**Trans** Complementation of Variant Cre Proteins for Defects in Cleavage and Synapsis*

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A. C. Shaikh‡ and Paul D. Sadowski§

From the Department of Molecular and Medical Genetics, University of Toronto, Toronto M5S 1A8, Canada

The Cre recombinase is a member of the integrase family of conservative site-specific recombinases. These proteins share five conserved catalytic residues, one of which is a tyrosine that acts as the nucleophile to attack the scissile phosphodiester bond in the DNA target. Recombination by the Cre recombinase takes place in a supramolecular structure called a synapse that consists of four molecules of Cre bound to two DNA target sequences called lox sites. The synapse is held together by an intricate network of protein-protein interactions. They bend the two sites into square planar structure that resembles a Holliday intermediate. We have studied three mutant Cre proteins that appear to have defects in synapsis (Cre A36V, Cre T41F, and Cre G314R). We found that they were unable to carry out strand cleavage but that cleavage occurred if they were mixed with a cleavage-defective Cre protein that lacks the catalytic nucleophilic tyrosine residue. The three variant proteins could also be complemented for the formation of a novel synaptic intermediate. We suggest that these three mutant proteins have a defect in DNA bending and discuss the relationship between bending, synapsis, and cleavage.

The integrase family members comprise a large family of recombinases that share a common mechanism of catalysis (1–4). Although they do not show a great deal of sequence homology, the common mechanism of catalysis is shown by the conservation of five residues that bring about cleavage and rejoicing of specific phosphodiester bonds in the target DNA (5–11). One of these conserved amino acids is an absolutely conserved tyrosine that carries out DNA strand cleavage. Hence, this family is also called the tyrosine family of recombinases that share a common mechanism of catalysis with the E helix of the cleaving monomer. Three “cross-core” interactions occur between two Cre monomers bound to the same lox site. These consist of 1) an NH2-terminal interface between the E helix of the cleaving monomer with the A helix of the non-cleaving monomer, 2) a COOH-terminal interaction that involves the A helix of a non-cleaving monomer and the B helix of the cleaving monomer, and 3) the contact of the β and β strands and K201 of the non-cleaving monomer with the E helix of the cleaving monomer.

Two “synaptic” interactions occur between Cre monomers bound to two different lox sites: 1) The E helix of a non-cleaving monomer interacts with the A helix of a cleaving monomer, and 2) the N helix of a non-cleaving monomer is buried in a COOH-terminal hydrophobic pocket of a cleaving monomer. The induction of the asymmetric bend is thought to be due to these protein-protein interactions, although Gly-314 contacts the backbone at the kink through a water molecule.

Wierzbicki et al. (17) have previously isolated two mutations (A36V, T41F) encoding mutant Cre proteins that were recombinase-defective. The A36V protein failed to form α-intermediates and was therefore thought to have a defect in synapsis (18). To gain further understanding of the mechanisms of synapsis and bending by Cre, we studied the Cre A36V and T41F proteins as well as another protein, A312T (17), which is recombination-competent but accumulates α-intermediates (19). We also created a G314R mutation in Cre. This glycine residue is highly conserved in all integrase family members (4); the same mutation in the Flp recombinase causes a severe bending defect (20). We performed in vitro complementation studies on the A36V, T41F, and G314R mutant proteins and found that

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§ To whom correspondence should be addressed: Dept. of Molecular and Medical Genetics, Medical Sciences Bldg., University of Toronto, Toronto M5S 1A8, Canada. Tel.: 416-978-6061; Fax: 416-978-6885; E-mail: p.sadowski@utoronto.ca.

1 The abbreviations used are: bp, base pair(s); PAGE, polyacrylamide gel electrophoresis; PCR, polymerase chain reaction; nt, nucleotide(s); cV, complex V; c–IV, complexes I through IV; covCre, Cre-dependent covalent complex; covHCRe, His-tagged Cre-dependent covalent complex; FRT, Flp recognition target.
they all show defects in cleavage. Their cleavage defects can be corrected by provision of a cleavage-defective, binding-competent Cre protein. We postulate that these three proteins may be defective in DNA bending. We discuss the relationship among DNA bending, synapsis, and strand cleavage in the Cre reaction.

EXPERIMENTAL PROCEDURES

Enzymes—All enzymes were obtained from New England BioLabs (unless otherwise stated) and used according to the manufacturer’s instructions.

Plasmids—The pShe1, pShe5, pShe6, pShe20, pShe21, and pRH43 plasmids have been described previously (21–25). 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 Banting Institute, University of Toronto. They were cartridge-purified. Where needed, the oligonucleotides were 5′-labeled with [γ-32P]ATP and T4 polynucleotide kinase as described previously (24). They were then annealed to the appropriate complementary oligonucleotide by heating and slow cooling in 0.1 M NaCl and 5 mM MgCl2.

Proteins—Native Cre protein was purified from an induced culture of pShe11 and Cre25 from an induced culture of pShe5. His-tagged Cre was purified from an induced culture of pShe20. Purification conditions for all proteins have been described previously (21, 22).

Construction of Expression Vectors Encoding Variant Cre Proteins—A common strategy was used to introduce mutations into the Cre gene contained in the His-Cre expression plasmid pShe6. Briefly, a PCR primer was designed to introduce the desired mutation and used together with another primer to amplify a DNA fragment. This PCR1 product was then used as a primer together with a second primer in another PCR reaction to amplify a second PCR product. The pShe6 vector and the PCR2 products were cut with the appropriate restriction enzymes (NdeI and XhoI), and the fragments were purified and ligated together to give the appropriate mutationally altered construct containing the variant Cre gene in-frame with the 10′ His-tag leader sequence. To obtain non-His-tagged variant Cre expression vectors, the pShe11 vector and the PCR2 products were similarly combined together. DNA sequencing of the entire Cre gene confirmed the mutation. A summary of the primers used in the mutagenesis is given in Table I.

Construction of pRecon II Vector—We designed a DNA substrate with which we could monitor by native PAGE the excision of the DNA segment between two directly oriented lox sites. A similar substrate was used to analyze η structures generated by Cre (19). The pRH43 plasmid (25) has two directly oriented lox sites separated by 1.6 kilobase pairs of DNA. We treated the plasmid with HindIII and BamHI enzymes to remove this intervening region and replaced it with the 309-bp HindIII-BamHI fragment from pET19b (Novagen) to give the plasmid pReconI. We further shortened the DNA between the two lox sites by first treating the pReconI with BamHI and filling in the recessed ends with Klenow enzyme. We then digested the plasmid with EcoRV, isolated the large vector fragment, and ligated the ends to give pReconII. The pReconII plasmid contains two directly oriented lox sites separated by 186 bp (see Fig. 1d).

Table I: Primers and primer pairs

| Primera | Sequence | Restriction site |
|---------|----------|-----------------|
| CNS     | See Ref. 22 | NdeI           |
| CTXb    | 5′-CTCTGAGCCCTGGATTAAAGCGTCCCACCTGCCAACCC-3′ | XhoI           |
| V36A    | 5′-GCTTCGAGAAACTCGGTAATCTGCCGACACTGTCCT-3′ |                 |
| F41T    | 5′-CGGAGGCTAGCATTTGCCGGAAATGGTCGAGAACCGA-3′ |                 |
| RG314   | 5′-CGGAGATTCGACGAGGCTGGCTGGCAC-3′ |                 |
| TA312   | 5′-CCGGGATCTGAGAAAATCGGGTCGG-3′ |                 |

a Primers used to construct variant Cre genes.

b In CTX, the underlined nucleotides indicate the XhoI site. The bold letters indicate the sites of the mutations.

c The primer pairs used to amplify the PCR1 and PCR2 fragments are shown for each of the mutations.

d They all show defects in cleavage. Their cleavage defects can be corrected by provision of a cleavage-defective, binding-competent Cre protein. We postulate that these three proteins may be defective in DNA bending. We discuss the relationship among DNA bending, synapsis, and strand cleavage in the Cre reaction.

e Assays with the pRecon II Substrate—We isolated the 285-bp EcoRI-XhoI DNA fragment from the pReconII vector and labeled the 3′-DNA ends with [α-32P]cytidine triphosphate (NEN Life Science Products) using terminal transferase (Life Technologies, Inc.). We purified the labeled substrate from unincorporated nucleotides using P6 spin columns (Bio-Rad).

Binding assays were done with His-tagged versions of Cre, and the Cre variants as described above except 6.5% native polyacrylamide gels were used and the gel was run for 14 h at 200 V. The complex in the gel was isolated after incubation of DNA with His-Cre or His-Cre A312T on ice for 1 min, after which the sample was immediately run on the gel at 4 °C. We exposed the wet gel to X-ray film for 4 h at 4 °C and isolated the complexes from the gel by the “crush and soak” method (28). We then digested the DNA with HindIII. After phenol-chloroform extraction and ethanol precipitation, the samples were resuspended in sequencing dye (80% formamide, 10 mM NaOH, 1 mM EDTA, and 0.01% of xylene cyanol and bromphenol blue dyes) and analyzed on an 8% urea-denaturing gel.

RESULTS

Substrates and Proteins Used—Previously, Wierzbicki et al. (19) described variant Cre proteins with alterations at positions 36, 41, and 312 (17). The Cre A312T variant accumulates Holliday intermediates in the recombination reaction. In fact, analysis of such intermediates led to the conclusion that Cre initiates recombination on the bottom strand of the lox site (Fig. 1a). In contrast, two Cre variants,Cre A36V and Cre T41F are recombination-defective. These Cre proteins bind to the lox site, but fail to generate higher order complexes indicative of synapsis (17, 22). Surprisingly, Cre A36V and Cre T41F can resolve the η structures generated by Cre A312T and synthetic Holliday intermediates at the lox site (Ref. 19 and data not shown).

A glycine residue at position 314 in Cre is highly conserved among most of the integrases (4). The Flp variant, Flp G328R, has a change in the corresponding residue. This protein is both recombination- and cleavage-defective (20, 29) but can donate its tyrosine in trans to another cleavage-defective Flp variant, Flp Y343F. The Flp G328R protein shows a severe defect in bending of the FRT site. We have constructed the Cre G314R variant and found that it is also defective for recombination activity (data not shown). In an effort to learn more about Cre-mediated cleavage, synapsis, and DNA bending, we have characterized further the in vitro phenotypes of these Cre variants.

Action of Cre Proteins on the Suicide lox Substrate—Although the Hoess group reported that the A36V and Cre T41F proteins were catalytically active in the resolution of Holliday intermediates (19), they did not assay these proteins directly for cleavage activity. We therefore designed the suicide lox site, 
The substrates used in this study. For a–c, the sequences of the oligonucleotides are shown. Symmetry elements (horizontal arrows) are derived from the lox site or the FRT site. Core-proximal regions are underlined. lox core region are shown by boxes. Vertical arrows indicate the cleavage sites by Cre on the native lox site. Labeled DNA ends (5’ or 3’) are denoted with asterisks. 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, sloxT DNA substrate. This is a suicide substrate to monitor cleavage at the top strand cleavage site. Cleavage at the vertical arrow on the top strand results in the covalent attachment of the Cre protein to the 3’-phosphoryl dA and liberates a TG dinucleotide. The shorter length of the adjacent oligonucleotide prevents Cre-mediated ligation of the top strand, and the covalent intermediate is trapped. c, sArkP DNA substrate. The hybrid suicide 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 symmetry element and the core-distal 9 bp of the lox symmetry element (24). This suicide substrate monitors cleavage and covalent attachment at the top strand. d, Recon II DNA substrate. The excision substrate (see “Experimental Procedures”) contains two directly oriented lox sites separated by 180 bp; the sizes of DNA regions in the substrate are shown. EcoRI and XhoI restriction ends and an internal HindIII restriction site are shown.

sloxT (Fig. 1b) to test the four Cre variants for binding and cleavage activity. Cre-mediated cleavage at the top strand cleavage site results in covalent attachment of the Cre protein to the 3’-end of the scissile phosphate and liberates a TG dinucleotide preventing religation and trapping the covalent complex. The covalent complex can be detected by SDS-PAGE.

The various Cre proteins were incubated with the sloxT substrate, and we found that, although Cre cleaved robustly (Fig. 2, lanes 2 and 3), the recombination-defective Cre variants Cre A36V, Cre T41F, and Cre G314R were all severely defective in cleavage activity (Fig. 2, lanes 4–7 and 10–11). Histagged versions of Cre A36V and Cre T41F were also cleavage-defective (data not shown). Consistent with previous results, the tyrosine-deficient Cre Y324C variant and the Cre COOH-terminal peptide Cre25 were also cleavage-defective (data not shown). Consistent with previous results, Cre25 bound the sloxT site with 10-fold reduced affinity compared with the full-length Cre proteins (23, 30). The only Cre variant that showed any cleavage activity was Cre A312T (Fig. 2, lanes 12 and 13). Note that the presence of the His-tag on Cre A312T slows the mobility of the covalent complex (covHCre) compared with the covalent complex generated by non-His-tagged Cre (covCre). By PhosphorImager (Molecular Dynamics) quantitation, Cre A312T was one-third as efficient in cleaving the sloxT substrate as wild-type Cre (data not shown). We have also tested a suicide lox site that assays cleavage at the bottom strand of the lox site and found that the cleavage activities of Cre, Cre A36V, Cre T41F, Cre G314R, Cre Y324C, and Cre25 at the bottom cleavage site were identical to those observed on the top strand cleavage site (data not shown). Interestingly, Cre A312T cleaved the bottom strand as well as wild-type Cre (data not shown). The decreased ability of Cre A312T to cleave the top strand scissile phosphate may explain the accumulation of Holliday intermediates by this Cre variant. Cre A312T should be able to form the Holliday intermediate efficiently by exchanging the bottom strands, but the intermediates would accumulate due to inefficient cleavage at the top strands.

The absence of cleavage activity by Cre A36V, Cre T41F, and Cre G314R could not be explained by a reduced affinity for the target site, because all the Cre proteins generated similar levels of monomeric (cI) and dimeric (cII) complexes (Fig. 3). However, neither the cleavage-defective Cre variants nor the Cre25 peptide formed higher order complexes (HO, Fig. 3, lanes 4–11 and 14). The catalytically active Cre and Cre A312T proteins did generate these higher order complexes with wild-type Cre forming about three times as much higher order complex as Cre A312T (by PhosphorImager quantitation, data not shown). Because these higher order complexes are believed to result from synaptic interactions between two recombination sites containing two Cre molecules (15, 18, 22), their absence confirms the synapsis defect of the Cre A36V, Cre T41F, and Cre25 proteins (18). Furthermore, we demonstrate that Cre G314R and Cre Y324C are also defective in synapsis.

Trans Complementation of Cre Variants by Cre Y324C Cleavage—To gain insight into the mechanism of cleavage and synapsis, we tested whether the Cre A36V, Cre T41F, and Cre G314R proteins could be complemented for cleavage activity by another cleavage-defective Cre protein. Cre Y324C lacks the catalytic tyrosine residue but can activate a scissile phosphodiester bond for trans cleavage by a tyrosine donated from a complementing Cre molecule (22). Such complementation would require the Cre Y324C protein to establish protein-protein interactions with the Cre A36V, Cre T41F, or Cre
Cre Proteins Defective in Synapsis and Cleavage

G314R proteins. We therefore combined each of the three Cre variants with Cre Y324C and assayed covalent attachment to the sloxT substrate by SDS-PAGE analysis.

Cre Y324C dramatically stimulated the cleavage by Cre A36V and Cre T41F variants (Fig. 4a, cf. lanes 6–8 with 9–11, and cf. lanes 12–14 with 15–17). Note that the covalent complexes have an electrophoretic mobility of a non-His-tagged protein (covCre) and were therefore not due to the activation of a latent nucleophile in the His-tagged Cre Y324C protein. Although these experiments were not designed to address the position of the cleaving monomer (i.e. Cre A36V or Cre T41F) within a protein-DNA complex, the crystal structure and recent in vitro studies showed cis-cleavage by Cre (15, 24). This would suggest that Cre A36V and Cre T41F were bound adjacent to the top strand scissile phosphate and exhibit cleaving in cis. The level of stimulation of Cre A36V and Cre T41F cleavage increased as greater amounts of Cre Y324C protein were added (Fig. 4a, lanes 9–11 and 15–17). By PhosphorImager quantitation, the maximum levels of Cre Y324C-dependent stimulation of Cre A36V and Cre T41F cleavage activity were 6- and 4-fold (data not shown). We also observed similar levels of stimulation of His-tagged Cre A36V and His-tagged Cre T41F cleavage activity that was dependent on addition of Cre Y324C (data not shown).

Cre Y324C also stimulated cleavage by Cre G314R to a maximum of 6-fold (Fig. 4b, cf. lanes 6–8 with 9–11). Although both proteins in these mixed reactions are His-tagged, the inactivity of His-Cre Y324C by itself suggests that the His-Cre covalent complex (covHCre) was mediated via the tyrosine of His-Cre G314R. The Cre25 peptide did not stimulate the cleavage activity of either Cre A36V or Cre T41F and very weakly (1.2-fold) stimulated Cre G314R activity (data not shown).

Cre Y324C could have stimulated cleavage activity of Cre A36V, Cre T41F, and Cre G314R by enhancing their binding to the sloxT substrate. To exclude this possibility, we examined the binding profile of the mixed reaction using gel mobility shift analysis. We found that addition of the Cre Y324C protein did not affect the binding of Cre A36V to the lox site (Fig. 5), and interestingly, the mixed reaction generated a heterodimeric complex (cII-Mix) that was consistent with the binding of one monomer of Cre A36V and one monomer of His-Cre Y324C to the same substrate molecule (Fig. 5, lanes 8–10). Furthermore, this mixed dimeric complex was favored over the homodimeric complexes generated by both Cre A36V (cII-Cre) and His-Cre Y324C (cII-HCre). This is most obvious in Fig. 5, lane 9, where equal amounts of each protein were added (cf. lanes 3, 6, and 9). Similar results were also obtained using the Cre T41F protein in the mixed reaction (data not shown). We therefore conclude that the assembly of the mixed dimeric complex containing Cre Y324C and either Cre A36V, Cre T41F, or Cre G314R accounts for the stimulation of Cre A36V, Cre T41F, and Cre G314R cleavage activities.

Action of Individual Cre Proteins on the Recon II Substrate—Because the Cre A36V, Cre T41F, and Cre G314R proteins fail to generate higher order complexes, they have putative defects in synapsis (17, 18). We wished to test whether complementation by Cre Y324C, which also fails to generate higher order complexes (see Fig. 2), could form a synapse with either Cre A36V, Cre T41F, or Cre G314R proteins. The binding profile of the mixed reaction (see Fig. 5) did not show any higher order complexes indicative of synapsis. However, this assay may not be sensitive enough to detect the intermolecular association of two mixed dimeric complexes. The intermolecular association of two lox sites may be less efficient or the synaptic complex less stable than with wild-type Cre.

Previously, Hoess et al. (18) used a substrate containing two directly oriented lox sites and non-denaturing gels to detect the
formation of α structures by both Cre and Cre A312T. We constructed an analogous substrate (the Recon II substrate, Fig. 1e) to monitor protein-protein interactions between two directly oriented lox sites on the same DNA molecule. Cre can rearrange the 286-bp Recon II substrate into a synaptic complex in which the two lox sites are in aligned. 2 Cre-mediated cleavage at the bottom strand cleavage sites of both lox sites, and subsequent exchange of DNA strands (Fig. 6, c and d) will result in the formation of an α structure (Fig. 6e), which is essentially a Holliday intermediate where two of the four arms are linked. The α structure can be resolved by Cre into two recombinant products, a 66-bp, 3’-end-labeled, linear DNA fragment and the excised unlabeled 220-bp DNA circle (Fig. 6f).

We incubated the Recon II substrate with His-Cre, His-tagged versions of the Cre variants or Cre25 and analyzed the complexes by a gel shift assay (Fig. 7). Cre and Cre A312T both generated complexes consistent with the binding of four His-Cre molecules to the substrate (cIV-HCre) and the slowly migrating α structure (Fig. 6e, and Fig. 7, lanes 2, 3, 9, and 10). Only wild-type His-Cre generated the 66-bp recombinant product (R) consistent with the results of Hoess et al. (18). Cre Y324C, Cre A36V, Cre T41F, and Cre G314R all generated cIV bands similar to the Cre and Cre A312T binding profiles (Fig. 7, lanes 5–8, 11, and 12), but no recombinant products or α structures were found. Thus the binding activities of Cre and of these Cre variants are comparable. Similarly, the Cre25 peptide also generated a complex IV (cIV-Cre25) (Fig. 7, lane 4) but no recombinant products or α structures.

Trans Complementation of Cre Variants by Cre Y324C Synapsis—Because Cre Y324C could complement the Cre variants for cleavage, we used the Recon II substrate to look for complementation of formation of α structures and recombination. We incubated Cre Y324C or Cre25 with Cre A36V, Cre T41F, or Cre G314R and analyzed the formation of complexes (Fig. 8). The addition of Cre Y324C to reactions containing Cre A36V, Cre T41F, or Cre G314R did not generate recombinant prod-

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2The analogous product detected previously by Hoess et al. (18) was called “Complex III” and contained a mixture of recombinant product, unrecombined substrate, and α structures.
from both these reactions showed the 80- and 206-nt fragments, indicating the absence of recombination (Fig. 10, lanes 6 and 7). However, the cv complex also showed a 42-nt (Fig. 10, CP) band, indicating that cleavage of the bottom strand had occurred (Fig. 10, lanes 6 and 7, see Fig. 6c). We conclude that the complementation of the cleavage activity of Cre A36V by Cre Y324C results in the cv complex, which we propose is a synaptic complex similar to that described in Fig. 6c. Furthermore, the formation of the cv complex by wild-type Cre confirms that this synaptic complex is in fact an intermediate in the Cre recombination pathway where Cre-mediated cleavage but not strand exchange has occurred.

Trans Complementation of Cre Variants by the Fre Chimeras—Although Cre Y324C complemented Cre A36V, Cre T41F, and Cre G314R for cleavage, Cre25 did not (Fig. 8 and data not shown). The two NH$_2$ termini of a Cre dimer bind to the lox site to form an interface that may be involved in coordinating cleavage activity within a Cre dimer (see Ref. 15, reviewed in the introduction). Cre25 might fail to complement, because it would not have made such an interface. Alternatively, Cre25 may not generate the asymmetric kink in the lox DNA that may be involved in stimulating cleavage activity (15, 16).

To assay the effect of supplying a full-length Cre-based protein that could not provide a homologous NH$_2$-terminal interface, we mixed Cre A36V or Cre G314R with the Fre protein (21, 24). Fre is a chimeric protein in which the NH$_2$-terminal P13 domain of Flp is fused to the COOH-terminal, Cre25 region of Cre. As a result of this fusion, Fre binds specifically to a symmetry element (fox) that is distinct from those of the lox site (Fig. 1c and Fig. 11). More importantly, despite the heterology between their NH$_2$ termini, Fre and Cre form stable dimeric complexes that show cleavage activity of both Fre and Cre (24).

We used gel shift assays to confirm the binding specificities of Cre A36V and Cre T41F alone or in combination with the Fre chimera (Fig. 11). Consistent with previous results (24) the lox and fox symmetry elements contained in the arcR substrate (Fig. 1c) are specific binding sites for Cre and Fre, respectively. The Cre variants bound with the same affinity as wild-type Cre and generated predominantly cI (Fig. 11, cf. lanes 2 and 3 with lanes 8 and 9, and with lanes 12 and 13). Fre forms predominantly cII as shown previously (Fig. 11, lanes 4 and 5). The mixed reactions combining His-tagged Fre with non-His-tagged Cre, Cre A36V, or Cre T41F showed the same complexes generated by the individual proteins but, in addition, showed heterodimeric Cre-Fre complexes (cII-Mix) (Fig. 11, cf. lanes 6 and 7 with lanes 10 and 11, and with lanes 14 and 15). The Cre G314R protein also bound the arcR hybrid substrate with a similar affinity to Cre (data not shown), but because His-Cre G314R has a molecular weight similar to His-Fre, the mixed dimeric complex from this combination could not be separated from the homodimeric complexes.

We then assayed the mixed Cre-Fre reactions for cleavage and covalent attachment of the arcR substrate by SDS-PAGE as before (Fig. 1c and Fig. 12). As expected from previous results (24), reactions containing only one type of protein showed no covalent product (Fig. 12, lanes 2, 3, 5, and 7). The mixed reaction containing Cre and Fre showed abundant covalent attachment by Cre (covCre) but no covalent complex indicative of trans cleavage by Fre as expected from previous results (Fig. 12, lane 4 (24)). The covalent complexes are thought to result from cleavage of the top strand by Cre in cis.

The combination of Cre A36V and Fre generated approximately one-third the amount of covalent complex (Fig. 12, lane 6) generated by wild-type Cre. Because homodimeric complexes of Cre A36V are catalytically inactive, the cleavage activity...
results from the mixed dimeric complex containing both Cre A36V and Fre.

The mixed reaction containing Cre G314R and Fre (Fig. 12, lane 8) showed a 4-fold lower amount of covalent complex (covHCre) than the Cre A36V. The inactivity of Fre or Cre G314R alone suggests that the covalent complex arises from mixed dimeric complexes containing the Cre variant and the Fre protein.
DISCUSSION

We have studied three Cre variants (A36V, T41F, and A312T) previously partially characterized (17–19) in addition to a novel mutation (G314R). We have assayed the variant proteins for DNA cleavage, synapsis (formation of higher order complexes), and strand exchange (formation of α-intermediates). We have confirmed and extended the findings of the Hoess group (17–19). Although these authors found that these proteins failed to synapse and form α-intermediates they did not assay directly for DNA cleavage. We have showed that A36V and T41F are defective in cleavage, synapsis, and strand exchange, whereas the A312T protein is competent for all of these reactions. Interestingly, the G314R mutation caused the identical phenotype to the A36V and T41F proteins, namely, it was also defective in cleavage, synapsis, and strand exchange.

Complementation by Cre Y324C—The cleavage defects in the A36V, T41F, and G314R proteins could all be complemented by mixing with Cre Y324C. Complementation also occurred for the formation of the novel cV intermediate but not for synapsis or strand exchange.

What Is the Nature of the Defects in A36V, T41F, and G314R?—Because they have identical phenotypes, it is tempting to speculate that these proteins also share a common defect. We constructed the G314R mutation, because the G314 residue is conserved in the integrase family members (4) and the analogous mutation in the FLP protein produces a severe defect in bending of the FRT site (20). Hoess et al. (19) observed that the A36V and T41F proteins were able to resolve Holliday intermediates, suggesting that the proteins were catalytically competent and that their defect lay in some early step of the reaction. It is possible that the A36V and T41F mutations also cause a bending defect. Although the cocrystal structures of Cre show that the DNA in the synapse is bent as much as 110°, it has been difficult to detect DNA bending by Cre in solution...
using conventional circular permutation assays (18). In the Cre synapse, cross-core interactions occur between the A helix of the non-cleaving monomer and the E helix of the cleaving monomer bound to the same lox site (15). The Ala-36 residue is located actually after the A helix, and the Thr-41 residue is in the B helix that contacts the DNA. It is possible that the mutations perturb the cross-core interactions and cause a bending defect. It should be noted that a number of hydrogen-bond interactions occur between residues of the two Cre monomers that comprise the catalytically active dimer. Interestingly, Ala-36 of the non-cleaving monomer exhibits backbone interactions with the Arg-118 residue of the cleaving monomer (15). Substitution of a valine residue at this position might disrupt this interaction for steric reasons. It should also be noted that NH2-terminal mutations of integrases leading to a DNA bending defect have been observed. Indeed an NH2-terminal lesion in Flp (Y60F) also causes a bending defect (32, 33).

The crystal structure of the Cre synaptic complex shows that the kink in the lox core region is asymmetrically placed 5 bp across the core from the activated scissile phosphodiester bond (16). The kink is apparently needed to stimulate cleavage distant from it or to suppress cleavage adjacent to it. The kink does not seem to derive primarily from direct protein-DNA interactions involving the NH2-terminal residues (16).

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3 L. G. Beatty and A. Obuchowska, unpublished observations.
How Does Y324C Complement the Defects in A36V, T41F, and G314R?—If indeed these proteins are unable to generate the kink, the Y324C protein may supply the kink by binding across the core, thereby allowing these proteins to cleave in cis. In the case of the A36V and T41F proteins, Y324C may induce bending by providing a properly positioned A helix for the A-E helix interaction. In the case of the G314R protein, Y324C may kink the DNA directly across the core. It is known from the crystal structure that mutation of Y324 does not affect the protein’s ability to induce the kink (16).

Synapsis and Cleavage—The three protein variants, Cre A36V, Cre T41F, and Cre G314R all showed defects in cleavage and synapsis. Complementation of Cre A36V, Cre T41F, and Cre G314R by Cre Y324C also generated putative synaptic complex (eV) that showed evidence of cleavage activity. Although it is tempting to conclude that synapsis requires synapsis, this is not clear. Previous experiments showing cleavage by Cre in dimeric complexes suggest that synapsis is not needed for cleavage (21, 24). Similarly for the Flp/FRT system, experiments also suggest that a dimer of Flp bound to a single FRT site is the active species for cleavage (34). It is possible that impairment of cross-core interactions leads to a defect in cleavage (possibly accompanied by a bending defect). Because of the cyclic nature of the protein-protein interactions in the synapse, the same forces required for cross-core interactions are also needed for synaptic interactions. These would include contact between the A and E helices at the NH₂ termini and the burying of the N-helix in the in the hydrophobic pocket at the COOH termini. Thus the Cre A36V, Cre T41F, and Cre G314R proteins may have defects in both cross-core interactions (causing absent cleavage) and synapsis. This does not necessarily mean, however, that synapsis requires cleavage. In fact, the crystal structures of the Cre synapse were derived from Cre proteins that were cleavage-competent (16).

Complementation by Fre—The Fre protein, like Cre Y324C, was able to complement the Cre A36V and Cre T41F proteins, whereas Cre 25 was not. Because we find it unlikely that the heterologous P13 domain of Fre could establish an NH₂-terminal interface with the Cre13 region of a full-length Cre protein, it is possible that Fre also acts to provide a kink 5 bp distal to the scissile phosphodiester bond. Catalysis may be aided by the interactions between the homologous COOH-terminal domains of Cre and Fre. Cre Y324F was able to complement the cleavage defect of Cre G314R, whereas Fre was not. Because the Gly-314 residue is very close to the Trp-315 residue, which is thought to play a role in catalysis (15), it is possible that the G314R mutation affects the active site as well as DNA binding. It is interesting to note that, although the A36V and T41F proteins are able to cleave Holliday junctions (19), the G314R protein cannot. This suggests that the latter protein may be defective in catalysis.

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4 A. C. Shaikh, data not shown.