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Genetics

Isolation and characterization of intermediates in site-specific recombination

(Holliday structure/*cre* mutants/*lox* sites/strand exchange)

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ABSTRACT **Cre, the site-specific recombinase from bacteriophage P1, catalyzes a recombination reaction between specific DNA sequences designated as *lox* sites. The breakage and rejoining of partners during this recombination process must be highly concerted because it has not been possible to detect intermediates of the reaction with wild-type Cre. Several mutant Cre proteins have been isolated that produce significant amounts of a possible intermediate product of the recombination reaction. The product has been identified as a Holliday structure in which one set of the DNA strands of the recombining partners has been exchanged. Wild-type Cre protein is capable of acting on this structure to form recombinant products, which is consistent with this being an intermediate in the recombination reaction. Characterization of the Holliday structure indicated that one set of strands in the recombining partners was always exchanged preferentially before the other set. In addition, it has been found that certain Cre mutants that are unable to carry out recombination *in vitro* are able to resolve the intermediate. This suggests that these mutants are defective in a step in the reaction that precedes the formation of the Holliday intermediate.**

Genetic recombination requires the physical exchange of DNA strands among partners in the reaction. The development of well-defined *in vitro* recombination systems has offered the possibility of biochemically dissecting this complex reaction (reviewed in refs. 1 and 2). Of considerable importance in understanding the mechanism of recombination is the identification and isolation of intermediates in the reaction. Because of its relative simplicity, we have chosen the Cre *lox* site-specific recombination system of bacteriophage P1 as a model to study this reaction. The system consists of a single protein, Cre, which mediates the recombination process between two 34-base-pair (bp) sequences designated as *lox* sites (3). Each *lox* site is comprised of two 13-bp inverted repeats separated by an 8-bp spacer region (4). Upon binding to the inverted repeats, Cre cleaves the DNA in the spacer region to initiate strand exchange with an equivalent partner in the reaction (5).

Early concepts of genetic recombination involved a simple breakage and rejoining mechanism, in which double-stranded breaks were formed and all partners in the reaction were exchanged at once. The discovery of gene conversion in fungi, which could not be adequately explained by simple breakage and rejoining, stimulated the formulation of an alternative model (6). This model proposed that recombination took place by sequential exchange, with first one set of strands being cleaved and exchanged followed by similar reactions with the second set of strands. The model predicts that between those two events a cross-stranded structure or Holliday intermediate will be formed (6). A large body of both genetic and physical evidence has implicated the Holliday structure as an intermediate in recombination. Perhaps most convincing are the direct observation of such intermediates by electron microscopy (reviewed in ref. 7).

The Holliday intermediate appears to be quite applicable to certain site-specific recombination systems. In the Cre *lox* system (8) and in the bacteriophage λ integrase system (9, 10), there is genetic evidence that site-specific recombination stimulates gene conversion of nearby markers. In both these systems, studies of linking number changes in DNA during recombination (11, 12) and the ability of the recombinases to act as type I topoisomerases (13, 14) are consistent with a model of sequential single-stranded breaks and exchanges. In addition, artificial Holliday intermediates have been formed using the DNA of the bacteriophage λ *att* site, and it has been demonstrated that such structures are acted upon by the recombinase Int (15).

MATERIALS AND METHODS

Cre Mutants. The isolation and characterization of the three Cre mutants HA136, HA15, and NA14-2 have been described (16). Each protein was overproduced by placing the mutant *cre* gene under the control of the λ P_L promoter and inducing cells containing this construct at 42°C for 3 hr (17). The proteins were partially purified (70–90% pure) by phosphocellulose column chromatography as reported (16).

DNA Substrate. For all *in vitro* recombination assays, the plasmid pRH43, containing two *loxP* sites in a directly repeated orientation, was used (3). Digestion of this plasmid DNA with restriction enzymes (New England Biolabs and Bethesda Research Laboratories) was carried out according to the manufacturers' specifications. End-labeling of the 5′ termini of restriction fragments with [γ-^{32}P]ATP (3000 Ci/mmol; 1 Ci = 37 GBq; New England Nuclear) using polynucleotide kinase (Boehringer-Mannheim) has been described (18).

Recovery of DNA Fragments. To locate the appropriate DNA fragment, agarose gels were briefly stained with an ethidium bromide solution (0.5 μg/ml), and the DNA was visualized with ultraviolet light. A gel piece containing the DNA fragment was mascerated in an equal volume of phenol, frozen 15 min at −70°C, and then centrifuged. The resulting aqueous phase was reextracted twice with an equal volume of phenol, and the DNA was precipitated with ethanol.

***In Vitro* Recombination Reactions.** Conditions for *in vitro* recombination reactions have been described (17).

Electron Microscopy. Recombination intermediates were isolated from agarose gels as described above. DNA samples were spread in a solution of cytochrome *c* using the basic protein film technique (19). Grids containing DNA were shadowed with platinum and observed by transmission electron microscopy.

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RESULTS

To assay for Cre-mediated recombination *in vitro*, a ^{32}P-labeled DNA fragment containing two directly repeated *loxP* sites was used as a substrate (Fig. 1*A*). Intramolecular recombination results in the excision of a circular molecule representing the DNA between the *loxP* sites and a 2.4-kilobase (kb) linear fragment containing the ^{32}P-labeled ends. Using this assay, mutant Cre proteins (16) were tested for their ability to carry out recombination *in vitro*. Three Cre mutants, HA136, HA15, and NA14-2, generated a fragment not observed in reactions with wild-type Cre. This unusual fragment, designated as α (Fig. 1*B*), migrated more slowly in gels than the unrecombined linear DNA.

Restriction Analysis of the α Band. To determine its structure, the α band was eluted from gels and digested with a number of restriction enzymes and compared with restriction digests of the unrecombined linear fragment. Fig. 2 shows that when the unrecombined linear substrate (lane B) is restricted with *Bgl* II, which cleaves between the two *loxP* sites, two fragments (3.0 and 1.3 kb) were produced (lane D). The α band (lane A) did not change in its migration when cleaved with *Bgl* II (lane C). Cleavage of the unrecombined linear with *Xho* I resulted in the predicted 1.9-kb and 2.4-kb fragments (lane F). However, the α band cleaved with *Xho* I resulted in a doublet comigrating with the 2.4-kb fragment (lane E). Thus, the α band represented a distinctly different DNA structure from the unrecombined linear substrate. The observed restriction pattern can best be explained if we assume that the α band represents a DNA intermediate in recombination, such that one set of strands has undergone recombination while the complementary partners have not. Such an intermediate or Holliday structure (6) would in this case be a circular DNA molecule with two tails, one of 40 bp and the other 2.4 kb long. Cleaving this structure with *Bgl* II would simply open the circle but leave the Holliday structure intact, converting the DNA into a χ form. Such structures have been shown to migrate anomalously in gels (20). Cleavage by *Xho* I simply removes the 2.4-kb tail while leaving the Holliday structure intact. Thus, a linear fragment of 2.4 kb as well as a circular species, which migrates slower than its 1.9-kb linear counterpart, are generated. Finally,

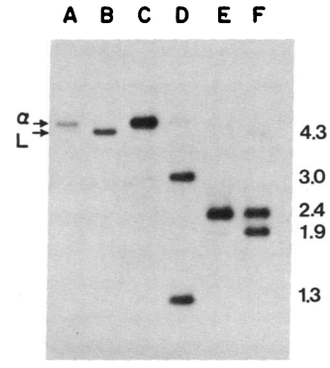

FIG. 2. Analysis of the α band. Autoradiograph of a 0.8% agarose gel containing DNA isolated from the α band (lanes A, C, and E) or the unrecombined linear substrate (lanes B, D, and F). Prior to electrophoresis, DNA in lanes C and D was restricted with *Bgl* II, and DNA in lanes E and F was restricted with *Xho* I. The position of molecular size markers in kb is indicated.

electron microscopy of the gel-isolated α band confirmed our prediction based on restriction analysis, where >95% of the observed structures had the appearance shown in Fig. 3. Contour length measurements of 23 such molecules gave a circle size of 1980 bp, based on a total size of 4300 bp for pRH43. This is the expected length for a loop formed between the two *lox* sites. The 40-bp tail is not resolved under these conditions. It should be noted that because the *lox* sites are embedded in regions of nonhomology, the Holliday junction is confined within the *lox* sites and is not free to branch migrate off of the α structure.

Is the α Structure an Intermediate in Cre–*lox* Recombination? Since the α structure is not observed in a recombination reaction using wild-type Cre, an important question is whether it represents an intermediate of the reaction or is simply an abnormal byproduct generated by the mutant Cre proteins. Two observations support the hypothesis that the α structure is a true intermediate. First, mutant Cre proteins that produce the α structure in addition produce recombinant products. Second, in the presence of wild-type Cre, the α structure is

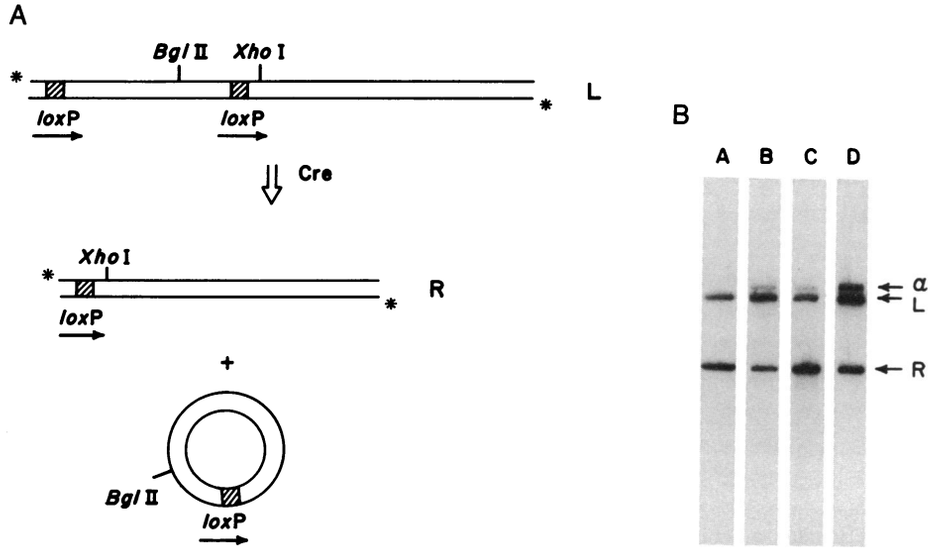

FIG. 1. Substrate for *in vitro* Cre-mediated recombination. (*A*) Plasmid pRH43 was linearized with *Eco*RI (L) and the 5′ ends of the molecule were labeled with ^{32}P (*). Intramolecular recombination of the *loxP* sites by Cre results in a ^{32}P-labeled recombinant linear molecule (R) and an unlabeled circular molecule. (*B*) Labeled plasmid DNA was incubated with either wild-type Cre or a mutant Cre for 30 min at 30°C, electrophoresed in a 0.8% agarose gel, and then autoradiographed. The position of the linear substrate (L) and linear recombinant (R) are indicated for reactions with wild-type Cre (lane A), Cre HA136 (lane B), Cre HA15 (lane C), and Cre NA14-2 (lane D). Note that in reactions using mutant Cre proteins an additional band appears designated α.

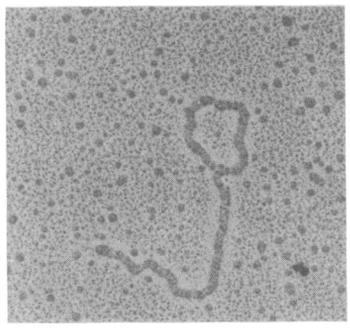

FIG. 3. Electron micrograph of the α structure. Gel-purified DNA from the α band was spread on grids and shadowed with platinum (19).

converted to recombinant products. This is shown in Fig. 4*B* (lane D), where cleavage with *Hin*cII followed by incubation of the α structure with wild-type Cre generates a 1890-bp fragment. This is the expected size fragment if the α structure diagrammed in Fig. 4*A* is resolved into recombinant products. We have also determined that the mutant CreNA14-2 used to generate the α structure is also able to resolve such a structure to recombinant products (data not shown). This suggests that the α structure is not an aberrant side product of the recombination reaction.

Resolution of the Intermediate. An interesting feature of the Holliday junction is its potential structural symmetry (21). Because of this symmetry, resolution of such a structure by a protein carrying out a strand-exchange reaction has two possible outcomes (Fig. 4*A*). First, the protein can exchange the set of strands that were not previously exchanged, thereby generating recombinant products. Alternatively, it can exchange the set of strands that were initially crossed over to produce the original recombination substrate. In our experiments in which wild-type Cre underwent reaction with the α structure, it was observed that a bias existed in the products generated. Careful quantitation of the products indicated that the structure treated with wild-type Cre favored exchange of the unrecombined strand over the strand that had already been recombined by about a 4:1 ratio (compare amount of 1890-bp with 1300-bp fragments Fig. 4*B*, lane D). To determine whether this bias is an intrinsic feature of Cre or reflects some feature of the topology of the DNA intermediate, the topology of the α structure was altered by cleaving it with *Bgl* II to generate a χ structure. When the χ structure underwent reaction with Cre, the two products were produced in equal amounts (lane C). Clearly, the topology of the DNA intermediate affects its processing.

The Nature of Strand Exchange. During recombination both strands of a double helix are exchanged. By examining the structure of the intermediate, we can determine whether there is a preference as to which strand is exchanged first. In Fig. 5*A*, the plasmid pRH43 was linearized and labeled at the *Eco*RI ends, reacted *in vitro* with Cre NA14-2, and the α structure was isolated. The DNA was then cleaved with *Xho* I and the products were separated on a denaturing gel. As shown in Fig. 5*A* there are two possible outcomes. If the light strand was not recombined (upper diagram), cleavage with *Xho* I would result in a 1.9-kb fragment. If, however, the light strand had been exchanged (lower diagram in Fig. 5*A*), then cleavage with *Xho* I should yield a 63-bp fragment. The autoradiograph (lane 1) reveals two products, a 2.4-kb and a 1.9-kb fragment. This result implies that the heavy strand had undergone recombination. To show that in fact this was the case, the reciprocal experiment was done in which the plasmid was first cleaved and labeled at the *Xho* I site,

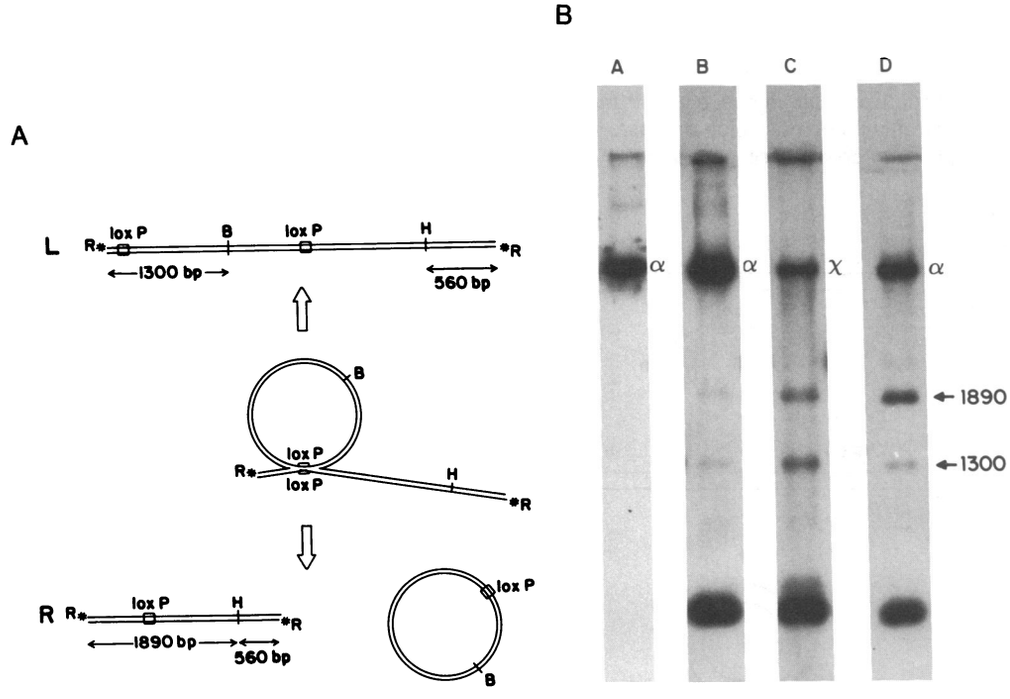

FIG. 4. Resolution of the Holliday structure by Cre. (*A*) Schematic diagram of the two possible outcomes of Cre acting on an α structure pictured in the center. If the strands that were initially crossed over to generate the α structure are again recombined, the structure is converted back to the original linear substrate (L). Cleavage with *Bgl* II (B) yields a diagnostic 1300-bp fragment. If, however, Cre exchanges the unrecombined strand of the α structure, the small linear recombinant (R) is formed, which yields a 1890-bp fragment when cleaved with *Hin*cII (H). *, ^{32}P-labeled 5′ ends. (*B*) Labeled α structure was incubated with wild-type Cre and the appropriate restriction enzyme followed by electrophoresis and autoradiography. The following reactions are shown: lane A, α structure; lane B, α structure cleaved with *Hin*cII; lane C, α structure cleaved with *Hin*cII and *Bgl* II and then reacted with Cre; lane D, α structure cleaved with *Hin*cII, reacted with Cre, and then cleaved with *Bgl* II.

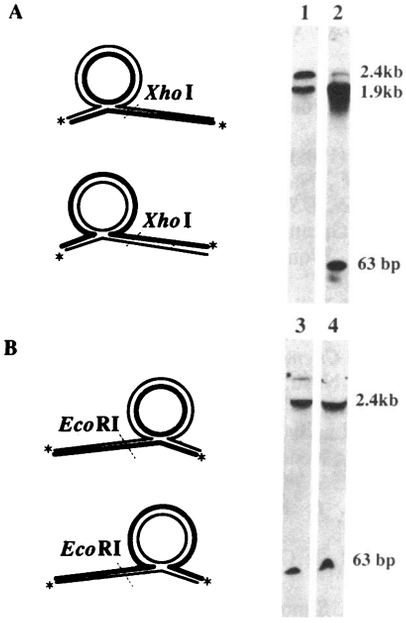

FIG. 5. Asymmetry of strand exchange. (*A*) Diagram depicts two possible *Eco*RI ^{32}P-labeled (*) α structures with the *Xho* I cleavage site indicated. The structure was cleaved with *Xho* I, boiled in the presence of 7 M urea, and electrophoresed on a 5% acrylamide gel containing 7 M urea. The gel was autoradiographed and lane 1 shows the *Xho* I-cleaved α structure and lane 2 shows both unrecombined and recombined *Eco*RI ^{32}P-labeled pRH43 cleaved with *Xho* I as marker. (*B*) Diagram depicts two possible *Xho* I ^{32}P-labeled (*) α structures. The structure was cleaved with *Eco*RI and treated as described in *A*. Autoradiograph shows the products in lane 3. Lane 4 contains the recombinant linear cleaved with *Eco*RI to serve as marker.

reacted with Cre NA14-2, and the α structure was isolated. This material was then cleaved with *Eco*RI and analyzed as described above. The possible outcomes are shown in Fig. 5*B*. From the previous experiment, we would predict that the heavy strand would have been exchanged (upper diagram in Fig. 5*B*) and cleavage with *Eco* RI should yield a 63-bp fragment. The autoradiograph (lane 3) of the denaturing gel shows two bands representing a 2.4-kb and a 63-bp fragment. These results indicate that strand exchange with wild-type *loxP* sites is asymmetric, with one strand being preferentially exchanged first.

Complementation of *Cre*$^-$ Mutants with Holliday Structures. Previously, we identified a number of *cre* mutants defective for *in vitro* recombination (16). The majority of these mutants still bind the *lox* site and must therefore be defective for a step after recognition of *lox*. Since the Holliday structure represents a possible intermediate in the reaction, we can ask whether the defect in any of the mutant Cre proteins can be complemented by using the Holliday structure as a substrate for the *in vitro* reaction. Three mutant Cre proteins HA16-1, HA208, and HA127 were identified that did not recombine a linear substrate containing two *lox* sites but were able to complete the recombination reaction if presented with a Holliday structure (data not shown). Interestingly, these mutants are clustered near the amino terminus of Cre, with HA16-1 and HA208 representing two different changes of amino acid residue 36 and HA127 representing a change at amino acid residue 41.

DISCUSSION

We report here the isolation of a possible reaction intermediate in the Cre *lox* site-specific recombination system. This intermediate is produced by certain mutant Cre proteins in an *in vitro* recombination reaction. We believe this to be an intermediate based on our observation that wild-type Cre is able to convert it to recombinant products. Analysis of the intermediate has revealed that it is a Holliday structure in which one of the DNA strands of each partner has been exchanged. While recent work in the bacteriophage λ Int system has shown that Int can efficiently resolve artificial Holliday structures made from phage *att* sites (15), we demonstrate here that the Cre *lox* system can generate such structures as well as resolve them.

Our previous studies that measured changes in DNA linking number during Cre–*lox* recombination were consistent with two different models for strand exchange (11). In one, two *lox* sites align in an anti-parallel orientation and strands are exchanged by a double-strand break. In the other model, two *lox* sites align in a parallel orientation and strands are exchanged in a series of two single-strand breaks. This second model of single-stranded exchange is supported by the observations that Cre acts as a type I topoisomerase (13) and that DNA homology is required within the spacer region of the *lox* sites (22). The demonstration that Holliday structures are intermediates during Cre–*lox* recombination lends additional support for this model.

A perplexing result is the difference observed between the resolution of the α structure versus the χ structure. By model building Sigal and Alberts (21) demonstrated the stereochemical feasibility of the Holliday junction and proposed that the crossed-strand structure could be interconverted, or isomerized, to lead to strand equivalence during genetic recombination. Because of strand equivalence, a recombination protein acting on such a structure should yield either recombinant products or the starting substrate with equal efficiency. This appears to be the case when Cre is presented with a χ structure. The α structure, on the other hand, is clearly biased toward being resolved to recombinant products. There are a number of possibilities that might explain such a result. One is that the Holliday junction contained within the α structure is unable to isomerize with the same efficiency as in the χ structure. From inspection of models, it is not intuitively obvious why this should be. If we assume strand equivalence in the α structure, why should the outcome be biased? A possible explanation might be that the closed circular portion of the α structure presents a torsional barrier for the Cre reaction. For example, if strand exchange in one direction requires that a twist be introduced into the circular portion of the α structure, this might be energetically less favorable and bias the reaction in the other direction.

The observation that strand exchange is asymmetric, one must assume, is a consequence of the only asymmetric feature of the *lox* site, the sequence within the spacer region. Since we have shown that bases within the spacer region can be altered without adversely affecting recombination (22), we assume that specific protein–nucleic acid contacts involving this region are not essential for recombination. What might be of importance here is the local geometry of the DNA helix. Parameters influenced by sequence, such as groove width, have been shown to affect the cutting efficiency of various nucleases (23). By making various base substitutions within the spacer region and determining the preference in strand exchange from isolated α structures, one should be able to test whether such parameters are important.

The question of why certain mutant Cre proteins yield these recombination intermediates needs to be addressed. Two of the mutants, HA136 and HA15, have been shown qualitatively to be altered in binding to the *loxP* site (16). A plausible explanation might be that the mutant protein is still able to recognize the *loxP* site, but its dissociation rate is increased so that it completes only half the recombination reaction before leaving the site. More puzzling is NA14-2,

which yields the largest amount of α structures, $\approx$30% of the initial substrate. This mutant qualitatively appears no different from wild-type Cre in binding to *loxP* (A.W. & R.H., unpublished data). *In vivo* this mutant behaves the same as wild-type Cre, suggesting that under the *in vivo* conditions either the intermediates are not formed or the cell efficiently resolves them into recombinant products.

Finally, we find that some mutant Cre proteins that are unable to recombine a linear substrate *in vitro* are able to complete the recombination reaction given an α structure. These mutants are presumably not defective in catalytic function (cleaving and exchanging strands) but in some step prior to this. Possibly, they are defective in bringing sites together (synapsis), and the Holliday structure is able to complement such a defect since there would be no requirement for the protein to bring the recombining sites in close proximity to one another. We feel that, in the long term, this sort of analysis will be of considerable value in elucidating the various steps in the recombination reaction and also help in assigning structure–function relationships for the Cre protein.

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