Identification of Cre Residues Involved in Synapsis, Isomerization, and Catalysis* [S]

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The Cre protein of bacteriophage P1 is a tyrosine recombinase and catalyzes recombination via formation of a covalent protein-DNA complex and a Holliday junction intermediate. Several co-crystal structures of Cre bound to its target lox site have provided novel insights into its biochemical activities. We have used these structures to guide the mutagenesis of several Cre residues that contact the lox spacer region and/or are involved in intersubunit protein-protein interactions. None of the mutant proteins had significant defects in DNA binding, DNA bending, or strand-specific initiation of recombination. We have identified novel functions of several amino acids that are involved in three aspects of the Cre reaction. 1) Single mutation of several NH2-terminal basic residues that contact the spacer region of loxP caused the accumulation of Holliday junction (HJ) intermediates but only a modest impairment of recombination. These residues may be involved in the isomerization of the Holliday intermediate. 2) We identified three new residues (Arg-118, Lys-122, and Glu-129) that are involved in synapsis. Cre R118A, K122A, and E129Q were catalytically competent. 3) Mutations E129R, Q133H, and K201A inactivated catalysis by the protein. The function of these Cre residues in recombination is discussed.

The Cre protein catalyzes recombination at its target sequence, the loxP site (Fig. 1a). This 34-bp sequence consists of two identical 13-bp inverted repeats (symmetry elements) that surround an asymmetrical 8-bp spacer region (15). The Cre protein binds specifically to each symmetry element and induces DNA bending (10, 16–19). The cleavage sites are separated by 6 bp within the spacer region (20). Cleavage occurs by covalent attachment of the protein to the 3'-phosphoryl group at the site of the nick via the conserved catalytic Tyr-324 (12, 20). We refer to the nucleotides that are immediately 5' of the cleavage sites as the scissile nucleotides. The bottom strands are cleaved and exchanged first to form a four-armed Holliday HJ intermediate that is then resolved on the top strands to form two reciprocally recombining molecules (17, 21–24).

Cre folds into a two domain structure (Fig. 1b): the small NH2-terminal domain interacts with the inner portion of the symmetry element and the spacer region, while the COOH-terminal catalytic domain contacts the entire symmetry element (8–13). The two Cre subunits bound to the lox site in slightly different conformations: the “cleaving” subunit is in position to cleave the scissile phosphate, whereas the “non-cleaving” subunit is in an inactive mode (Fig. 1c). While the cleaving and non-cleaving Cre subunits make similar contacts with the symmetry elements, they interact differently with the spacer region (Fig. 1d) (10). In particular, several NH2-terminal basic residues in the cleaving subunit directly contact the DNA phosphate backbone opposite the activated scissile phosphate. As a result, the continuous strand (containing the inactive scissile phosphate) is tightly bound in the Cre-DNA interface, while the solvent-exposed crossing strand (containing the activated scissile phosphate) points toward the center cavity of the synapse poised for strand exchange after cleavage (10).

Recombination takes place within a roughly square-planar synaptic complex consisting of four Cre molecules and two lox sites (Fig. 1c) (8–13). The Cre-lox synaptic complex is stabilized by an intricate network of cyclic protein-protein interactions between the Cre molecules bound to the same lox site (“cross-spacer” interactions) and those bound to two different lox sites (“synaptic” interactions). We hereafter refer to the cross-spacer/synaptic protein-protein interactions as simply “intersubunit” interactions, unless otherwise stated. These intersubunit interactions consist of: 1) an NH2-terminal interface primarily between helices A and E, 2) interaction between helix E and the β-loop between β2 and β3 strands (β2/β3-loop), and 3) the burying of the COOH-terminal helix N in a hydrophobic pocket of the adjacent subunit (8, 12). The flexible β2/β3-loop contains the conserved catalytic Lys-201 residue (Fig. 1b) (6, 12, 25).

In this article we studied the functions of the Cre residues that contact the lox spacer region and/or are involved in intersubunit protein-protein interactions. We find that the mutations of these residues have variable effects on recombination and have divided them into three groups (Table I): 1) those that have moderate impairment of recombination and accumulate...
Cre subunits are drawn as colored ovals: non-cleaving (A, A'), cleaving (B, B'). The light blue arrows designate the orientation of the lsoxP site. The crossing strands (thick lines) contain the activated scissile phosphate (green circles), whereas the continuous strands (thin lines) contain the inactive scissile phosphate (magenta circles). The R118- and K122-DNA interactions and the R118-A36 intersubunit interactions occur within a lsox site (cross-spacer, green and blue dashed arrows) and between two lsox sites in a synaptic complex (synaptic; magenta and red dashed arrows). The boxed section represents the region shown in panel d, d. The Cre contacts with the lsox spacer region observed in the Cre R173K/loxS (4CRX) structure (10). The distances between non-hydrogen atoms involved in the contacts shown are less than 3.5 Å. Similar contacts are seen in the other Cre-lox structures. Note that Arg-118 and Lys-122 interact with the distal end of the spacer region from where the Cre molecule is bound (also see panel a). For simplicity, we have excluded most water (blue dots) mediated contacts (for more details, see Guo et al., Ref. 10). Also shown are intersubunit protein-protein interactions (->) involving the residues studied in this article. The Cre residues are colored as in a, magenta, non-cleaving subunit A; red, A'; green, B. The conserved catalytic residues are indicated with asterisks.

**EXPERIMENTAL PROCEDURES**

**Construction of Cre Mutations**—The construction of the His-tagged mutant cre genes is described in Tables S1 and S2 (see “Supplementary Data”). The Q133H mutation was discovered as a secondary mutation during the construction of Cre R101A, and was subsequently cloned as a single mutation (see Table S2). The construction of the non-His-tagged Cre A36V was described previously (22). The Cre K201A expression plasmid was a gift from Dr. Greg Van Duyne, University of Pennsylvania.

The mutant Cre proteins were expressed in *Escherichia coli* BL21 (DE3 pLysS) and purified essentially as described (26). Analysis on SDS-PAGE showed that the proteins were greater than 90% pure. Protein concentration was determined using the Bradford assay (27) with IgG as protein standard (Bio-Rad). The purified proteins were stored at -70 °C.

**Binding, Cleavage, Recombination, Resolution, and Phasing Assays**—These assays were performed as described in previous publications (19, 24, 28).

**RESULTS**

We are interested in studying the regulation of the order of strand exchange by Cre. We have previously reported that the order of strand exchange and the position of the Cre-induced Holliday intermediates, 2) those that fail to carry out synopsis, and 3) those that are catalytically inactive.

**Holliday intermediates, 2) those that fail to carry out synopsis, and 3) those that are catalytically inactive.**
enhanced sensitivity to OP-Cu suggests that these Cre mutant molecules bound to a single substrate; complexes cI and cII correspond respectively to one and two reactions had reached equilibrium (data not shown). The reaction was room temperature for 30 min as described (24). At this time point, the 32P-labeled 82-bp DNA substrate containing either the (loxP site was incubated with 0.25 nM Cre R100A, R101A, R106A, and R121A accumulated twice the amount of Holliday intermediates as the wild-type Cre protein. All four of these mutant proteins were also able to cleave loxP linear suicide substrates (Fig. 4) and resolve synthetic loxP Holliday structure (Fig. 5) with less than 2-fold reduction in efficiency. Note that Cre R121A actually enhanced cleavage on both strands of loxP by about 2-fold relative to the wild-type Cre protein.

Cre R101A was the most defective in recombination (5-fold reduction) among the Group I Cre mutant proteins (Fig. 3). Arg-101 is involved not only in a protein-DNA interaction, but also in a cross-spacer intersubunit interaction with Asn-111 (Fig. 1d) (8–13). The side chain of Arg-101 in the cleaving Cre subunit contacts the spacer region, but in the non-cleaving Cre subunit it forms a hydrogen bond with Asn-111 (OD1) in the adjacent cleaving Cre subunit. The mutant phenotype of Cre R101A could result from a disruption of the protein-DNA and/or protein-protein interaction. To distinguish between these two possible functions of Arg-101, we mutated Asn-111 to Ala (N111A). We found that Cre N111A can bind to loxP (Fig. 2, lane 7) and was less than 2-fold impaired in recombination (Fig. 3), placing it with the Group I mutant proteins. The N111A mutation was less deleterious than the R101A mutation (Figs. 3–5), demonstrating that the more severe R101A phenotype is not due solely to the disruption of the Arg-101-N111A interaction, and that the interaction of Arg-101 with the DNA is also important for function.

We have shown previously that the wild-type Cre protein initiates recombination adjacent to the scissile G nucleotide, but preferentially resolves HJ and cleaves linear suicide substrates adjacent to the scissile A nucleotide (24, 28). To determine whether the asymmetric protein-DNA interactions in the lox spacer region contribute to the order of strand exchange and asymmetric cleavage, we isolated the Holliday intermediates generated in the recombination reaction and analyzed them by denaturing PAGE (Fig. 3c). Like those generated by the wild-type Cre protein, the Holliday intermediates generated by all five Group I Cre mutant proteins had exchanged the bottom strands of the loxP site adjacent to the scissile G nucleotide (Fig. 3c). These Cre mutant proteins also preferentially cleaved linear suicide substrates and resolved Holliday structures on the top strand of loxP (Figs. 4 and 5). Therefore, these NH2-terminal arginine residues (and presumably their asymmetric interactions with the spacer region) and Asn-111 do not dictate the strand preference during strand cleavage and exchange in the loxP sites.

We previously found that the order of strand exchange catalyzed by wild-type Cre is reversed when the position of the scissile base pairs is interchanged as in the lox4 site (Fig. 1a; compare Figs. 3c and 6c, lane 3) (24, 28). We therefore examined the activity of the Cre variant proteins on the lox4 site. The Group I Cre mutant proteins bound efficiently to the lox4 site (Fig. 2b). Like the wild-type protein, the Group I Cre mutant proteins generally recombined the lox4 sites less efficiently and accumulated slightly more Holliday intermediates than they did with the loxP sites (compare Figs. 3 and 6, a and b). Interestingly, Cre R101A and N111A formed two lox4 Holliday species (Fig. 6a, lanes 5 and 7); the major χ1 species co-migrated with those generated by the other Cre proteins, while the novel faster migrating minor species (χ2) may repre-
sent an alternate HJ isomer. The minor χ2 species was also formed by Cre R101A, R106A, N111A, and R121A in the loxP recombination reaction, but at substantially lower amounts than with the loxP substrates (Fig. 3a, upper panel). The Cre residues in Group I may be involved in the isomerization of the Holliday intermediate (see “Discussion”).

Arg-100, Arg-101, Arg-106, Asn-111, and Arg-121 do not influence the order of strand exchange in the lox4 site. Like wild-type Cre, these mutant proteins predominantly initiated strand exchange adjacent to the scissile G nucleotide on the top strand of lox4 (Fig. 6c). They also preferentially cleaved linear suicide substrates (Fig. 7a) and resolved the χ1 synthetic Holliday structure (Fig. 7b) near the scissile A nucleotide on the bottom strand of lox4. In conclusion, as with the wild-type Cre protein, the scissile base pairs primarily dictate the order of strand exchange by the Group I Cre mutants.

**Group II Cre Proteins (R118A, K122A, E129Q) Are Synapsis-defective—**While the NH2-terminal Arg residues in the Group I proteins interact with the half of the spacer region adjacent to the bound Cre molecule, we also noticed that the side chains of Arg-118 and Lys-122 in helix E of Cre are positioned across the spacer region and interact with the distal end of the spacer region (“cross-spacer” interactions, Fig. 1, c and d) (8–10). The latter protein-DNA interactions are also seen within a synaptic complex between a Cre molecule bound to one lox site and the spacer region of an adjacent lox site (“synaptic” interactions). These Arg-118- and Lys-122-DNA interactions form a cyclic network of cross-spacer and synaptic protein-DNA interac-

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### Table I

| Protein         | His-tag | Binding | Cleavage | Recombination | Resolution | Phasing $L_{min}$ |
|-----------------|---------|---------|----------|---------------|------------|------------------|
|                 |         | cl & cl | HO       | Recombinants  | of $\chi$ |                 |
| Wild type       | N&Y     | +++     | +++      | +++           | 115.8 ± 0.1| 115.1 ± 0.1     |
| Group I         |         |         |          |               |            |                  |
| R100A           | Y       | +++     | +++      | +++           | 115.9 ± 0.1| 115.1 ± 0.1     |
| R101A           | Y       | +++     | +++      | +             | 115.9 ± 0.1| 115.2 ± 0.1     |
| R106A           | Y       | +++     | +++      | +             | 115.8 ± 0.1| 115.3 ± 0.1     |
| N111A           | Y       | +++     | +++      | +             | 115.7 ± 0.2| 115.1 ± 0.1     |
| R121A           | Y       | +++     | +++      | +             | 115.3 ± 0.2| 115.2 ± 0.1     |
| Group II        |         |         |          |               |            |                  |
| R118A           | Y       | +++     | +        | +             | 115.8 ± 0.1| 115.0 ± 0.1     |
| K122A           | Y       | +++     | +        | +             | 115.5 ± 0.2| 115.1 ± 0.1     |
| E129Q           | Y       | +++     | +        | +             | 115.8 ± 0.1| 115.2 ± 0.1     |
| Group III       |         |         |          |               |            |                  |
| R118A           | Y       | +++     | +        | +             | 115.8 ± 0.1| 115.0 ± 0.1     |
| K122A           | Y       | +++     | +        | +             | 115.6 ± 0.1| 115.2 ± 0.1     |
| E129Q           | Y       | +++     | +        | +             | 115.3 ± 0.2| 115.2 ± 0.1     |

* N, no tag; Y, has an NH2-terminal His-tag.

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### Table II

| Protein | His-tag | Binding | Cleavage | Recombination | Resolution | Phasing $L_{min}$ |
|---------|---------|---------|----------|---------------|------------|------------------|
|         |         | cl & cl | HO       | Recombinants  | of $\chi$ |                 |
| Wild type | N&Y   | +++     | +++      | +++           | 115.3 ± 1.1| 116.1 ± 1.2     |
| Group I  |         |         |          |               |            |                  |
| R100A   | Y       | +++     | +++      | +++           | 115.3 ± 0.7| 115.6 ± 0.0     |
| R101A   | Y       | +++     | +++      | +             | 115.4 ± 0.0| 116.1 ± 0.0     |
| R106A   | Y       | +++     | +++      | +             | 115.6 ± 0.1| 116.0 ± 0.0     |
| N111A   | Y       | +++     | +++      | +             | 115.5 ± 0.2| 115.1 ± 0.1     |
| R121A   | Y       | +++     | +++      | +             | 115.3 ± 0.2| 115.2 ± 0.1     |
| Group II |         |         |          |               |            |                  |
| R118A   | Y       | +++     | +        | +             | 115.2 ± 0.2| 115.3 ± 0.1     |
| K122A   | Y       | +++     | +        | +             | 115.0 ± 0.0| 116.0 ± 0.0     |
| E129Q   | Y       | +++     | +        | +             | 115.4 ± 0.0| 116.1 ± 0.0     |
| Group III |       |         |          |               |            |                  |
| R118A   | Y       | +++     | +        | +             | 115.2 ± 0.0| 115.3 ± 0.1     |
| K122A   | Y       | +++     | +        | +             | 115.0 ± 0.0| 116.0 ± 0.0     |
| E129Q   | Y       | +++     | +        | +             | 115.4 ± 0.0| 116.1 ± 0.0     |

* Table legend similar to Table I.

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### Notes:

- The asterisk (*) indicates an abolished or reversed resolution bias (see Fig. 7b).
- Not done.
Arg118 is also involved in an intersubunit interaction with Ala-36 in the loop following helix A of the adjacent Cre subunit (8, 12). Ala-36 has been found to be required for synapsis since mutation to Val (A36V) resulted in defects in the formation of higher order complexes, recombination and strand cleavage but not in the resolution Holliday junctions (17, 21, 22).
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22, 29) (also see Figs. 3–5). We therefore examined the properties of the R118A and K122A proteins.

We found that Cre R118A and K122A exhibited similar mutant phenotypes to Cre A36V. They bound efficiently to loxP, but failed to form stable HO complexes that are the presumed synaptic complexes of two loxP sites (17) (Fig. 2a). This suggests that Arg-118 and Lys-122 also contribute to stabilization of the synaptic complex. In addition, Cre R118A and K122A were severely defective in recombining loxP sites and forming Holliday intermediates (Fig. 3). Cre R118A and K122A cleaved linear suicide substrates about 8-fold and 3-fold, respectively, less efficiently than the wild-type Cre protein (Fig. 4). The severe cleavage defect of R118A and A36V relative to K122A implies that the Arg-118–Ala-36 interaction is likely more important than the interactions made by Lys-122. Like the wild-type Cre protein, both Cre R118A and K122A cleaved the top strand of loxP more efficiently than the bottom strand. The inability of Cre R118A and K122A to mediate recombination and strand cleavage is not due to a defect in catalysis per se, since they both resolved Holliday structures efficiently (Fig. 5) and could recombine loxP sites (see below). Like wild-type Cre, these synopsis-defective Cre mutants resolved the Holliday junction preferentially on the top strands of loxP. In fact, the bias for Cre A36V in favor of top strand resolution was even more pronounced than the wild-type Cre protein. Therefore, Ala-36, Arg-118, and Lys-122 do not regulate the strand preference during strand cleavage and resolution of loxP. We have also mutated Arg-118 to Gln, Ser, Val, and Trp, and these Cre Arg-118 mutant proteins are also defective in synapsis (data not shown).

Although Cre R118A and K122A could not recombine loxP sites, they were able to recombine lox4 sites (Fig. 6, a and b). The R118A and K122A mutations reduced recombination between lox4 sites only by 2-fold or less compared with the wild-type Cre protein. However, Cre A36V was unable to recombine lox4 sites. Cre R118A and K122A were able to produce some lox4 Holliday intermediates (Fig. 6, a and b) and analysis of these intermediates showed that, like wild-type Cre, the top strands of lox4 had been predominantly exchanged (Fig. 6c). It is unclear how interchanging the scissile base pairs partially suppressed the recombination defect of Cre R118A and K122A, since these proteins still failed to form stable HO complexes with lox4 (Fig. 2b) and cleaved lox4 poorly (Fig. 7a). All three Group II Cre mutants efficiently resolved the \( \chi_h \) Holliday structure (Fig. 7b). Interestingly, the R118A and K122A mutations essentially abolished the strand bias in the resolution of \( \chi_x \) though not of \( \chi_b \) (compare Figs. 5 and 7b).

Glu-129 is located in the long linker connecting the NH2-terminal and COOH-terminal domains of Cre (E/F-linker; Fig. 1b). In the 3CRX structure (9), Glu-129 contacts the catalytic Lys-201 residue that is located in the \( \beta/\beta \)-loop of the adjacent Cre subunit (Fig. 1d). Substitution of Glu-129 by Gln (E129Q) impaired the formation of HO complex, the initiation of recombination and cleavage of linear suicide substrates (Figs. 2–4, lane 14). Nonetheless, the E129Q protein is proficient in resolution of synthetic Holliday structures (Fig. 5, lane 14). Therefore, Glu-129 is also important for synapsis. The E129R mutation was more detrimental to recombination than the conservative E129Q mutation (Figs. 2–5, lane 15). As discussed in the section below, Cre E129R is catalytically inactive (Group...
III) in addition to being defective in synapsis. Another Glu-129 mutation, E129P, is also impaired in synapsis and catalysis like E129R (data not shown).

Group III Proteins (E129R, Q133H, and K201A) Are Defective in Catalysis—The Group III proteins are catalytically inactive, being dramatically defective (>20-fold reduction) in all the enzymatic reactions assayed including recombination, cleavage, formation, and resolution of Holliday intermediates for both the loxP and lox4 sites (Tables I and II; Figs. 3–7). In addition to E129P/R mentioned above, the Q133H and K201A mutations also disrupt catalysis. Like Glu-129, Gln-133 is located in the E/F-linker of Cre (Fig. 1b). The side chain of Gln-133 is positioned close to the active site (see “Discussion”). Unlike the Glu-129 mutant proteins, however, Cre Q133H formed some higher order complexes (Fig. 2, lane 11). Possible roles for Glu-129 and Gln-133 in catalysis are proposed in the “Discussion.” Lys-201, which is located in the H2/H3-loop, is conserved in the Int family members (6, 25). Guo et al. (10) have also found that Lys-201 is important for catalysis. We found that Cre K201A was also defective in synapsis (Fig. 2, lane 11), possibly due to disruption of intersubunit interactions involving the H2/H3-loop (see “Discussion”) (8, 12).

Lys201 of the cleaving subunit, but not the non-cleaving subunit, makes a minor groove contact with the scissile nucleotide adjacent to the activated scissile phosphate (Fig. 1d) (8–13). We therefore examined whether Lys201 also contributes to distinguishing the scissile base pairs during the resolution of Holliday structures (Fig. 8). Although this protein gave a very low level of Holliday resolution, we found that Cre K201A exhibited similar strand bias to the wild-type Cre protein in the resolution of the loxP Holliday structure χp (Fig. 5a, lane 12 and Fig. 8, lane 9). Preferential resolution by Cre K201A on the top strands of the loxP HJ was also observed for the χex structure in which the orientation of the loxP site was inverted relative to that in the χp structure (Fig. 8, lane 10). The strand bias was essentially abolished in symmetric lox Holliday structures such as χ SA (Fig. 8, lane 11). However, unlike wild-type Cre, interchanging the scissile base pairs did not reverse the strand bias in the resolution of lox4 Holliday structure (Fig. 7b; Fig. 8, compare lanes 8 and 12). Cre K201A resolved both loxP and lox4 preferentially on the top strands, suggesting that the scissile base pairs do not determine the strand preference in Holliday resolution by Cre K201A. Because of the extremely low efficiency of resolution by Cre K201A, it is difficult to assess the significance of the observed bias in resolution.

The Cre Mutant Proteins Do Not Significantly Alter DNA Bending—The Cre-lox crystal structure revealed that Cre induced an asymmetric DNA bend in the lox site (10). We have confirmed this finding using phasing and circular permutation analyses (19). The Cre-induced asymmetric bend is thought to be due to cross-spacer protein-protein interactions between the Cre subunits (10). Alternatively, the asymmetric protein-DNA interactions in the spacer region may contribute to DNA bending due to asymmetric neutralization of the phosphate charges of the DNA backbone (30–32).

To examine whether any of the Cre residues studied here contributes to DNA bending, we analyzed the DNA bends in...
duced by the Cre mutant proteins using phasing analysis (19, 33). The results for loxP and lox4 are summarized in Tables I and II. We detected minor differences in DNA bending when only one molecule of Cre R121A, R118A, K122A, or Q133H is bound to the loxP site (cI bend). However, because of the small magnitude of the cI bend, it was difficult to assess the significance of these minor changes. None of the Cre mutations dramatically altered the DNA bends in the loxP and lox4 sites when two Cre molecules are bound (cII bend), and this is consistent with the fact that the site of initial strand exchange remained unchanged (Figs. 3c and 7c). The Cre mutant proteins also induced similar DMS methylation protection patterns to the wild-type Cre protein for loxP (data not shown) (19).

DISCUSSION

We have characterized several Cre residues that interact with the lox spacer region or are involved in intersubunit protein-protein interactions. Although the majority of the Cre mutations did not drastically alter DNA binding, DNA bending or the strand preference, some of them did affect certain aspects of the recombination reactions (Table I). We present in Fig. 9 possible roles for the Cre residues in recombination.

Group I Residues May Promote Isomerization of the Holliday Intermediate—The Group I Cre mutants (R100A, R101A, R106A, N111A, and R121A) all formed Holliday intermediates efficiently, and in fact all except R100A accumulated more HJ than wild-type Cre. Arginines 100, 101, 106, and 121 interact as asymmetrically with the phosphate backbone of the continuous (non-cleaved) strand in the lox spacer region (Fig. 1d). However, because of the small magnitude of the cI bend, it was difficult to assess the significance of these minor changes. None of the Cre mutations dramatically altered the DNA bends in the loxP and lox4 sites when two Cre molecules are bound (cII bend), and this is consistent with the fact that the site of initial strand exchange remained unchanged (Figs. 3c and 7c). The Cre mutant proteins also induced similar DMS methylation protection patterns to the wild-type Cre protein for loxP (data not shown) (19).

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that the individual asymmetric protein-DNA contacts and the Arg-101-Asn-111 interactions are not essential for recombination. Cre R100A may not have shown increased accumulation of HJ because Arg-100 contacts the same phosphate(s) as Arg-101 and Arg-106 (Fig. 1d).

Resolution is believed to be coupled to an isomerization of the HJ in which the continuous and crossing strands in the HJ switch roles (Fig. 9) (9, 34–36). Isomerization of the HJ would require switching the asymmetric protein-DNA and intersubunit interactions as well. The increased accumulation of the χ1 Holliday intermediates and the appearance of the novel χ2 species by Cre R101A, R106A, N111A and R121A may arise from a slow isomerization of the Holliday intermediate (Fig. 9) and/or a slight decrease in resolution. We speculate that charge neutralization of the DNA phosphates on the continuous strands by the arginine residues would be ideal mediators of

![Graph](image1)

**FIG. 7.** Strand cleavage and resolution of loxP by Cre mutant proteins. a, graph of the percentage of cleavage on the top (solid bars) and bottom (striped bars) strands of loxP suicide substrates as described (24) (also see Fig. 4). b, graph of the results from the resolution of synthetic loxP (χ4) structure. The 87-bp R1 (solid bars) and 75-bp R2 (striped bars) products result from resolution on the top and bottom strands of loxP, respectively (28) (also see Fig. 5).

![Graph](image2)

**FIG. 8.** Resolution of the synthetic χ structures by the wild-type and K201A Cre. a, the lox spacer sequence in the χ structures. Note that the χrev structure contains the loxP site in the reverse orientation relatively to that in the χp structure. b, 2 nM synthetic χ substrate was incubated with 0.25 μM of the indicated Cre protein for 1 h at 30 °C as described (28) (also see Fig. 5). The 87-bp R1 and 75-bp R2 products result from resolution on the top and bottom strands, respectively, of the lox site as illustrated in a (24). The right panel is a ~5-fold longer exposure of the autoradiogram showing the resolution products from lanes 9–12.
the isomerization of the Holliday intermediate. The cross-spacer protein-protein interactions between Arg-101 and Asn-111 (Fig. 1d) may also contribute to the isomerization. Even though mutations of these residues did not affect the cII bend, they may nevertheless influence the isomeric state of the HJ.

**Arg-118 and Lys-122 Are Required for Synapsis**—The second group of Cre proteins (R118A and K122A) are defective in synapsis, strand cleavage and the recombination of **loxP** sites, but they resolve Holliday structures efficiently. These phenotypes are similar to those of the synapsis-defective Cre A36V protein (17, 21, 22, 29), and support the importance of the cII Ala-36-Arg-118 intersubunit interactions in synapsis. Arg-118 and Lys-122 also form a cyclic network of protein-DNA interactions (Fig. 1, c and d), which may also contribute to stabilization of the synapse. Despite the defect in synapsis, Cre A36V, R118A, and K122A were proficient in DNA bending, suggesting that synapsis is not essential for DNA bending at least in cII.

**The E/F-linker Is Essential for Recombination**—The long E/F-linker connecting the NH2-terminal and COOH-terminal domains of Cre is situated close to the active site and interacts with the flexible β2/β3-loop in the adjacent Cre subunit (8, 12). We found that Glu-129 and Gln-133, located in the E/F-linker are essential for catalysis and Glu-129 is required for synapsis as well. Mutation of Glu-129 to Arg (or Pro, data not shown) impaired both synapsis and catalysis, whereas the conservative E129Q mutation was able partially to suppress the catalytic defect but not the synaptic defect.

In the 3CRX structure, Glu-129 of the cleaving Cre subunit contacts Val-85 in helix D within the same Cre subunit and Lys-201 of the non-cleaving subunit (Fig. 1d) (9). Disruption of these intra- and/or intersubunit interactions may be responsible for the synaptic/catalytic defects. Unlike the other Cre residues studied, Gln-133 does not directly contact the DNA nor does it contact the adjacent Cre subunit. However, it does make a water-mediated contact with the phosphate 5’ of the inactive scissile phosphate in the high-resolution 4CRX structure (Fig. 1d) (10). Within the same Cre molecule, the side chain of Glu-133 is positioned close to helix M (which contains the nucleophilic Tyr-324) (8, 9). Gln-133 may function either directly or indirectly in positioning Tyr-324 and/or other catalytic residues in the active site. Other mutations in helix E and the following E/F-linker (namely, V125F and G128D) have also been reported to impair recombination (29). In addition, an insertion of two amino acids (Val-Asp) at position 182 altered the topology of recombination products, implying that synapsis was affected (37). This insertion is close to Asp-184 that interacts with Arg-130 in the E/F-linker of the adjacent Cre subunit.

It is possible that Glu-129, Gln-133, and other residues in the E/F-linker contribute to synapsis and catalysis either directly or indirectly.

We previously proposed that the NH2-terminal domain of Cre allosterically masks the COOH-terminal domain and this inhibitory effect is released upon DNA binding (38, 39). The NH2-terminal domain of XerD also appears to hinder the activity of the COOH-terminal domain (40, 41). Subramanya et al. (41) proposed that a large conformational change may accompany DNA binding and/or synapsis. A conformational change was indeed observed in λ Int upon DNA binding and cleavage (42–45). We propose that the E/F-linker in Cre is likely flexible and functions as a hinge to uncover the active site upon activation. The mutations in the E/F-linker may interfere with this allosteric regulation. The E/F-linker may also be important for communication between the two Cre domains as well as with other Cre subunits in the synaptic complex.

**Lys201 Is Required for Catalysis**—Lys-201 is conserved among the tyrosine recombinases and Type IB topoisomerases (6, 46). The equivalent lysine residue in the vaccinia topoisomerase is important for catalysis, acting as a general acid to protonate the leaving 5’-hydroxyl group during cleavage (46–48). The corresponding Lys residue is also essential for catalysis in XerD (25, 49) and Flp (50). We have confirmed the finding of Van Duyne and co-workers (10) that Lys-201 is important for catalysis in Cre. We also found that mutation of Lys-201 disrupts the formation of the synaptic complex. This supports the involvement of the intersubunit interactions between the β2/β3-loop and helix E (and the E/F-linker) in synapsis (8–13).

Martin et al. (13) observed that Lys-201 interacts asymmetrically with the scissile nucleotides in the Cre-**loxP** HJ crystal structure and proposed that Lys-201 may function to distinguish the scissile nucleotides. However, even with the very low level of resolution that we did detect, we found that Cre K201A exhibited a similar strand bias to the wild-type Cre protein in the resolution of **loxP** Holliday structures. In addition, since Cre K201A induced similar bends to the wild-type Cre protein and we previously found that DNA bending correlates with the site of initiation (19), we believe that Lys-201 likely does not determine the site of strand initiation. Unfortunately, a direct assay of this function is precluded because Cre K201A failed to form any detectable amount of HJ.

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