The Cre recombinase of bacteriophage P1 cleaves its target site, loxP, in a defined order. Recombination is initiated on one pair of strands to form a Holliday intermediate, which is then resolved by cleavage and exchange of the other pair of strands to yield recombinant products. To investigate the influence of the loxP sequence on the directionality of resolution, we constructed synthetic Holliday (χ) structures containing either wild-type or mutant lox sites. We found that Cre preferentially resolved the synthetic wild-type χ structures on a particular pair of strands. The bias in the direction of resolution was dictated by the asymmetric loxP sequence since the resolution bias was abolished with symmetric lox sites. Systematic substitutions of the loxP site revealed that the bases immediately 5′ to the scissile phosphodiester bonds were primarily responsible for the directionality of resolution. Interchanging these base pairs was sufficient to reverse the resolution bias. The Cre-lox co-crystal structures show that Lys86 makes a base-specific contact with guanine immediately 5′ to one of the scissile phosphates. Substituting Lys86 with alanine resulted in a reduction of the resolution bias, indicating that this amino acid is important for establishing the bias in resolution.

The Cre recombinase of bacteriophage P1 belongs to the integrase family of conservative site-specific recombinases whose members include the integrase of bacteriophage λ (1), XerC/XerD of Escherichia coli (2), and the Flp protein of the 2μm plasmid (3). Cre assists in the efficient segregation of the low copy P1 plasmid during bacterial cell division by resolving dimeric lysogenic P1 plasmids into monomeric units (4).

Cre breaks and rejoins DNA at specific sequences called the loxP sites (Fig. 1a). The 34-bp1 loxP site is composed of two 13-bp symmetry elements surrounding an 8-bp asymmetric AT-rich region (5). Since the symmetry elements in loxP have identical sequences, the orientation of the loxP site is dictated by the asymmetric central region. A 6-bp overlap region separates the two cleavage sites. This organization of the recombination site is common to the integrase family members, although the interval of the overlap region varies (1–3). Certain recombinases such as λ-integrase and XerC/XerD have additional accessory sequences and require accessory protein factors (1, 6).

The integrase family members share a common mechanism of catalysis that involves the formation of a four-way branched DNA intermediate known as a Holliday junction (7–13). Formation and resolution of the Holliday intermediate occur via two sequential sets of single-strand DNA cleavages and exchanges (Fig. 1b). DNA cleavage is mediated by a tyrosine residue that is absolutely conserved among the integrase family members, and hence, members of this family are also known as tyrosine recombinases (14–16). The catalytic tyrosine makes a nucleophilic attack upon the scissile phosphodiester bond, resulting in the covalent attachment of the protein via a 3′-phosphotyrosine linkage and the release of a free 5′-hydroxyl DNA end. The phosphotyrosine bond is thought to conserve the energy of the scissile phosphodiester bond. The cleaved strands from two loxP sites involved in the recombination are exchanged and ligated, resulting in the formation of a Holliday junction. The Holliday intermediate is then resolved by a second set of strand cleavages and exchanges. Depending on which pair of strands is cleaved, the Holliday intermediate may be resolved to either the parental or the recombinant configuration.

Several tyrosine recombinases such as the λ-integrase, XerC/XerD, and Cre proteins catalyze recombination with a defined order of strand exchange in which one pair of strands is preferentially cleaved and exchanged first (6, 8–10, 13, 17, 18). Hoess et al. (8, 19) isolated the α Holliday intermediate formed during a Cre-mediated excision reaction. The bottom strands2 of the loxP sites had been exchanged in these Holliday intermediates, suggesting that Cre initiated recombination on the bottom strands. The α structure was preferentially resolved on the top strands to yield recombinant products (8). The order of strand exchange was further confirmed by studies on the effect of heterologies in the loxP overlap region on recombination. Lee and Saito (20) found that homology near the bottom strand cleavage site (positions 2 and 3) (Fig. 1a) was required for the formation of the Holliday intermediate, whereas homology near the top cleavage site (positions 3′ to 1) was required for resolution. Therefore, Cre appears to initiate strand cleavage and exchange on the bottom strands to generate a Holliday junction, and the Holliday intermediate is subsequently resolved on the top strands to yield recombinant products.

A key question regarding the tyrosine recombinase family is what are the factors that regulate the order of strand exchange and the directionality of resolution of the Holliday intermediate. Because the central 8 bp are the only asymmetric feature of the loxP site, we investigated the influence of this asymmetric sequence on the directionality of resolution of synthetic
Resolution of Holliday Structures by Cre

31093

Holliday (χ) structures. We designed synthetic χ structures containing either wild-type or mutant lox sites. We found that Cre preferentially resolved the wild-type χ structures on the top strands. This bias was dictated by the asymmetric loxP sequence, in particular by the bases immediately 5′ to the scissile phosphodiester bonds (positions 4′ and 4). We found that Lys86, which contacts the O-6 group of guanine at position 3′, is important for establishing the resolution bias of the Cre protein.

EXPERIMENTAL PROCEDURES

Oligonucleotides and Construction of χ Synthetic Structures—Each χ structure was constructed from four complementary oligonucleotides (see Fig. 2a and Tables I and II). The deprotected oligonucleotides were synthesized by Life Technologies, Inc. and were purified by electrophoresis on a denaturing 6% polyacrylamide gel. After extraction from the gel by the "crush-and-soak" method (22), the oligonucleotides were purified by reverse-phase chromatography on a Waters Sep-Pak C18 cartridge.

Strand 1 of each χ structure was 5′-radioactively labeled with [α-32P]ATP using T4 polynucleotide kinase (New England Biolabs Inc.) to detect the R1 and R2 resolution products (Fig. 2a). In certain cases, strand 3 was 5′-radioactively labeled instead of strand 1 to detect the R3 and R4 resolution products. The four complementary oligonucleotides that constituted a particular χ structure were annealed by heating to 80 °C and slow cooling in 100 mM NaCl and 5 mM MgCl2. The three unlabelled oligonucleotides were added in 5-fold molar excess (5 pmol) over the radioactively labeled oligonucleotide (1 pmol) in a total volume of 50 μl.

Resolution Assay—2 nm χ structure was incubated with 250 nm Cre in 50 mM Tris-HCl (pH 7.4), 30 mM NaCl, 2 mM MgCl2, 3% glycerol, and 0.05 mg/ml denatured calf thymus DNA at 30 °C for 1 h, by which time the reaction had reached equilibrium (data not shown). Reactions were stopped by treatment with 0.1% (w/v) SDS and 0.05 mg/ml proteinase K (final concentrations) at 50 °C for 30 min. The resolution products were analyzed on a nondenaturing 5% polyacrylamide gel. An aliquot of the resolution reaction was analyzed on a denaturing 8% polyacrylamide gel to verify the sizes of the products.

The radioactivity in the bands on the nondenaturing gels was quantitated using a Molecular Dynamics PhosphorImager. The amount of each resolution product was calculated as the counts in the product divided by the total counts in the product and substrate bands. A background correction was applied from the lane without Cre added. The resolution bias is calculated as the ratio of the amounts of R1 product to R2 product (R1/R2).

Construction of the His-Cre K86A Expression Vector—The Cre K86A mutant protein was constructed by a His tag at the N terminus for ease of purification using the His-Cre expression vector, pSh6e. pSh6e contains the wild-type cre gene fused to a 10-histidine N-terminal tag in a pET19b vector (23). Lys86 of His-Cre was mutated to alanine by polymerase chain reaction mutagenesis using the following primers: T7 pro, 5′-TAATAGCTACTATAGAGGGTAAGG-3′ (hybridizes to the T7 promoter of vector pShe6); CA86-Top, 5′-GGCCTACGCTAGACCTAGACCGCGCGGCGGCGGCGGCGG-3′ (mutated nucleotides are in boldface, the mutated codon 86 is underlined, and the silent NheI site is in italics); CA86-Bot, 5′-AGTTGGTACTGCTGCTAGCCGGCGGCGGCGGCGG-3′ (mutated nucleotides are in boldface, the mutated codon 86 is underlined, and the silent NheI site is in italics); and 115LD, 5′-GATTTGACCTTATCCGTCATCAGC-3′ (hybridizes to sequences encoding amino acids 231–237). The mutagenesis was done in three polymerase chain reaction steps as described by Higuchi (24). The final polymerase chain reaction product was digested with NcoI and BamHI and cloned into the NcoI/BamHI-digested pSh6 vector to form the His-Cre K86A variant expression vector, pLC86A. Mutagenesis and the accuracy of cloning were further verified by DNA sequencing. T7 pro and 115LD were synthesized by the Hospital for Sick Children Biotechnology Service Center at the University of Toronto. CA86-Top and CA86-Bot were synthesized by Life Technologies, Inc. The T7 DNA polymerase (PCR SuperMix high fidelity) used in the polymerase chain reactions was obtained from Life Technologies, Inc. The restriction enzymes and T4 DNA ligase were from New England Biolabs Inc. The shrimp alkaline phosphatase used to dephosphorylate the vector was from U. S. Biochemical Corp.

Expression of Cre, His-Cre, and His-Cre K86A Constructs—Wild-type Cre and His-Cre were expressed from pSh6e11 and pSh6e2, respectively, constructed by Shaikh and Sadowski (23), whereas His-Cre K86A was expressed from pLC86A. Each construct was expressed in E. coli BL21(DE3) pLy85 as described by Shaikh and Sadowski (23).

Purification of Cre, His-Cre, and His-Cre K86A Proteins—The wild-type Cre, wild-type His-Cre, and His-Cre K86A proteins were purified essentially as described by Shaikh and Sadowski (23). Analysis on SDS-polyacrylamide gel showed that the proteins were >90% pure. Protein concentration was determined using the Bradford assay (25) with IgG (Bio-Rad) as the protein standard. The purified proteins were stored at −70 °C.

RESULTS

Cre Preferentially Resolves the Top Strands of the Wild-type Synthetic χ Structures—To investigate the resolution of the Holliday structure by the Cre recombinase, we constructed synthetic Holliday (χ) structures containing two loxP sites (Fig. 2a). The four complementary DNA oligonucleotides used to construct the wild-type χ0 structure are listed in Table I, and a diagram of the central 8-bp region of the loxP sites at the Holliday junction is shown in Fig. 2b. In the case of χ0, strands 1 and 3 correspond to the top strands of the loxP site, whereas strands 2 and 4 correspond to the bottom strands. The loxP sites in the synthetic χ structure are flanked by 12–32 bp of non-homologous sequences in the arms to prevent branch migration beyond the loxP sites. The arms are of variable lengths so that the direction of resolution can be deduced from the size of the resolution products. For example, resolution by cleavage of strands 1 and 3 would generate the 87-bp R1 and 70-bp R3 products. Resolution on strands 2 and 4 would generate the 75-bp R2 and 82-bp R4 products. Upon incubation of the χ substrate with Cre, two rapidly migrating bands appeared that comigrated with linear R1 and R2 DNA markers (Fig. 2e). Only the 87-bp R1 and 75-bp R2 resolution products are detectable on the autoradiogram because only strand 1 of the T7 DNA polymerase reaction was radioactively labeled at its 5′-end. The other resolution products (R3 and R4) could be detected when strand 3 was 5′-radioactively labeled (data not shown). The sizes of the resolution products and the cleaved intermediates were further verified by analysis on denaturing polyacrylamide gels (data not shown).
Resolution of Holliday Structures by Cre

**Fig. 2. Resolution of the synthetic Holliday structure.** a, schematic diagram of the synthetic Holliday structure and its resolution. Each synthetic Holliday (χ) substrate was constructed from four complementary DNA oligonucleotides (see Table I). The two lox sites are located at the center of the χ structure with a symmetry element in each arm and flanked by non-homologous sequences to prevent branch migration beyond the lox sites. The χ structure is drawn with the lox sites in antiparallel alignment as indicated by the two middle black arrows. The 5′-ends of the DNA strands are numbered 1–4, and the arms are designated by Roman numerals. The nucleotide length of each strand on either side of the cleavage site is indicated in italic numbers. The sizes of the resolution products are shown on the right. The R1 and R2 products generated when the χ substrate was cleaved on strands 1 (red) and 3 (black) are shown on the top right. Resolution on strands 2 (green) and 4 (blue) gave the R4 and R3 products shown on the bottom right. Since strand 1 was 5′-radiolabeled (*), only the R2 and R1 products (in boldface) could be detected. The substrates and products are not drawn to scale. b, the central 8-bp sequence of the loxP sites in the wild-type χp structure. Strands 1 (red) and 3 (black) of χp correspond to the top strands of loxP, whereas strands 2 (green) and 4 (blue) correspond to the bottom strands. Arms I and III contain symmetry elements a, whereas arms II and IV contain symmetry elements b. The other synthetic χ structures differ from χp in the sequences of the central 8-bp region of the lox sites (see Table I). c, an autoradiogram of the resolution of χp. The radiolabeled χp substrate was incubated either without (−) or with (+) Cre for 1 h at 30 °C and then analyzed by nondenaturing 5% polyacrylamide gel electrophoresis (see “Experimental Procedures”). The slowly migrating band at the top of the gel corresponds to the χp substrate. The two quickly migrating bands at the bottom of the gel are the 87-bp R1 and 75-bp R2 products resulting from resolution on the top and bottom strands, respectively.

We found that Cre preferentially resolved the wild-type χp substrate on the top strands of loxP. At equilibrium, −4-fold more of the top strand resolution product (R1) was formed than the bottom strand resolution product (R2). The bias toward resolution on the top strands was accentuated early in the reaction (data not shown). When strand 3 was 5′-radioactively labeled to detect the reciprocal resolution products, the other top strand resolution product (R2) predominated over R1 by −4-fold also (data not shown). The biased resolution that we observed for our synthetic χp substrate was unexpected considering previous studies by Hoess et al. (8). They isolated a closed Holliday intermediate (α structure) that was accumulated by certain Cre mutant proteins during an in vitro excise recombination reaction with a 4.3-kilobase pair linear substrate. As with our synthetic χp structure, they observed an −4-fold preference for resolution of the α structure on the top strands of the loxP sites over the bottom strands. However, when the closed loop of the α structure was cleaved to generate an open χ structure, Hoess et al. observed unbiased resolution of the χ structure. This result differs from our findings with the synthetic χp structure. A possible explanation for the discrepancy is that the sequences flanking the loxP sites may have influenced the direction of resolution. Our synthetic χp structure is smaller and has different flanking arm sequences compared with the α-derived χ structure characterized by Hoess et al.

To determine whether the flanking sequences in the arms of our synthetic χ structure were responsible for the biased resolution, we constructed the χsym structure, in which the orientation of the loxP sites is reversed relative to χp, but the flanking arm sequences remain the same as in χp (Table III). The χsym structure still contains wild-type loxP sites, but strands 1 and 3 now correspond to the bottom strands, whereas strands 2 and 4 are the top strands. If the resolution bias was determined by the loxP sequence, we expected that reversing the orientation of the loxP sites would reverse the R1/R2 ratio. On the other hand, if the flanking non-loxP sequences were primarily responsible for the observed resolution bias, reversing the loxP orientation should not affect the R1/R2 ratio. We found that Cre preferentially resolved the χsym substrate on the top strands, giving predominantly the Rp product and thus reversing the R1/R2 ratio (Fig. 3, lane 4). Therefore, we concluded that for our synthetic χ structures, the bias in the direction of resolution was not dictated by the flanking non-homologous sequences in the arms. Nonetheless, we cannot rule out the possibility that the flanking sequences in the arms of the α-derived χ substrates or some other factors could be responsible for the unbiased resolution observed by Hoess et al. (8) (see “Discussion”).

**Effects of Mutations in the Central 8-bp Region of the loxP Site on the Direction of Resolution**—Because the central 8-bp sequence is the only asymmetric feature of the loxP site, we investigated how this asymmetric sequence influences the direction of resolution. We constructed synthetic χ structures containing altered sequences in the central 8-bp region, but the same flanking sequences in the arms as χp (Table II). The results for the resolution of these altered χ structures are shown in Fig. 3, and the resolution biases (expressed as the R1/R2 ratios) are summarized in Table II.

The χsr and χsh structures contain symmetric lox sites corresponding to either the left or right half of the wild-type loxP site, respectively. Cre resolved both these symmetric substrates without bias, giving a R1/R2 ratio of close to 1 (Fig. 3, lanes 6 and 8). This indicates that the asymmetric loxP spacer sequence is responsible for the observed bias in the direction of resolution.

To narrow down the sequences within the loxP central region responsible for the bias in the direction of resolution, we first focused on positions 4/3′ and 4/3 surrounding the scissile phosphates. In the wild-type loxP site, an AT dinucleotide step surrounds the top cleavage site (positions 4′ and 3′), whereas a GC dinucleotide step surrounds the bottom cleavage site (positions 4 and 3). When the dinucleotide steps at these positions were interchanged in the χsr structure, the direction of resolution was changed (Fig. 3, lane 10). Most of the resolution bias was abolished when we placed either a GC step (χGC) (Fig. 3, lane 12) or an AT step (χAT) (lane 14) at both cleavage sites, although χGC still exhibited a small bottom strand bias. The Cre-lox co-crystal structures show that the protein-DNA
Resolution of Holliday Structures by Cre

**TABLE I**
Oligonucleotides used to construct the wild-type Holliday structure, χp

| Strand | Length (nt) | Sequence (5’ to 3’) |
|--------|-------------|---------------------|
| 1       | 75          | aatctggctacgtcgtgctATTACTTCCATATATAATTAGTGATGCATACGAGTTATgactcagtacctgcattagctccc |
| 2       | 70          | cgggtgagatctaatctcggtctATTACTTCCATATATAATTAGTGATGCATACGAGTTATgactcagtctgctgctgctgcc |
| 3       | 82          | gatcagcgacctcggtATATTCCATATATAATTAGTGATGCATACGAGTTATgactcagtctgctgctgctgcc |
| 4       | 87          | tggagactggctacgtcgtgctATTACTTCCATATATAATTAGTGATGCATACGAGTTATgactcagtctgctgctgctgcc |

The loxP sequences are in uppercase letters with the central 8-bp sequence indicated in boldface, and the arm sequences flanking the loxP sites are shown in lowercase letters.

**TABLE II**
Resolution of χ structures by wild-type Cre and Cre K86A

| Name            | Central 8-bp sequence | R1:R2 ratio | WTCre<sup>a</sup> | Cre K86A<sup>a</sup> |
|-----------------|------------------------|-------------|-------------------|----------------------|
| χp              | 5′-ATGTATGC-3′          | (3.8 ± 0.8):1 | (1.5 ± 0.4):1 |                      |
| χrev            | GCGATACAT              | 1:(3.3 ± 0.7) | 1:(1.0 ± 1.0) |                      |
| χaa             | ATGTAGTA               | (1.2 ± 0.2):1 | (1.3 ± 0.1):1 |                      |
| χab             | GCATATCC               | (1.1 ± 0.2):1 | (1.3 ± 0.1):1 |                      |
| χs              | GTGTATAT               | 1:(2.6 ± 0.4) | (1.3 ± 0.3):1 |                      |
| χGc             | GCGATAGC               | 1:(1.8 ± 0.4) | 1:(1.7 ± 0.4) |                      |
| χ4T             | TACATACG               | (1.4 ± 0.3):1 | (1.9 ± 0.2):1 |                      |
| χ4A             | ATGTAGTAC             | 1:(3 ± 1)      | 1:(2.5 ± 1)      |                      |
| χ4C             | TACATACG               | 1:(1.2 ± 0.1) | 1:(2.1 ± 0.2) |                      |
| χ4G             | CACATAGC               | (1.7 ± 0.2):1 | (1.0 ± 0.04):1 |                      |
| χ4AC            | ATGTAGTACC            | (1.0 ± 0.1):1 | 1:(3.6 ± 0.2) |                      |
| χ4AT            | TACATACCT             | (5 ± 1)       | 1:(1.4 ± 0.2) |                      |
| χ4C             | CGATACACC             | (4.6 ± 0.8):1 | (4.7 ± 0.8):1 |                      |
| χ4T             | TGTGATGC              | 1:(2.5 ± 0.5) | 1:(1.8 ± 0.6) |                      |

<sup><sup>a</sup></sup>The sequences of the symmetry elements and the flanking arms of the mutant χ structures are the same as χp. Only the central 8-bp loxP sequences for the mutant χ structures are different from χp; sequences that differ from wild-type χp are in boldface, and the symmetrical positions are underlined. Strands 1 and 3 of the χ structure contain the sequences shown in the upper line, whereas strands 2 and 4 contain that shown in the lower lines.

<sup><sup>b</sup></sup>The resolution bias is expressed as the ratio of the amounts of R<sub>1</sub> to R<sub>2</sub> resolution products [R<sub>1</sub>:R<sub>2</sub>], averaged from several experiments. The amount of each resolution product in the autoradiogram (see Fig. 3) was quantitated using a Molecular Dynamics PhosphorImager and corrected for background in the lane without any Cre protein added.

<sup><sup>c</sup></sup>WTCre, wild-type Cre.

<sup><sup>d</sup></sup>The Cre K86A mutant protein has an N-terminal His<sub>6</sub> tag. The wild-type His-tagged Cre protein exhibited the same resolution bias as the wild-type untagged Cre protein, indicating that the His tag did not influence the direction of resolution (Fig. 5, compare lanes 2 and 3).

Thus far, we studied only transition substitutions at positions 4′ and 4 and found that Cre preferentially resolved adjacent to an A over a G at these positions. We wondered how a pyrimidine at the corresponding positions would affect the directionality of resolution. To answer this question, we made transversion substitutions at these positions. The χ structures are named χ<sub>4A</sub> (where the number (#) refers to the mutated position and the base N refers to the mutated base 5′ to the scissile phosphate (Table II). For example, χ<sub>4A</sub> contains a 4G → C substitution adjacent to the bottom cleavage site. This χ<sub>4Al</sub> substrate was resolved equally in either direction (Fig. 3, lane 22). Comparison between the minor top strand bias of χ<sub>4A</sub> and the unbiased resolution of χ<sub>4Al</sub> suggests that resolution at 4C is preferred slightly over 4A. This was further evident for χ<sub>4C</sub> in which a 4′A → C substitution enhanced the top strand bias to a small extent (Fig. 3, lane 26) compared with χ<sub>4A</sub>. A 4G → T substitution (χ<sub>4T</sub>) also resulted in a stronger top strand bias (Fig. 3, lane 24) than χ<sub>4A</sub>, suggesting that resolution at 4T is less favored than at 4G. This was indeed the case for χ<sub>4T</sub>, which displayed 2.5-fold bias toward the bottom strand. The resolution of these substrates indicates that Cre appears to possess the following hierarchical resolution preference for the base that is immediately 5′ to the scissile phosphate: C ≈ A > G > T.

**Effect of the Altered lox Spacer Sequence on Overall Resolution**—The mutations in the lox spacer region not only affect the resolution bias, but can also affect the total amount of resolution products. Fig. 4 is a graphic representation of the total amounts of R<sub>1</sub> and R<sub>2</sub> resolution products that accumulated at equilibrium (Table II). These results indicate that Cre appears to possess the following hierarchical resolution preference for the base that is immediately 5′ to the scissile phosphate: C ≈ A > G > T. However, the χ<sub>4A</sub> and χ<sub>4C</sub> structures were resolved 2.5-fold less effectively than wild-type χ<sub>p</sub> and χ<sub>rev</sub>. Both χ<sub>4A</sub> and χ<sub>4C</sub> contain a GC dinucleotide step at positions 4′/3′ and 4/3, and it is possible that the GC dinucleotide step at these positions inhibits cleavage (see “Discussion”).

**Fig. 3. Resolution of the synthetic χ structures by the wild-type Cre protein.** Each χ substrate was incubated either without (−) or with (+) Cre as described under “Experimental Procedures.” Only the 87-bp R<sub>1</sub> and 75-bp R<sub>2</sub> resolution products are shown (see Fig. 2a). The sequences of the central 8-bp region and the R<sub>1</sub>:R<sub>2</sub> ratios for the various χ substrates are listed in Table II.
Resolution of the Synthetic Χ Structures by the Cre K86A Mutant Protein—The crystal structures of Cre bound to a symmetric lox site (containing the same lox sequence as in the Χ structure) showed that Lys86 hydrogen bonds to the O-6 carbonyl group of G at positions 4 and 4 in the major groove (21). Guo et al. (21) predicted that in the wild-type loxP site, Lys86 would make a base-specific contact with G at position 4, but not with A at position 4', which has an N-6 amino group. To investigate whether this putative asymmetric contact of Lys86 is involved in the direction of resolution, we mutated Lys86 to alanine (Cre K86A). The Cre K86A mutant protein was constructed with a His<sub>6</sub> tag at the N terminus for ease of purification. The His tag did not significantly affect the kinetics and yields of recombination by wild-type His-tagged Cre relative to wild-type untagged Cre (data not shown). The wild-type His-tagged Cre protein exhibited similar resolution bias compared with the wild-type untagged Cre protein (Fig. 5, compare lanes 2 and 3), indicating that the His tag did not influence the direction of resolution. The Cre K86A protein resolved the synthetic Χ structures on average 2-fold less efficiently than the wild-type Cre protein (Fig. 5, lane 4). Cre K86A is not defective in binding to the loxP site, but is impaired in recombination by ~2-fold relative to wild-type Cre.  

The K86A mutation also affected the directionality of resolution. A comparison of the resolution bias of the various Χ structures by wild-type and K86A Cre proteins is listed in Table II. The Cre K86A protein displayed little or no bias in the resolution of the wild-type Χ<sub>p</sub> and Χ<sub>rev</sub> substrates, respectively (Fig. 5, lane 4). The K86A mutation also reduced or abolished most of the bias in the resolution of Χ<sub>sa</sub>, Χ<sub>ab</sub>, and Χ<sub>XT</sub>. At least for these Χ structures, Lys86 appeared to be required for the bias in resolution. Surprisingly, the K86A mutation failed to eliminate the bias in the resolution of the Χ<sub>T</sub> structure. The K86A mutant protein also possessed similar bias compared with the wild-type protein in the resolution of Χ<sub>sa</sub>, Χ<sub>ab</sub>, Χ<sub>GC</sub>, Χ<sub>XT</sub>, Χ<sub>4C</sub>, and Χ<sub>T</sub>. Interestingly, the K86A mutation induced a bias toward the bottom strands in the resolution of the Χ<sub>4C</sub> and Χ<sub>GC</sub> structures, whereas the wild-type protein did not exhibit any bias.

For the majority of the Χ structures studied in this work, the Cre K86A protein exhibited a bias in the direction of resolution of 2-fold or less. The exceptions are Χ<sub>4C</sub> and Χ<sub>4T</sub>, where Cre K86A possessed a strong preference for resolving adjacent to a 3 C residue. In general, the K86A mutation appears to reduce the ability of Cre to discriminate between A, G, and T at positions 4 and 4', but it did not abolish the preference for a C residue over the other bases (C > A, T, G).

DISCUSSION

The Cre protein and other tyrosine recombinases catalyze site-specific recombination via a Holliday intermediate. We have demonstrated in this study that Cre preferentially resolves synthetic Χ structures on the top strands of the loxP sites. The bias in the direction of resolution is dictated by the asymmetric loxP sequence, in particular the bases at positions 4 and 4'. Lysine 86, which is known from the Cre-lox co-crystal structures to contact the guanine at position 4 (21, 26, 27), is important for establishing the resolution bias by the Cre protein. Hoes et al. (8) also observed a 4-fold top strand resolution bias, but only in the context of an α structure. The α structure is a Holliday intermediate in which two of the four arms form a closed loop. Hoes et al. isolated the α structure formed during a Cre-mediated excisive recombination between two direct loxP sites on a 4.3-kilobase pair linear substrate. However, when the closed α structure was converted to an open Χ structure by restriction digestion, they found that the bias in resolution was abolished. DNA topology has been shown to influence the directionality of resolution of Holliday junctions by E. coli RuvC and bacteriophage T7 endonuclease I (28). Our results suggest that the loxP sequence also contributes to directional resolution of Holliday junctions by Cre. Other factors may have overridden the intrinsic top strand bias during the resolution of the Χ structure of Hoes et al. (8). Although we have shown that the sequences flanking the loxP sites appear not to affect the directionality of resolution of our synthetic Χ structures, the substantially longer and more flexible arms of the Χ structure studied by Hoes et al. may have contributed to the resolution bias. Alternatively, branch migration into the flanking sequences may have been responsible for the loss of resolution bias. The loxP sites in the pRH43 substrate (used in the resolution studies by Hoes et al.) shared some homology on either side of the loxP sites (29), and so the Holliday junction might be able to branch migrate beyond the loxP sites in the unconstrained Χ structure. However, branch migration would be restricted to the loxP sites in our synthetic Χ structures.

Since Cre initiates strand cleavage on the bottom strands of the loxP site (8, 19), preferential resolution on the top strand is consistent with the order of strand exchange required to generate recombinant products. However, the resolution bias ob-

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3 L. Lee and P. D. Sadowski, unpublished data.
Resolution of Holliday Structures by Cre

The mutant protein has the hierarchical preference $C$ over $H_1$ immediately preceding substrates containing the same mutant sequence. Tribble et al. (30) noticed that cleavage on the top strand of the linear $loxP$ substrate is more efficient than cleavage on the bottom strand. We have also observed a similar difference between cleavage on the top and bottom strands using suicide substrates to trap the covalent intermediate.

Importance of the Nucleotides at Positions 4’ and 4 in Directing Resolution—The direction of resolution is dictated by the asymmetric $loxP$ sequence. The resolution bias was abolished when the Holliday structure contained symmetric $loxP$ sites. In particular, the direction of resolution was primarily influenced by the base pairs at positions 4’ and 4. Positions 4’ and 4 are 5’ to the scissile phosphates to which Cre becomes covalently attached via a 3’-phosphotyrosine bond upon strand cleavage. Mutagenesis of positions 4’ and 4 revealed that Cre has the following hierarchical resolution preference for the base that is immediately 5’ to the scissile phosphate: $C > A > G > T$. Lee and Saito (20) found that substitutions at position 4’ or 4 of the $loxP$ site significantly reduced recombination efficiency and suggested that these positions may be involved in defining the order of strand exchange. Although we have not examined the first cleavage event in this study, we found that substitutions at positions 4’ and/or 4 did indeed affect the resolution bias. However, they did not significantly affect the total yield of resolution products. Therefore, the low recombination efficiency observed by Lee and Saito may have been due to defects in the first cleavage event(s) and/or to the reversal of the first strand exchange to give parental (non-recombinant) products.

The base pairs immediately 5’ to the cleavage sites may also influence the directionality of resolution for other tyrosine recombinases. The $\lambda$-integrase catalyzes strand exchange in a defined order, initiating cleavage on the top strands and resolving on the bottom strands (9, 10, 17, 18). As in the $loxP$ site, the base pairs immediately 5’ to the cleavage sites in the $\lambda$-integrase core-binding sites are different: there is a T adjacent to the top cleavage site and an A adjacent to the bottom cleavage site. Azaro and Landy (31) found that interchanging the base pairs at these positions changed the resolution bias, similar to our findings for Cre. Furthermore, the base pairs at the analogous positions in the Flp recognition target site are identical, and Flp does not appear to possess a preference in the directionality of resolution (32). However, the directionality of resolution may be controlled by other means. For example, the order of strand exchange in the XerC/XerD recombination system is regulated by a complicated mechanism involving two recombinases and additional accessory proteins (6, 33, 34).

Role of Lys$^{86}$ in Resolution—The crystal structures of Cre bound to a symmetric $loxP$ site show that Lys$^{86}$ contacts the O-6 group of guanine at position 4 (and 4’), but Lys$^{86}$ is predicted not to contact the N-6 group of adenine at position 4’ in the wild-type $loxP$ site (21). It is possible that Cre senses the bases at positions 4’ and 4 via their asymmetric contact with Lys$^{86}$. We found that mutation of Lys$^{86}$ to Ala generally reduced the resolution bias. Whereas the wild-type protein has the hierarchical resolution preference $C > A > G > T$, the Cre K86A mutant protein has the hierarchical preference $C > A$, $T$, $G$ for the nucleotide immediately 5’ to the scissile phosphate. Cre K86A still possessed a strong preference for resolving adjacent to a C residue, suggesting that this Lys$^{86}$-independent bias may be intrinsic to the DNA. However, the K86A mutation reduced the ability of Cre to discriminate an A from a G or a T at positions 4’ and 4. Therefore, Lys$^{86}$ is important for establishing the resolution preference for the strands with an A 5’ to the cleavage site over a G or a T. Lys$^{86}$ may also have roles in resolution in addition to distinguishing between the two cleavage sites since the K86A mutation also affected the overall levels of resolution.

There were certain cases where the resolution bias was not entirely abolished either by the K86A mutation or by having identical base pairs at positions 4’ and 4, suggesting that other factors such as the overlap sequence and the DNA conformation of the $\gamma$ or other amino acid residues may also influence the direction of resolution. For example, the small resolution bias of $\chi_{GC}$ (by both wild-type Cre and Cre K86A) may be caused by the asymmetric sequence at position 2 (and 2’) and/or by the dinucleotide step at positions 3 and 2 (and 3’ and 2’). Positions 3 and 2 (or 3’ and 2’) correspond to the location of the asymmetric DNA bend observed by Guo et al. (21) in the crystal structure of the Cre-lox synaptic complex; and so, it is possible that the sequences at these positions may affect DNA bending. The Cre-lox crystal structures reveal several residues other than Lys$^{86}$ that interact asymmetrically with the central 8-bp region of the $loxP$ site, and these asymmetric protein-DNA contacts may also influence the order of strand exchange (21).

In summary, we have shown that the asymmetric $loxP$ sequence is important in determining the direction of resolution of Holliday structures by Cre. The bias in resolution can partially be attributed to the asymmetric interaction between Lys$^{86}$ and the bases immediately 5’ to the cleavage sites. Other factors such as DNA conformation, DNA topology, and other protein-DNA contacts may also influence the direction of resolution of Holliday structures.

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Resolution of Holliday Structures by Cre

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