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The role of the *loxP* spacer region in P1 site-specific recombination

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ABSTRACT

The lox-Cre site-specific recombination system of bacteriophage P1 is comprised of a site on the DNA where recombination occurs called loxP, and a protein, Cre, which mediates the reaction. The loxP site is 34 base pairs (bp) in length and consists of two 13 bp inverted repeats separated by an 8 bp spacer region. Previously it has been shown that the cleavage and strand exchange of recombining loxP sites occurs within this spacer region. We report here an analysis of various base substitution mutations within the spacer region of loxP, and conclude the following: (1) Homology is a requirement for efficient recombination between recombining loxP sites. (2) There is at least one position within the spacer where a base change drastically reduces recombination even when there is homology between the two recombining loxP sites. (3) When two loxP sites containing symmetric spacer regions undergo Cre-mediated recombination in vitro, the DNA between the sites undergoes both excision and inversion with equal frequency.

INTRODUCTION

Bacteriophage P1 encodes a site-specfic recombination system consisting of a site, loxP, at which recombination takes place, and a protein, Cre, which carries out the recombination reaction (1). We are interested in elucidating the detailed mechanism by which this simple system carries out recombination. Of particular interest is the question of how recombining sites are aligned with one another during the breakage and rejoining of DNA strands and what role DNA homology plays in this process.

The loxP site is a 34 bp sequence consisting of two 13 bp inverted repeats separated by an 8 bp spacer region (2). Recent experiments have demonstrated that prior to strand exchange Cre cleaves the DNA of the spacer region to generate a 6 bp staggered cut (Fig. 1)(3). The single-stranded ends formed by

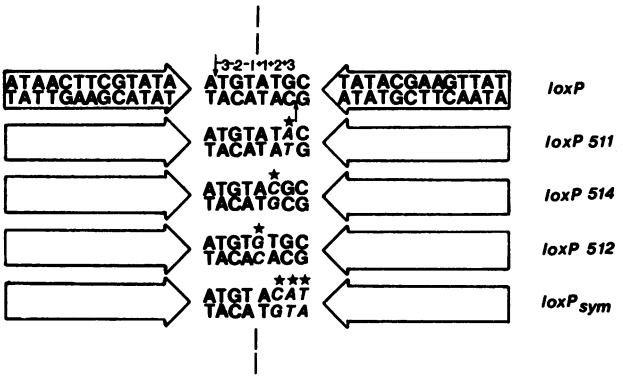

Fig. 1 Mutant loxP sites with altered spacer regions. The top line shows the wild type loxP site with the inverted repeats enclosed within the large arrows and separated by the 8 bp of the spacer region. The points at which Cre cleaves the loxP site during recombination is denoted by the small vertical arrows. The positions of the bases with respect to the axis of dyad symmetry of the site are numbered. Mutant loxP sites are depicted below with the base change indicated by (★).

the cleavage reaction must then pair with the complementary single-stranded sequences of the other loxP partner in recombination. We have designated the 6 bp single stranded sequences as the overlap region since following recombination the DNA in this region is comprised of one strand from each partner. Two types of experiments have shown that sequences outside of the 34 bp loxP site are not required for recombination. First, analysis of DNA deletions has shown that sequences outside of the inverted repeats can be removed without loss of recombination function (4). Second, DNA footprinting experiments using purified Cre have shown that the recombinase is not in contact with sequences outside of the inverted repeats (5). Thus the 8 bp spacer region is the only asymmetric feature of the loxP site. This has important consequences for the outcome of recombination between loxP sites. When two loxP sites on the same DNA molecule are directly repeated, recombination results in the excision of the DNA between the sites. If, however, the sites are in an inverted orientation with respect to each other, then upon recombination, the DNA between the sites undergoes inversion rather then excision. A question then is how does the asymmetry of the 8 bp

spacer region confer directionality on the site? Is the directionality achieved by DNA - DNA homology between the two recombining sites?

To address these questions we have constructed a number of mutant loxP sites containing alterations in the spacer region. These experiments have revealed that DNA-DNA homology is required but not necessarily sufficient for recombination to take place. We have also shown that a loxP site with a completely symmetric spacer region will in vitro both excise and invert DNA when it recombines with another symmetric loxP site.

MATERIALS AND METHODS

Mutant loxP sites

Mutations in loxP were constructed using chemically synthesized oligonucleotides. A 36 bp oligonucleotide was synthesized which represented the sequence of one strand of the wild-type loxP site with a CpG dinucleotide at the 5' end. Complementary strands containing a single base substitution in the spacer region were also synthesized with the dinucleotide CpG at the 5' end. Mutant and wild-type strands were then annealed. The CpG sequences at the 5' ends serve as single-stranded tails which allow direct cloning into the ClaI site of the vector pZ152. A symmetric loxP site was constructed in a similar fashion. A 36 bp oligonucleotide was synthesized in which the 8 bp spacer region was now made symmetric. Because of the self-complementary nature of the sequence, the oligonucleotide was simply heated and allowed to self anneal prior to cloning. Following transformation and outgrowth (6), transformants were selected on plates containing ampicillin. Mini-plasmid preparations (7) were made from the transformed colonies and tested for the presence of a loxP site in the vector. Mutant candidates were then confirmed by DNA sequence analysis.

Construction of loxP X loxP Plasmids

An outline of the construction of the plasmids containing two loxP sites is shown in Fig. 2. All mutant loxP sites were cloned into the unique ClaI site of pZ152 (8). To construct plasmids that contained a wild type loxP site in addition to a mutant site, a SmaI - XhoI fragment from pRH43 (9) was inserted

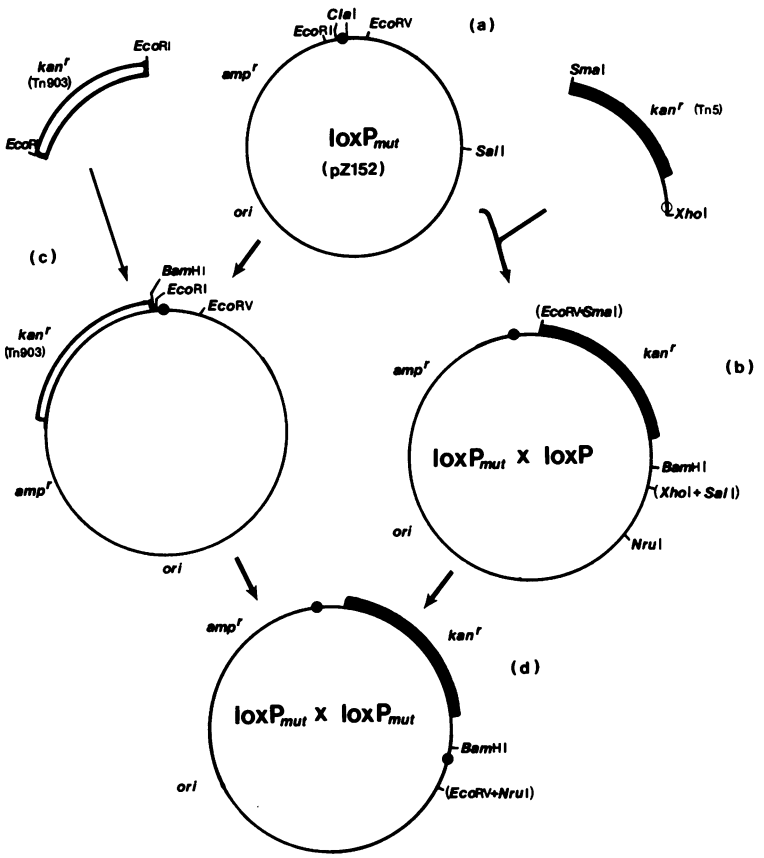

Fig. 2 Construction of loxP mutant substrates. (a) loxP mutants (●) were cloned into the ClaI site of pZ152. A fragment containing the kanamycin resistance gene of Tn5 and a wild type loxP site ( ) was then inserted to form a plasmid (b) containing both a mutant and wild type loxP sites. In a separate cloning a cassette containing the kanamycin resistance gene from Tn903 was inserted into the EcoRI site of the plasmid containing the mutant loxP site (c). This construction allowed the mutant loxP site to be moved as a BamHI-EcoRV fragment and inserted in place of the wild type loxP site resulting in a plasmid (d) containing two directly repeated mutant loxP sites.

replacing the EcoRV - SalI fragment of the vector (Fig. 2b). This inserted fragment contains the gene for kanamycin resistance from Tn5, and a wild type loxP site. Plasmids containing two mutant loxP sites were derived from this construct by digestion with BamHI and NruI to remove the wild type site, followed by

replacement of this with the appropriate mutant site contained on a BamHI - EcoRV fragment (Fig. 2d).

Assays for Recombination

The in vivo assay for recombination is described in the Results. Standard conditions for the in vitro recombination assay using purified Cre have been described previously (10).

Enzymes

Restriction enzymes were purchased from either New England Biolabs or Bethesda Research Laboratories, and were used under the conditions recommended by the vendor. Klenow fragment of DNA polymerase I of Escherichia coli was from Boehinger-Mannheim, and S1 nuclease was from Sigma.

DNA Sequencing

All plasmids used in this study were derived from pZ152; a hybrid vector containing both the pBR322 origin of replication and the M13 origin of replication (8). Infection of strains containing this vector with M13(IR-1) results in the encapsulation of single-stranded copies of the plasmid into the phage capsids. This single-stranded DNA was extracted and sequenced using the chain termination method (11). Primers for the reactions were EcoR (GTATCACGAGGCCCT) and BamH (CCACTATCGACTAC) from New England Biolabs.

S1 Reactions

Approximately 2 μg of supercoiled pRH529 were treated with 114 units of S1 nuclease in a 200 μl reaction volume at 30° C for 30 min. The reaction was terminated by the addition of stop solution (6X, 3% Sodium dodecyl sulfate, 0.13 M EDTA pH 8.0, 0.2% Bromophenol blue, 0.2% Xylene cyanole, 38% Sucrose) followed by electrophoresis on a 1% agarose gel. The resulting S1 cleaved fragments were extracted from the gel with phenol and precipitated with 2 volumes of ethanol.

RESULTS

Base Substitution Mutations

Using chemically synthesized oligonucleotides we have constructed mutant loxP sites containing single base substitution mutations in the spacer region, shown in Fig. 1. For each mutant two types of plasmids were constructed. The first type contained

Table 1 Recombination of mutant loxP sites.

Plasmid	loxP sites	Recombination in vivo[a]	in vitro[b]
pRH43	loxP x loxP	100	61
pRH515	loxP511 x loxP	0	1
pRH517	loxP514 x loxP	7	18
pRH516	loxP512 x loxP	0	0
pRH525	loxP511 x loxP511	100	68
pRH527	loxP514 x loxP514	100	65
pRH526	loxP512 x loxP512	0	0

[a]The percentage of colonies that have become sensitive to kanamycin as a result of the excision of the kanamycin resistance gene via Cre mediated recombination between loxP sites.
[b]Percentage of plasmid DNA in which recombination between loxP sites has resulted in the formation of two new products. The products of the Cre reaction were separated on agarose gels and quantitated by densitometry.

the kanamycin resistance gene from Tn5 bounded on one side by the mutant loxP site and on the other side by a wild type loxP site. Both sites were in a directly repeated orientation so that recombination between the two loxP sites would excise the kanamycin resistance gene from the plasmid. The second type of construction was similar except that the wild type loxP site was replaced by the corresponding mutant loxP site, resulting in a plasmid with two mutant loxP sites.

Plasmids were introduced into NS2114 (9), a strain expressing a functional cre gene constitutively, by transformation and selection for resistance to ampicillin. The ampicillin resistant colonies were then tested for their ability to confer resistance to kanamycin. When a plasmid containing two wild type loxP sites was tested in this manner, 100% (100/100) of the transformants were found to be sensitive to kanamycin (9). Table 1 shows the results from such an in vivo test using plasmids containing both a mutant loxP site and a wild type loxP site. The presence of the mutant sites clearly reduces the efficiency of recombination as measured by the loss of the kanamycin marker. Only the mutant change in position +2 shows a low level of recombinational activity in crosses with a wild type loxP site. The results were dramatically different when plasmids containing two identical mutant sites flanking the kanamycin marker were tested. As shown in Table 1, efficient recombination is restored when both loxP

sites are mutants at the +3 postion (pRH525) or at the +2 position (pRH527). Thus it appears that the defect observed when these mutant sites fail to recombine with a wild type site can be overcome by restoring homology during recombination. However, the mutant in position +1 is an exception to this rule, since it remains totally devoid of recombinational activity even when crossed with itself (pRH526) (Table 1).

All of the plasmids with mutant loxP sites were also tested for their ability to recombine in vitro using purified Cre protein. The results obtained in vitro parallel those observed in vivo. All mutant sites were defective in recombination with a wild type site. Sites with a mutation in position +2 and +3 recombined well with the identical site, but a site with a mutation at position +1 did not recombine with an identical mutant at position +1.

A Symmetric loxP Site

As we have shown earlier using deletion analysis (4) as well as footprinting experiments (5) the spacer region of the loxP site is the only asymmetric sequence in the site. We reasoned that the sequence of the spacer must in some way dictate the directionality of recombination. A mutant loxP site has been constructed which contains a spacer region which is now symmetric (Fig. 1). Essentially the 17 bp to the left of the axis of dyad symmetry of the site were inverted resulting in a perfect 34 bp palindromic sequence.

Plasmids were constructed similar to those used for testing the base substitution mutants, and were assayed in vivo and in vitro. Constructs containing a symmetric loxP site and a wild type loxP site (pRH518) failed to show any recombination either in vivo or in vitro (Table 2). This result is not surprising in light of the results obtained with the single base substitution mutations. Since the loxP symmetric site and the wild type loxP site do not share complete homology over their spacer regions, they would not be expected to recombine with each other.

A plasmid containing two symmetric loxP sites (pRH529) was also constructed. When transformed into NS2114 the in vivo efficiency, as measured by the loss of kanamycin resistance, was that of two wild type sites recombining (Table 2). While the two

Table 2 Recombination of symmetric loxP sites

Plasmid	loxP sites	Recombination in vivo[a]	Recombination in vitro[b]
pRH43	loxP X loxP	100	65
pRH518	loxP X loxPsym	0	0
pRH529	loxPsym X loxPsym	100	0[c] 54[d]

[a]The percentage of colonies that have become sensitive to kanamycin as a result of the excision of the kanamycin resistance gene via Cre mediated recombination between loxP sites.
[b]Percentage of plasmid DNA in which recombination between loxP sites has resulted in the formation of two new products. The products of the Cre reaction were separated on agarose gels and quantitated by densitometry.
[c]Amount of recombination observed when the initial substrate molecule is supercoiled.
[d]Amount of recombination observed when the plasmid substrate is linearized by restriction enzyme digestion prior to the in vitro recombination reaction.

symmetric loxP sites appear to undergo a very efficient excision reaction the inversion product, which would theoretically be expected with equal frequency, has not been detected in vivo. We believe the reason is that even if inversion can occur as frequently as excision, the inversion product has a high probability of undergoing a second round of intramolecular recombination that could lead to either inversion again or excision. Conversely, the excision products have a low probability of undergoing an intermolecular recombination to restore the original plasmid or the inversion product. In addition, the excision product containing kanamycin resistance is unable to replicate and is diluted out of the population. Thus, with time, the excision product containing ampicillin resistance and the origin of replication becomes the majority plasmid in the population. The behavior of the plasmid containing two symmetric loxP sites was also tested in an in vitro Cre reaction. A rather surprising result was that starting with a supercoiled plasmid no recombinant products could be detected, either as free supercoiled products or catenated products. However, if the plasmid is linearized prior to incubation with Cre, then recombinant products can be observed (Fig. 3). These recombinant products included both the expected excision product (lane B) as well as the inversion product (lane C). Both the excision reaction and

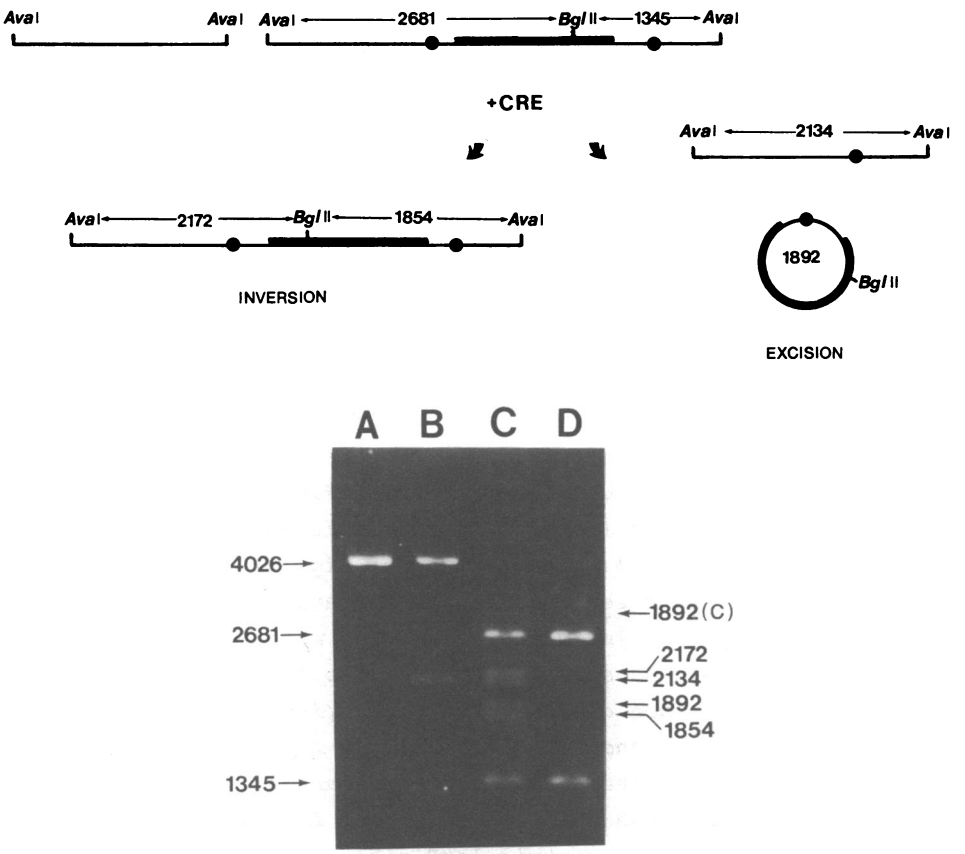

Fig. 3 Recombination of symmetric loxP sites results in both excision and inversion. Plasmid pRH528 was digested with AvaI and the larger (4026 bp) fragment containing the two symmetric loxP sites was gel purified (lane A). When incubated with Cre two new bands appear, a 2134 bp linear fragment, and a 1892 bp (c) circle (lane B). If the 4026 bp fragment is digested with BglII, two fragments of 2681 bp and 1345 bp are produced (lane D). When the 4026 bp fragment was first treated with Cre and then subsequently digested with BglII, four new bands 2172 bp, 2134 bp, 1892 bp, and 1854 bp, appeared (lane C). As shown in the drawing these would be the expected products if both excision and inversion were taking place.

the inversion reaction appear to work with comparable efficiency. Overall, the symmetric loxP sites recombine with one another in vitro with efficiencies comparable to those observed for two wild type loxP sites (Table 2).

The question arises as to why a supercoiled plasmid containing two symmetric loxP sites shows no detectable recombination in vitro. One possibility is with the increase in symmetry, the symmetric loxP site is more likely to be extruded into a cruciform structure then the wild type loxP site, and Cre is unable to act on a loxP site that is a cruciform. Previous work has shown that cruciforms extruded in supercoiled plasmid DNA are sensitive to cleavage by S1 nuclease (12,13). To test the hypothesis that a supercoiled plasmid containing two symmetric loxP sites has the loxP sites extruded as cruciforms,pRH529 was cleaved with nuclease S1 so as to linearize the plasmid. Interestingly, while about half of the DNA appeared to be full length linear, the remainder formed two discrete smaller sized fragments of 4850 bp and 1900 bp in size (Fig. 4, lane A). The sizes of these smaller fragments were consistent with S1 cleaving at both loxP sites (See schematic top of Fig. 4). To confirm this, both the linearized full length DNA and the two smaller fragments were gel isolated and restricted with the appropriate restriction enzyme. As shown in Fig. 4, the full length linear fragment (lane D) when cleaved with BglII (lane E) resulted in fragments of 1200 bp and 700 bp as well as fragments of 6050 bp and 5550 bp (not resolved on this particular gel). This would indicate that the large full length linear fragment results from S1 cleavage at either symmetric loxP site. The two smaller S1 generated fragments (lanes F and H) were also cleaved with the appropriate restriction enzyme. The 4850 bp fragment was cleaved with PstI (lane G), resulting in two fragments 4100 bp and 750 bp in size. The 1900 bp fragment when cleaved with BglII (lane I) yields fragments of 1200 bp and 700 bp in length. These results are consistent with the two smaller S1 cleavage products (4850 bp and 1900 bp) being a consequence of simultaneous cleavage by S1 at both loxP sites. If the same experiments are done with a plasmid containing a wild type loxP site, very little S1 cleavage at the loxP site is observed compared to the pBR322 major S1 cleavage site (data not shown). It seems reasonable to conclude that the inhibition of recombination in vitro using supercoiled DNA containing the symmetric loxP sites is a result of cruciform extrusion.

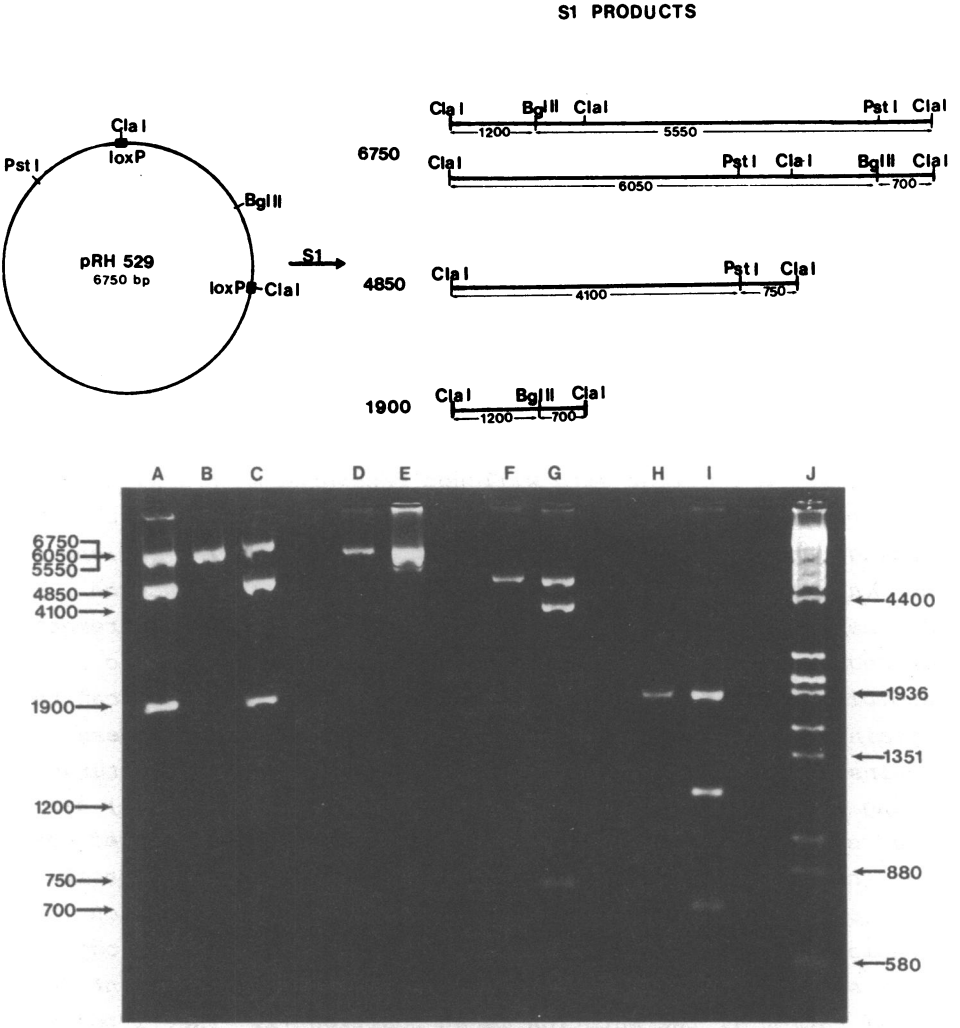

Fig. 4 The symmetric loxP sites are sensitive to S1 nuclease. Plasmid pRH529 was cleaved with S1 nuclease (lane A) resulting in a full length linear fragment (6750 bp) as well as two discrete smaller fragments (4850 bp and 1900 bp). Following gel purification of each of these fragments shown in lanes D, F, and H respectively, each was restricted with the appropriate diagnostic restriction enzyme. The full length linear was digested with BglII (lane E), the 4850 bp fragment with PstI (lane G), and the 1900 bp fragment with BglII (lane I). Digests of the gel isolated fragments were incomplete, so the undigested fragment is present in each of these lanes. Molecular weight markers are: lane B, pRH529 digested with BglII; lane C, pRH529 digested with ClaI; and lane J, bacteriophage lambda digested with HinIII and EcoRI.

DISCUSSION

Previously we have argued for the importance of the loxP spacer region as a key element in Cre mediated site-specific recombination. The spacer region serves as both substrate for Cre cleavage (3) as well as imparting directionality to loxP recombination (4,5). A number of mutant loxP sites have been constructed which change the sequence within the 8 bp spacer region. The behavior of these mutants during recombination has led us to a number of important observations regarding the mechanism of recombination.

First, sequence homology in the 6 bp overlap region is a requirement for recombination between loxP sites. This has been demonstrated by the base changes at positions +2 and +3, where a mutant site will not recombine with a wild type site, but a mutant site will recombine with itself. These findings are analogous to those found for the overlap regions of the λatt site (14,15), the yeast 2 μFlp site (16), and the Salmonella Hin system (17). Thus it seems that the requirement for sequence homology appears common to many site specific recombination systems. How this requirement for homology can be explained in physical terms during the recombination process remains unclear. Models invoking four stranded DNA structures during synapsis (18,19,20), or Holliday intermediates (14) all require homology for their formation. It should be pointed out that the mutations rescued by homology both represent transition mutations. Future work will determine whether transversion mutations in these positions can also be rescued by homology.

A second observation is that homology is not sufficient for recombination if the mutation is at position +1. The +1 position then appears critical for recombination. If one aligns the two halves of the loxP site and derives a consensus sequence, this is the only conserved position in the spacer region. Why is this position a critical one? Perhaps it is a base where there is an essential specific contact between nucleic acid and protein. Footprinting data suggests that at least some of the spacer region must be in contact with Cre (5). A second possibility is that certain sequences themselves play a crucial physical role during the recombination process. Note that the +1 position and

its mirror image across the dyad axis of symmetry, position -1, form the dinucleotide TpA. Recently Drew and coworkers have proposed that this sequence is predisposed to melting (21). This offers the attractive possibility that the TpA sequence is critical for unwinding of the loxP site either during the pairing or the strand exchange step of recombination.

We have presented here additional evidence that indeed the sequence of the spacer region dictates the directionality of loxP recombination. By creating a loxP site with a completely symmetric spacer region, recombining sites now are capable of both excision and inversion in the same construct. Since both occur with approximately equal efficiency we assume the choice is simply the way in which the two loxP sites come together. The in vitro experiments indicate that Cre will not act on loxP symmetric sites if they are extruded as cruciform structures, as in the case when the initial substrate is a supercoiled plasmid. The fact that the supercoiled substrate recombines in vivo is not surprising since it is unlikely that cruciform structures such as this form efficiently in vivo (22,23).

Finally, these results suggest an interesting way in which a site-specific recombination system could potentially undergo diversification. For instance, we have already created two new functional loxP sites, as represented by the changes at positions +2 and +3, where a mutant site can only recombine with itself. Thus, one could imagine that a cell could potentially have multiple sets of loxP sites, perhaps one set controlling an invertible segment of DNA while another set would be responsible for excising a piece of DNA. Neither set would be able to interact with the other, and yet both sets would recombine using the identical recombinase. This suggests an alternative to changing protein structure in order to change specificity of the system.

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