The Cre Recombinase Cleaves the *lox* Site in *trans**

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The Cre protein is a conservative site-specific recombinase that is encoded by bacteriophage P1. Its function *in vivo* is to resolve dimeric lysogenic P1 plasmids that arise by general recombination. In this way Cre facilitates effective partition of the P1 prophage.

Cre is a member of the integrase family of conservative site-specific recombinases. Cleavage of the DNA by the integrases involves covalent attachment of a conserved nucleophilic tyrosine to the 3'-phosphoryl end at the site of the break.

We have used *in vitro* complementation tests to show that the Cre protein, like the Flp protein of the 2-μm plasmid of *Saccharomyces cerevisiae*, cleaves its target *lox* site in *trans*. Moreover, the data are compatible with two modes of cleavage; one requires the reconstitution of a pseudo full-site from half-sites and the other requires the assembly of a higher order complex that resembles a synaptic complex.

Site-specific recombination occurs in a multiprotein-DNA complex whose proper assembly ensures that the reaction progresses in an orderly manner (1–5). Conservative site-specific recombinases bind to specific sequences in the DNA targets, bring together the target sites in an act called synapsis,† and covalently attach to the DNA. Recombination occurs following two pairs of strand exchanges and ligation of the DNAs in a novel (recombiant) form.

The canonical DNA target site for conservative site-specific recombinases consists of two inverted recombinase-binding sites that surround an “overlap” or “core” region. DNA cleavage and subsequent strand exchanges take place on the top or bottom strands at the margins of this core. (In the *lox* site, the target of the Cre recombinase, we define this core region as the 6 bp between the top and bottom strand cleavage sites.) The DNA cleavage event promoted by site-specific recombinases is catalyzed by a nucleophilic hydroxylated amino acid. A serine is used by the resolvase/invertase family members (6), whereas the integrase family members use tyrosine (7, 8). The nucleophilic attack on a specific phosphodiester bond is followed by the covalent attachment of the recombinase to the target sequence through a phosphoamino acid linkage.

An interesting issue in the chemistry of site-specific recombination is the position of the donor of the nucleophile in the synaptic complex. Does the recombinase that donates the nucleophilic amino acid bind immediately adjacent to the site of cleavage (cis cleavage) or does the donor lie somewhere else in the synaptic complex (trans cleavage)? For the *γ* resolvase, the answer was that cleavage took place in *cis*, as intuitively expected (6, 9). However, Chen *et al.* (10) showed that the Flp recombinase, a member of the integrase family, cleaved in *trans*. *Trans* cleavage may occur in one of three ways. *Trans* horizontal cleavage means that the nucleophile donor is across the core from the site of cleavage but on the same recombination target molecule. *Trans*-Vertical and *trans*-diagonal cleavage mean that the nucleophile donor resides on a different DNA target site in the synaptic complex from the one being cleaved (see Refs. 10 and 11). These latter two modes of cleavage imply that a synaptic complex must assemble before cleavage can occur. Recent evidence from the Jayaram laboratory (12) suggests that *trans* cleavage by Flp takes place by a *trans*-horizontal mechanism, i.e. it does not require prior synopsis of the two Flp recognition target sites. *Trans* cleavage is also observed for the R recombinase of the 2-μm-like plasmid of *Zygosaccharomyces rouxii* (13). Flp has recently been shown to resolve synthetic Holliday junctions in *trans* (14).

These studies have stimulated an examination of the mode of cleavage by other site-specific recombinases of both the transpositional and conservative varieties (for discussion see Refs. 15, 16). The phage Mu transposase executes both cleavage and strand transfer by a *trans*-mechanism (17–19). Although initial evidence supported a *trans* cleavage mode for the *λ* integrase protein acting on the *attL* site (20), subsequent studies showed that *λ* integrase resolves Holliday intermediates by *cis* cleavage (21). Likewise the XerC/XerD recombinase, also a member of the integrase family, is thought to cleave in *cis* (22).

Because of the apparent diversity among members of the integrase family with respect to their mode of cleavage, it was of interest to examine other integrase family members for their mechanism of cleavage. The Cre protein of bacteriophage P1 is a well characterized recombinase of the integrase family (23). Its biological function is to resolve dimeric P1 plasmids to monomers and hence to aid partition of the plasmid (24). Cre catalyzes reciprocal recombination between its *lox* sites (Fig. 1). The *lox* sites are similar to the Flp recognition target sites of Flp in both overall architecture and actual sequence (25). Furthermore, both the Cre and Flp proteins promote efficient recombination in *vitro* without the requirement for any accessory proteins.

We have used half- and full-*lox* sites to show that the Cre protein, like the Flp protein, executes cleavage in *trans*. These studies extend the diversity of the cleavage mechanisms among the members of the integrase family. This is the first example of a prokaryotic member of the integrase family that cleaves in *trans*.

MATERIALS AND METHODS

Enzymes—All enzymes were obtained from New England Biolabs and used according to the manufacturer’s instructions.

Plasmids—The pET19b plasmid was obtained from Novagen. Plasmid pRH200 was used as the source of the Cre coding sequence and was the gift of Dr. R. Hoess (Merck, DuPont NEN). Plasmids were prepared...
using the Qiagen plasmid isolation kit.

Oligonucleotides—Oligonucleotides were synthesized at the Hospital for Sick Children Biotechnology Service Center at the Banting Institute, University of Toronto. They were purified using the OPC cartridge before removal of the trityl group. Where needed the oligonucleotides were labeled with [γ-32P]ATP and polynucleotide kinase. Each labeled oligonucleotide was annealed to its respective unlabeled complementary strand oligonucleotide to give the DNA substrates as depicted in Fig. 1.

Reactions with a Single Half-site or Lox Site—One-tenth pmol of labeled half-fox site or full-fox site were incubated with 1 and 10 pmol of Cre protein (CreHis, Cre, CreHis Y324C, or Cre25) in a 40-μl mixture containing 50 mM Tris-Cl (pH 7.4), 30 mM NaCl, 3% SDS, and 1 mM dithiothreitol in one of two ways. In Method 1, the substrate was incubated with the protein for 15 min at room temperature, at which point a 15-fold excess of the same unlabeled substrate over the amount of the labeled substrate was added, and the reaction was continued for 5 min at room temperature. In Method 2, the initial incubation of the substrate with the protein occurred on ice for 3 min (prebinding), at which point a 15-fold excess of the same unlabeled substrate was added, and the reaction was continued for 5 min on ice (quenching). In both methods the reaction was then continued for 25 min at room temperature. Reactions were stopped by the addition of SDS sample buffer to give final concentrations of 10% glycerol, 3% SDS, 60 mM Tris-Cl (pH 6.8) and 5% β-mercaptoethanol. Samples were boiled for 5 min and then run on a 15% SDS-polyacrylamide gel (27), which was soaked in 50% methanol, 20% glycerol solution, dried, and exposed to x-ray film.

Reactions involving two different half-site substrates were done as above except that 0.05 pmol of each half-site were mixed together before incubation with protein.

Complementation reactions were set up essentially as with the single half-site reactions. Each reaction contained 0.05 pmol of a single half-site in a 20-μl volume and 0.5 and 5 pmol of Cre protein. After a preincubation step an excess of cold site was added, and 2 min later the two reactions of a complementing pair were combined to give a final reaction volume of 40 μl that was incubated at room temperature for an additional 25 min. Reactions were terminated, processed, and analyzed as above.

Binding Reactions—All binding reactions were done as described above except they were terminated by adding stop dye (1 mM Tris-Cl (pH 7.4), 0.1 mM EDTA, 10 mg/ml bovine serum albumin, 2% glycerol, and 0.1% sodium dodecyl sulfate (SDS)). Reactions were then run on an 8% nondenaturing polyacrylamide gel at 4°C (28).

Construction of CreHis Expression Vector—The cre gene was cloned into the pET19b vector to give a 10-histidine N-terminal fusion plus 11 amino acids from the linker of the vector. A fragment containing the Cre coding sequence was synthesized by PCR using the plasmid pRH200 as template. The 5′-Terminal primer, CN5, was 35 nucleotides long and contained an NdeI restriction site for later cloning steps, and had the sequence 5′-TATGAGCCATATGGTCAAATTCGACCCGCTAC3′. The 3′-Terminal primer, CN6, was 33 nucleotides long and contained a NdeI site and had the sequence 5′-TCTAGGATCATATGTAATCCGGCATTCTTGACC3′. The underlined sequence in both cases represents the NdeI restriction site. The DNA was purified by chloroform extraction, followed by the Tip-5 PCR Clean-up Kit (Qiagen). The PCR product was digested with NdeI and ligated to the pET19b vector that had been digested with NdeI and dephosphorylated with calf intestinal phosphatase. The DNA from both digestions was purified using an Ultra-free Probon 0.45-μm filter unit (Millipore). The ligation mixture was transformed into competent XL1-Blue cells (Stratagene: F lacIq RecA1 hsdR17 (rK Km− mKm−) ) as described by Sambrook et al. (29). One isolate contained a plasmid that had the cre gene fused to the His-10 tag as expected and was named pShe6.

Construction of Y324C CreHis Variant Expression Vector—PCR mutagenesis was used to change the tyrosine 324 of CreHis to cysteine. The 3′ primer (PLB) had the sequence 5′-TCTAGGACGCTTACAT-CGATAAGC3′. It hybridized to pET19b subclone 320 bp downstream from the 3′ end of the cre gene in pShe6. The 5′ primer (PCY) had the sequence 5′-GCTAAGCTTCATCCTAATACCTGATGTTAGCACCGG-GCGC5′. The resulting 1350-bp fragment, PCR 2, was digested with PstI and enzyme to verify that an additional PstI site was introduced by the mutagenesis. PCR 2 and pShe6 were each digested with BstBI and then HindIII. The 5.9-kilobase fragment from pShe6 and a 930-bp fragment from PCR 2 were ligated together to give pShe9. The mutagenesis and the accuracy of cloning were verified by DNA sequencing.

Construction of Cre Expression Vector—To construct a vector that contained no N-terminal leader sequence, the His tag region in the pET19b vector was removed by cutting with NcoI and NdeI and ligating an adaptor that contained ends compatible with these two enzymes as well as a new SalI site. This vector was called pShe1. It was cleaved with NdeI, dephosphorylated, and used to reclone the entire Cre coding sequence from pShe6 as an NdeI-NdeI fragment. The plasmid, pShe11, contained the cre gene in frame with the ATG start site of the pShe1 vector.

Construction of Cre25 Expression Vector—Construction of the Cre25-containing vector was done exactly as described by Hoess et al. (30). The source of the Cre25 coding fragment was pRH200, and all manipulations of the fragment into pET3c vector were described previously (30). The plasmid carried the Cre25 coding region in frame with 10 amino acids derived from the N terminus of the gene 10 protein of phage T7 and a translation stop signal. Sequencing of all cloned plasmids was done using the Circumvent Thermal Cycle DNA Sequencing Kit (New England BioLabs).

Testing Expression of CreHis, CreHis Y324C, Cre, and Cre25 Constructs—Each construct was transferred into Escherichia coli BL21 (DE3 pLysS) (31). The transformants were grown at 37°C; Cre protein expression was induced for 4 h at 37°C in the presence of 1 mM isopropyl-β-D-thiogalactoside, and the cell pellets were analyzed by SDS-PAGE. The solubility of each protein was assayed by sonication and low speed centrifugation followed by SDS-PAGE and Coomassie Blue staining. In all cases the proteins were at least 85% soluble.

Purification of CreHis and CreHis Y324C Proteins—Histidine-tagged CreHis and CreHis Y324C were purified in a single step by nickel affinity chromatography. The cell pellet from 500 ml of isopropyl-β-D-thiogalactoside-induced culture was resuspended in 3 volumes of sonication buffer (50 mM sodium phosphate (pH 8.0), 300 mM NaCl). Sonication was done with six 20-s bursts (40% gain, Vibra Cell sonicator, Sonic Materials) on ice with 2-min intervals between bursts. The sonicate was centrifuged at 100,000 × g for 1 h at 4°C. All subsequent manipulations were done at 0°C. The supernatant was applied to a 2-mL Ni-NTA agrose column (Qiagen) previously equilibrated with wash buffer (50 mM sodium phosphate (pH 8.0), 300 mM NaCl, 10% glycerol). The column was washed with 5 volumes of wash buffer and then with 3 column volumes of wash buffer containing 50, 75, 100, 125, 150, and 175 mM imidazole to remove proteins binding nonspecifically to the column. The CreHis or CreHis Y324C protein was eluted with 200 mM imidazole-containing wash buffer, and 2-mL fractions were collected.
Both proteins were greater than 95% pure as assayed by SDS-PAGE. The imidazole was removed by passing the Cre proteins through a desalting 10DG column (Bio-Rad) previously equilibrated with wash buffer. Protein concentrations were determined using the Bradford assay (32) with IgG as standard (Bio-Rad). From 500 ml of induced culture, 15 mg of CreHis and 12 mg of CreHs Y324C protein were obtained. The purified proteins were stored at −70°C.

**Purification of Cre and Cre25 Proteins**—Both the Cre and Cre25 proteins were purified essentially as described by Hoess et al. (30). The cell pellet from 500 ml of induced culture was resuspended in 3 volumes of TSE buffer (20 mM Tris-Cl (pH 7.5), 1 mM EDTA). Sonication and centrifugation were done as with CreHis and CreHs Y324C. The supernatant was applied to a 4-ml phosphocellulose column (Whatman) previously equilibrated in 0.05 TSEG (50 mM NaCl, TSE buffer, 10% pernatant was applied to a 4-ml phosphocellulose column (Whatman) centrifugation were done as with CreHis and CreHis Y324C. The su-

FIG. 1. *The *lox* sites used in this study.* The sequences and lengths of the oligonucleotides are shown. The labeled 5′-end is shown by the asterisk. A, the full-lox site (She4). The horizontal arrows are the two identical inverted repeats (symmetry elements) to which Cre binds. The vertical arrows are the sites of Cre cleavage and covalent attachment. They flank a 6-bp core or overlap region. B, the X25 half-site corresponds to the left side of the lox site. Cleavage at the vertical arrow results in the covalent attachment of the Cre protein to the 3′-phosphoryl dA terminus. C, the B half-site corresponds to the right half of the lox site. Cleavage at the vertical arrow would result in the attachment of Cre to the 3′-phosphoryl dG residue. nt, nucleotide.

Activity Assays of Cre Proteins—CreHis, CreHis Y324C, Cre, and Cre25 were assayed for recombination, binding to full- and half-lox sites, cleavage, and ligation of activated DNA substrates as described previously (33–35). Cre and CreHis showed similar specific activities in all these assays. All proteins were free of nuclease activity as assayed by denaturing polyacrylamide gel electrophoresis using labeled oligonucleotide substrates.

RESULTS

**Substrates and Proteins Used to Demonstrate trans Cleavage**—The full-lox site (She4) is illustrated in Fig. 1a. It contains two 13-bp Cre-binding elements and is recombination-compe-
be assayed, covalent attachment could be detected by the appearance of a \(^{32}\)P-labeled band that migrates more slowly than the starting oligonucleotide after SDS-PAGE. A further aspect of our strategy was that the polyhistidine tag on the CreHis protein caused the covalent product to migrate more slowly in SDS-PAGE than that formed by the Cre protein alone.

An important requisite for the half-site complementation assay was that the half-site be cleaved only when complemented by another site to which an appropriate protein had been bound. We therefore compared the ability of the Cre and CreHis proteins to cleave the X25 half-site and found that while the Cre protein was able to cleave and attach to the cleavable strand (Fig. 2a, lanes 2 and 3), CreHis (lanes 4 and 5) was unable to cleave the X25 site, whereas the Cre protein was still unable to cleave in spite of the presence of the B half-site in the mixture would influence the pattern of cleavages, we did experiments in which the Cre proteins were incubated with both the X25 and the B half-sites together. Only the Cre protein was able to cleave the X25 site, whereas the CreHis protein was still unable to cleave in spite of the presence of the B half-site in the reaction (Fig. 2b). In none of these reactions was the B half-site cleaved. Although the X25 half-site was cleaved by Cre in these reactions, the experiments do not speak to the mode of cleavage, whether cis or trans.

trans Complementation of CreHis Y324C by Cre or CreHis—To determine which Cre molecule was covalently attached to the cleaved substrate, we carried out complementation tests between two distinguishable Cre proteins bound to two different half-sites (Fig. 3). The CreHis Y324C protein was prebound to the X25 substrate, and the Cre or CreHis protein was prebound to the noncleavable B site with a cleavage-competent Cre protein (top). After the two reactions are mixed, the Cre protein bound to the B site donates its tyrosine 324 which cleaves the X25 site and covalently attaches the protein to the \(^{32}\)P-labeled top strand (middle). The covalent complex is detected by SDS-PAGE (bottom). Squares, CreHis Y324C; triangles, CreHis; asterisk, \(^{32}\)P radioactive label.
protein is providing the tyrosine and excludes the possibility that the complementation has somehow activated a surrogate nucleophile in the CreHis Y324C protein. When the positions of the proteins were reversed, i.e. the X25 site contained the CreHis protein and the B site contained CreHis Y324C, no cleavage and covalent attachment occurred (Fig. 4a, lanes 8 and 9). This was because the CreHis Y324C protein was unable to donate a tyrosine in trans. This experiment served as an important control that the proteins were not dissociating from the site to which they were originally bound and then reassociating with the partner site. Had this been occurring, we would have observed some covalent attachment of the CreHis protein to the X25 site (as in lanes 4 and 5). Further evidence for the stability of the Cre half-site complexes and the effectiveness of the cold competitor is presented in Fig. 5.

To determine whether the Cre25 protein was also able to act as an acceptor for a complementing tyrosine residue, we assayed for the ability of Cre25 to stimulate cleavage of the X25 site by CreHis. When the CreHis Y324C protein was replaced in the complementation scheme by Cre25 peptide, robust complementation by CreHis was also seen (Fig. 4a, lanes 6 and 7). We speculate that the polyhistidine tag on the N terminus may interfere with the ability of CreHis to cleave the X25 site but that the absence of 13 kDa from the N terminus of the Cre protein allows the Cre25 more flexibility to accept the tyrosine donated by CreHis in trans.

When the complementation test was done by loading the half-sites with the same amounts of proteins but then diluting the final mixture into a larger reaction volume, the covalent attachment declined (data not shown). This result is consistent with an intermolecular cooperation between the two half-sites. We conclude that both Cre and CreHis are able to complement the CreHis Y324C or Cre25 proteins in trans.

trans Complementation of CreHis by Cre—Since variants of Cre which were defective in cleavage could be complemented by the Cre protein, we wished to learn whether Cre might stimulate the capacity of CreHis to cleave the X25 half-site. Recall that this site is not cleaved following incubation with CreHis alone. Accordingly, CreHis was bound to the X25 site and Cre was bound to the B site and the two reactions were mixed. As can be seen in Fig. 4b (lanes 2 and 3), we saw the presence of abundant covalent complexes of Cre to the X25 half-site. We assume that these arose by cleavage and covalent attachment carried out by Cre bound to the B half-site just as Cre complemented the CreHis Y324C protein in trans (Fig. 4a). We observed covalent complexes of CreHis to the X25 site in addition to the expected complexes of Cre. Thus the presence of Cre bound to the B half-site stimulated markedly the cleavage of the X25 site by the CreHis. Note that when the positions of the two proteins were reversed (Cre bound to X25 and CreHis to B), we saw trans cleavage of the X25 site by CreHis. In addition, the cleavage of the X25 site by the Cre protein has also been markedly stimulated (lanes 4 and 5). It is possible that this stimulation is due to the assembly of a higher order complex and that it takes place by a trans-vertical or -diagonal mechanism (see “Discussion”).

Stable Association of Cre Proteins with Half-sites during Complementation—The above experiments support a trans mode of cleavage by Cre. However, the validity of these conclusions depends on the assurance that the respective proteins, once bound to a particular half-lox site, do not dissociate from that site and bind to another one. The protocol (see “Materials and Methods”) included the addition of a 15-fold excess of cold half-site to sequester any protein that might dissociate from further participation in the reaction. To show that the proteins did not dissociate from the respective half-sites after the reac-
tions were combined, we analyzed the products on a native polyacrylamide gel. When the X25 site was incubated with Cre on ice, about 20% of the complex was disrupted by subsequent incubation with an excess of the cold site (Fig. 5, lanes 5 and 6, determined by PhosphorImager analysis in triplicate). When the cold competition was followed by incubation with the longer B half-site, no complex with the B site was seen (I(B), cf. lanes 2, versus 8 and 9). However, if the cold competitor was omitted, incubation of both sites with the Cre protein gave the expected complexes with the individual X25 and B half-sites (lanes 2, 4, and 10). Both half-sites also showed the presence of higher order complexes (ho, Fig. 5). We believe these may comprise dimers and tetrarmers of the respective half-site with Cre (data not shown). We did a similar experiment in which the B half-site was loaded with Cre protein. After addition of a 15-fold excess of cold B site the labeled X25 site was added (without a 15-fold excess of cold X25 half-site), and the reaction was analyzed by SDS-PAGE. No covalent complex of Cre with the X25 site was detected, again showing that the Cre protein bound stably to the B half-site during the complementation experiments (data not shown). Thus we are confident that our complementation results are not attributable to dissociation of Cre during the experiment.

**Trans Cleavage between a Full- and Half-lox Site**—The finding that Cre bound to the B half-site markedly stimulated cleavage by CreHis (Fig. 4) suggested that trans cleavage might be occurring in a synaptic complex. To detect such cleavage, a linear full-lox site (She4, Fig. 1) and the X25 half-site were each loaded separately with the Cre proteins to be tested. After incubation at 0 °C and addition of the cold site competitor, the two preformed complexes were mixed and incubated at room temperature for 25 min (Fig. 6). Whereas Cre gave little and CreHis gave no covalent complex, respectively, when incubated with the X25 site alone (lanes 2 and 3), addition of the She4 full-site loaded with the respective Cre protein gave a very large amount of cleavage by either protein (lanes 8 and 9). Note that covalent attachment to the full-site was not detectable in these experiments, presumably because cleavage was followed by rapid religation (lanes 5 and 6). When the X25 site contained bound Cre but the She4 site contained CreHis, the CreHis covalent complex predominated, although a small amount of the Cre covalent complex was also present (lane 10). **trans** complementation of the CreHis Y324C mutant protein was readily apparent (lane 11). However, when the positions of the two proteins were reversed (lane 12), i.e. CreHis bound to the X25 site and CreHisY324C to the She4 full-site, there was still a substantial amount of cleavage and covalent attachment of X25 by the CreHis (cf. lanes 2 versus 12). Thus the presence of the CreHis Y324C protein bound to the full-lox site stimulated the ability of the CreHis to cleave the X25 site, perhaps by stabilizing it in a synaptic complex.

**FIG. 6.** Influence of full-lox site on cleavage of half-lox site by Cre proteins. SDS-PAGE. The labeled lox site (She4) and/or the half-lox site (X25) were incubated with the Cre proteins (10 pmol) as indicated at the top of the figure according to Method 2 (“Materials and Methods”). The same symbols were used as in Fig. 4. The X25 substrate ran off the front of this gel. Substrates used were as follows: lanes 1–3, X25 only; lanes 3–6, She4 only; lanes 7–12, both X25 and She4.

**FIG. 7.** Assembly of synaptic complexes from half-lox sites. a, synaptic complex of half-site homodimers. Cre (circles) bound to the X25 site forms homodimers and cleaves the site trans-horizontally. Synapsis with a dimer of B sites loaded with the CreHis protein (triangles) allows trans-vertical cleavage of the X25 site by CreHis. Reversal of the positions of the proteins would give a similar result. b, synaptic complex of half-site heterodimers. A heterodimer of an X25 half-site loaded with Cre (circle) forms a heterodimer with a B site loaded with CreHis (triangle) which cleaves the X25 site trans-horizontally. The Cre protein then cleaves another X25 site in the synaptic complex trans-vertically.

**Cre-X25/CreHis-B**

- **a**
  - X25
  - CATACA
  - TG
  - ACATAAC
  - X25
  - *5’

- **b**
  - X25
  - TG
  - ACATAAC
  - X25
  - *5’
Finally, the levels of covalent complex generated in the reactions of both combinations of the CreHis and CreHis Y324C complementation tests (lanes 11 and 12) were lower than the levels in the reactions where both substrates were prebound with CreHis (lane 8). Indeed phosphorimage quantitation of the complex in lane 8 showed that it was approximately equal to the sum of those seen in lanes 11 and 12. Thus it is possible that the complexes in lanes 8 and 9 arose from two modes of complementation, trans-vertical/diagonal (as in lane 11) and trans-horizontal (as in lane 12). These possibilities will be discussed further below.

**DISCUSSION**

The results presented in this paper support a trans cleavage mechanism for the Cre recombinase. A cleavage-competent Cre protein (either Cre or CreHis) when bound to a noncleavable half-lox site (B) was able to complement the cleavage defect of CreHis Y324C or Cre25. This effect was not due to dissociation of the cleavage-competent Cre from its half-site and its reassociation with the partner site (Figs. 4a and 5). The results were strengthened by the finding that cleavage of the X25 site by the CreHis protein was greatly stimulated by the presence of the Cre protein bound to a full- or half-lox site. (Figs. 4 and 6). Finally, we found that the stimulation of cleavage was sensitive to dilution; diluting the prebinding mixtures into a greater volume caused a diminution of cleavage. This suggests that cleavage requires an intermolecular reaction and hence does not occur in cis.

Do our results provide any information about the mode of cleavage, i.e. does it occur by a trans-horizontal, trans-vertical, or trans-diagonal mechanism? The use of half-sites makes an assignment of a cleavage mode difficult and, in part, semantic. However, the fact that Cre (although not CreHis) cleaves the X25 half-site is compatible with the formation of a homodimeric complex (Fig. 7a) followed by cleavage in a trans-horizontal mode. Cre bound to the X25 half-site is able to form homodimers, and cleavage occurs in such dimers (data not shown). The CreHis protein can form such dimers (data not shown) but cannot carry out cleavage in them. Mixing of the CreHis-bound B half-site with the Cre-bound X25 site had two effects: cleavage by the CreHis and by Cre of the X25 half-site were both greatly stimulated. As illustrated in Fig. 7a, Cre would cleave the X25 site trans-horizontal, whereas CreHis bound to the B half-site would be stimulated to cleave the X25 site trans-vertically, perhaps by virtue of its incorporation into a synaptic complex. If the positions of the proteins are reversed (not shown, CreHis on X25 site and Cre on B), trans-horizontal cleavage of the X25 site by CreHis might be stimulated by its incorporation into a synaptic complex with a homodimer of the Cre-bound B site. Alternatively, mixed dimers of X25-bound Cre and B-bound CreHis might assemble, and trans-horizontal cleavages of X25 by CreHis and trans-vertical cleavages of X25 by Cre might occur (Fig. 7b). Reversal of the locations of the two proteins would lead to the opposite result.

The use of a full-site removes some of the ambiguity caused by the use of complementing half-sites. The full-lox site bound by CreHis dramatically stimulated the cleavage of the X25 site by CreHis (Fig. 8a). Here the cleavages could be both trans-vertical/diagonal or trans-horizontal. CreHis bound to the full-lox site (Fig. 8b) clearly complemented the cleavage defect of the CreHis Y324C protein bound to the X25 site. By definition this must have occurred by a trans-vertical or -diagonal mechanism. But, interestingly, the presence of a full-site loaded with a cleavage-incompetent Cre protein also greatly stimulated the cleavage of the X25 site by CreHis (Fig. 8c). We hypothesize that the occupied lox site may stabilize the X25 sites bound with CreHis in a higher order complex or synaptosome (Fig. 8c). In this structure the CreHis can now cleave, probably trans-horizontally. It should be noted that Qian and Cox (36)
have recently proposed that an asymmetric complex consisting of three bound molecules of Flp protein is responsible for cleavage in the synaptic complex. In their model, cleavage takes place by both trans-horizontal and trans-vertical modes. Such a mixed mode of cleavage is compatible with our data for Cre.

Thus the mechanism of the Cre protein seems most closely parallel to the Flp paradigm. Flp was shown to cleave half-Flp recognition target sites in trans (10), and recent experiments support a trans-horizontal mechanism (12). The Flp protein cleaves Holliday junctions in trans (14), whereas the λ integrase cleaves such structures in cis (21). A recent alignment of the integrase family members by Blakely and Sherratt (37) suggested a possible correlation between the spacing of a conserved glycine residue (314 of Cre) and the nucleophilic tyrosine (324 of Cre) and the ability to cleave in trans. The spacing was 10–11 amino acids for the prokaryotic members of the family, two of which are known to cleave in cis, but was 14 amino acids for the eukaryotic members (Flp and Flp-like proteins), two of which have been shown to cleave in trans. However, the Cre protein cleaves in trans in spite of a spacing of 11 amino acids between the glycine and the nucleophilic tyrosine. On the other hand, both Cre and Flp have simple target sites, have relaxed topological requirements, and can perform the entire reaction in vitro without addition of accessory factors. It is possible that the ability to cleave in trans is a reflection of the relative simplicity of the Cre and Flp reactions.

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REFERENCES
1. Craig, N. L. (1988) Annu. Rev. Genet. **22**, 77–105
2. Landy, A. (1989) *Annu. Rev. Biochem.* **58**, 913–949
3. Mizuuchi, K. (1992) *Annu. Rev. Biochem.* **61**, 1011–1051
4. Sadowski, P. D. (1990) *FASEB J.* **7**, 769–767
5. Jayaram, M. (1994) *Trends Biochem. Sci.* **19**, 78–82
6. Grindley, N. D. F. (1993) *Science* **262**, 738–740
7. Argos, P., Landy, A., Abremski, K., Egan, J. B., Haggard-Ljungquist, E., Hoess, R. H., Kahn, M. L., Kalionis, B., Narayana, S. V. L., Pierson, L. S., III, Sternberg, N., and Leong, J. M. (1986) *EMBO J.* **5**, 433–440
8. Abremski, K. E., and Hoess, R. H. (1992) *Protein Eng.* **5**, 87–91
9. Droge, P., Haffull, G. F., Grindley, N. D. F., and Cozzarelli, N. R. (1990) *Proc. Natl. Acad. Sci. U. S. A.* **87**, 5336–5340
10. Chen, J.-W., Lee, J., and Jayaram, M. (1992) *Cell* **69**, 647–658
11. Sadowski, P. D. (1995) *Proc. Natl. Acad. Sci. U. S. A.* **92**, 5346–5354
12. Yang, S. H., and Jayaram, M. (1994) *J. Biol. Chem.* **269**, 12789–12796
13. Dixon, J. E., Shaikh, A. C., and Sadowski, P. D. (1995) *Molec. Microbiol.* **18**, 449–458
14. Stark, W. M., and Boocock, M. R. (1995) *Trends Genet.* **11**, 121–123
15. Jayaram, M., and Lee, J. (1995) *Trends Genet.* **11**, 432–433
16. Savilahiti, H., and Mizuuchi, K. (1996) *Cell* **85**, 271–279
17. Aldaz, H., Schuster, E., and Baker, T. A. (1996) *Cell* **85**, 257–269
18. Yang, J.-Y., Jayaram, M., and Harshay, R. M. (1996) *Cell* **85**, 447–448
19. Han, Y. W., Gumpori, R. I., and Gardner, J. F. (1993) *EMBO J.* **12**, 4577–4584
20. Nunes-Du¨by, S. E., Tirumalai, R. S., Dorgai, L., Yagil, E., Weissberg, R. A., and Landy, A. (1994) *EMBO J.* **13**, 4421–4430
21. Arciszewska, L. K., and Sherratt, D. J. (1995) *EMBO J.* **14**, 2112–2120
22. Abremski, K., Hoess, R., and Sternberg, N. (1983) *Cell* **32**, 1301–1311
23. Austin, S., Ziese, M., and Sternberg, N. (1981) *Cell* **25**, 729–736
24. Vetter, D., Andrews, B. J., Roberts-Beatty, L., and Sadowski, P. D. (1983) *Proc. Natl. Acad. Sci. U. S. A.* **80**, 7284–7288
25. Andrews, B. J., Beatty, L. G., and Sadowski, P. D. (1987) *J. Mol. Biol.* **205**, 60–89
26. Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual*, 2nd Ed., pp. 1.79–1.81, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
27. Landmli, U. K. (1970) *Nature* **227**, 680–685
28. Andrews, B. J., Beatty, L. G., and Sadowski, P. D. (1987) *J. Mol. Biol.* **193**, 345–358
29. Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual*, 2nd Ed., pp. 1.79–1.81, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
30. Hoess, R. H., and Abremski, K. (1984) *Proc. Natl. Acad. Sci. U. S. A.* **81**, 1026–1029
31. Studier, F. W., Rosenberg, A. H., Dunn, J. J., and Dubendorff, J. W. (1990) *Methods Enzymol.* **185**, 60–89
32. Bradford, M. M. (1976) *Anal. Biochem.* **72**, 248–254
33. Abremski, K., and Hoess, R. (1984) *J. Biol. Chem.* **259**, 1509–1514
34. Yuan, J., Kirkaldy, D., Abremski, K., and Hoess, R. (1987) *J. Mol. Biol.* **195**, 785–794
35. Pan, G., Luetke, K., Juby, C. D., Brousseau, R., and Sadowski, P. D. (1993) *J. Biol. Chem.* **268**, 3683–3689
36. Qian, X., and Cox, M. M. (1995) *Genes Dev.* **9**, 2053–2064
37. Blakely, G. W., and Sherratt, D. J. (1996) *Mol. Microbiol.* **20**, 234–237