Bacteriophage P1 Site-specific Recombination

PURIFICATION AND PROPERTIES OF THE Cre RECOMBINASE PROTEIN

Ken Abremski and Ronald Hoess

From the Laboratory of Molecular Biology, Basic Research Program—LBI, Frederick Cancer Research Facility, Frederick, Maryland 21701

Bacteriophage P1 encodes a site-specific recombination system that consists of two components: a site (loxP) and the Cre protein, which carries out recombination. The loxP-Cre system has been shown to be of in vitro reaction. No high energy cofactors are needed. The purified Cre protein binds to loxP-containing DNA and makes complexes that are resistant to heparin. Cre efficiently converts 70% of the DNA substrate to products and makes complexes that are topologically linked. Several models to account for the ability of Cre to produce free supercoiled products are discussed.

In the lysogenic state in Escherichia coli, bacteriophage P1 exists as a unit-copy plasmid (1). P1 possesses a site-specific recombination system, the loxP-Cre system, which consists of two components: loxP is the site on the phage DNA at which recombination occurs and Cre is the phage-encoded protein that carries out this recombination between two loxP sites (2). This system serves two roles in the viral life cycle. The first role involves circularization of P1 DNA. The P1 DNA in the phage head is a linear double-stranded molecule and must be circularized when it enters the host. Because 10–20% of P1 DNA is terminally redundant, some of the entering P1 DNA molecules contain two loxP sites and can be efficiently circularized by Cre (3, 4). The second role involves the breakdown of dimer P1 DNA molecules that may form as a result of replication or homologous recombination. If P1 dimers were to exist at cell division, then only one of the daughters would inherit the P1 DNA and it would be lost quickly in the lysogenic state. This loss occurs very rarely, partly because of the loxP-Cre system (5).

In order to study this system at the biochemical level, we cloned both the loxP site and the cre gene into phage λ vectors and plasmids (6). The loxP site consists of a 34-bp sequence containing two 13-bp inverted repeats separated by an 8-bp spacer region (7). Only these 34 bp appear to be required for efficient recombination (6). An in vitro system to carry out loxP × loxP recombination has been established that uses a partially purified extract derived from a P1 lysogen of E. coli (6). This extract can efficiently carry out recombination between two loxP sites, with only Mg2+ or spermidine needed as a cofactor. The extract can recombine two loxP sites intramolecularly whether they are in direct or inverted orientation and can recombine loxP sites intermolecularly. The form of the DNA substrates containing the loxP sites (i.e., whether it is a linear, supercoiled, or nicked circular molecule) does not appear critical for efficient recombination. This is in contrast to other site-specific recombination systems such as λ (8, 9) and Tn3/γ (10), where the form of the DNA substrate is critical for the recombination reaction. When a supercoiled loxP2 substrate is used, the resulting products are found both as free supercoiled circles and as catenated supercoiled circles in approximately equal numbers (6). This differs with results found in other systems where the large majority of products are catenated molecules (10–12). We proposed several models that could account for the appearance of free products in the loxP-Cre reaction (6).

A very important step in determining the mechanism of P1 site-specific recombination is the isolation and characterization of the Cre protein, which carries out this recombination. In this paper, we report the cloning and overproduction of the Cre protein, its purification, and a characterization of its activities that are involved in loxP recombination.

EXPERIMENTAL PROCEDURES

Materials

Bacteria and DNA—E. coli OR1265 is Fstrhis−su−leu−
galP308::IS2 (λcl857TN53cro27 cryptic) (13). This contains a defective λ phage and a temperature-sensitive CI repressor. K1353 is OR1265/pRK13.

Plasmid pHK12 is a derivative of a λ pBR322 plasmid expression vector (14) in which the cloning sites downstream from pBR322 have been changed. In pRK12, there is an Xhol site 320 bp downstream from pBR322 and an EcoRI site 1080 bp downstream. The cre gene was cloned into plasmid pBR322 (pRH100) (6). A derivative of pRH100 (pRH100(3)) contains the cre gene on a 1500-bp Xhol-EcoRI fragment. Plasmid pRH43 contains two loxP sites in direct orientation, and pRH44 contains a single loxP site. Both plasmids have been described previously (6).

Proteins—Bovine serum albumin (fraction V, reagent grade) and myoglobin were purchased from Miles. Ovalbumin and chymotrypsinogen were obtained from Sigma. Restriction enzymes and molecular weight marker proteins were purchased from Bethesda Research Laboratories.

Other Materials—Phosphocellulose P-11 was obtained from Whatman. Sephadex G-75 was purchased from Pharmacia. Bio-Gel P-60 (fine) was purchased from Bio-Rad. Streptomycin sulfate and heparin were purchased from Sigma.
were obtained from Sigma. For the filter binding assays, Schleicher & Schuell type BA85 nitrocellulose membranes were used.

**Methods**

**Assay for Recombination in Vitro**—The restriction fragment assay for loxP recombination has been described previously (6). Recombination reactions were carried out in 30 µl of the reaction mixture, which contained 50 mM Tris-HCl, pH 7.5, 35 mM NaCl, 5 mM spermidine, 500 µg of bovine serum albumin/ml, 0.25 µg of pRH3 DNA, and various amounts of Cre protein. Reactions were incubated at 37 °C for 15 min and stopped by heating to 70 °C for 5 min, which inactivates Cre. The samples were chilled, and 10 mM MgCl₂ was added with the appropriate restriction enzyme. After the samples were digested, they were extracted with 1 volume of a chloroform/isoamyl alcohol (24:1) mixture and then run on an agarose gel.

The DNA used in these reactions was supercoiled pRH43 (4300 bp in length), which contains two loxP sites. When Cre-mediated intramolecular recombination occurs between the two loxP sites, the resulting products are supercoiled molecules, one that is 2300 bp in length and another that is 2000 bp. Between the loxP sites on one side is a single EcoRI site and on the other side is a single BamHI site. Digestion of pRH43 with either enzyme results in a single linear 4300-bp fragment. If recombination has occurred, two new product bands will appear on an agarose gel. One of these products is cleaved by EcoRI, and the other is cleaved by BamHI. The use of [³H]-thymidine-labeled substrate DNA allowed us to quantitate the amount of recombination. After recombination, restriction enzyme digestion, and gel electrophoresis, the bands were cut out of the gel and the radioactivity was determined as described previously (6).

The percentage of recombination equals 100 x [³H] counts per min in the product bands/total [³H] counts per min in all bands. One unit of Cre activity is defined as the minimum amount of Cre needed to produce a maximum level of loxP x loxP intramolecular recombination (~75%) under the assay conditions described above.

**Protein-DNA Filter Binding**—Filter binding reactions were carried out in 50 µl of the reaction mixture, which contained 50 mM Tris-HCl, pH 7.5, 35 mM NaCl, 500 µg of bovine serum albumin/ml, 1 mM Na₂EDTA, 0.05 µg of [³H]mimydine-labeled DNA, and various amounts of Cre protein. After 15 min at 30 °C, 2 µl of heparin (125 µg/ml) was added to some samples for 2 min. One ml of binding buffer containing 50 mM Tris-HCl, pH 7.5, 1 mM Na₂EDTA, 50 mM KCl, and 10% glycerol was added, and the sample was filtered through nitrocellulose filters, which were equilibrated with binding buffer. The filter was washed with 1 ml of binding buffer, dried under a heat lamp, and counted using a liquid scintillation counter. The percentage of DNA bound equals 100 x [³H] counts per min bound to the filter/total [³H] counts per min added. In the absence of Cre, less than 1% of the total counts per min binds to the filters.

**Other Methods**—Polyacrylamide gel electrophoresis of proteins was carried out using the Laemmli system (15) except that the running gel was composed of a 10-15% gradient of acrylamide and a 5-15% gradient of sucrose. The samples were electrophoresed at 60 V for 16 h and stained for 1 h in a solution containing 7.5% acetic acid, 50% methanol, 0.25% Coomassie brilliant blue, and 0.1% cupric acetate. Gels were destained in 7.5% acetic acid and 10% methanol.

Glycerol gradient centrifugation was performed in a SW 50.1 rotor at 48,000 rpm for 24 h at 4 °C. The gradients were 10-30% glycerol in 50 mM Tris-HCl, pH 7.5, 1 mM Na₂EDTA, 100 mM NaCl, and 1 mM β-mercaptoethanol, in a total volume of 4.6 ml. Twenty µg of Cre protein is a volume of 150 µl was layered on top. After centrifugation, the gradients were collected in 25 fractions and 4 µl of each was assayed for Cre recombination activity.

Protein concentration was determined using the method of Bradford (16) with bovine serum albumin as standard. DNA concentrations were determined using 33258 Hoechst fluorochrome with sonicated calf thymus DNA as standard (17).

**RESULTS**

**Cloning and Overproduction of Cre**

The cre gene was cloned into pBR322 and the resulting plasmid (pRH100) was shown to make a functional cre gene product which could be assayed in _vivo_ (6). Initial attempts at demonstrating Cre activity in _vitro_ using this plasmid suggested that the amount of Cre produced was very low. We, therefore, decided to overproduce Cre by cloning it into a high level expression vector. The vector we chose (pRK12) contains the bacteriophage λ_p promoter and appropriate cloning sites. A 1500-bp XhoI-EcoRI fragment of pRH103 (a derivative of pRH100) that contains the cre gene was cloned into pRK12 between the XhoI and EcoRI sites (Fig. 1). In the resulting plasmid, pRK13, transcription begins at _p, and continues into the cre gene. This plasmid is kept in strain OR1285, which contains a temperature-sensitive λ cl repressor that represses transcription from _p on the plasmid when the cells are grown at 32 °C. To turn on the _p promoter, the temperature is raised to 42 °C, which inactivates the cl857 repressor and leads to high level expression of Cre.

**Enzyme Purification**

**Growth of Cells**— _E. coli_ strain KR1333 was grown at 32 °C in 2 liters of L broth containing 25 µg/ml of ampicillin. When the culture reached _A_650 = 0.5, the temperature was raised to 42 °C and incubation was continued for 4 h. The cells were then chilled and centrifuged. The cell pellet was resuspended in 2 volumes of ice-cold TSE buffer (TSE contains 20 mM Tris-HCl, pH 7.5, 1 mM Na₂EDTA, and 50 mM NaCl) and stored at -80 °C. Approximately 8 g of cells, wet weight, were obtained from each 2-liter culture.

**Crude Sonicate**—Frozen cells (8 g wet weight) were thawed at room temperature. The cell suspension was sonicated in five 1-min bursts and was cooled in ice water between each burst. The sonicate was centrifuged at 17,000 rpm for 30 min.

All centrifugations were performed in a Sorval SS44 rotor and the sample was filtered through a nitrocellulose filter. The supernatant (fraction I, Table I) was collected.

**Streptomycin Sulfate/Ammonium Sulfate Extract**—A solution of 20% streptomycin sulfate in TSE buffer was stirred drop by drop into fraction I to a final concentration of 4%.

![FIG. 1. Plasmid expression vector for overproduction of Cre. An XhoI-EcoRI fragment, which contains the cre gene, from pRH103 was cloned into the _p vector pRK12 downstream from the _p promoter. The dashed arrow indicates the direction of transcription from the _p promoter.](http://www.jbc.org/)

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Volume</th>
<th>Protein</th>
<th>Cre</th>
<th>Specific activity</th>
<th>Recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>19.0</td>
<td>405.0</td>
<td>1,140</td>
<td>2,800</td>
<td>100%</td>
</tr>
<tr>
<td>II</td>
<td>4.8</td>
<td>129.0</td>
<td>390</td>
<td>2,800</td>
<td>92%</td>
</tr>
<tr>
<td>III</td>
<td>9.1</td>
<td>6.9</td>
<td>340</td>
<td>49,300</td>
<td>30%</td>
</tr>
<tr>
<td>IV</td>
<td>7.7</td>
<td>3.3</td>
<td>132</td>
<td>40,000</td>
<td>12%</td>
</tr>
</tbody>
</table>
This suspension was stirred for 15 min and then centrifuged at 11,500 rpm for 20 min. Solid ammonium sulfate was added to the resulting supernatant (1.76 g/10 ml), and the mixture was stirred for 15 min and then centrifuged at 11,500 rpm for 15 min. The resulting pellet was resuspended in 4 ml of TSEG(0) buffer (TSEG(X) buffer contains 20 mM Tris-HCl, pH 7.5, 1 mM Na3EDTA, 10% (v/v) glycerol, and X M NaCl (X refers to the appropriate NaCl concentration in each buffer)). The resuspended pellet was dialyzed against 2 liters of TSEG(0.05) buffer for 2 h with 1 change of buffer. Following dialysis, the material was centrifuged at 11,500 rpm to remove a light precipitate. The resulting supernatant (fraction II, Table I) was frozen at -80 °C.

**Phosphocellulose Chromatography**—Fraction II was thawed and diluted with an equal volume of TSEG(0) buffer. This material was applied to a 4-ml column (1 X 5 cm) of phosphocellulose equilibrated with TSEG(0.05) buffer. The column was washed with 10 column volumes of equilibration buffer and then washed with 4 column volumes of TSEG(0.4) buffer. Cre activity (fraction III, Table I) was eluted with TSEG(0.6) buffer.

**Sephadex G-75 Gel Filtration**—Fraction III (volume = 9.1 ml) was precipitated by the addition of 2.85 g of solid ammonium sulfate. The mixture was stirred for 15 min and centrifuged at 11,500 rpm for 20 min. The pellet was resuspended in 1 ml of TSEG(0) buffer and dialyzed against 500 ml of TSEG(0.2) buffer for 1 h. This dialyzed material was then applied to an 80-ml column (1.5 X 45 cm) of Sephadex G-75 that had been equilibrated with TSEG(0.2) buffer. The flow rate was 4 ml/h, and 1-ml fractions were collected. Active fractions were pooled (fraction IV, Table I) and stored at -80 °C. For everyday use, Cre (fraction IV) was diluted to a concentration of 200 μg/ml in a buffer containing 20 mM Tris-HCl, pH 7.5, 1 mM Na3EDTA, 100 mM NaCl, and 25% glycerol. This is stored at -20 °C, and no loss of activity has been observed over a 9-month period.

**Physical Properties of the Purified Protein**

Cre protein has been purified to greater than 98% homogeneity, and it migrates as a single polypeptide band on an SDS-polyacrylamide gel (Fig. 2, lane d). Even in the crudest fraction derived from induced cells (Fig. 2, lane a), a major band can be seen at the position of Cre. From the data in Table I, Cre appears to represent 7% of the total soluble protein in the crude extract following induction of cells containing the plasmid pRK13.

The *M*<sub>r</sub> of denatured Cre appears to be 35,000, as determined by SDS-polyacrylamide gel electrophoresis and comparison of its relative mobility with a set of standard proteins (Fig. 3). To determine the native form of the Cre protein in solution, its behavior was analyzed by velocity sedimentation and gel filtration. Fig. 4 indicates that Cre has a sedimentation coefficient of 3.0 S, and gel filtration on Bio-Gel P-60 (Fig. 5) demonstrates that Cre elutes as expected for a monomer of *M*<sub>r</sub> = 35,000. To learn something about the shape of the Cre molecule in solution, we calculated several physical parameters using the above results; these are summarized in Table II. Cre behaves as a monomer under the conditions described in Figs. 4 and 5, and it is also an asymmetric protein. However, the sedimentation coefficient of Cre changes from 3.0 to 4.0 S if Cre is centrifuged in the presence of 10 mM MgCl<sub>2</sub>, suggesting that the protein may be dimerizing under these conditions. Table III shows the results of an amino acid analysis of purified Cre protein. From these data, we can calculate a minimum *M*<sub>r</sub> of approximately 32,000, which agrees with the molecular weight determined above.

**Interaction of Cre with DNA**

Purified Cre is a DNA-binding protein. Fig. 6 shows that Cre can bind nonspecifically to pBR322 DNA. If the DNA contains a loxP site, then Cre binds to it with 20-fold higher efficiency. When the Cre-DNA complexes are challenged with heparin, which should compete for any nonspecific interactions between Cre and DNA, DNA containing loxP is still bound by Cre with the same efficiency, but DNA without a loxP site is no longer bound. This result suggests that Cre can
Purification and Properties of Cre Recombinase Protein

TABLE I

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>Mol fraction</th>
<th>Mol residue/mol Cre</th>
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<tbody>
<tr>
<td>Ala</td>
<td>0.109</td>
<td>32</td>
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<tr>
<td>Arg</td>
<td>0.102</td>
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<tr>
<td>Asx</td>
<td>0.122</td>
<td>36</td>
</tr>
<tr>
<td>Glx</td>
<td>0.095</td>
<td>28</td>
</tr>
<tr>
<td>Gly</td>
<td>0.071</td>
<td>21</td>
</tr>
<tr>
<td>His</td>
<td>0.020</td>
<td>6</td>
</tr>
<tr>
<td>Ile</td>
<td>0.044</td>
<td>13</td>
</tr>
<tr>
<td>Leu</td>
<td>0.105</td>
<td>31</td>
</tr>
<tr>
<td>Lys</td>
<td>0.041</td>
<td>12</td>
</tr>
<tr>
<td>Met</td>
<td>0.027</td>
<td>8</td>
</tr>
<tr>
<td>Phe</td>
<td>0.027</td>
<td>8</td>
</tr>
<tr>
<td>Pro</td>
<td>0.031</td>
<td>9</td>
</tr>
<tr>
<td>Ser</td>
<td>0.068</td>
<td>20</td>
</tr>
<tr>
<td>Thr</td>
<td>0.051</td>
<td>15</td>
</tr>
<tr>
<td>Tyr</td>
<td>0.021</td>
<td>6</td>
</tr>
<tr>
<td>Val</td>
<td>0.065</td>
<td>19</td>
</tr>
</tbody>
</table>

*Tryptophan and cysteine were not determined.
*Calculations are based on the assumption that Cre contains 294 residues.

Recombination with Purified Cre Protein

In the in vitro system for loxP recombination, Cre is the only protein required. No high energy cofactors are needed and recombination between two loxP sites occurs in the presence of only buffers and simple salts. Under the standard reaction conditions, 75% of the substrate is converted to products. Spermidine can be replaced by 10 mM MgCl₂ in the

Fig. 4. Glycerol gradient sedimentation of Cre. Purified Cre protein (fraction IV, 25 µg) was centrifuged in a 10-30% glycerol gradient for 24 h at 48,000 rpm. In another gradient, a set of standard proteins with the following $s_{20w}$ values was centrifuged: bovine serum albumin, 4.3; ovalbumin, 3.6; and myoglobin, 2.0. The position of Cre in the gradient is indicated.

Fig. 5. Gel filtration of Cre. Fraction IV, 100 µg, was run on a Bio-Gel P-60 column (25 x 0.7 cm) in TSEG(0.2) buffer. The following proteins, with the indicated diffusion coefficients (D), were used as markers: ovalbumin, 7.76 x 10⁻⁷ cm²/s; chymotrypsinogen, 9.5 x 10⁻⁷ cm²/s; and myoglobin 11.3 x 10⁻⁷ cm²/s. Fractions, 0.275 ml, were collected and assayed for Cre recombination activity. The peak of Cre activity is indicated by the arrow.

Fig. 6. Binding of Cre to DNA. Binding reactions were carried out as described under "Experimental Procedures." Purified Cre (fraction IV) was added in the amounts indicated. To some samples, heparin was added following the incubation of Cre with DNA. Total ³H counts per min added to pRH44 DNA, 5,000 cpm; pBR322, 15,000 cpm. The number of DNA molecules added to each reaction was kept constant. O—O, pRH44 (lox⁺) + Cre; ●—●, pRH44 (lox⁺) + Cre + heparin; □—□, pBR322 + Cre; ■—■, pBR322 + Cre + heparin.

Table IV compares the effect of SDS on the binding of Cre to DNA with one or two loxP sites. When Cre is treated with SDS before the DNA is added, no binding is observed. However, when Cre is bound to the DNA before the SDS is added, there is a significant level of binding of DNA containing two loxP sites but no binding of DNA containing one loxP site. This suggests that the complex formed between Cre and two loxP sites is different from the complex formed between Cre and DNA with a single loxP site.
reaction. However, if both spermidine and MgCl₂ are omitted, the amount of recombination falls to approximately 50%. All of the characteristics of the in vitro system described previously when crude extracts were used (6) are also true when the purified protein is used.

Cre can carry out intermolecular and intramolecular recombination between two loxP sites. The form of the DNA substrate does not seem important, and recombination occurs efficiently over a wide range of ionic strengths. For intramolecular recombination, if the two loxP sites are oriented in opposite directions, then the DNA between the sites is inverted. If the two loxP sites are oriented in the same direction, then the DNA between the sites is excised by recombination. If this loxP² substrate is supercoiled, then the resulting products are also supercoiled; 50% of the products are catenated and 50% are free molecules. Thus, purified Cre is the only protein needed to produce these free product circles.

The kinetics of recombination are fast: maximal conversion of substrate to products occurs within 2–3 min at 37 °C. Table V shows the levels of Cre needed for recombination. If suboptimal amounts of Cre are used, only low levels of recombination are observed, even if the incubation is continued for several hours. If the level of DNA in the reaction is raised, then the amount of recombination decreases; however, if the amount of Cre is also increased, the levels of recombination increase. The recombination values in Table V measure the extent of the reaction. These results suggest that Cre is acting stoichiometrically, and, from the data in Table V, it appears that 25–30 molecules of Cre are required to recombine 1 substrate molecule. This value is uncertain because we do not know what percentage of the Cre molecules in the purified preparation are active.

Cre protein was also examined for several other activities. We tested nuclease activity by looking for the conversion of supercoiled DNA to linear or nicked DNA in the presence of Cre. DNA with and without a loxP site was used, and there was no detectable nuclease activity under the standard conditions. We also assayed for topoisomerase activity by looking for the relaxation of supercoiled DNA substrates on 1% agarose gels containing the intercalating agent chloroquine. No relaxation activity was observed under standard conditions regardless of whether the supercoiled DNA substrates had no, one, or two loxP sites (in either direct or opposite orientations). We have not been able to detect any intermediates in the recombination reaction.

**DISCUSSION**

We describe here the purification and characterization of the P1 site-specific recombinase Cre, which mediates recombination between loxP sites on the P1 genome. The inherent simplicity of the Cre-lox system by comparison with other well characterized site-specific recombination systems such as λ and Tn3/γ (10, 11) makes this an ideal system for studying the mechanism of site-specific recombination. Unlike the λ Int protein or the Tn3 resolvase protein, the Cre protein will bind and carry out recombination regardless of the conformation of the DNA, be it supercoiled, relaxed circle, or linear. High energy cofactors are not required, nor are accessory proteins encoded by P1 or the host E. coli needed for efficient recombination.

The Cre protein, in solution, behaves as a monomer. In the presence of MgCl₂, the protein appears to form dimers, as evidenced by an increase in sedimentation velocity. Whether this is the active form of the protein in recombination is unclear at present because the in vitro reaction does not require MgCl₂. However, recent DNA footprinting results using purified Cre suggest that a minimum of 2 Cre molecules must bind to each loxP site. From the sedimentation and gel filtration data, Cre appears to be an asymmetric molecule that can be represented as a prolate ellipsoid with an axial ratio of 3:1. From the physical measurements an approximation of the overall dimensions of Cre can be calculated, and these indicate that the 34-bp loxP site could easily be covered by 2 Cre molecules.

In addition, we have found that the binding of Cre to a DNA molecule containing two loxP sites differs from the binding to a molecule with one site: Cre molecules attached at different sites on the same DNA molecule result in a protein-DNA complex that is less sensitive to dissociation by detergent. This finding suggests a protein-protein interaction between Cre molecules that is unique to complexes formed on DNA molecules with two lox sites. In vitro, we have found that intramolecular recombination (two lox sites on the same DNA molecule) is considerably more efficient than intermolecular recombination. Perhaps the protein-DNA complexes insensitive to dissociation by detergent are a reflection of Cre molecules actively recombining loxP sites, an event more likely to be observed when a substrate with two loxP sites is used.

The recombination data obtained with the use of various amounts of Cre suggest that Cre acts stoichiometrically during recombination. However, these results may represent an inactivation of Cre by some unknown mechanism during recombination. It should be noted that λ Int (18), Xis (19), and IHF (20) along with the γδ resolvase (10) all appear to act stoichiometrically during recombination.

Previously, we reported that free supercoiled product molecules are found after the intramolecular recombination of a supercoiled loxP² substrate (6). This result was unexpected because, in the bacteriophage λ and the Tn3γ6 site-specific recombination systems, more than 95% of the product molecules are catenanes (10–12). At this time, we presented several models to account for the production of free supercoiled products, including a topoisomerase model, a nucleosome model, and a protein tracking model (6). From the observations reported here, we can only address the topoisomerase model. According to this model, all of the products from intramolecular recombination of a supercoiled loxP² substrate would initially be catenated. Then, a topoisomerase activity would act to unlink the products, causing loss in the superhelical density of the free product molecules. Several lines of evidence rule this out. Free supercoils are observed in vitro when purified Cre is used; therefore, these free molecules cannot result from a separate topoisomerase activity in the original crude extracts. In addition, under the standard conditions for in vitro recombination, we cannot demonstrate

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**Table V**

Amount of Cre required for recombination

<table>
<thead>
<tr>
<th>Amount of Cre used (μg/ml)</th>
<th>pH43 DNA (μg/ml)</th>
<th>% Recombination</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.175 (5.97)</td>
<td>2.27 (0.95)</td>
<td>14</td>
</tr>
<tr>
<td>0.35 (11.9)</td>
<td>2.27 (0.95)</td>
<td>41</td>
</tr>
<tr>
<td>0.70 (23.9)</td>
<td>2.27 (0.95)</td>
<td>74</td>
</tr>
<tr>
<td>1.40 (47.8)</td>
<td>2.27 (0.95)</td>
<td>75</td>
</tr>
<tr>
<td>2.00 (60.6)</td>
<td>2.27 (0.95)</td>
<td>68</td>
</tr>
</tbody>
</table>

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2 R. Hoess and K. Abremski, unpublished data.
any topoisomerase activity for the Cre protein. Previously, we showed that free product molecules resulting from in vitro intramolecular recombination have not lost a significant amount of superhelicity (6). Therefore, it seems unlikely that such a model plays a significant role in this recombination system. With the purified system described in this paper, further elucidation of the mechanism of site-specific recombination will be possible.

Acknowledgments—We thank N. Sternberg for many helpful discussions, S. Oroszlan and L. Henderson for the amino acid analysis, and R. Grafstrom and P. Benfield for their comments on the manuscript.

REFERENCES