Cre initiates recombination by preferentially exchanging the bottom strands of the \textit{loxP} site to form a Holliday intermediate, which is then resolved on the top strands. We previously found that the scissile AT and GC base pairs immediately 5’ to the scissile phosphodiester bonds are critical in determining this order of strand exchange. We report here that the scissile base pairs also influence the Cre-induced DNA bends, the position of which correlates with the initial site of strand exchange. The binding of one Cre molecule to a \textit{loxP} site induces a \(-55^\circ\) asymmetric bend adjacent to the scissile GC base pair. The binding of two Cre molecules to a \textit{loxP} site induces a \(-55^\circ\) asymmetric bend near the center of the spacer region with a slight bias toward the scissile A. Lys-86, which contacts the scissile nucleotides, is important for establishing the bend near the scissile GC base pair when one Cre molecule is bound but has little role in positioning the bend when two Cre molecules are bound to a \textit{loxP} site. We present a model relating the position of the Cre-induced bends to the order of strand exchange in the Cre-catalyzed recombination reaction.

The Cre recombinase of bacteriophage P1 assists in the efficient segregation of the low copy P1 plasmid by resolving dimeric lysogenic P1 plasmids into monomeric units (1). Cre is a member of the \(\lambda\) integrase or tyrosine recombinase family of conservative site-specific recombinases (2–5). The tyrosine recombinases share a common mechanism of catalysis. A conserved tyrosine (Tyr-324 in Cre) serves as the catalytic nucleophile that cleaves a specific phosphodiester bond in the DNA target sequence and attaches the recombinase to the DNA via a \(-phosphotyrosine bond (see Fig. 1). A 5' symmetric phosphotyrosine bond (see Fig. 1a below) (3-9). Recombination proceeds via two sequential strand exchanges, forming a four-armed Holliday structure as an intermediate (10-17).

The DNA target sequence for the Cre protein is called \textit{loxP} (Fig. 1b) and consists of two identical 13-bp inverted symmetry elements surrounding an 8-bp asymmetric sequence (18). This 8-bp sequence defines the orientation of the \textit{loxP} site, and we refer to it as the “spacer” region. Each symmetry element serves as a site for sequence-specific binding of a Cre monomer (19–21). The scissile phosphodiester bonds are 6 bp apart, and the Cre protein attaches covalently to the 3'-phosphoryl A residue on the top strand and the 3'-phosphoryl G residue on the bottom strand (22). We refer to these nucleotides as the “scissile A” and “scissile G,” respectively.

Several crystal structures of the Cre-\textit{lox} complexes have provided remarkable insights into the conformations of the various intermediates in the Cre-\textit{lox} reaction (21, 23–27). Two Cre-bound \textit{loxP} sites are brought together by a cyclic network of protein-protein interactions to form an approximately square-planar synaptic complex (Fig. 1a) (21, 23–30). The two Cre molecules bound to a \textit{lox} site are conformationally and functionally different: one is poised to cleave the DNA (“cleaving” subunit), and the other is in an inactive conformation (“non-cleaving” subunit; see Fig. 1a) (21, 23–27).

Cre catalyzes recombination with a defined order of strand exchange (see Fig. 1, a and b): it first cleaves and exchanges the two bottom strands adjacent to the scissile G nucleotide to form the Holliday intermediate (11, 29–32) that is then resolved preferentially on the top strand adjacent to the scissile A nucleotide to generate reciprocal recombinant products (11, 31–33). We found previously that the order of strand exchange was dictated primarily by the scissile base pairs at positions 4’ and 4 in the \textit{loxP} site (32, 33). The order of strand exchange was reversed when the scissile base pairs were interchanged in the mutated \textit{lox4} site (see Fig. 1c). Furthermore, we found that Lys-86, which contacts the scissile nucleotides (21, 23–27), is important for establishing the strand selectivity in the resolution of the \textit{lox}-Holliday intermediate but not in the initiation of recombination between the \textit{loxP} sites (32, 33).

The crystal structure of the Cre-\textit{lox} synaptic complex reveals the presence of an asymmetric DNA bend in the \textit{lox} spacer region, positioned 5 bp away from the activated cleavage site (25). Guo \textit{et al.} (25) proposed that the position and/or direction of the DNA bend dictate the site of initial strand exchange. In this report we have further characterized the roles of the scissile base pairs and the Lys-86 residue in the Cre-induced DNA bending to better understand the basis for the order of strand exchange. We find that Cre induces asymmetric bends in the \textit{loxP} site, and the position of the bends is dictated by the scissile base pairs: the binding of one Cre molecule (complex I, CI)\(^1\) induces a bend near the margin of the spacer region adjacent to the scissile G nucleotide, whereas the binding of two Cre molecules (complex II, CII) induces a bend near the center of the spacer region with a slight bias toward the scissile A (see Fig. 1, b and c). Lys-86 has a role in positioning the Cre-induced bend in the \textit{loxP} CI but not in the \textit{loxP} CII. Changes in the Cre-induced DNA bends within CII correlate with the site of initial strand exchange as originally proposed by Guo \textit{et al.} (25). We present a model relating the position of the Cre-induced bends to the order of strand exchange in the Cre-catalyzed recombination reaction.

\(^1\)This work was supported in part by grants from the Medical Research Council (MRC) of Canada and the Canadian Institutes for Health Research (CIHR) (to P. D. S.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

\(^5\) The on-line version of this article (available at http://www.jbc.org) contains Supplemental Figs. S1–S8.

\(^\text{‡}\) Supported by Studentships from the MRC and CIHR and by a University of Toronto Open Fellowship.

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\(^\text{\textregistered}\) The abbreviations used are: cl, complex I; cII, complex II; DMS, dimethyl sulfate; EMSA, electrophoretic mobility shift assay; OP-Cu, 1,10-phenanthroline-copper; Wt, wild type.
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**EXPERIMENTAL PROCEDURES**

Oligonucleotides, DNA Constructs, and Proteins—The SS2 substrates used in the chemical footprinting experiments were constructed by ligating the 40-nt, two-compartment, oligonucleotide long repeats into the NotI site of plasmid pK10LPA. The OP-Cu footprinting reactions were performed using the ImageQuaNT 5.0 software program. The marker M bands in each lane were aligned. The phasing analysis was conducted according to the method of Zinkel and Crothers (35) as follows. The relative electrophoretic mobility (μ) of a protein-DNA complex was calculated as the mobility of the complex divided by the mobility of the unbound substrate to correct for small variations in the mobility of the unbound substrate. The relative electrophoretic mobility (μ) of the particular complex from all six phasing substrates (μ(avg)) was calculated as the distance (bp) between the center of the kinetoplast DNA that contains the A-tracts (35) and the middle of thelox site. The curve was plotted to the best fit polynomial curve (Microsoft Excel).

**RESULTS**

Guo and colleagues (25) have proposed that the position and/or direction of the asymmetric DNA bend in thelox spacer region revealed in the crystal structure of the Cre-lox synapic complex determined the site of initial strand exchange. We therefore investigated whether the scissile base pairs and Lys-86 dictate the order of strand exchange by affecting the Cre-induced DNA bending. We have probed the DNA conformation of theloxP andlox4 sites (Fig. 1) bound to Cre using chemical footprinting, circular permutation, and phase-sensitive analyses.

**Cre Increases the Sensitivity to DMS Methylation in theloxP andlox4 Spacer Region on the Strand Containing the Scissile G**—DNA footprinting provides information about protein-DNA contacts and distortions in the DNA conformation such as bending and unwinding (40–44). To detect subtle changes in the DNA conformation, we probed the Cre-bound DNA using small chemical compounds. We first analyzed the sensitivity of guanine and adenine residues in theloxP andlox4 sites to methylation by dimethyl sulfate (DMS). DMS methylates the N7 group of guanine in the major groove of DNA and, to a lesser extent, the N8 group of adenine in the minor groove (41).

An 82-bp DNA substrate (S82) containing either the wild-typeloxP or the mutatedlox4 site was treated with DMS in the absence and presence of the wild type Cre protein (Fig. 2). The DMS footprints for the top and bottom strands are shown in Fig. 2 (a and b) and summarized in Fig. 2c. The two strands of theloxP andlox4 sites differed in the sensitivity of their spacer region to DMS modification upon binding to Cre, and the differences were dependent on the location of the scissile G. The scissile G at position 4 on the bottom strand ofloxP (Fig. 2b, lane 2) was protected by the wild type Cre protein, and this protection was dependent on Lys-86 (see below). Bases 3′ to the scissile G on the bottom strand ofloxP (most notably position 3′A) exhibited enhanced reactivity to DMS upon binding to Cre. These enhanced sensitivities suggested that Cre causes structural alterations in thelox spacer region, possibly due to DNA bending (see “Discussion”).

Cre also protected the scissile G and enhanced the DMS methylation of downstream bases (2′G, 1′A, 3′G, and 5′T) on the top strand oflox4 (Fig. 2a, lane 6). The increased sensitivity to

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*Note: The text is extracted from a scientific publication, which is not fully visible due to the cropping of the image. The full text is available in the reference.*
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Fig. 1. Cre-mediated site-specific recombination. a, schematic diagram of the mechanism of Cre-loxP recombination. Two Cre molecules bind to and induce an asymmetric bend in each of the loxP sites (25). We refer to the Cre subunits and the DNA strands using the nomenclature of Van Duyne and coworkers (24, 25). One of the two Cre molecules bound to the lox site is poised to cleave the adjacent scissile phosphate (“cleaving” subunit, dark ovals), whereas the other “non-cleaving” monomer (gray) is in the inactive conformation (21, 23, 25). The “crossing” strand (thin lines in i–iii) is defined as the strand containing the activated scissile phosphate and is pointed toward the center cavity of the synapase ready for strand exchange after the first strand cleavage (25). The “continuous” strand (thick lines in i–iii) contains the inactive scissile phosphate and is on the concave side of the DNA bend (25). Cleavage initiates on the crossing strands (bottom strand of loxP, thick lines) and results in covalent attachment of the Cre dimer. Bases 3' to the scissile G on the bottom strand of loxP and on the top strand of lox4 remained sensitive to DMS methylation when bound to Cre K86A. Furthermore, the K86A mutant protein generally increased the sensitivity of the central region of the loxP and lox4 spacer to OP-Cu compared with the wild type protein (Fig. S1). The Cre K86A-induced DMS and OP-Cu footprinting patterns in the symmetry elements were similar to the wild type Cre protein.

Results

Lys-86 Contacts the Scissile G in Both loxP and lox4—Because Lys-86 influences the order of strand exchange (32, 33), we investigated the role of Lys-86 in the conformation of the Cre-lox complexes by mutating Lys-86 to Ala (K86A). Although the Cre K86A mutant protein is fused to a N-terminal His10 tag, the His-tagged wild type (HisWt) Cre protein exhibited similar properties to the non-tagged wild type Cre protein (Figs. 2 and S1) (32, 33).

We found that the K86A mutation relieved the protection of the scissile G from DMS methylation in both loxP (Fig. 2b, lane 4) and lox4 (Fig. 2a, lane 8). This suggests that the observation from several Cre-lox crystal structures that Lys-86 contacts the scissile G nucleotide (23–25). The K86A mutation not only relieved the protection but strongly increased the reactivity of the scissile G to DMS methylation compared with the substrate without any protein added. The hyper-reactivity of the scissile G in the presence of Cre K86A required DMS treatment (data not shown), indicating that the hyper-reactivity was not due to Cre-catalyzed cleavage. The Lys-86-dependent protection of the scissile G was also observed in the DMS footprint of the substrate “se b” that contains only symmetry element b (Table I; data not shown). This suggests that the interaction between Lys-86 and the scissile G nucleotide does not require the binding of a Cre dimer. Bases 3' to the scissile G on the bottom strand of loxP and on the top strand of lox4 remained sensitive to DMS methylation when bound to Cre K86A. Furthermore, the K86A mutant protein generally increased the sensitivity of the central region of the loxP and lox4 spacer to OP-Cu compared with the wild type protein (Fig. S1). The Cre K86A-induced DMS and OP-Cu footprinting patterns in the symmetry elements were similar to the wild type Cre protein.

Cre Bends loxP Toward the Major Groove Near the Middle of the Spacer Region—Because the chemical footprinting analyses showed evidence for Cre-induced conformational changes in the spacer region of the lox site, we used circular permutation and phasing analyses to detect the Cre-induced DNA bending in the lox site. Bent DNA migrates more slowly than unbent DNA in a polyacrylamide gel due to its shorter end-to-end distance (46).

We first used circular permutation analysis to map the position and angle of the Cre-induced DNA bends (Table II, Fig. 1b and c, and Supplementary Data, Figs. S2–S6) (46, 47). The element (se a or se b; Table I). The error in estimating the position of the bend center was about ±4 bp (see Supplementary Data, Fig. S7). The direction of the Cre-induced bend (either toward the major or minor groove of the DNA) at the bend center is given in parentheses. The direction of the cl bend at position 8' in loxP and se a is ambiguous, but we speculate that it is probably toward the minor groove like the other cl bends (see “Results” c, the lox4 site. In the mutated lox4 site, the scissile base pairs at positions 4' and 4 have been interchanged relative to the wild type loxP site. The schematic of the lox4 site and the Cre-induced bends within the lox4 site are illustrated in a similar manner to loxP in b.
binding of a Cre monomer (cI) induces a bend of $35 \pm 5^\circ$ in the symmetry element generally near the margin of the spacer region ("cI bend"). The binding of two Cre molecules (cII) induces a bend of $55 \pm 5^\circ$ in the lox site near the center of the spacer region ("cII bend"). However, the small bend angles made it difficult to position the bend center accurately (see Fig. 2).

**Fig. 2.** DMS methylation protection of loxP and lox4 by wild type and K86A Cre proteins. A 82-bp DNA (S82) containing either the loxP site (lanes 1–4) or the lox4 site (lanes 5–8) was 5'-radioactively labeled on the top strand (a) or the bottom strand (b). 2 nm DNA was incubated without (lanes 1 and 5) or with 0.5 mM of wild type (Wt), His-tagged wild type (HisWt), or HisK86A Cre protein, then treated with DMS as described under "Experimental Procedures." The DMS-treated DNA was cleaved at methylated G and A residues with alkali and analyzed on an 8% denaturing polyacrylamide gel. Representative autoradiograms are shown. The loxP sequence corresponding to the cleaved bands are indicated to the left of the autoradiogram. The residues mutated to create lox4 are indicated in parentheses. The symmetry elements (normal type) are represented by vertical arrows, and the spacer region (boldface type) is boxed with the small arrow denoting the cleavage site. The band intensities were quantified as described under "Experimental Procedures." The average ratios (from at least three experiments) of the normalized band intensities in the presence to absence of Cre (+ to − Cre ratio) are represented graphically on a log scale to the right of the autoradiogram. The nucleotides are numbered from the center of the lox site as shown in Fig. 1b, with the nucleotides in the spacer in boldface type and the flanking non-lox sequences in lowercase. The dotted vertical lines indicate 1.5-fold protection or enhancement (hypermethylation). Solid blue bars, loxP plus Wt Cre (non-tagged and His-tagged); striped blue bars, loxP plus K86A; solid red bars, lox4 plus Wt Cre; striped red bars, lox4 plus K86A. a, summary of the DMS footprints of the loxP and lox4 spacer region and proximal nucleotides by wild type (Wt) and K86A. Sites of protection (green diamonds) and enhancement (yellow triangles) greater than 1.5-fold are indicated. Although the scissile G at position 4' in lox4 was protected by wild type Cre by slightly less than 1.5-fold, the protection was reproducible.
Supplementary Data, Fig. S7) and to interpret the significance of small differences between loxP and lox4. In addition, circular permutation cannot distinguish between a directed bend and a non-directed protein-induced flexure of the DNA (35, 47, 48).

We therefore turned to phasing analysis to magnify the differences in electrophoretic mobility as well as to determine the direction of the Cre-induced bends. The Cre-induced bend was analyzed relative to a sequence-directed A-tract bend (Fig. 3) (35). We varied the helical phasing of the two bends (and hence the end-to-end distance of the DNA fragment) by varying the linker length between the lox site and the A-tract in 2-bp increments through one helical turn (Fig. 3a). When the two bends are in-phase (in the same direction), the end-to-end distance would be the shortest in the cis isomer, and so the protein-DNA complex would have the minimum mobility (Fig. 3b) (35, 46). In contrast, maximum mobility is obtained in the trans isomer when the two bends are out of phase (in the opposite direction). We define the linker length as the number of base pairs between the center of the kinetoplast DNA that contains the A-tracts (35) and the middle of the lox site. We chose the middle of the lox site as an arbitrary reference center for comparing the various lox sites. The sequences of the various lox sites studied are listed in Table I, and the results of the phasing analysis are summarized in Table II.

We found that Cre induced a directed bend in the loxP site. The electropherogram of wild type Cre bound to the loxP-containing phasing fragments is shown in Fig. 3b (lanes 1–6). The unbound substrates themselves exhibited a slight phasing in their mobility, suggesting that the lox-containing DNA possessed an intrinsic bend. Both cl and cII exhibited phase-dependent variations in gel mobility with a periodicity of about 10 bp indicative of a directed bend (Fig. 3c). The amplitude for the cII phasing curve was about 2-fold greater than that obtained for cl (cl, 0.04 versus cII, 0.08), suggesting that cII exhibited a larger bend angle than cl and supporting the relative bend angles measured by circular permutation analysis (Fig. 3c and Table II). The relative mobility of the Cre-loxP cl and cII reached a minimum mobility at a linker length (L_{min}) of 116 and 115 bp, respectively (Fig. 3c), both of which correspond to about 11 helical turns with a helical repeat of 10.5 bp/turn. Because the A-tract is bent toward the major groove at the center of the kinetoplast DNA and the two in-phase bends are separated by an integral number of turns, we concluded that the Cre-induced bends in cl and cII were also toward the major groove in the middle of the loxP site (the arbitrarily designated reference center). This is further confirmed by analysis of the trans isomer in which the two out-of-phase bends were separated by ~121 (cl) or 120 bp (cII), corresponding to 11.5 turns (Fig. 3c).

From the circular permutation analysis (Fig. 3c), we estimated that the Cre-induced bend center in cl is close to position 4, about half a helical turn from the reference phasing center, and so, Cre bends the loxP in cl toward the minor groove at position 4. In contrast, the bend center in cII is near position 1' (close to the reference phasing center), and so, the loxP in cII is bent toward the major groove near the middle of the spacer region.

Cre Induces an Asymmetric Bend in loxP—An asymmetrically positioned DNA bend was observed in the crystal structure of the Cre-lox synaptic complex (25). We assessed the asymmetry of the Cre-induced bend in the loxP site by comparing the relative phasing of the loxP site in the forward and reverse orientations. The 80-bp test fragment containing the loxP site precisely in its middle was inserted into the phasing vectors in either the forward (F) or the reverse (R) orientation (Fig. 3a). Therefore, the linker length between the A-tract and the middle of the lox site was the same in both orientations. Because the position and magnitude of the Cre-induced bends should be identical regardless of the orientation of the fragment, differences in the relative mobility between the two orientations would reflect a difference in the position of the actual Cre-induced bend center relative to the A-tracks. If the center of the Cre-induced bend is exactly in the middle of the lox site (i.e. at the reference center) and the DNA is bent toward one of the grooves, then the phasing curves should be the same for both orientations (46). On the other hand, if Cre induces an asymmetric DNA bend, then the actual distance between the two bends would be different in the two orientations and hence, the phasing curves would be altered (unless the difference is a full helical turn).

We found that wild type Cre does indeed bend the loxP site asymmetrically and that the asymmetric bend is dictated by the asymmetric spacer sequence. The gel mobility shown in Fig. 3b (b and c) was for the forward orientation, “loxP-F,” which corresponds to the orientation of the loxP site illustrated in Fig. 1b. In Fig. 3d, we compare the relative mobility of the Cre-loxP cII bound to the “loxP-F” substrates and the reverse “loxP-R” substrates. We found that the two phasing curves were slightly different: the “loxP-R” curve was consistently shifted by about +1 bp relative to the “loxP-F” curve. The “loxP-F” and “loxP-R” cII had L_{min} values of 115 and 116 bp, respectively, both of which still correspond to a bend toward the major groove in the middle of the loxP sequence (11 turns, 10.5 bp/turn). Therefore, reversing the orientation of the loxP site causes a small but highly reproducible shift in the phasing curves, suggesting that the Cre-induced bend in the cII was asymmetrically positioned in the loxP site. Asymmetry in the cl bend was also seen (Table II) and is discussed in more detail in a later section.

If the apparent asymmetry in the Cre-induced DNA bend is indeed due to the asymmetric loxP site and not to differences in the flanking non-lox sequences, then a symmetric lox site should abolish this asymmetry and the phasing curves would be the same in both the forward and reverse orientations. In fact, we found that the Cre-induced bend was indeed positioned symmetrically in both the symmetric loxSA and loxSB substrates (Table I), with an L_{min} of around 115.5 bp in both orientations for cl and cII (Table II and Fig. S8). Therefore, the asymmetric Cre-induced bend in the loxP site is dictated by the asymmetric spacer sequence.

The Scissile Base Pairs Determine the Cre-induced DNA Bending: the Cre-induced Bends in lox4 Resemble Those for loxP in the Reverse Orientation—The DMS footprint induced by Cre in the lox4 spacer region was almost the reverse of that for loxP (Fig. 2). To investigate whether the Cre-induced bends in loxP and lox4 exhibit a similar inverse relationship, we also constructed two sets of lox4 phasing substrates with the lox4-containing DNA inserted in the forward (“lox4-F”) and reverse (“lox4-R”) orientations. The electrophoretic mobility shift assay of the “lox4-F” phasing substrates is shown in Fig. 3b (lanes 7–12) and the phasing curves are plotted in Fig. 3e. We observed differences between the phasing of the Cre-lox4 and the
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### Table II

Summary of the Cre-induced DNA bends in various lox sites

| lox<sup>a</sup> | Orientation<sup>b</sup> | Wild Type Cre<sup>c</sup> | Cre K86A<sup>d</sup> | Cre K86A<sup>e</sup> |
|---------------|-----------------|-----------------|-----------------|-----------------|
|               |                 | L<sub>min</sub><sup>f</sup> | L<sub>min</sub><sup>f</sup> |                  |
|               |                 | Bend center<sup>g</sup> | Bend angle<sup>g</sup> | Bend center<sup>g</sup> | Bend angle<sup>g</sup> |
|               |                 | bp               | degrees          | bp               | degrees          |
| 1 loxP        | Forward         | 115.9 ± 0.1      | 4                | 35               | 115.0 ± 0.1      | 55               | 114.8 ± 0.1 | 8'<sup>h</sup> | 40               | 115.1 ± 0.1 | 3' 60           |
| 2             | Reverse         | 115.3 ± 0.3      | 116.4 ± 0.3      | 55               | 116.6 ± 0.2      | 40               | 116.0 ± 0.2 | 40               | 115.7 ± 0.1 | 1' 60           |
| 3 lox4        | Forward         | 115.3 ± 0.1      | 5'               | 35               | 116.1 ± 0.1      | 1               | 55               | 116.0 ± 0.2 | 40               | 115.7 ± 0.1 | 1' 60           |
| 4             | Reverse         | 115.7 ± 0.1      | 114.7 ± 0.1      | 1               | 55               | 116.0 ± 0.2      | 40               | 115.7 ± 0.1 | 1' 60           |
| 5 loxSA       | Forward         | 115.4 ± 0.1      | ND<sup>i</sup>    | ND               | 115.4 ± 0.1      | ND               | 115.4 ± 0.1 | ND               | 115.4 ± 0.1 | ND               |
| 6             | Reverse         | 115.4 ± 0.1      | 115.2 ± 0.1      | ND               | 115.6 ± 0.1      | ND               | 115.4 ± 0.1 | ND               | 115.4 ± 0.1 | ND               |
| 7 loxSB       | Forward         | 115.5 ± 0.1      | ND               | ND               | 115.6 ± 0.1      | ND               | 115.7 ± 0.1 | ND               | 115.7 ± 0.1 | ND               |
| 8             | Reverse         | 115.6 ± 0.1      | 115.4 ± 0.1      | 1               | 55               | 115.7 ± 0.1      | 115.5 ± 0.1 | ND               | 115.5 ± 0.1 |                 |
| 9 se a        | Forward         | 115.1 ± 0.1      | 8'               | 35               | 114.9 ± 0.1      | 8'               | 40               | 116.3 ± 0.1 |                 |                 |
| 10            | Reverse         | 116.1 ± 0.1      | 6                | 35               | 116.5 ± 0.1      | 40               | 116.5 ± 0.1 | 40               | 114.8 ± 0.1 |                 |
| 11 se b       | Forward         | 116.4 ± 0.1      | 6                | 35               | 114.9 ± 0.1      | 6                | 35               | 116.5 ± 0.1 | 40               | 114.8 ± 0.1 |                 |
| 12            | Reverse         | 114.9 ± 0.1      | 114.9 ± 0.1      | 115.1 ± 0.1      | 115.1 ± 0.1      | 115.1 ± 0.1      | 115.1 ± 0.1 | ND               | 115.1 ± 0.1 |                 |

<sup>a</sup> Similar results were obtained for the untagged and His-tagged wild type Cre proteins.<br>
<sup>b</sup> The Cre K86A protein was fused to an N-terminal His tag.<br>
<sup>c</sup> Similar results were obtained for the two cIs of Cre K86A (data not shown). The values shown are the average for the two cIs.<br>
<sup>d</sup> The lox sequences are listed in Table I.<br>
<sup>e</sup> The orientation of insertion of the 90-bp lox-containing fragment in the phasing vectors (see Fig. 3a).<br>
<sup>f</sup> The linker length is the number of base pairs between the center of the kinetoplast DNA that contains the A-tracts (35) and the middle of the lox site. The L<sub>min</sub> for all the Cre-induced bends in cl and cII examined were ~115–116 bp, which corresponds to ~11 turns (helical repeat, ~10.5 bp/turn). This suggests a bend toward the major groove at the arbitrary reference center, the middle of the lox site. The direction of the bend at the bend center estimated from circular permutation analysis is shown in Fig. 1 (b and c). The S.D. for L<sub>min</sub>, was from at least three experiments.<br>
<sup>g</sup> The position of the bend center (using the lox site numbering scale in Fig. 1b) was extrapolated from circular permutation analysis. Errors in estimating the bend centers were about ±0.1 bp (see Supplementary Data, Figs. S2 and S7).<br>
<sup>h</sup> The magnitude of the Cre-induced bends (degrees) was estimated from the circular permutation assays as described in Supplementary Data (Fig S2). The error in estimation is about ±5°.<br>
<sup>i</sup> The His-tagged proteins (WT and K86A) generally induce similar bend angles, and the 5° difference relative to the untagged wild type protein is likely due to the His tag.<br>
<sup>j</sup> ND, not done.

Cre-loxP complexes with the most notable difference being in cII (Fig. 3d). We discuss the differences in cl at a later section. Although the cII bend centers in loxP and lox4 differed by only 1 bp by circular permutation (Figs. 1b, c; S2, and S3), we confirmed by phasing analysis that they were indeed different and asymmetric (Fig. 3d). Recall that the relative mobility of the "loxP-F" cII had a L<sub>min</sub> of 115 bp (Fig. 3b, lane 3). In contrast, the "loxP-F" cII reached an apparent minimum gel mobility at a linker length of 117 bp (Fig. 3b, lane 10). After we corrected for the intrinsic bend in the substrate, the phasing curve for the "loxP-F" cII reproducibly had a L<sub>min</sub> of 116 bp, as +1 bp shift relative to "loxP-F" (Fig. 3d). Therefore, although Cre appears to bend the loxP site in the same general direction as loxP (i.e. toward the major groove at the center of the loxP site), the 1-bp difference in phasing may reflect the 1-bp difference in the position of the bend center measured by circular permutation (Fig. 1, b and c). The Cre-induced DNA bend in lox4, like that in loxP, was asymmetrically positioned, because the cII phasing curve for "lox4-F" was shifted by almost ~1.5 bp relative to "loxP-F" (Fig. 3d). Interestingly, the phasing curve for "lox4-F" cII coincided with that for loxP in the reverse orientation ("loxP-R") and correspondingly, the "lox4-R" curve resembled the "loxP-F" curve. Therefore, the effect of interchanging the 4' and 4 bp is analogous to reversing the entire loxP site.

Single Symmetry Elements and cl: Wild Type Cre Bends loxP and lox4 Near the Scissile G Base Pair in cl—We wished to examine whether the asymmetric lox spacer sequence also influences the Cre-induced bend in cl. Because cl formed on a full lox site may consist of a mixture of complexes of a Cre molecule bound to either the left or the right symmetry element, we constructed lox half-sites in which we replaced one or the other symmetry element with a random sequence to disrupt Cre binding (Table I and Fig. 4a). The "se a" substrates contain an intact left symmetry element a and spacer region, but the right symmetry element had been disrupted. Similarly, the "se b" substrates have a mutated left element and an intact right symmetry element b. Note that symmetry elements a and b have identical sequence and are defined by their position relative to the asymmetric spacer sequence (Fig. 1b).

We detected a difference in the phasing of the cl bound to "se a-F" and "se b-F" (forward orientation): the L<sub>min</sub> for "se a-F" was at 115.1 bp, whereas that for "se b-F" was at 116.4 bp (Fig. 4b, lanes 3 and 10, and Fig. 4c). The distance of L<sub>min</sub> was calculated using the middle of the spacer region as the reference center, which does not correspond to the bend centers determined by circular permutation: position 8' for se a and position 6 for se b (Fig. S4). For example, the bend center in se b is separated by (115.1 + 5.5) or ~121 bp from the A-tracts in the cis isomer, and 121 bp corresponds to 11.5 turns with a helical repeat of ~10.5 bp/turn, implying that Cre bends se b toward the minor groove at position 6.

The se a-F and se b-F phasing substrates differ in the distance of the functional symmetry element relative to the A-tracts: symmetry element a is closer to the A-tracts than the more distal symmetry element b in the forward orientation (Fig. 4a). The 1-bp difference in the L<sub>min</sub> for the phasing curve of se a-F and se b-F may simply reflect this difference in relative distance. To better evaluate the two symmetry elements, we compared substrates in which the distance of the functional symmetry element relative to the A-tracts was the same, but the orientation of the spacer sequence was reversed. For example, the functional symmetry element in the "se a-F" and the "se b-R" substrates are proximal (closer) to the A-tracts than the functional symmetry element in the "se a-R" and "se b-F" substrates, which are more distal to the A-tracts (Fig. 4a). The relative mobility profile of the "se a-F" substrates was similar to the proximal "se b-R" substrates, and conversely, the distal...
FIG. 3. Phasing analysis of the Cre-induced bends in loxP and lox4. a, schematic diagram of the phasing substrates. A 90-bp DNA containing the lox site (blue box) flanked by non-lox sequences (striped boxes) was inserted into the BamHI site of a set of phasing vectors (pK10, 12, 14, 16, 18, and 20) constructed by Zinkel and Crothers (35) (see “Experimental Procedures”). The phasing vectors were named such that the number refers to the length of the variable region (10–20 bp). Hence, the linker length (the distance between the center of the kinetoplast DNA that contains the A-tracts (35) and the middle of the lox site) was varied in 2-bp increments (111–121 bp) by one helical turn. The small arrow within the blue box indicates the orientation of the inserted 90-bp fragment as well as the lox site: the forward (F) orientation corresponds to the orientation of the lox site as shown in Fig. 1b and in Table I. Rs, RsaI; S, SacI; B, BamHI; N, NcoI; P, PvuII. b, gel mobility of the Cre-DNA complexes bound to the phasing substrates containing loxP (lanes 1–6) and lox4 (lanes 7–12) inserted in the forward orientation. 2 nM 32P-labeled DNA substrate was incubated with 0.05 μM wild type Cre protein and analyzed on a 4% native polyacrylamide gel as described under “Experimental Procedures.” cI and cII, one or two Cre molecules bound to loxP, respectively (45); M, a 464-bp RsaI vector fragment that was contaminated with the 347- to 357-bp RsaI/PvuII phasing substrates (S). The M fragment was not bound by Cre (data not shown) and served as a useful non-phased size marker. Note that the 347- to 357-bp phasing substrates S migrated more slowly than the larger M fragment on the polyacrylamide gel likely as a consequence of its intrinsic DNA bend. This mobility anomaly was not observed on an agarose gel (data not shown). The cis isomer (the A-tract bend and the Cre-induced bend are in phase) and the trans isomer (the two bends are out of phase) are diagrammed to the left of the gel. The Cre molecule is represented as an oval. The asterisk marks the lane with the slowest relative mobility (cis isomer) for cII (see d). c, graph of the relative mobility of Wt Cre-loxP cI (open triangles and dashed line) and cII (filled squares and solid line) as a function of the linker length. The loxP site is inserted in the forward orientation. The vertical arrows denote the linker length at which the Cre-induced bend and A-tract bend are in cis (minimum relative mobility). The error bars represent the experimental errors from at least four experiments. The graphs were plotted to the best fit polynomial curve (Microsoft Excel). d, comparison of the relative mobility of cIIIs formed by Wt Cre bound to loxP and lox4 in the forward (F) and reverse (R) orientations. loxP-F, filled squares and solid blue line; loxP-R, open diamonds and dashed green line; lox4-F, filled triangles and solid red line; lox4-R, open circles and dashed magenta line. e, graph of the relative mobility of Wt Cre-lox4 cI (open circles and dashed line) and cII (filled triangles and solid line) as a function of the linker length. The lox4 site is inserted in the forward orientation. The vertical arrows denote the linker length at which the Cre-induced bend and A-tract bend are in cis.
pairs “se a-R” and “se b-F” were similar to each other (Fig. 4d). Therefore, Cre appears to bend se a and se b toward the minor groove near the margin of the spacer region independently of the spacer sequence.

However, in a full lox site with two competing symmetry elements, the spacer sequence influences the predominant cI species. The cI bends induced by wild type Cre in loxP and lox4 mapped to opposite ends of the spacer region by circular permutation (Fig. 1, b and c, and Supplemental Figs. S2 and S3) and phasing analyses (Fig. 4d). The cI bend in the loxP site was at the scissile G adjacent to symmetry element b, and its phasing curve resembled that of the se b substrate more closely than that of the se a substrate. In contrast, the cI bend in the full lox4 site was in symmetry element a (also adjacent to the scissile G), and its phasing curve resembled that of the se a substrate. The differences in the cI phasing between the forward and reverse orientations were smaller (~0.5 bp) for the full lox substrates than the individual single symmetry elements (~1 bp; Table II). This may have been due to the shuffling of the Cre monomer between the left and the right symmetry elements during electrophoresis (37, 49). In conclusion, the wild type Cre bends loxP and lox4 near the scissile G base pair in cI, and this suggests preferential binding of the first Cre molecule to the adjacent symmetry element (see “Discussion”).

Lys-86 Is Important for the Positioning of the Cre-induced DNA Bend in loxP and lox4 cI—Because Lys-86 contacts the scissile bases (21, 23–27) and affects the order of strand exchange (32, 33), we investigated its role in the position and direction of the Cre-induced bends. Mutation of Lys-86 to Ala (K86A) dramatically altered the position of the DNA bend in cI, shifting the bend center (determined by circular permutation) by 11 bp from position 4 to position 5 in loxP (Figs. 1b and S5b) and shifting the \( L_{min} \) in the phasing analysis by ~1 bp to a linker length of about 115 bp (Fig. 5b). Note that the phasing curve for Cre K86A-loxP cI resembled that of the substrate se a (Table II), positioning the Cre K86A-induced cI bend in loxP in symmetry element a adjacent to the scissile AT base pair. The K86A mutation did not significantly affect the position of the cI bend in the single symmetry element substrates se a and se b (Fig. 1b and Table II), suggesting that Lys-86 is not involved in actually bending the DNA but rather in selecting the symmetry element to be occupied by the first Cre monomer within a full lox site (see “Discussion”). The K86A-loxP cII exhibited a similar phasing profile to wild type Cre-loxP cII with a \( L_{min} \) of about 115 bp (Fig. 5c), suggesting that both these proteins induced a similar cII bend in loxP. Circular permutation anal-
Flanking sites revealed that Cre K86A also bends loxP at similar position to the wild type Cre protein in cII (Figs. 1b and S5c). Therefore, Lys-86 is important for positioning the cl band in loxP but has little if any role in directing the cII bend.

In contrast to loxP, Lys-86 influences both the cl and cII bends in lox4 (Figs. 6 and S6). As in loxP, the cl bend centers for the wild type and K86A Cre protein were located at opposite ends of the lox4 spacer region (Wt Cre, at position 5' in the left symmetry element; Cre K86A, position 4 near the right margin of the spacer; Figs. 1c and S6b). The Lmin for the phasing curve of the Cre K86A-lox4 cl was 116 bp, a difference of ~1 bp from that for the wild type Cre-lox4 cl (Fig. 6b) and close to that for the substrate se b (Table I). Hence, Cre K86A bends both the loxP and lox4 sites near the scissile AT base pair, whereas wild type Cre bends the DNA near the scissile GC base pair in cl.

Unlike loxP, the K86A mutation also altered the cII bend in lox4, shifting the phasing curve by ~0.4 bp relative to wild type Cre with the lox4-F substrates (Fig. 4c). Although the difference in the cII bend between the wild type and K86A Cre proteins is small, this difference was also evident and slightly larger (~0.7 bp) in the lox4-R substrates (Table I). The K86A-lox4 cII appeared to be bent symmetrically around the middle of the lox4 site, analogous to the bends in the symmetrical loxSA and loxSB sites (Fig. S7). The difference in Lmin for the Cre K86A cII bound to the lox4-F and lox4-R substrates was only ~0.3 bp (Fig. 6d), which is smaller than the 1.4-bp difference in Lmin between the two orientations for the wild type Cre-lox4 cII (Fig. 3d). This symmetric cII bend induced by Cre K86A correlates with its loss of strand preference in the initiation of strand exchange in lox4 (see “Discussion”) (32).

**DISCUSSION**

The Scissile Base Pairs Determine the Cre-induced DNA Bends in cl and cII—The Cre recombinase has long been known to initiate recombination by preferentially exchanging the bottom strands of the loxP site and resolving the Holliday intermediate on the top strands (11, 29–32). We previously found that the scissile base pairs at positions 4' and 4 were critical in determining this order of strand exchange (31, 32). The evidence we report here links the scissile base pairs to their influence on the Cre-induced DNA bends within cl and cII.

In the Cre-lox crystal structures, the two DNA strands adopt different conformations and are contacted differently in the spacer region by the cleaving and non-cleaving Cre subunits (21, 23–27). The “continuous” strand contains the inactive scissile phosphate and is on the concave side of the DNA bend (Fig. 1a) (25). It makes numerous direct contacts with the N-terminal domain of the cleaving Cre subunit directly opposite the activated scissile phosphate. The more extended “crossing” strand contains the activated scissile phosphate and is contacted directly by the Cre molecules only at the activated scissile phosphate and scissile nucleotide but not throughout the rest of the spacer region (25). The crossing strand points toward the center cavity of the synapase ready for strand exchange after cleavage (Fig. 1a).

The major differences in the Cre-induced footprints of loxP and lox4 were in the spacer region (Fig. 2). The footprints within the lox4 site were similar to those for the loxP site in the reverse orientation. Cre increased the sensitivity to DMS methylation of the bases in the spacer region on the strand containing the scissile G nucleotide in both loxP and lox4. We propose that the DMS-sensitive, scissile G-containing strand corre-

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**Fig. 5.** Phasing analysis of the Cre K86A-induced bends in loxP. a, EMSA of His-tagged wild type Cre (H intoler (Wt; lanes 1–6) or H intoler (K86A) (lanes 7–12) Cre protein bound to loxP-F phasing substrates. b, comparison of the phasing curves of loxP-F cl bound by H intoler (Wt and H intoler (K86A). The arrows indicate the linker length at which the minimum mobility is reached. c, comparison of the phasing curves of loxP cII bound by H intoler (Wt and H intoler (K86A). For b and c, H intoler (Wt, open squares and dashed line; H intoler (K86A, filled circles and solid line. Note that the phasing curves for H intoler (Wt are similar to those of the untagged Wt Cre protein (Fig. 3c). The His-tagged proteins gave two cl bands, although H intoler (Wt gave predominantly the lower cl band (see a). The two cl bands may be due to the His tag for the following reasons. (i) They were also observed using substrates containing only a single symmetry element (see a and se b, data not shown) and so did not appear to correspond to the binding of a Cre monomer to different symmetry element within the lox site. (ii) The two cls exhibit similar circular permutation and phasing profiles (data not shown), suggesting that their bend centers are located at similar positions. (iii) Analysis of the complexes by two-dimensional electrophoresis (first, native PAGE; second, SDS-PAGE) did not detect a covalently attached Cre in either cls (data not shown). We show in b the phasing curves for the lower cl, but the upper cl band exhibits similar phasing profiles (data not shown).
The conformational alterations seen in the chemical footprints are supported by changes in the Cre-induced DNA bending measured by circular permutation and phasing analyses (Fig. 1, b and c, and Table II). The CII bends induced by the wild type Cre protein in loxP and lox4 were near the middle of the spacer region with a slight bias toward the scissile A. In fact, interchanging the scissile base pairs as in the lox4 site resulted in changes similar to those seen by inverting the entire loxP site by 180°. Therefore, the scissile base pairs dictate the Cre-induced DNA bending and the order of strand exchange (32).

We propose that the first Cre molecule binds preferentially to the symmetry element adjacent to the scissile G and induces a CII bend near the margin of the spacer. We speculate that because this Cre molecule is bound adjacent to the initial cleavage site, it may be in a "pre-activation/cleaving" conformation even prior to the binding of the second Cre monomer. The binding of a second Cre molecule redirects the bend asymmetrically toward the middle of the spacer region away from the scissile G. Within the CII and/or synaptic complex, the strand containing the scissile G adopts the "crossing" configuration (as suggested by the footprinting analysis) and is cleaved adjacent to the scissile G. Interchanging the scissile base pairs as in the lox4 site reverses the DNA bend induced by Cre from those in loxP and, as a consequence, alters the order of strand exchange (32).

The proposed preferential binding of Cre to a particular symmetry element in CII remains to be verified. Kinetic and equilibrium studies indicate that Cre binds the individual symmetry elements a and b with similar affinity (50). In addition, we did not detect preferential protection of one symmetry element relative to the other element on a full lox site by footprint analyses of the isolated CII (data not shown).

We found that Cre induced a 35° CII bend in both loxP and lox4; this small CII bend may correspond to the −25° smooth
bend observed within each symmetry element in the crystal structures of the Cre-lox synaptic complex (21, 23–27). We measured a cII bend of \(-55^\circ\) toward the major groove at position 1' inloxP and at position 1 inlox4. Our cII bends resemble most closely the DNA bend observed in the Cre-lox crystal structures for the covalent cleavage intermediate and the Holliday intermediate in which the DNA was bent near the center of the spacer region toward the major groove (23, 24). However, our results differ to some extent from the asymmetric DNA bend observed in the crystal structure of the Cre-loxS synaptic complex in which the DNA was bent 78° toward the minor groove between positions 2/3 (or 2'/3') (25). It is possible that the DNA bend angles within a cII in solution and a crystalized synaptic complex are different. The latter consists of two lox sites and four Cre molecules. The act of synopsis and/or crystallization may alter the bend angle and position. Furthermore, because the bend angle measured by circular permutation analysis was small, the positioning of the bend was subject to appreciable error (see Supplementary Data, Fig. S7). The circular permutation analysis may also underestimate the magnitude of the Cre-induced DNA bend (48, 51). Other possible reasons for the discrepancies include the type of Cre proteins and lox sites used. The crystal structures were solved using mutant Cre R173K and Y324F proteins to block cleavage (25), whereas we used wild type and K86A Cre proteins. In addition, Guo et al. (25) used a symmetric loxS, which corresponds to our loxSB sequence (Table I). Although we did not determine the position of the cII bend center in our symmetrical lox substrates, we observed symmetric bends in these substrates by phasing analysis. It is possible that the symmetric phasing curves represent an average of rapidly alternating, asymmetric bends as seen in the crystal structures (25). Correspondingly, the Cre-loxP and Cre-lox4 cII may also consist of rapidly alternating isomers whose bend center and direction may not correspond precisely to the overall average measured. Nevertheless, the predominant cII species is different for loxP and lox4.

**Lys-86 and DNA Bending**—The scissile base pairs of the lox spacer region are critical in determining the position of the Cre-induced bends and the order of strand exchange, but how does Cre distinguish between the scissile base pairs? We confirmed by DMS methylation that Lys-86 makes major groove contacts with the scissile G nucleotide in both loxP and lox4 as observed in several of the Cre-lox crystal structures (23–25).

We observed this contact even when only symmetry element \(b\) was present (data not shown), indicating that the Lys-86-scissile G interaction can occur within cII. In a recent crystal structure of the wild type Cre protein bound to a loxP-Holliday junction, Lys-86 of the cleaving Cre subunit contacts the scissile A at N7, and the adjacent scissile phosphate was activated for cleavage (27). However, Lys-86 of the non-cleaving Cre subunit did not contact the adjacent scissile G. Because resolution occurs preferentially adjacent to the scissile A (31, 32), this structure may represent the conformation of the Cre-lox synaptic complex just prior to resolution, and this may differ from the initial cII or the synaptic complex containing two linear lox sites.

Mutation of Lys-86 to Ala significantly altered the position of the cII bend, shifting the bend center by 8–11 bp to position 8' inloxP and to position 4 inlox4 (Fig. 1, b and c, and Table I). The cII bends induced by Cre K86A in both loxP and lox4 were near the scissile AT base pair and were at the opposite half of the lox site relative to the cII bends induced by the wild type Cre protein. Mutation of Lys-86 to Ala did not significantly alter the position of the cII bend in the single symmetry element substrates se a and se b. We suggest that Lys-86 is not involved in actually bending the DNA, but is responsible for directing the Cre-induced cII bend and possibly the first Cre molecule toward the scissile GC base pair on a full lox site. The interaction between Lys-86 and the scissile G may help stabilize the cII in which the wild type Cre protein is bound adjacent to the scissile G.

Although important for positioning the cII bend, Lys-86 was not critical for establishing the cII bend in loxP (Table I). Despite the fact that the wild type and K86A Cre proteins bent loxP at opposite ends of spacer region in cII, they induced similar cII bends. This implies that, unlike wild type Cre, the first or predominant Cre K86A molecule to bind to the loxP site in cII does not necessarily become the cleaving subunit in cII and/or the synaptic complex. The similarities in the circular permutation and phasing analyses of the loxP cII bends induced by the wild type and K86A Cre proteins further complement the footprinting results. Like the wild type Cre protein, Cre K86A also increased the sensitivity of the spacer region on the bottom strand of loxP to DMS and OP-Cu modifications (Figs. 2 and S1). This could imply that the bottom strand is the incipient crossing strand in the wild type and K86A Cre-loxP complexes as discussed above (Fig. 7). The K86A mutation may not have dramatically altered the DNA conformation in cII but rather simply revealed the already exposed crossing strand, and this would account for the hypermethylation of the scissile G. Therefore, although the scissile base pairs are still critical for directing the cII bend in loxP, it appears that their interaction with Lys-86 is not essential. This is consistent with the fact that Lys-86 is not important for determining the strand preference in loxP during the first strand exchange event (32).
Although Lys-86 did not determine the cII bend in loxP, it did contribute to the cII bend in lox4. Whereas the wild type Cre protein induced an asymmetric cII bend in lox4, the cII bend induced by the Cre K86A protein was more symmetrical. The symmetric K86A-lox4 cII bend is consistent with our previous observation that the K86A mutation abolished the strand bias for the initiation of strand exchange in lox4 (32). Therefore, Lys-86 may be responsible for establishing the asymmetric cII bend and the site of initial strand exchange in lox4.

In summary, the location of the scissile base pairs is important for dictating the asymmetric Cre-induced cI and cII bends in loxP and lox4 as well as the site of the first strand exchange event. Lys-86 is critical for directing the cI bend in loxP and lox4 toward the scissile GC base pairs as well as the position of the cII bend and the site of initiation in lox4. In contrast, the cII bend and the site of initiation in loxP are not dependent on Lys-86. Additional bases in the lox spacer region as well as other residues in Cre may also contribute to the Cre-induced DNA bending and the order of strand exchange.

Acknowledgments—We thank Barbara Funnell, Brigitte Lavoie, and Linda Beatty for helpful comments.

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