Chimeras of the Flp and Cre Recombinases: Tests of the Mode of Cleavage by Flp and Cre

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The Flp and Cre recombinases are members of the integrase family of tyrosine recombinases. Each protein consists of a 13 kDa NH$_2$-terminal domain and a larger COOH-terminal domain that contains the active site of the enzyme. The COOH-terminal domain also contains the major determinants for the binding specificity of the recombinase to its cognate DNA binding site. All family members cleave the DNA by the attachment of a conserved nucleophilic tyrosine residue to the 3'-phosphate group at the sites of cleavage.

In order to gain further insights into the determinants of the binding specificity and modes of cleavage of Flp and Cre, we have made chimeric proteins in which we have fused the NH$_2$-terminal domain of Flp to the COOH-terminal domain of Cre ("Fre") and the NH$_2$-terminal domain of Cre to the COOH-terminal domain of Flp ("Clp"). These chimeras have novel binding specificities in that they bind strongly to hybrid sites containing elements from both the Flp and Cre DNA targets but poorly to the native target sites.

In this study we have taken advantage of the unique binding specificities of Fre and Clp to examine the mode of cleavage by Cre, Flp, Fre and Clp. We find that the COOH-terminal domain of the recombinases determines their mode of cleavage. Thus Flp and Clp cleave in trans whereas Cre and Fre cleave in cis. These results agree with the studies of Flp and with the cocrystal structure of Cre bound to its DNA target site. They disagree with our previous findings that Cre could carry out trans cleavage. We discuss the variations in the experimental approaches in order to reconcile the different results.

Keywords: Flp; Cre; site-specific recombination; DNA binding; chimeric proteins

Introduction

The integrase family of conservative site-specific recombinases is comprised of over 100 members (Abremski & Hoess, 1992; Argos et al., 1986; Esposito & Scocca, 1997; Nunes-Duby et al., 1998). These proteins all use a conserved catalytic tyrosine residue to break and covalently attach to their target sequences at specific phosphodiester bonds (Craig, 1988; Evans et al., 1990; Gronostajski & Sadowski, 1985; Landy, 1989; Sadowski, 1993, 1995). The target sequences contain two inverted binding sites each of which binds a molecule of the recombinase. Although cleavage can take place in a dimeric structure consisting of two recombinase molecules bound to one target sequence (Andrews et al., 1987; Lee et al., 1994; Qian et al., 1990), recombination takes place in a synaptic structure consisting of two target sites each bound by two molecules of recombinase (Amin et al., 1991; Guo et al., 1997, 1999; Hamilton & Abremski, 1984; Hoess et al., 1990b). The crystal structure of the Cre synapse reveals an extensive series of cyclic NH$_2$-terminal and COOH-terminal interactions that establish both "cross-core" interactions between the two protomers bound to the same lox site and "synaptic" interactions between the Cre molecules bound to the two lox sites in the synapse (Guo et al., 1997).

Two of the most extensively characterized members of the integrase family are the Flp protein encoded by the 2 μ plasmid of yeast (Sadowski, 1995) and the Cre protein of the bacteriophage P1 (Hoess & Abremski, 1990). The first step in the recombination reaction is the site-specific binding
of the recombinase to its cognate recognition site, the FRT site for the Flp protein and the lox site for the Cre protein (Figure 1(a) and (b)). Although the sequences of the sites differ, they share an identical organization consisting of two inverted 13 bp sequences ("symmetry elements") surrounding an 8 bp core region (Hoess et al., 1984, 1986; Lee & Saito, 1998; Vetter et al., 1983). Early gel mobility shift and footprinting experiments established the 13 bp symmetry elements as the recognition elements for Flp and Cre (Andrews et al., 1987; Mack et al., 1992). Hoess et al. (1990a) showed that the NH2-terminal domain of Cre protected the core region of the lox site whereas the COOH-terminal domain bound to the symmetry element. Missing contact probing of a single symmetry element showed that the core-proximal 4 bp of the symmetry were important for the binding of intact Cre but not Cre25, the COOH-terminal domain (Hoess et al., 1990a). The core-distal 9 bp were needed for binding of both intact Cre and Cre25 (Shaikh, 1997). Crosslinking studies using the NH2 and COOH-terminal domains of Flp showed a similar bipartite structure of the FRT symmetry element. The core-proximal 4 bp were important for the binding of the NH2-terminal domain, P13 and the COOH-terminal domain, P32 bound to the core-distal 9 bp (Panigrahi & Sadowski, 1994).

In order to gain further insight into the mechanism of binding and cleavage by the Flp and Cre recombinases, we have constructed chimeras between the two recombinases. The Fre protein contains the NH2-terminal 13 kDa of Flp fused to the COOH-terminal 25 kDa of Cre. The Clp protein contains the NH2-terminal 13 kDa of Cre fused to the 32 kDa COOH-terminal domain of Flp. Each of the chimeras binds site-specifically to hybrid sites composed of sequence elements from the target site of Flp and Cre, the FRT and lox sites. These novel binding specificities enabled us to examine the mode of cleavage by Flp, Cre, Fre and Clp.

The members of the integrase family of recombinases all employ a conserved nucleophilic tyrosine that breaks the scissile phosphodiester bond and covalently attaches the protein to the 3'-phosphoryl end at the site of the break (Argos et al., 1986; Esposito & Scocca, 1997; Nunes-Duby et al., 1998). However the family members display a diversity of mechanisms of cleavage (Jayaram, 1997; Jayaram & Lee, 1995). Cis cleavage means that the molecule of the recombinase that donates the catalytic tyrosine is bound immediately adjacent to the scissile bond. Trans cleavage implies that the recombinase molecule that donates the tyrosine is not bound next to the scissile bond but resides elsewhere in the synaptic structure.

The Flp recombinase was the first to be studied in this respect and all tests have showed that it cleaves in trans (Chen et al., 1992; Lee et al., 1994, 1999; Pan et al., 1993). This implies the active site of Flp is formed by contributions from two Flp protomers: the Flp molecule bound next to the bond to be cleaved activates that bond to receive the catalytic tyrosine from another Flp protomer bound to the target FRT sequence. While initial experiments on the λ integrase were compatible with a trans mode of cleavage (Han et al., 1993), subsequent experiments using Holliday junctions gave unequivocal evidence of cis cleavage (Nunes-Duby et al., 1994). The XerC/XerD recombinase also gave clear evidence for cis cleavage (Arciszewska & Sherratt, 1995). Our initial complementation experiments also indicated that the Cre recombinase could cleave in trans (Shaikh & Sadowski, 1997). However, the cocrystal structure of Cre that was covalently attached to its DNA target showed that the cleavage had occurred in cis (Guo et al., 1997).

The studies showing trans cleavage by Flp and Cre made use of complementation experiments in which a given molecule of recombinase was positioned upon a full or half-target site (Chen et al., 1992; Shaikh & Sadowski, 1997). Trans cleavage was demonstrated when one molecule of recombinase donated its catalytic tyrosine to another recombinase protomer that was unable to cleave due to a mutation and was bound to a different site.

Such complementation experiments are subject to the possible artifact that trans cleavage occurs because the design of the experiment precludes cis cleavage. The experiments with λ integrase and Holliday junctions gave unequivocal results because they made use of two integrases (λ and HK022) of different binding specificities and the authors were thereby able to position the proteins at known sites in the Holliday substrate (Nunes-Duby et al., 1994). Likewise the XerC and XerD proteins have different binding specificities and therefore one can be certain of their position during the cleavage experiment (Arciszewska & Sherratt, 1995).

In the present study we have made use of the chimeric recombinases Clp and Fre to re-examine the mode of cleavage by Cre and Flp. We exploited the altered binding specificities of the Clp and Fre proteins to position them on hybrid binding elements called lrt and fox. We isolated heterodimeric complexes containing one molecule of each recombinase and assayed for covalent attachment of the recombinases to the target site. We find that the Cre and Fre proteins cleave in cis whereas the Flp and Clp proteins cleave in trans. We conclude that the COOH termini of the Cre and Flp proteins determine their mode of cleavage.

Results

Production of chimeric proteins Fre and Clp

Proteolysis of the Cre and Flp proteins had suggested a bidomainal structure for each protein (Hoess et al., 1990a; Pan & Sadowski, 1993; Pan et al., 1991). Furthermore, previous studies (Hoess et al., 1990a; Panigrahi & Sadowski, 1994; Shaikh,
Substrates used to characterize binding of native and chimeric recombinases

Before attempting complementations between Cre or Flp and Fre or Clp, we used gel mobility shift assays to characterize the binding activities of the chimeras on a variety of substrates. These experiments provided an estimate of the relative binding affinity of each hybrid protein for the lox and FRT sites and allowed us to design composite binding substrates that were used later in the tests of the mode of cleavage.

The target sequence of Cre is called lox (Figure 1(a)) and consists of two inverted 13 bp symmetry elements (inverted arrows) surrounding an 8 bp core region (open box). The sequences of the lox symmetry elements are shown in green, with the core-proximal 4 bp underlined. The lox core region is shown in magenta. The two cleavage sites for Cre are within the core and are 6 bp apart (vertical arrows, Figure 1(a)). The minimal recombination sequence for Flp is called FRT (Figure 1(b)) and has a similar organization to lox. The FRT symmetry elements are shown in red with the core-proximal 4 bp underlined. The 8 bp core region is in blue and the cleavage sites for Flp are 8 bp apart (vertical arrows, Figure 1(b)).

The test substrates ArkZ, ArkP, ArkF, ArkL and fox39 (Figure 1(c)-(g)), contained portions of both lox and FRT as indicated. A hybrid symmetry element consisting of the core-proximal 4 bp of the right-hand FRT symmetry element and the core-distal 9 bp of the lox symmetry element is called fox. The hybrid symmetry element consisting of the core-proximal 4 bp of the lox symmetry element and the core-distal 9 bp of the FRT symmetry element is called lrt.

Binding of proteins to the ArkZ substrate

The substrate ArkZ (Figure 1(c)) contains a single Cre binding element and a Flp binding element flanking a lox core. We incubated ArkZ with Fre, Clp, Cre, Flp, Cre25 and P32, and analyzed the complexes using gel mobility shift assays. As expected, Cre and Flp each formed a single complex (cI Cre and cI Flp) caused by binding of a single protein molecule to its cognate symmetry element (Figure 4, lanes 2 and 3, 6 and 7). Interestingly, incubation of ArkZ with both proteins produced the expected cI complexes of Cre and Flp, but very little of the mixed complex II (cII, lanes 12 and 13). The amount of complex formation can be compared with the binding of the same amounts of Cre and Flp to the full-lox and full-FRT sites (Figure 5(a) and (b)), sites shown in Figure 1(a) and (b), where both proteins readily generate dimeric complexes (cII) and where Cre also generates higher order (HO) complexes that are most likely the result of protein-dependent synaptic assemblies (Shaikh, 1997; Wierzbicki et al., 1987).

The His-Fre and His-Clp proteins bound weakly to the ArkZ substrate but, like their parental recombinases, generated only complex I (Figure 4, lanes 4 and 5, 8 and 9). Note that the presence of the His-tag on both chimeras allowed us to resolve the migration of the four different protein-dependent complex IIs in the polyacrylamide gel. Since only the COOH-terminal domains of both Cre and Flp are able to form protein:DNA complexes in vitro (Hoess et al., 1990a; Pan & Sadowski, 1993), the Fre and Clp proteins were most likely interacting with the core-distal lox and FRT elements in the substrate. To be sure that the ArkZ substrate was capable of interacting with the COOH-terminal domains of Fre and Clp, we incubated the substrate with isolated Cre25 or His-P32 peptide. Not only did Cre25 and His-P32 generate monomer complexes with the ArkZ substrate, but each protein demonstrated apparently greater affinity for the binding sites in this substrate than the comparable chimera (Figure 4, lanes 20-23). As the native Cre and Flp proteins have a five to ten times greater affinity for their cognate sites than their COOH-terminal domains (Hoess et al., 1990a; Pan & Sadowski, 1993), the reduced binding affinity of the Fre and Clp proteins for the lox and FRT elements may represent an inhibitory effect of the heterologous NH2-terminal domain on the COOH-terminal binding domains of Cre and Flp in the chimeric proteins (see Discussion).

As noted above, incubation with Cre and Flp revealed the complex IIs of each protein as well as
a weak slower migrating complex, cII (Figure 4, lanes 12 and 13). Binding of a molecule of Cre or Flp seems to exclude binding of a second molecule. Alternately, the absence of cII may reflect the absence of cooperativity between the two proteins (see Discussion). A small amount of mixed complex II was observed when His-Fre and Flp were incubated together with ArkZ (Figure 4, lanes 16 and 17). The slightly slower migration of this complex II is due to the presence of the additional His-tag in the Fre-Flp mix. In contrast, Cre and His-Fre generated no mixed complex IIs (Figure 4, lanes 10 and 11) and a barely detectable amount of cI with His-Fre. Similar results were observed with a mixture of Flp and His-Clp (Figure 4, lanes 18 and 19). We had expected the mixture of Cre and His-Clp to generate a heterodimeric complex II, but only complex I of each protein was observed (Figure 4, lanes 14 and 15), perhaps due to the very weak binding of Clp to the substrate.
Binding of proteins to the lox substrate

We next used a full-lox site (Figure 1(a)) to test the abilities of Fre and Clp to bind to the lox site. As expected, Cre readily forms monomer (cI), dimer (cII) and higher order (HO) complexes on its cognate site (Figure 5(a), lanes 2 and 3). The Flp protein forms the analogous complex I and II on its cognate FRT site, though little higher order complex is seen (Figure 5(b)). As with the single lox element-containing site ArkZ, the His-Fre protein bound weakly to the lox site compared to Cre and the Cre25 peptide (Figure 5(a), lanes 4 and 5, lane 7). Despite the decreased binding affinity of His-Fre for the lox site, the binding of the chimera was cooperative, as formation of complex II (cII Fre) was favored over complex I (cI Fre). In contrast to the binding of Cre25, protein-protein interactions between Fre monomers also generated some higher order complexes.

The Clp protein was unable to bind the lox site, consistent with both the absence of a Cre COOH-terminal binding domain in the fusion protein and the inability of the NH2-terminal domain to confer affinity to the heterologous COOH-terminal domain (Figure 5(a), lane 6).

Binding of the proteins to the ArkP substrate

We then examined the ability of the native and chimeric recombinases to bind to the ArkP substrate (Figure 6). The substrate contains a lox core flanked by the left lox symmetry element and a fox element (Figure 1(d)). The fox element contains the 4 bp FRT core-proximal sequence and the 9 bp core-distal sequence of the lox site. This fox symmetry element should provide the binding regions for both the P13 and Cre25 portions of the Fre chimera.

Neither Flp nor Clp was able to bind to the ArkP substrate because of the absence of the core-distal sequence of the FRT symmetry element in the substrate (Figure 6, lanes 8-10).

The modified lox symmetry element contained in the ArkP substrate dramatically affected Cre binding (Figure 6, lanes 2 and 3). The degree of cooperativity previously observed with Cre binding to the full-lox substrate (Figure 5(a), lanes 2 and 3) was diminished as the Cre-bound complexes favored the monomeric cI over the dimeric cII. Furthermore, no Cre-dependent higher order complexes were observed. In contrast, the His-Fre protein exhibited cooperativity (Figure 6, lanes 4 and 5) and bound with two to four times higher affinity to the ArkP substrate than to the full-lox substrate (Figure 5(a), lanes 4 and 5). Incubation with both Cre and His-Fre generated a complex II band (cII Mix, Figure 6, lanes 6 and 7) that was distinct from both the Cre and Fre homodimer complexes. The intermediate migration of the mixed complex between the homodimer complexes of Cre and His-Fre was consistent with the binding of one monomer each of Cre and His-Fre to the lox and fox elements of the ArkP substrate. Indeed, the heterodimeric cII of Cre and His-Fre was favored over each of the homodimer species (Figure 6, cf. lanes 2, 4 and 6).

Binding of proteins to the ArkF substrate

We designed a second hybrid substrate to test the binding of the native and chimeric proteins. The ArkF substrate was similar to the ArkP substrate described above, except that the fox site was replaced with another composite symmetry element, lrt (Figure 1(e)). This lrt element contained the core-proximal region of a lox symmetry element and the core-distal region of an FRT element and therefore contained the recognition elements for both the NH2 and COOH-terminal domains of the Clp protein. We performed gel mobility shift assays with this substrate using the Cre, Flp, Cre25, His-P32, His-Fre and His-Clp proteins and the results are shown in Figure 7.

Cre generated copious amounts of the monomeric cI and, interestingly, formed some dimeric cII as well (Figure 7, lanes 2 and 3) when higher levels of Cre protein were used. As with the single lox symmetry element-containing ArkZ substrate,

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Figure 1. The lox and FRT-based substrates used in this study. The sequences of the oligonucleotides are shown. Each was 35 nucleotides in length. Where required, the 5’-end was labeled as described in Materials and Methods. Symmetry elements (horizontal arrows) are derived from the lox site (green) or the FRT site (red). Core-proximal regions are underlined. lox and FRT core regions (open boxes) are magenta and light blue. Vertical arrows indicate the putative cleavage sites by Cre or Flp on the native lox and FRT sites. (a) Full-lox DNA substrate. The cognate site for the Cre protein contains two identical, inverted 13 bp lox symmetry elements that flank an 8 bp lox core region. The cleavage sites are 6 bp apart. (b) Full-FRT DNA substrate. The cognate site for the Flp protein contains two inverted 13 bp FRT symmetry elements (a and b) that flank an 8 bp FRT core region. The cleavage sites are 8 bp apart. The two symmetry elements differ by a single base-pair in the core-proximal region. (c) ArkZ DNA substrate. The hybrid substrate consists of a lox core region flanked by the left symmetry element of the lox site and the a symmetry element from the FRT site. (d) ArkP DNA substrate. The hybrid substrate consists of a lox core region flanked by the left symmetry element of the lox site and a composite binding element, fox, which is derived from the core-proximal 4 bp of the FRT a symmetry element and the core-distal 9 bp of the lox symmetry element. (e) ArkF DNA substrate. The hybrid substrate consists of a lox core region flanked by the left symmetry element of the lox site and a composite binding element, lrt, which is derived from the core-proximal 4 bp of the lox symmetry element and the core-distal 9 bp of the FRT symmetry element. (f) ArkL DNA substrate. The hybrid substrate consists of an FRT core region flanked by the left symmetry element of the FRT site and the composite binding element, fox. (g) fox2b DNA substrate. The substrate contains two inverted 13 bp fox symmetry elements flanking an FRT core region.
Cre25 generated a single complex with the ArkF substrate and His-Fre bound weakly to form a monomeric cI (Figure 7, lanes 10-13). No complex was formed when the ArkF substrate containing the \textit{lrt} symmetry element was incubated with Flp (Figure 7, lanes 8 and 9). In contrast, the His-Clp protein formed copious amounts of monomer complexes with the ArkF substrate (Figure 7, lanes 4 and 5): by phosphorimager quantification, the amount of complex I generated by Clp on the \textit{lrt} site was about 20 times that observed in the Clp reaction with the FRT symmetry element in the ArkZ substrate (cf. Figure 4, lanes 8 and 9). Clp formed no dimeric complex II, showing that the protein does not bind to the \textit{lox} symmetry element of the ArkF substrate.

The incubation of Cre and His-Clp with the ArkF substrate generated abundant amounts of a heterodimeric complex (cII Mix) in addition to the individual complex I bands attributable to the binding of Cre and His-Clp (Figure 7, lanes 6 and 7). The mixed complex II consists of one Cre monomer bound to the \textit{lox} symmetry element and one His-Clp protein bound to the \textit{lrt} symmetry element in the ArkF substrate.

**Binding of proteins to the ArkL substrate**

While both the ArkP and ArkF substrates were essentially modified \textit{lox} substrates, it was also of interest to examine the binding of the chimeric proteins to an altered FRT substrate. The ArkL sub-
strate contains the FRT b symmetry element, the FRT core and a fox symmetry element (Figure 1(f)). We conducted gel mobility shift assays to measure the binding of Cre, Flp, Cre25, His-P32 and the two chimeric proteins to the ArkL substrate. As expected, both Flp and His-Fre formed only complex Is on the ArkL substrate (Figure 8, lanes 2-5). The Cre25 and His-P32 proteins also generated a single complex band by binding to the fox and the FRT symmetry elements contained in the ArkL substrate (Figure 8, lanes 8-10). The His-P32 peptide exhibited a decreased binding affinity to the ArkL substrate compared to the Flp protein, as observed previously with the FRT symmetry element contained in the ArkZ substrate. In contrast, the His-Fre chimera bound the ArkL DNA with a higher affinity than the Cre25 peptide.

Both Cre and His-Clp exhibited weak interactions with the substrate (Figure 8, lanes 11-14). Modifications of the core-proximal nucleotides of the lox symmetry element severely impaired the binding of Cre, while the absence of the core-proximal nucleotides of the lox symmetry in the FRT symmetry element severely impaired the binding of the Clp.

A mixed complex II band (cII Mix) was observed when the Flp and His-Fre proteins were incubated with the ArkL substrate (Figure 8, lanes 6 and 7). This heterodimeric complex II reflects the binding of one monomer each of Flp and His-Fre to the FRT and fox symmetry elements of ArkL.

The combined results of the binding assays illustrate the altered binding affinity of the Fre and Clp chimeras compared to the Cre and Flp proteins. Cre and Flp bound with high affinity to the lox and FRT sites. In contrast, the Fre and Clp chimeras bound weakly to these symmetry elements. The composite symmetry elements fox and lrt, were high-affinity binding sites for the Fre and Clp proteins. Native Cre and Flp recombinases bound weakly to these novel composite target symmetry elements. The Cre25 and P32 proteins bound their respective native and composite symmetry elements with equal affinity; hence the sequence of the core-proximal nucleotides in a target symmetry element did not affect the binding of the isolated COOH-terminal peptides of Cre or Flp. Finally, incubation of the native and chimeric recombinases with the hybrid substrate containing both the corresponding native and composite target symmetry elements generated distinct mixed dimer complexes. The combinations of Cre and His-Fre with the ArkP substrate, Cre and His-Clp with the ArkF substrate and the incubation of Flp and His-Fre with the ArkL substrate, are all examples of such mixed reactions combining a native protein with a chimeric protein that generate heterodimeric complexes.

**Mode of cleavage: experimental design**

Previous experiments designed to illustrate trans cleavage by Cre and Flp made use of a comple-
mentation strategy whereby a protein was bound to a target site and trans complementation for cleavage was measured (Shaikh & Sadowski, 1997). In a previous paper we described experiments where a half-site that was non-cleavable was occupied with a Cre molecule that acted as the tyrosine-donor (site B, see Figure 9). The site that was destined to be cleaved (X25) was occupied by a Cre protein that was cleavage-incompetent due to a mutation in the catalytic tyrosine.

A more rigorous protocol for examining the mode of cleavage is to use whole recombination sequences to which proteins can be targeted by site-specific DNA binding. The hybrid recombination sites ArkP, ArkF and ArkL allowed us to place native and chimeric proteins at known symmetry elements within the substrates and thereby permitted us to address directly whether the various proteins cleave in cis or trans (see Figure 1(d)-(f)). We labeled the top or bottom strand of each substrate, incubated it with the appropriate pair of proteins and isolated the mixed heterodimeric complexes (cIIs). After analysis of the complex by SDS-PAGE, we were able to determine the mode of cleavage for the Cre, Fre, Flp and Clp proteins.

Cleavage of the ArkP substrate by Cre and His-Fre

We incubated the labeled ArkP substrates (containing a lox and a fox symmetry element, Figure 1(d)) with a mixture of Cre and His-Fre, and isolated the band containing the mixed complex II. Fortunately, the preparative reactions gave no Cre and His-Fre homodimeric complex IIs. As controls, we also isolated the complex I band formed by Cre as well as the homodimeric cII bands formed from reactions containing only Cre or Fre. After electrophoresis, the labeled complexes were analyzed by SDS-PAGE and the results are shown in Figure 10((a) top strand labeled; (b) bottom strand labeled).

The Cre complex I bands showed no covalent attachment (Figure 10(a) and (b), lanes 2). In contrast, the homodimeric complex II bands isolated after treatments with Cre or His-Fre alone showed robust or very weak cleavage, respectively (Figure 10(a) and (b), lanes 3 and 4). While the more slowly migrating His-Fre covalent complex was barely detectable, the results illustrate that both strands of the ArkP substrate were cleavable by both proteins. Coupled with the absence of
covalent attachment in the Cre monomeric complexes, these results demonstrate that the cleavage activity of Cre is dependent upon establishing protein-protein interactions between monomers (Guo et al., 1997). These results also show the importance of preparative isolation of the heterodimeric complex II to avoid possible contamination by homodimeric complex IIs.

When the top strand was labeled, the isolated heterodimeric complex showed only a Cre covalent complex (Figure 10(a), lane 5). Similarly, when the bottom strand was labeled and the mixed dimeric complex was isolated, only His-Fre covalent attachment was observed (Figure 10(b), lane 5). Since the covalent complex results from attachment of the protein after cleavage of the labeled DNA strand, the location of the cleaving protein in the complex allows us to determine the mode of cleavage. Since Cre is bound adjacent to the cleavage site on the labeled top strand (Figure 6), the covalent attachment of Cre solely to the top strand indicates a cis mode of cleavage. Similarly, as the His-Fre chimera was bound adjacent to the cleavage site on the labeled bottom strand, and only a His-Fre covalent complex was detected, Fre was also cleaving in cis. Since covalent attachment of His-Fre to the top strand or Cre to the bottom strand was not detected, the results exclude a trans mode of cleavage for Cre and His-Fre.

Cleavage of the Ark F substrate by Cre and His-Clp

We then used the ArkF substrate to monitor top strand cleavage adjacent to the Cre binding site and bottom strand cleavage adjacent to the Clp binding site (see Figure 1(e)). The preparative reaction combining Cre, His-Clp and the ArkF substrate generated the same pattern of complexes as the analytical scale reaction (Figure 7, lane 6). The
SDS-PAGE analysis of the isolated mixed complexes and the complex I bands formed by Cre and Clp is shown in Figure 11.

No covalent complexes were observed in the Cre and His-Clp complex I bands isolated after treatment with single proteins. This again shows the dependence of the catalytic activity on the formation of multimeric protein-DNA complexes.

Most surprisingly, the isolated mixed dimer complex in which the top strand was labeled showed, in addition to Cre-dependent covalent attachment, a large excess of the His-Clp covalent complex (Figure 11(a), lane 6). These results, coupled with the binding assignments of Cre and Clp on the ArkF substrate, demonstrate that Cre cleaves the top strand in cis, while the Clp chimera is cleaving the same site in trans. A number of additional bands appear that are also seen in assays of covalent attachment by Flp (Figure 12). These most likely result from proteolytic fragments of Clp covalently attached to the cleaved labeled strand. By phosphorimager analysis, we determined that Clp-dependent covalent attachment exceeded that of Cre by a factor of 10-15 times (data not shown). The Cre complex I band isolated from a mixed reaction showed barely detectable levels of covalent attachment (Figure 11(a), lane 4). However, the isolated His-Clp complex I band showed a slightly greater amount of cleavage product than that observed from the isolated dimer (Figure 11(a), lane 5). As the complex I bands from the unmixed protein reactions showed no covalent attachment, the presence of cleavage in these complex I bands may be attributable to the disassembly of the heterodimeric complex after cleavage.

The results for the isolated mixed dimeric complex II in which the bottom strand was labeled are shown in Figure 11(b). A Cre covalent complex was detectable (Figure 11(b), lane 6). But by phosphorimage quantification, the level of Cre-dependent cleavage was of the order of 50 times lower than the amount of Cre covalent complex observed on the top strand. While the amount of Clp-dependent covalent complex far exceeded the amount of Cre cleavage product as before, the level of Clp cleavage was also reduced by 100-fold compared to the top strand cleavage results. This reduction in cleavage product from the Cre-His-Clp dimer was also evident in the complex I bands isolated from the mixed reaction (Figure 11(b), lanes 4 and 5). Here, the amount of Clp-dependent covalent complex was decreased by 100 times, while no cleavage product was detectable from the isolated Cre complex I band. However, despite the reduced levels of cleavage by both Cre and Clp of the bottom strand, these results are consistent with trans...
cleavage by Cre and a cis mode of cleavage by Clp. The small amount of trans cleavage by Cre is consistent with our previous experiments (Shaikh & Sadowski, 1997).

Cleavage of the ArkL substrate by Flp and His-Fre

Incubation of the ArkL substrate with Flp and Fre allowed us to examine the mode of cleavage by the Flp and Fre proteins (Figure 1(f)). ArkL contains one FRT and one fox symmetry element. The complex I bands isolated after incubation with the Flp or His-Fre alone generated no covalent complexes with either the top or bottom strand of the ArkL substrate (Figure 12(a) and (b), lanes 2 and 3). We were unable to isolate Flp and His-Fre complex I bands from a mixed reaction without contamination by the other complex I bands (data not shown).

The labeled top strand of the ArkL substrate monitored cleavage adjacent to the Flp binding site. The heterodimeric complexes showed a barely detectable protein-dependent complex whose origin could not be determined. We could not be certain whether it was indeed a Flp covalent complex when comparing it to the position of the Flp-FRT covalent complex (Figure 12(a), lanes 4 and 5). However, no His-Fre-dependent covalent complex was observed. Hence, Fre was unable to cleave the top strand in trans and little, if any, cis-cleavage by Flp occurred.

The mixed dimeric complex generated both Flp and His-Fre covalent complexes with the bottom strand (Figure 12(b), lane 4). By phosphorimager quantification, we found that Flp and Fre produced equal amounts of covalent complex. The level of Flp-dependent cleavage was greatly reduced compared to the activity of Flp on the FRT substrate. Interestingly, the Fre protein was more active in the Flp-His-Fre heterodimer than in the Fre homo-

Figure 7. Binding of Cre and Flp-derived proteins to the ArkF substrate. Gel mobility shift assay. Complexes named as in Figures 4 and 5.
dimer (Figure 12(b), lane 6). Since the labeled bottom strand detects cleavage adjacent to the Fre binding site, the results illustrate a trans mode of cleavage for Flp and cis cleavage for the Fre chimera.

In summary, these results show that Cre cleaves predominantly, if not exclusively, in cis while Fre cleaves only in cis. We also found that Flp cleaves in trans and that Clp cleaves predominantly in trans.

Discussion

The rationale for the design of the Fre and Clp chimeras originated from the studies of the proteolysis of the Cre and Flp proteins. Both recombinases can be cleaved into analogous NH$_2$ and COOH-terminal domains (Hoess et al., 1990a; Pan & Sadowski, 1993). The COOH-terminal domains of both Cre and Flp contain the four conserved catalytic residues and exhibit the binding specificity of the full-length proteins for their cognate DNA target sites. Panigrahi & Sadowski (1994) identified the core-proximal and core-distal regions of the FRT site as the cognate sites for the NH$_2$-terminal P13 and COOH-terminal P32 domains of Flp (see Figure 3). Similarly, the lox symmetry element can be subdivided into a core-proximal site that is bound by the NH$_2$-terminal Cre13 and a core-distal site that is bound by the COOH-terminal Cre25 (Shaikh, 1997). The common domainal structure of Cre and Flp and the parallel organization of the symmetry elements of the lox and FRT sites allowed us to construct novel proteins, Fre and Clp, through swaps of the NH$_2$-terminal regions of Cre and Flp.

The Fre and Clp chimeras qualify as novel proteins distinct from their Cre and Flp progenitors. The addition of the heterologous NH$_2$-terminal domain in both chimeras greatly reduced their bind-
ing affinity for the cognate DNA target sites of Cre and Flp. In addition, the composite recognition target sites developed for Fre and Clp were specific for each chimera and did not permit binding of the parental Cre and Flp proteins. Secondly, the Fre and Clp proteins were not simply new versions of the COOH-terminal peptides with relatively large NH2-terminal tags. Fre and Clp both exhibited greater binding affinity for their cognate sites than the Cre25 and P32 peptides. Furthermore, Clp showed a level of cleavage activity similar to intact Flp and much greater than that generated by P32 (Shaikh, 1997).

Figure 9. Rationale of the previous complementation test used (Shaikh & Sadowski, 1997, Reproduced with permission from Journal of Biological Chemistry (1997) 272, 5695-5702). The cleavable X25 site is loaded with the cleavage-competent CreHis Y324C protein and the non-cleavable 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 32P-labeled top strand (middle). The radioactive label covalent complex is detected by SDS-PAGE (bottom). Squares, CreHis Y324C; triangles, CreHis; asterisk, 32P radioactive label.
Similarly, the Fre protein demonstrated the ability to generate synaptic complexes and cleavage activity, in contrast to Cre25, which is defective for both activities (Hoess et al., 1990a; Shaikh, 1997).

The mechanism of binding by Fre and Clp

In gel mobility shift assays, the Fre and Clp chimeric proteins exhibited very weak binding to single \textit{lox} and FRT symmetry elements. Fre showed much less complex formation than the COOH-terminal domain of Cre with a single \textit{lox} symmetry element-containing site. Similarly, the P32 peptide of Flp bound a single FRT symmetry element much better than Clp. The decreased affinity of Fre and Clp for the \textit{lox} and FRT symmetry elements might suggest that the heterologous NH\textsubscript{2}-terminal domain masks the intrinsic binding capacity of the COOH-terminal domain. Similar regulation of DNA binding of the COOH-terminal domain of the \textit{\sigma}70 protein by its NH\textsubscript{2}-terminal region has been observed (Dombroski et al., 1993). This putative masking of the COOH-terminal binding domain by the NH\textsubscript{2} terminus may also occur in the native Flp and Cre proteins. Flp does not bind the \textit{irt} symmetry element (Figure 7, lanes 8 and 9), whereas P32 binds well (lanes 14 and 15). Likewise, native Cre binds poorly to the \textit{fox} symmetry element (Figure 8, lanes 11 and 12), whereas Cre25 does bind well (lanes 8 and 9).

The nature of the interactions between the NH\textsubscript{2} and COOH-terminal domains in Flp and Cre is unknown. Such an interaction is not apparent in the cocystal structure of Cre because the DNA of the \textit{lox} site is interposed between the two domains (Guo et al., 1997). However, Subramanya et al.
(1997) postulated that a large conformational change in the XerD protein might be needed to enable the DNA to contact the active site. Alternatively, the native and chimeric recombinases may simply bind weakly to their non-cognate sites because the NH2-terminal domain is repelled by the non-cognate 4 bp core-proximal sequence. The NH2-terminal domain would contribute a positive free energy of interaction with the core-proximal sequence, thereby lowering the affinity of the full-length protein for the non-cognate site.

Cross-core interactions and the formation of complex II

The crystal structure of the cleaved synaptic complex of Cre highlights the cooperative protein interactions that arise between the NH2-terminal domains of two opposing Cre monomers (Guo et al., 1997). These cross-core interactions are formed by the interface between the A and E helices of the two monomers. Our experiments show the importance of these NH2-terminal domain interactions in establishing a stable dimer complex. When Cre and Flp were incubated with the ArkZ substrate (containing inverted lox and FRT symmetry elements) we detected only Cre and Flp complex I bands and barely detectable levels of a Cre-Flp heterodimeric complex. Therefore, the failure of Cre and Flp to form significant amounts of complex II on ArkZ may reflect a steric clash between the Cre13 and P13 regions of Cre and Flp that deters mixed complex formation. This observation is further supported by the observation that

![Figure 11](image-url)
incubation of the ArkZ substrate with Cre and P32 from Flp or with Flp and Cre25 generated much greater levels of a mixed dimer complex (data not shown). On the other hand the failure to form mixed complexes on this substrate may simply reflect the absence of cooperative interactions between the heterologous NH2-terminal and COOH-terminal domains of Flp and Cre.

In contrast, Cre and Clp readily formed mixed dimeric complexes when incubated with the ArkF substrate (containing inverted lox and lrt symmetry elements). Likewise, heterodimeric complexes were easily seen when Flp and Fre were incubated with the ArkL substrate (containing inverted FRT and fox symmetry elements). Although all three substrates would position the heterologous COOH-terminal domains of Cre and Flp opposite each other, only the Cre+Clp and the Flp+Fre paired mixtures would position the same NH2-terminal domains together across the core. In the case of ArkF, a Cre13-Cre13 interaction is created between the two protein monomers bound in an inverted configuration on the substrate. In the case of the ArkL substrate, the two P13 domains are opposed.

**Figure 12.** Covalent attachment of Flp and Fre to the ArkL substrate. Equal counts of isolated complexes and substrate bands were analyzed by SDS-17% PAGE. The labeled (asterisk) ArkL substrate is shown at the top. (a) No covalent attachment by Flp or Fre. Lane 1 (S), isolated ArkL substrate. Lanes 2 and 3, isolated Flp complex I (cIFlp) and His-Fre complex I (cIHFre) from Flp- and His-Fre-only reactions. Lane 4, isolated Flp-Fre heterodimeric complex (cIMix) from the mixed Flp and His-Fre reaction. Lane 5, isolated complex II band of Flp (cIIFlpFRT) generated with a top strand labeled full-FRT substrate (see Figure 1(b)). Lane 6, isolated complex II band of His-Fre (cIIHFretfox2) generated with a top strand labeled fox2b substrate. This substrate contains two inverted fox symmetry elements surrounding an FRT site core. (Figure 1(g)). covFlp, covalent complex generated by cleavage of the labeled DNA strand by Flp. covHFre, covalent complex generated by cleavage of the labeled DNA strand by His-Fre. Note that the absence of covFlp and covHFre in lane 4 means no cis cleavage by Flp or trans cleavage by Fre occurred. (b) Covalent attachment by Fre in cis, covalent attachment by Flp in trans. Lane 1 (S), isolated ArkL substrate. Lanes 2 and 3, isolated Flp complex I (cIFlp) and His-Fre complex I (cIHFre) from Flp- and His-Fre-only reactions. Lane 4, isolated Flp-Fre heterodimeric complex (cIMix) from the mixed Flp and His-Fre reaction. Lane 5, isolated complex II band of Flp (cIIFlpFRT) generated with a top strand labeled full-FRT substrate (Figure 1(b)). Lane 6, isolated complex II band of His-Fre (cIIHFretfox2) generated with a top strand labeled fox2b substrate. covFlp, covalent complex generated by cleavage of the labeled DNA strand by Flp. covHFre, covalent complex generated by cleavage of the labeled DNA strand by His-Fre. Note that the presence of both covFlp and covHFre in lane 4 indicates cleavage by Flp in trans and cleavage by Fre in cis.
Hence, the formation of the mixed dimer complex was dependent upon presenting two identical homologous NH₂-terminal domains cross-core at the dimer interface.

The Cre cocrystal structure showed that the Cre dimer was also stabilized by the insertion of the COOH-terminal N helix of the cleaving subunit into a COOH-terminal hydrophobic pocket of the opposed, non-cleaving monomer. Although pairing of the heterologous NH₂-terminal domains of Cre and Fre seems unlikely, the binding of Cre and Fre to the ArkP substrate (containing inverted lox and fox symmetry elements) resulted in the formation of a heterodimeric complex. It is possible that this complex was stabilized through protein-protein interactions involving the capture of the N helix of Cre by the Fre monomer bound across the core. Similarly, both Cre and Fre generated homodimeric complex IIs on the ArkP substrate likely due to protein-protein interactions between the COOH-terminal regions of the two proteins in the dimer.

Cre formed a small amount of homodimeric complex with the ArkF substrate (containing inverted lox and lrt symmetry elements) despite generating only a monomeric complex with ArkZ (containing inverted lox and FRT symmetry elements). It is likely that this homodimeric complex is mediated by cross-core interactions between two opposing Cre13 domains.

As observed from the crystal data and confirmed by these results, Cre relies on major protein-protein interactions between the NH₂ and COOH-terminal domains of Cre monomers to stabilize multi-protein-DNA complexes. The Flp protein, however, does not show the same degree of binding cooperativity that is shown by Cre (Ringrose et al., 1998). Formation of a stable heterodimeric complex when Flp and Fre were bound to the ArkL substrate (containing inverted FRT and fox symmetry elements) supports the idea that the P13-P13 interactions stabilize the mixed dimeric complex despite the heterology between the COOH-terminal domains bound cross-core on the same substrate.

The diminished level of cooperative cross-core interactions between the Flp COOH-terminal domains may relate to the trans mode of cleavage by Flp. The Cre cocrystal shows that the N helix of the cleaving subunit is buried in a hydrophobic pocket of the non-cleaving Cre monomer situated across the core (Guo et al., 1997). The catalytic tyrosine present in the M helix of the COOH-terminal domain of the cleaving molecule of Cre has attached to the scissile phosphate in cis. The Van Duyne group has modeled a trans cleaving mechanism in which the N helix of the trans-cleaving molecule (e.g. Flp) would have to be buried in cis to allow for donation of the active tyrosine in trans (Gopaul & Van Duyne, 1999; Guo et al., 1999). This arrangement might effectively remove the major protein-protein interaction that is evident between the COOH-terminal domains of Cre and may account for the apparent lack of cooperative interactions between the COOH-terminal domains of Flp and Clp.

Footprinting studies suggest that protein-protein interactions between the NH₂-terminal domains of opposing Cre monomers trigger a conformational change in the COOH-terminal domain of Cre that repositions the entire protein on the site (Guo et al., 1997; Hoess et al., 1990a; Shaikh, 1997). Hence, the core-proximal region of the lox site may function as a nucleation site for the NH₂-terminal domain of Cre that allows communication between protein monomers leading to the formation of catalytically active complexes. Similarly, the P13 domain of Flp is believed to induce a conformational change in the COOH-terminal domain of Flp upon binding the FRT site (Panighrahi & Sadowski, 1994). The core-proximal region in the FRT site may also function as an initiation site for complex formation by the Flp protein.

The mode of cleavage

Here, we have used the chimeric Fre and Clp proteins to test the mode of cleavage by the native Cre and Flp recombinases. We have also used the distinct site-specific binding activities of all four proteins to target them to specific symmetry elements in hybrid recombination sites. Furthermore, by directly isolating the mixed dimeric complex, we were certain of the location of the two proteins on the hybrid substrate and that only proteins in the mixed complex II could contribute to the covalent complexes observed on subsequent SDS-PAGE gels. This strategy allowed us to determine the mode of cleavage of both proteins in the heterodimeric complex. However, since the mode of cleavage was determined on dimeric complexes containing single target DNA sites, we cannot assess how the formation of a synaptic complex consisting of two sites might modify the mode of cleavage.

Our results showed that the native Cre protein in this protocol cleaved predominantly in cis whereas the Flp protein cleaved almost entirely in trans. The chimeric Fre protein also cleaved in cis while the Clp protein cleaved mostly in trans. Our results illustrate that the COOH-terminal domain determined the mode of cleavage of the protein and that the addition of the heterologous NH₂-terminal domain in the chimeric proteins did not alter the mode of cleavage defined by the catalytic COOH-terminal region of the protein.

In earlier experiments we showed that Cre cleaved in trans (Shaikh & Sadowski, 1997). What accounts for the discrepancy between the two sets of experiments? Previously we made use of a half-site complementation strategy whereby a Cre protein was pre-bound to a non-cleavable half-site and cleavage in trans was measured on another cleavable half-site (Figure 9). The protocol also made use of cleavage-incompetent Cre variants. The use of tyrosine-deficient and His-tagged versions of Cre
and the non-cleavable half-lox sites may have contributed to the trans cleavage by Cre.

One of the half-lox sites, X25, was cleavable only by the non-His-tagged version of Cre in the half-site reactions. Therefore, the His-tagged version of Cre may have been unable to act in cis in reactions with the X25 site alone. Furthermore, the other half-lox site, B, was not cleavable by either version of Cre. Hence, this site prevented cleavage in cis by the protein bound to it and acted solely as a carrier of catalytically active Cre proteins to an assembly with the Cre-X25 complex.

Most complementation experiments used to test the cleavage mode of Cre or Flp also relied on the use of protein variants that replace the catalytic tyrosine with an amino acid that may not form a hydrogen bond in the active site (Chen et al., 1992; Gopaul et al., 1998; Guo et al., 1997, 1999). While the Cre Y324F protein assumes the conformations in crystal structures similar to that of wild-type Cre (Guo et al., 1999), the effect of the Y324C mutation that we used may have allowed for trans complementation to occur in vitro. The crystal structure of a Cre synaptic complex shows that the active site of the cleaving Cre monomer is composed of the conserved catalytic amino acid residues Arg173, Arg292, His289 and Trp315 surrounding the scissile bond, with the catalytic tyrosine covalently attached at the cleavage site (Guo et al., 1997). The non-cleaving monomer of Cre shows a similar arrangement except that both the histidine and tyrosine residues are shifted away from the scissile bond. If cis attack were prevented by the cleavage-incompetent nature of the scissile bond, then the catalytic tyrosine of the Cre monomer bound to one half-site may have been directed towards a trans attack of an opposing cleavable half-site. Likewise the Cre monomer bound to the X25 site may have also adopted the conformation required for the acceptance of an incoming catalytic tyrosine residue.

It is possible that the original finding of trans cleavage by the phage λ integrase was attributable to the same explanation (Han et al., 1993). It should be noted that the crystal structure of the COOH-terminal domain of the λ integrase showed considerable mobility of the catalytic tyrosine that might lead to trans cleavage (Kwon et al., 1997). Construction of a Fre Y324C variant would allow a test of the prediction that such a mutation would change the mode of cleavage by Cre on the ArkP substrate to trans.

It is important to note that the experiments in our previous paper did not rule out cis cleavage by Cre (Shaikh & Sadowski, 1997). When both Cre and His-Cre were bound to their respective half-sites, His-Cre cleaved in trans, but cleavage by Cre was also observed. The design of the experiment did not allow us to distinguish cis from trans cleavage by Cre. The present results from the mixed Cre and Clp heterodimeric complex demonstrated that Cre could cleave in cis or support trans cleavage by Clp. Here, we show that although Cre cleaves predominantly in cis it can also cleave in trans even when the experimental protocol allows cis cleavage.

**Action of Cre and Fre on ArkP**

When Cre and Fre were both bound to the ArkP substrate, both proteins cleaved in cis although Cre cleaved more efficiently than Fre. This difference may be related to the efficiency with which each protein activates the scissile phosphate bond. Alternatively the substrate may be bent differently by the respective protein. In the Cre and Fre heterodimeric complex the homologous catalytically active COOH-terminal domains were positioned together. In the Cre cocrystal structure, the catalytically active mixed dimeric complex is comprised of cleaving and non-cleaving protein monomers (Guo et al., 1997). The cis-cleaving monomer inserts its N-helix into a hydrophobic pocket in the non-cleaving monomer. Cre cleaves the top strand of the ArkP substrate in cis and covalently attaches its catalytic tyrosine to the scissile phosphate adjacent to the Cre binding site. The N helix of the cleaving Cre monomer could have been donated in trans to the non-cleaving Fre protein. When Fre cleaves the bottom strand of ArkP in cis, its N helix might be donated in trans to the COOH-terminal docking site in the non-cleaving Cre protein.

**Assembly of Cre and Clp heterodimers on ArkF**

The Cre and Clp heterodimeric complexes were comprised of the cis-cleaving COOH-terminal domain derived from Cre and a trans-cleaving COOH-terminal domain of Flp. These complexes verified a cis cleavage mode for Cre (Figure 11(a)). This may also mean that the N helix of Cre is donated in trans to the non-cleaving Clp protein bound cross-core on the substrate. This would require that the COOH-terminal domain of Clp (derived from Flp) must accept the heterologous COOH-terminal N-helix from Cre.

The heterodimeric complex of Cre and Clp on the ArkF substrate showed that Clp cleaved the top strand in trans (Figure 11(b)). Since Clp is derived from the COOH-terminal domain of Flp, this result is consistent with the known cleavage mode of Flp. Surprisingly, Cre was able to activate the scissile phosphate and to accept the incoming catalytic tyrosine from a Flp-derived COOH terminus. The donation of the catalytic tyrosine of Clp to the acceptor Cre monomer might require a cis collapse of the N helix in both Cre and its equivalent in Clp (Gopaul & Van Duyne, 1999).

As both Cre cleavage in cis and Clp cleavage in trans occur at the top strand of the ArkF substrate, the scissile bond activated by the adjacent bound Cre should be the normal Cre cleavage site. In contrast, the putative cleavage site of the bottom strand of the substrate is in fact a Cre cleavage site, which may be inefficiently activated by the adjacent bound Clp protein. This may have contribu-
targeted to the inefficient trans cleavage by Cre and cis cleavage by Clp at this site. Although we consider it unlikely, it is possible that cleavages by Cre and Clp at this site occur after redistribution of the protein(s) that occurs after isolation of the mixed complex II.

**Action of Fre and Flp on ArkL**

In contrast to the robust cleavage activity demonstrated by both Cre and Clp in the heterodimeric complex with the ArkF substrate, the Flp and Fre heterodimeric complex on the ArkL substrate showed weak catalytic activity of both protein partners. The amount of covalent attachment of Flp to the bottom strand of the ArkL substrate was much lower than that generated by Flp on the full-FRT substrate. This may have been due to a decreased ability of Fre to activate the scissile bond for cleavage or to a defect in accepting the catalytic tyrosine in the active pocket from either a cis or trans-cleavage mode. However, it should be noted that Cre had no difficulty in accepting the catalytic tyrosine from Clp in trans. Therefore, we favor the former explanation.

In none of these experiments have we determined the actual location of the scissile bond. Although it is reasonable to assume that the protein bound next to the scissile bond would determine the position of cleavage, it is possible that the source of the catalytic tyrosine could also play a role. For example, in the Cre and Clp heterodimer, it is likely that the scissile bond is in the “normal” position on the top strand for cis cleavage by Cre. However, trans cleavage by Clp might shift the site of cleavage by the incoming tyrosine residue. Similarly, Flp cleavage at the bottom strand of the ArkL substrate might be inefficient if Fre had activated the phosphodiester bond one nucleotide core-proximal to the expected site of trans cleavage by Flp. The cleavage sites for Flp in the FRT site are 8 bp apart whereas the scissile bonds for Cre in the lox site are 6 bp apart. These differences in spacing may have influenced cross-core interactions between the native recombinases and the chimeras and hence the efficiency and sites of cleavage. Unfortunately, the SDS-PAGE analysis we used to distinguish the covalent complexes of the different proteins was not sensitive enough to determine the site of cleavage to one nucleotide resolution.

**Materials and Methods**

**Enzymes**

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

**Plasmids**

The pShe2, pShe3, pShe5, pShe6, pShe11 and pLD3 plasmids have been described previously (Shaikh, 1997; Shaikh & Sadowski, 1997). All plasmids were prepared using the Qiagen plasmid isolation kit.

**Oligonucleotides**

Oligonucleotides were synthesized at the Hospital for Sick Children/Pharmacia Biotechnology Service Center at the Baniting Institute, University of Toronto or at Dalton Chemical Laboratories, Toronto. They were purified using the OPC cartridge method. Where needed the oligonucleotide was 5’-labeled with [γ-32P]ATP with T4 polynucleotide kinase. After phenol/chloroform-extraction, the oligonucleotide was separated from unincorporated nucleotides using a BioRad P6 spin column (BioRad). It was then annealed to the appropriate complementary oligonucleotide by heating and slow cooling in 0.1 M NaCl, 5 mM MgCl2.

**Proteins**

Native Cre protein was purified from an induced culture containing pShe11 and Cre25 from an induced culture containing pShe5. His-tagged P32 and native Flp were purified from induced cultures containing pShe2 and pLD3, respectively. Purification conditions for all proteins have been described previously (Shaikh, 1997; Shaikh & Sadowski, 1997).

**Construction of His-Fre expression vector**

The Fre protein is a chimera of the NH2-terminal domain of Flp, P13, residues 1-123, and the COOH-terminal domain of Cre, Cre25, residues 119-343 (Figure 2(a) and (b)). The P13-coding region of Flp was isolated by PCR using as template the pShe2 plasmid that contains the Flp NH2-terminal coding region. The 5’ NH2-terminal primer, FLPN, was 44 nucleotides long and hybridized to the start of the P13 coding region. This primer contained an NdeI restriction site (underlined) for later cloning steps and had the sequence: 5’ TAGGGCACGATATGCGCAATTGTTGATATATTATG1AACAAC 3’.

The COOH-terminal primer was P32B and hybridized to the sequence encoding the amino acid residues 117-123 of the P13 coding region. This primer contained a BamHI restriction site (underlined) for later cloning steps (underlined) and had the sequence: 5’ TCTAGGGGATCCCGAACAAC-TACTCACAATACAGCTT 3’.

PCR reactions contained 10 mM KCl, 20 mM Tris-HCl (pH 8.8), 10 mM (NH4)2SO4, 2 mM MgSO4, 0.1% (v/v) Triton X-100, (ThermoPol buffer, New England Biolabs), 1 μM each primer FLPN and P13B, 0.8 μM pShe3 template, and 400 μM each dNTP (Pharmacia) in a total reaction volume of 100 μl. Reaction mixtures were pre-heated to 95°C for four minutes, two units of Vent DNA polymerase were added and the 95°C incubation was continued for 1.5 minutes. Cycling occurred in three stages. The first stage consisted of two cycles of 50°C, one minute, 72°C, 42 seconds, 95°C, 30 seconds. The second stage consisted of 30 cycles of 65°C, one minute, 72°C, 42 seconds, 95°C, 30 seconds. The third stage consisted of a 65°C, one minute annealing step and a 72°C, five minutes extension. Enzyme, dNTPs and oligonucleotide primers were removed by the QIAquick column. The purified 365 bp PCR product was double-digested with NdeI and BamHI enzymes, and was purified by the QIAquick column as before. The pShe6 vector, containing the full Cre coding sequence fused to a 10× His NH2-terminal tag, was digested with BamHI. The 710 bp
DNA fragment that contains the sequences from amino acid 119 of Cre to beyond the stop codon for Cre in pShe6 (Cre25-coding fragment) was isolated from a 1.0 % (w/v) agarose gel and purified using the QIAquick column. Similarly, the pShe6 plasmid was digested with NdeI and BamHI enzymes, and the 5.7 kbp vector fragment was isolated and purified as before. The 360 bp NdeI-BamHI digested PCR product, the 710 bp BamHI-BamHI Cre25-coding fragment and the NdeI-BamHI digested She6 vector fragment were ligated together using T4 DNA ligaase. An aliquot of the ligation mixture was used to transform competent XL1-blue cells (Stratagene: F′ LacZα recA1 hisD17 recF1 mcrA and a unique restriction site (underlined) immediately 5′ of the start codon of the Cre gene in pShe6. The primer used was exchanged for a buffer containing 20 mM Hepes (pH 7.8), 0.1 mM EDTA and 0.1 mM (NH₄)₂SO₄. Fractions were pooled and the imidazole-containing buffer was exchanged for a buffer containing 10 mM Hepes (pH 7.8), 0.1 mM EDTA and 0.1 mM (NH₄)₂SO₄ using 10DG desalting columns (BioRad). Initially, both chimeric proteins caused a non-specific stimulation of Cre binding as assayed in gel mobility shift assays (data not shown). This activity was removed by passing the His-Fre and His-Clp samples through a Centricon 50 followed by a Centricon 10 spin-concentrator (Millipore). Even though the SDS-PAGE profile did not differ from the original eluted fractions, apparently the molecular mass sieves used in the concentrators removed factors that stimulated Cre binding. These His-Fre and His-Clp samples were resuspended in buffer containing 20 mM Hepes (pH 7.8), 0.1 mM EDTA and 0.1 mM (NH₄)₂SO₄ and their concentrations were determined by the Bradford assay (Bradford, 1976) with reagents and IgG standard from BioRad. Both proteins were stored at −70 °C. An aliquot of this mixture was transformed into the XL1-Blue strain and the desired plasmid, encoding the Cre13-P32 chimera, Clp, in frame with an NH₂-terminal 10× His-tag, was denoted pShe20 and maintained in both the XL1-Blue strain and the BL21 expression strain. The predicted molecular mass of the chimera is approximately 48 kDa (45 kDa Clp + 3 kDa His leader sequence).

**Expression and purification of His-Fre and His-Clp**

The BL21 strains transformed with the Fre and Clp expression vectors were grown at 37 °C and protein expression induced with 1 mM isopropyl-β-D-thiogalactoside for 30 minutes at 37 °C; the culture was shifted to room temperature for four hours. When produced in 5 ml cultures the proteins were at least 85 % soluble as assayed by SDS-PAGE and Coomassie Blue staining after sonication and low-speed centrifugation. The cell pellet from a 500 ml culture was resuspended in three volumes of sonication buffer (50 mM sodium phosphate (pH 8.0), 300 mM NaCl). Sonication was done with six 20 second bursts (40 % gain, Vibra Cell sonicator) on ice with two minute intervals between bursts. The sonicate was centrifuged at 100,000 g for one hour at 4 °C. All subsequent manipulations were done at 4 °C. The supernatant was applied to a 2 ml Ni-NTA agarose column (Qiagen) previously equilibrated in wash buffer (50 mM sodium phosphate (pH 8.0), 300 mM NaCl, 10 % glycerol). The column was washed with five column volumes of wash buffer and then successively with three column volumes of wash buffer containing 50 mM, 75 mM, 100 mM and 125 mM imidazole to remove proteins binding non-specifically to the column. The His-Fre protein was eluted from the column with 175 and 200 mM imidazole in wash buffer. Similarly, the His-Clp protein was eluted between 150 and 200 mM imidazole-containing buffer (Figure 2(c)). Both proteins were greater than 95 % pure as assayed by SDS-PAGE and migrated as predicted by their respective molecular mass. The yields of His-Fre and His-Clp were 10 mg/l of induced culture. Fractions were pooled and the imidazole-containing buffer was exchanged for a buffer containing 20 mM Hepes (pH 7.8), 0.1 mM EDTA and 0.1 mM (NH₄)₂SO₄ using 10DG desalting columns (BioRad). The design of the substrates is described in detail in Results. Substrates were singly 5′-end labeled and the binding reactions were performed using the pShe6 plasmid as template. The 5′ terminal domain of Flp, P32, residues 124-423 (Figure 2).

**Construction of His-Clp expression vector**

The Clp protein is a chimera of the NH₂-terminal domain of Cre, Cre13, residues 1-122, and the COOH-terminal domain of Flp, P32, residues 124-423 (Figure 2). The Cre13-coding region of Cre was isolated by PCR using the pShe6 plasmid as template. The 5′ NH₂-terminal primer, NCC, was 32 nucleotides long and hybridized to the 10× His-leader sequence upstream of the start codon of the Cre gene in pShe6. The primer contained an NcoI restriction site (underlined) for later cloning steps and had the sequence: 5′ TAGGGCTACCAGGGCCATCATCATCATCAT 3′.

The 3′ COOH-terminal primer was Cre13B and hybridized to the sequence encoding amino acid residues 116-122 of the Cre protein. Cre13B was 43 nucleotides long and contained sequences that encode the amino acid residues 124-128 of the Flp protein as well as a BstXI restriction site (underlined) immediately 5′ to the start of the Cre portion (italics) of the primer. The sequence of this primer was: 5′ TCTAGGTTCGAACGGTAAATTGTTTTCGATCCGCGATACCC 3′.

A two-stage PCR was done as described before using Vent DNA polymerase, these two primers and pShe6 as the template. The first stage consisted of 30 cycles of 68 °C, one minute, 72 °C, 42 seconds, 95 °C, 30 seconds. The second stage consisted of a 68 °C, one minute hybridization and a 72 °C, five minutes extension. The PCR product was purified and the 455 bp Cre13-PCR product was digested with NcoI and BstXI. The pShe2 plasmid encodes the COOH-terminal P32 fragment of Flp in frame with an NH₂-terminal His-tag. This plasmid was digested with NcoI and then BstXI and the 5.8 kbp DNA vector fragment isolated from an agarose gel. The 450 bp NcoI-BstXI digested PCR product and the 5.8 kbp NcoI-BstXI digested pShe2 vector were ligated together.
in 20 μl containing 50 mM Tris-HCl (pH 7.4), 30 mM NaCl, 2 mM MgCl₂, 3 % glycerol and 0.33 mg/ml of sonicated and denatured calf thymus DNA as competitor. Binding reactions were done with 0.077 pmol of substrate and incubated at 30 °C for 45 minutes with Cre, Cre25, His-Fre, Flp, His-P32 peptide and His-C1p. Actual levels used for each protein are described in Results and the Figures. Reactions were ended by the addition of 2 μl of binding dye solution (1 mM Tris-HCl (pH 7.4), 0.1 mM EDTA, 10 mg/ml BSA, 2 % glycerol, and 0.01 % (w/v) xylene cyanol and bromophenol blue dyes) and then run on a non-denaturing 10 % (w/v) polyacrylamide gel at 4 °C (Andrews et al., 1987).

**Isolation and analysis of complexes**

Analytical binding reactions were scaled up for preparative reactions from 20 to 50 μl containing 100 mM Tris-HCl (pH 7.4) 60 mM NaCl, 4 mM MgCl₂, 6 % glycerol and 0.66 mg/ml of sonicated and denatured calf thymus DNA as competitor. Binding reactions were done with 0.385 pmol of substrate, and were incubated at 30 °C for 45 minutes and run on a polyacrylamide gel as described. For all scaled up reactions, the lower levels of protein used in the analytical reactions were increased by a factor of 5 for reactions containing both one and two proteins (see Results). After electrophoresis, substrate or complexes were located by a brief exposure of the wet gel to X-ray film. Complexes were electroeluted from the gel slices into Centricon 10 spin units (Millipore) in SDS reservoir buffer (25 mM Tris-HCl (pH 6.8), 250 mM glycine, 0.1 % (w/v) SDS); the sample volume was reduced to about 40 μl by centrifugation. SDS sample buffer was added to the samples. The samples were heated to 95 °C for five minutes and equal counts of each sample were analyzed by either 15 % or 17 % polyacrylamide SDS-PAGE. The gel was soaked in 50 % methanol, 20 % glycerol, dried and exposed to X-ray film.

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**References**


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