact, respectively)). The complexity of such a nucleoprotein structure and the fact that the DNA will follow a highly defined path through such a complex, provides a mechanism for resolution selectivity (see Stark *et al.* 1989) that has been well documented for the Tn3 resolvase and Gin invertase systems.

(*b*) *Recombination at the chromosomal site* dif *and chromosome segregation*

E. coli dif is located in the replication terminus region of the chromosome (see figure 3) (Blakely *et al.* 1991; Kuempel *et al.* 1991). Deletion of *dif* with about 2 kb of flanking DNA results in cells that filament and show abberant chromosome segregation, a phenotype indistinguishable to that of XerC$^-$ and XerD$^-$ cells. Reinsertion of a 33 b.p. fragment containing the *dif* core site into the deleted region results in cells that appear to divide and segregate their chromosomes normally. This same fragment is a competent recombination site *in vivo* when inserted into a plasmid. Reinsertion of *dif* into either of two ectopic sites (close to *oriC* and within *lacZ*) of chromosomes deleted for normally located *dif*, does not suppress the filamentation-abberant segregation phenotype, despite the fact that such sites are competent for Xer recombination – as judged by their ability to recombine with *dif* sites present in a plasmid. As yet we do not understand why *dif* does not allow normal chromosomal segregation when inserted at these ectopic sites.

Since recombination at *dif*, at least when inserted in a plasmid, does not show resolution selectivity, how

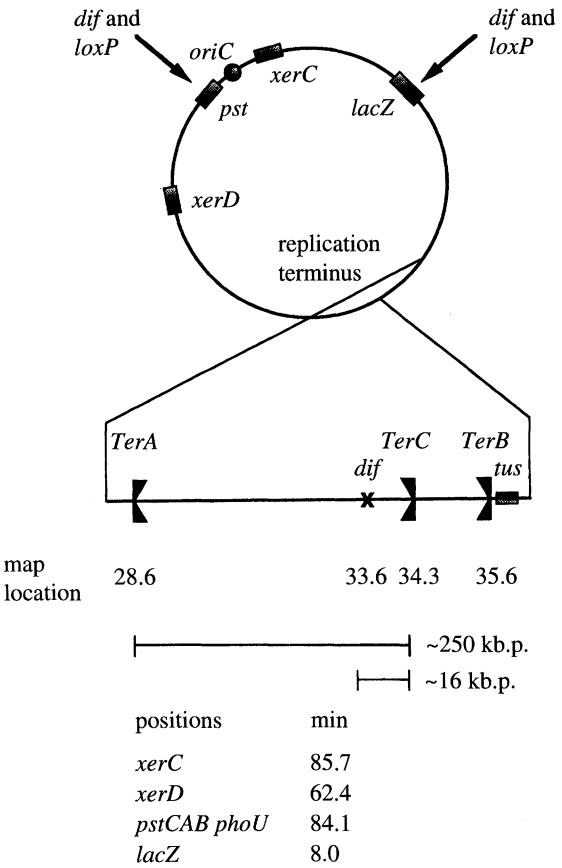

Figure 3. A diagrammatic map of the *E. coli* chromosome indicating the normal position of *dif* in the replication terminus region and two ectopic positions (constructed in a strain deleted for its normal *dif* site). The *loxP* site was also inserted at these positions. Neither *dif* nor *loxP* allowed normal chromosome segregation when inserted ectopically.

does such recombination ensure that chromosomes are monomeric prior to cell division? We have proposed two models (Sherratt 1993). In the first, rapid Xer-mediated exchanges occur after *dif* is replicated, so that rapid interconversion of monomers to dimers occurs. The partition mechanism will then have an opportunity to segregate monomers. In the second model, one pair of Xer-mediated strand exchanges occurs and generates Holliday junction irrespective of whether the completely replicated chromosomes are destined to be monomers or dimers. As the partition mechanism begins to separate the joined chromosomes, a second pair of exchanges occurs in the direction that will ensure monomer segregation. The second model is attractive because of its economy and the possible separate control of the two pairs of strand exchanges in this system, but we have no direct experimental evidence to support it.

(*c*) *The role of the two recombinases, XerC and XerD, in Xer site-specific recombination*

The best characterized site-specific recombination systems (e.g. lambda Int, FLP, Cre) use a single recombinase to mediate the recombination event. Why then does the Xer system require two? Earlier experiments (Blakely *et al.* 1993) have demonstrated

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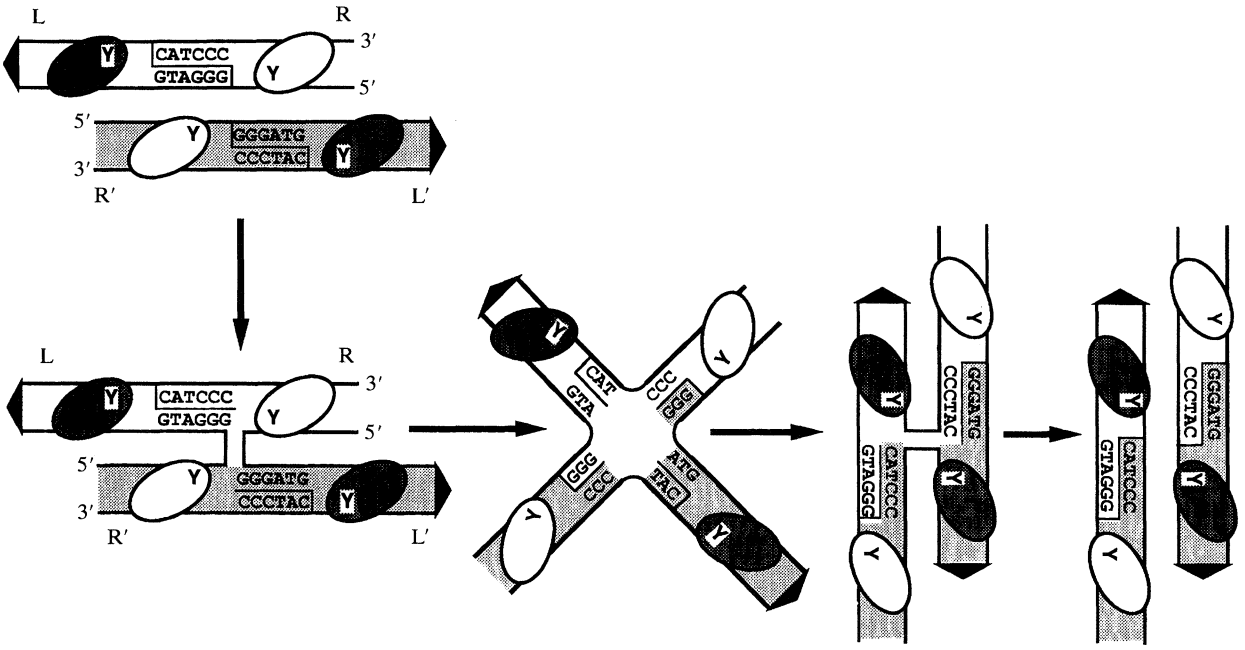

Figure 4. Cartoon showing a 'classical' view of the integrase family recombination mechanism as applied to the Xer system. XerC molecules are represented as unfilled ovoids while XerD molecules are shaded dark. The central region is that of *cer6.1*. After synapsis (here arbitrarily indicated in the antiparallel configuration) a pair of XerC-mediated strand exchanges give a Holliday junction containing intermediate. Branch migration/isomerization move the exchange point to where the XerD-mediated exchanges would be expected to occur. After such exchanges recombinant product forms.

that the asymmetry introduced into core recombination sites by having separate half-sites binding different recombinases (see figure 2), is used to ensure that only correctly aligned sites are recombined in the Xer system. In contrast, correct alignment is ensured in single recombinase systems by either using asymmetry outside of the core recombination site (e.g. with Tn3 *res*, Bednarz *et al.* 1990; Stark *et al.* 1992) or asymmetry at the centre of the core site in the region flanked by the strand exchanges (lambda Int, Gin; see Stark *et al.* 1992). We believe that the need for correct alignment does not provide the major reason for using two recombinases, since *dif* and *cer* sites contain symmetry in their central regions that in principle could be used to ensure that only correctly aligned sites recombine. We suspect, therefore, that the prime requirement for two recombinases is to be able to control the two pairs of strand exchanges differentially. Our data are consistent with each recombinase catalysing one pair of strand exchanges (Blakely *et al.* 1993). By having two recombinases, each pair of strand exchanges is potentially under separate biochemical and genetic control, thus allowing the production and resolution of Holliday junction intermediates to be functionally separated. How this relates to the biological role of this system remains unclear, though as indicated earlier, we have previously suggested how such functional separation of the pairs of strand exchanges could function in chromosome segregation (Sherratt 1993). Experimental evidence from both *in vivo* and *in vitro* experiments supports such functional separation. For example, Xer recombination between two directly repeated *cer* sites in a plasmid substrate *in vivo* in an *E. coli* strain, RM40, in which XerC expression is under the stringent control of the *lac* promoter, can lead to the production of XerC-mediated Holliday junction-containing intermediates that persist over several hours after induction of XerC expression (McCulloch *et al.* 1994), despite the fact that such cells are *xerD*$^+$. Similarly, *in vitro* assays using any of three different recombination substrates have demonstrated XerC-mediated strand exchanges in the absence of XerD-mediated exchanges in reactions that require the presence of both recombinases (see below). Nevertheless, both XerC and XerD mediated cleavages can be observed *in vitro* (see below), and have been inferred to occur *in vivo* (Blakely *et al.* 1993). Taken together, these results indicate that subtle changes in recombination site sequence and the structure of the complexes with recombination proteins can influence the outcome of the recombination reaction.

The separation of the XerC and XerD coding sequences on the *E. coli* chromosome, and the co-expression with other genes may also point to the importance of the functional separation of the XerC and XerD activity, though the genes co-expressed with XerC and XerD remain a bizarre collection with no apparent relation to the recombinase function: XerD is co-expressed with DsbC (previously XprA, see Lovett & Kolodner 1991) a periplasmic disulphide isomerase (Missiakas *et al.* 1994) and *RecJ*, a 5′ exonuclease involved in homologous recombination (Lovett & Kolodner 1991). XerC is co-expressed with a DapF (diaminopimelate epimerase) and two genes of unknown function (Richaud *et al.* 1987; Colloms *et al.* 1990). The functions of none of these other genes are essential for Xer recombination at *cer* or *dif* (our unpublished data).

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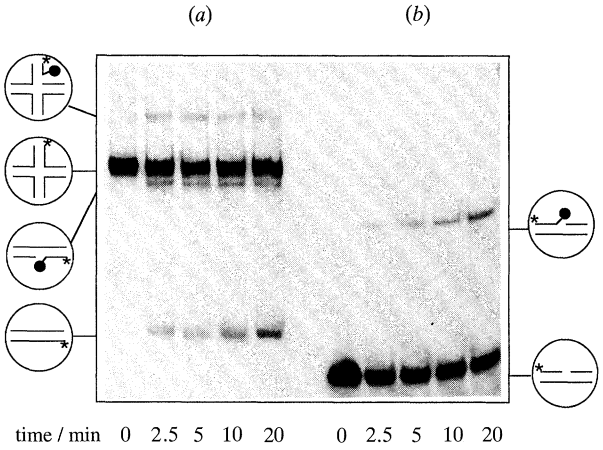

Figure 5. Xer site-specific recombination *in vitro* on Holliday junction substrates (*a*) and linear suicide substrates (*b*). Substrates contained the *cer6.1* core site and were incubated with purified XerC and XerD. The Holliday junctions were constructed as described in the text. The suicide substrates were constructed by annealing three oligonucleotides as shown in the diagram. The nick is between the third (G) and the fourth (A) nucleotide of the central region. XerC-mediated cleavage releases a trinucleotide and generates a stable covalent protein DNA intermediate (-(●)) between XerC and recombinase. In panel (*a*), the positions of duplex products and covalent intermediates are indicated. The gel is 4% polyacrylamide in TBE + 0.16% SDS. Visualization was by autoradiography. The position of ^{32}P atoms is indicated (*).

(*d*) *The Xer site-specific recombination strand exchange mechanism*

The classical view of integrase family-mediated site-specific recombination when applied to the Xer system is cartooned in figure 3. Two recombinase molecules (one each of XerC and XerD in the Xer system) bind specifically to each core site. Recombinase–recombinase interactions now lead to recombination synapse formation. In systems using no additional accessory sequences or accessory proteins (e.g. FLP, Cre, Xer recombination at *dif*) recombinase–DNA and recombinase–recombinase interactions lead to synapsis which occurs irrespectively of the configuration of the recombination partners (i.e. whether on separate molecules or in either direct or inverted configuration on the same molecule). Within synaptic complexes, phosphodiester bond activation followed by two transesterifications (using a recombinase tyrosine nucleophile and a 5′ OH nucleotide nucleophile, respectively) lead to the completion of one pair of strand exchanges. Holliday junction branch migration/isomerization now place the intermediate ready for the second pair of transesterifications (see figure 3).

In order to test this model for the Xer system and in particular to determine the functional roles of the two recombinases, we constructed synthetic Holliday junction molecules containing the *cer6-1* core recombination site. We reasoned that such structures would avoid the need for a potentially rate-limiting synapsis step and should be good candidates for *in vitro* recombination substrates. The *cer6-1* sequence was chosen because it can recombine with or without accessory sequences and accessory proteins. The initial substrates were produced by annealing four synthetic oligonucleotides of 76, 2 × 84 and 92 nucleotides. The resulting four-way junction could potentially branch migrate throughout the whole core region (but not beyond it) and each of the four arms has diagnostic restriction sites to allow product analysis. Subsequent substrates have had the junction position constrained to particular regions of the core by using heterology to block branch migration.

Recombinant product with any of these substrates requires that both XerC and XerD be present. In the presence of both proteins, recombinant products are observed (see figure 4, panel *a*). The products have the size expected for an XerC-mediated strand exchange (assuming XerC activates and cleaves the phosphodiester adjacent to its binding site, see figure 2). No evidence of XerD-mediated cleavage or substrates containing the *cer6-1* or *cer* cores sites has been observed for reasons we do not understand. The majority product from Xer-mediated cleavage is complete duplex in which both pairs of strand exchanges have been completed. Significant amounts of covalent complex between XerC recombinase and both duplex product and Holliday junction substrate were also observed. This has allowed mapping of the Xer-mediated cleavages in these intermediates (cleavage occurs between the last nucleotide of the XerC-binding site and the first nucleotide of the central region, see figure 2) and has allowed the demonstration that it is XerC rather than XerD that becomes covalently bound to DNA (by using fusion protein derivatives of both XerC and XerD that have different sizes to the parental protein, data not shown). By using mutants of XerC and XerD that are defective in their presumptive active site tyrosine nucleophile (Y converted to F) or defective in their presumptive activation domain (R converted to Q) or defective in both activation and nucleophile (Blakely *et al.* 1993), we have observed normal levels of XerC-mediated recombinant product when XerD is defective in nucleophile or activation domain, or both domains, as long as XerC is wild-type (though note that XerD protein needs to be present for reaction to occur). In contrast, mutation in either nucleophile or activation domain of XerC abolishes recombinant product formation on this substrate, confirming that XerC is mediating the catalysis we observed. We have observed no functional complementation between XerC and XerD mutant proteins. Moreover in mixtures containing XerD along with $XerC^{YF}$ and $XerC^{RQ}$ proteins we have observed no functional complementation between the XerC mutants (as measured by covalent complex production). All of these mutant proteins bind their cognate core recombination half-sites and can interact co-operatively. We therefore have found no evidence to support the fractional active site hypothesis of Jayaram and co-workers (Chen *et al.* 1992; Jayaram 1994) which suggests that for FLP recombinase, two recombinase protomers cooperate to form a catalytically-active molecule in which a single-strand exchange is mediated by the activation domain of one protomer and the nucleophile of the other protomer acting in

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trans. Certainly our data are inconsistent with the '*trans*-diagonal' and '*trans*-horizontal' models of Jayaram and co-workers as applied to Holliday junction resolution in the Xer system; moreover, we can find no support for their '*trans*-vertical' model in our system. We therefore favour the idea that on a Holliday junction substrate a single XerC molecule promotes both phosphodiester activation and nucleophilic attack at that activated phosphodiester.

We find essentially the same results with a 'suicide' substrate (see figure 5*b*). In this linear substrate a recombinase-mediated cleavage leads to the diffusion away of a trinucleotide (Pargellis *et al*. 1987), thus stabilizing the recombinase-DNA covalent intermediate. With such substrates we have observed XerC but not XerD-mediated catalysis. Attempts at complementation have yielded the same results as for the Holliday junction substrate.

In other experiments (data not shown), we have used supercoiled substrates containing two directly repeated *cer* sites or two directly repeated *psi* sites (see figure 2). Addition of two recombinases and appropriate accessory proteins leads to recombinant product formation. The recombinants have a unique topology, confirming our ideas about resolution selectivity. With the *cer* substrate, the products are Holliday junction-containing supercoils in which XerC-mediated catalysis has occurred. In contrast, the *psi* substrate undergoes both pairs of strand exchanges. This preliminary result further confirms how subtle changes in recombination sites can effect the outcome of Xer recombination.

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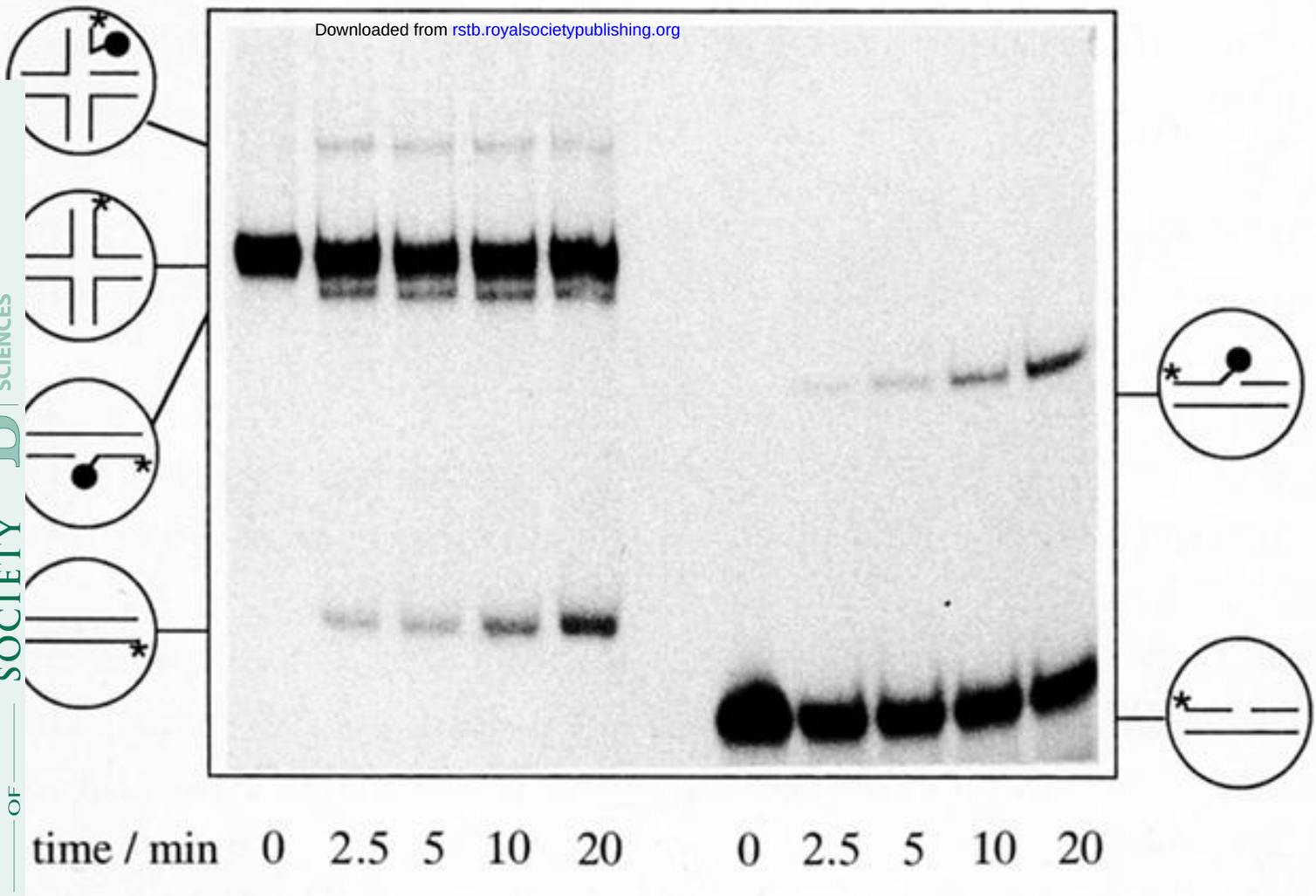

Figure 5. Xer site-specific recombination *in vitro* on Holliday junction substrates (*a*) and linear suicide substrates (*b*). Substrates contained the *cer6.1* core site and were incubated with purified XerC and XerD. The Holliday junctions were constructed as described in the text. The suicide substrates were constructed by annealing three oligonucleotides as shown in the diagram. The nick is between the third (G) and the fourth (A) nucleotide of the central region. XerC-mediated cleavage releases a trinucleotide and generates a stable covalent protein DNA intermediate ( ) between XerC and recombinase. In panel (*a*), the positions of duplex products and covalent intermediates are indicated. The gel is 8 % polyacrylamide in TBE + 0.16 % SDS. Visualization was by autoradiography. The position of ^{32}P atoms is indicated (*).

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