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Site-specific recombination by the β protein from the streptococcal plasmid pSM19035: minimal recombination sequences and crossing over site

Inés Canosa, Fernando Rojo and Juan C. Alonso*

Centro Nacional de Biotecnología, CSIC, Campus de la Universidad Autónoma de Madrid, Cantoblanco, 28049 Madrid, Spain

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ABSTRACT

The β recombinase from the broad host range Gram-positive plasmid pSM19035 catalyzes intramolecular site-specific recombination between two directly or inversely oriented recombination sites in the presence of a chromatin-associated protein (Hbsu). The recombination site had been localized to a 447 bp DNA segment from pSM19035. This segment includes a 90 bp region that contains two adjacent binding sites (I and II) for β protein dimers. Using *in vitro* recombination assays, we show that this 90 bp region is necessary and sufficient for β protein-mediated recombination; this defines the *six* site as the region required for β protein binding. The point of crossing over has been localized to the center of site I. Hbsu has a strong binding affinity for an unknown site located within the 447 bp segment containing the *six* site. We discuss the possibility that Hbsu recognizes an altered DNA structure, rather than a specific sequence, generated in the synaptic complex.

INTRODUCTION

The β recombinase, encoded by the Gram-positive broad host range plasmid pSM19035, mediates the conversion of plasmid multimers into monomers (DNA resolution) to maximize stable plasmid inheritance and is also involved in an inversion process probably required for correct plasmid replication (1–3). In the presence of the *Bacillus subtilis* chromatin-associated protein Hbsu, the purified β recombinase is able to catalyze both deletions (resolution) and inversions between two specific recombination sites, depending on their relative orientation (2,3). The role of Hbsu protein is to facilitate the formation of the synaptic complex, since in the absence of Hbsu, synaptic complexes are not formed (4). The chromatin-associated proteins HU from *Escherichia coli* or HMG-1 from mammals can substitute for Hbsu in the recombination reaction (4). HMG-1 shares neither sequence nor structural homology with Hbsu. Therefore, it is likely that these chromatin-associated proteins work by recognizing and stabilizing a bent or altered DNA structure at the recombination site, rather than acting as a bridge between β recombinase dimers through protein–protein interactions (4).

Sequence homology comparisons allow the inclusion of the β protein into the Tn*3* family of DNA recombinases (2,5), which includes three subfamilies: DNA resolvases, DNA invertases and resolvase-invertases. The β recombinase and the highly related Resβ protein of plasmid pAMβ1 are the only members of the last group that have been analyzed *in vitro* (2,3,6). DNA resolvases are highly specialized in catalyzing deletions between two directly oriented recombination sites, named *res* sites (reviewed in 7,8). DNA invertases mediate inversions between two inversely oriented target sites, but deletions between two directly oriented sites occur at a very low frequency (reviewed in 9,10). The β recombinase does not have such bias and can catalyze both deletions (resolution) and inversions between two specific recombination sites with comparable efficiency, depending on their orientation (2,3).

DNA resolvases bind to a 120 bp DNA segment termed *res* containing three adjacent binding sites with dyad axis symmetry (I, II and III; 11). DNA resolution occurs between two directly oriented *res* sites, at the center of site I. All three sites are required for efficient recombination, but no additional factors are needed (12; reviewed in 7,8). DNA invertases bind to a 26 bp site, within which recombination occurs, and require a 60 bp recombination enhancer sequence in *cis*, to which the Fis protein binds (reviewed in 9,10). The recombination site (*six* site) for β recombinase has been localized to a 447 bp DNA sequence immediately downstream of the plasmid replication origin (2; see Fig. 1). The β protein, which is a dimer in solution, binds cooperatively to two adjacent sites, named I and II, located within the *six* site (2; see Fig. 1). This binding does not require Hbsu or any other factor (2). By using different footprinting techniques, the sequences required for β protein binding have been shown to include a 10 bp inverted repeat at site I, with a 14 bp spacer and a non-symmetrical 32 bp long sequence at site II (Fig. 1; 13). We have now investigated whether the recombination determinants are just the sequences required for β protein binding or if additional DNA sequences are required. This information could be particularly relevant in unravelling the way in which Hbsu helps to form the synaptic complex. Hbsu protein, which is needed in stoichiometric amounts, can be competed out from the β-mediated synaptic complex by an excess of a supercoiled DNA containing the *six*

* To whom correspondence should be addressed

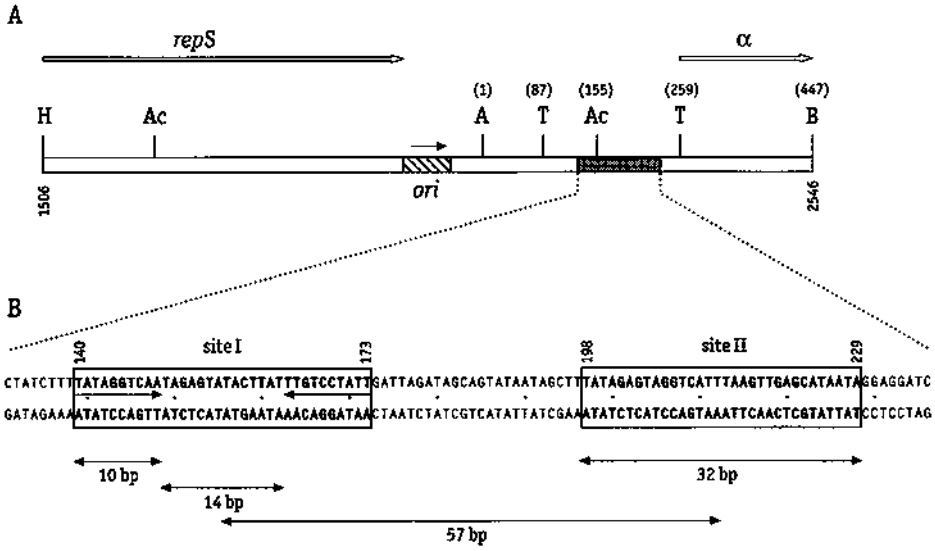

Figure 1. The β recombinase binding site of plasmid pSM19035. (**A**) Location of the β recombinase binding site. The positions of the *rep*S gene, the origin of replication (*ori*), the 447 bp *Ase*I–*Bbr*PI segment containing the *six* site (the *Ase*I site is taken as position 1 of this segment, while the *Bbr*PI site would be at position 447), the β protein binding site (filled) and *orf*α are indicated. Reading frames are indicated by open arrows and the filled arrow shows the direction of replication of the plasmid. Relevant restriction sites are shown. Coordinates on both ends of the DNA segment refer to the published sequence of pSM19035 (28). (**B**) Nucleotide sequence of the β protein binding region. Sites I and II for β recombinase binding, as defined by chemical footprinting (13), are boxed and in bold. The inverted repeated sequences recognized by β protein at site I (13) are denoted by convergent arrows. The lengths of selected segments are indicated. Numbering of positions at the *six* site refers to the cutting site for endonuclease *Ase*I. A, *Ase*I; Ac, *Acc*I; B, *Bbr*PI; H, *Hin*dIII; T, *Taq*I.

site, but not by a DNA lacking the *six* site (4). This suggests that Hbsu has a marked binding preference for a site located within the 447 bp DNA region from pSM19035 that contains the *six* site. Determination of the minimum sequences required for recombination could unravel whether Hbsu binds to a specific sequence. The results presented show that β-mediated DNA resolution requires two 90 bp recombination sites in direct orientation that contain sites I and II for β protein binding. This would be the smallest recombination site described so far for a DNA resolvase of the Tn*3* family. Furthermore, this suggests that no specific sequences are needed for Hbsu binding and that Hbsu would perform its task by recognizing or stabilizing an altered DNA structure at the recombination site. We have also investigated where the recombination reaction occurs; we found that it takes place at the center of site I.

MATERIALS AND METHODS

Bacterial strains and plasmids

The *Escherichia coli* strain XL-1 Blue was used (15). Plasmids pCB8 and pUC18Ω have been described previously (2). Plasmid pBT316A contains two directly oriented *six* sites, one from plasmid pSM19035 and the other from the highly related plasmid pAMβ1, separated by a marker gene coding for resistance to phleomycin. Plasmids pCB61, pCB62, pCB63, pCB64, pCB66 and pCB67 were obtained from pCB8 by making nested deletions with the exoIII enzyme in one of the two 447 bp DNA segments that include the *six* site (deletions were performed from the *Pst*I site; see Fig. 2). The deletion end points were determined by nucleotide sequencing. Deletions of 4 (pCB61), 27 (pCB62), 110 (pCB63), 121 (pCB64), 178 (pCB66) or 195 bp (pCB67) were obtained; numbering of positions considers the cutting site for *Ase*I as position 1 (see Figs 1 and 2B).

To obtain a DNA fragment containing just the minimal β protein binding sequences two partially complementary oligonucleotides containing sites I and II for β protein binding were synthesized. One of them (55mer) included positions 142–195 of the top strand and the second one (60mer) positions 174–233 of the bottom strand (see Fig. 1B). The oligonucleotides included two modifications. (i) By changing positions T189 to C and A190 to T, a new *Sca*I restriction site was introduced into the –10 box of the promoter for the *orf*α gene β operon, located between sites I and II (see Fig. 3; 14). This mutation ameliorated the deleterious effect of the promoter on the plasmid vector. (ii) The first two bases of the inverted repeat of site I were modified by changing positions T140 to A and A141 to C. The two oligonucleotides were allowed to hybridize and the recessive 3′-ends were filled-in with dNTPs and the Klenow enzyme. The resulting 91 bp dsDNA fragment (coordinates 142–233) was cloned into the *Sal*I site of plasmid pUC18Ω after filling in the ends as above. Two plasmids were obtained: in pCB103, the direction of the β protein binding site in the multicloning region was *Hin*dIII–site I–site II–*Eco*RI, while in pCB31 the direction was the opposite. Plasmid pCB105 was obtained by substituting the 447 bp *six* site of plasmid pCB8 located between sites *Afl*III (blunted) and *Kpn*I by a 136 bp *Kpn*I–*Hin*dIII (blunted) fragment from pCB103, containing the 91 bp *six* site. The two missing bases forming site I in plasmid pCB103 (bases 140 and 141) were re-introduced by substituting the 100 bp *Eco*RI–*Acc*I fragment including the upstream part of site I by an equivalent fragment from plasmid pREST that contains a complete site I (13), thereby obtaining plasmid pCB104. Plasmid pCB51 was obtained by cloning a 120 bp *Sca*I–*Kpn*I fragment from pCB104 into *Eco*RI (filled in)/*Kpn*I-cleaved-pCB8. Plasmid pCB106 derives from pCB105, by

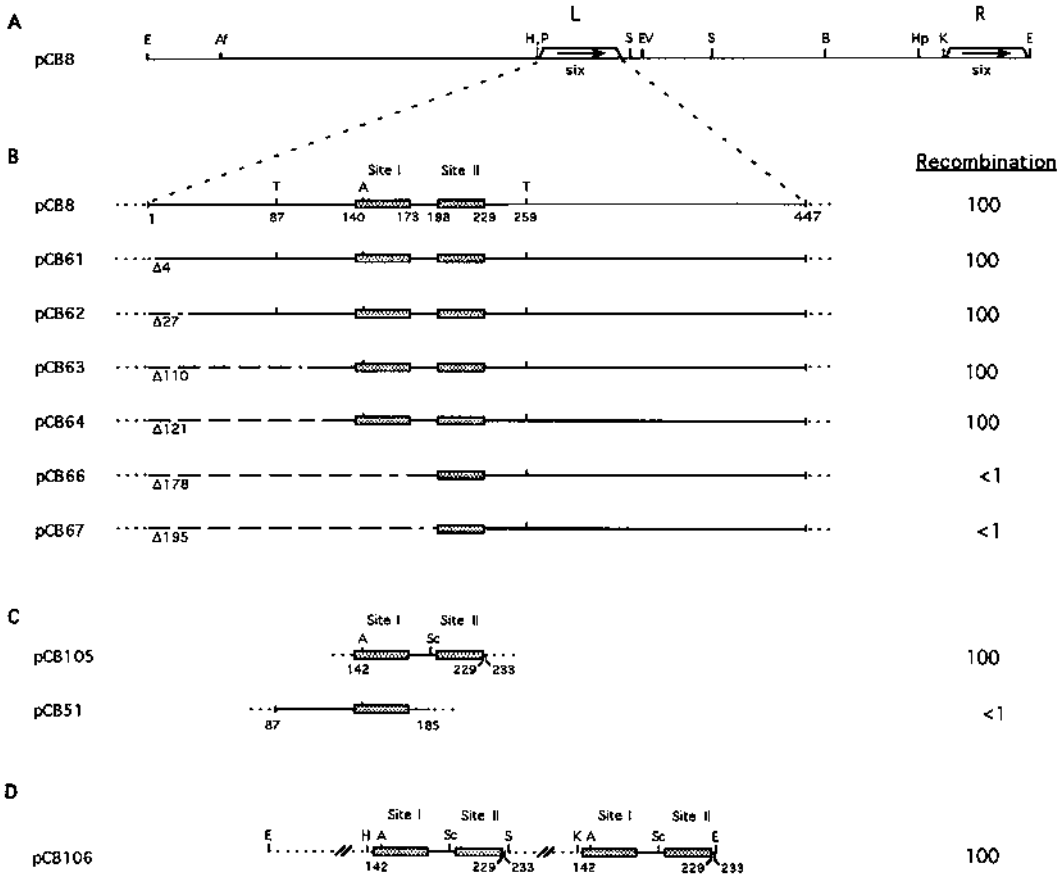

Figure 2. Determination of the minimal sequences required for β protein-mediated DNA recombination. (**A**) Map of the parental plasmid used, pCB8, which contains two identical 447 bp *Ase*I–*Bbr*PI *six* sites (marked L and R) located in direct orientation (open boxes with an arrow; see also Fig. 1). (**B**) Derivatives of plasmid pCB8 with deletions upstream of site I or affecting site I, within the left (L) *six* site. Only the 447 bp region in which the deletions were performed is shown; the other region remained unchanged. The dotted line indicates vector sequences and the discontinuous line denotes the sequences eroded by the exoIII enzyme. The shaded boxes correspond to sites I and II for β protein binding. Relevant positions are shown; numbering is as indicated in Figure 1. The ability of these plasmids to serve as recombination substrates for β protein is shown on the right hand side, expressed as percent relative to that observed for plasmid pCB8. (**C**) Derivatives of plasmid pCB8 in which one of the 447 bp boxes was substituted either by a 91 bp DNA fragment containing exclusively sites I and II for β protein binding (plasmid pCB105) or by a DNA segment containing only site I (plasmid pCB51). The first 2 bp of site I as defined by hydroxyl radical footprinting (13) are missing in plasmid pCB105. (**D**) Plasmid pCB106 contains two directly oriented 91 bp DNA segments containing sites I and II for β protein binding. A, *Acc*I; Afl, *Afl*III; B, *Bam*HI; E, *Eco*RI; EV, *Eco*RV; H, *Hin*dIII; Hp, *Hpa*I; K, *Kpn*I; S, *Sal*I; Sc, *Sca*I; T, *Taq*I.

substitution of the 447 bp region containing the *six* site (excised with *Hin*dIII and *Bam*HI) by the 91 bp region containing the *six* site (positions 142–233), obtained from pCB31 as a 127 bp *Hin*dIII–*Bam*HI fragment.

Proteins and enzymes

Protein β was purified and its concentration determined as previously described (2,16). Purified Hbsu protein was a gift from Prof. U. Heinemann (Max-Delbrück-Centrum für Molekulare Medizin, Berlin, Germany). Since both proteins are dimers in solution, their concentration is expressed as mol of protein dimers.

In vitro assays for site-specific recombination

Reaction mixtures to assay site-specific recombination contained 10.6 nM substrate plasmid, 20–335 nM purified β protein, 100 nM purified Hbsu protein, 50 mM Tris–HCl, pH 7.5, 50 mM NaCl, 10 mM $MgCl_2$, in a total volume of 25 µl. Reactions were initiated by the addition of β protein and incubated at 30°C for 30 min. To analyze the recombination products, the DNA was digested with two restriction enzymes, one cutting at the multicloning site of the vector (*Pst*I or *Hin*dIII) and the other one cutting at the region lying between the two *six* sites (*Sal*I, *Eco*RV or *Hpa*I). The DNA fragments generated were analyzed by agarose gel electrophoresis. The relative amounts of DNA present in any particular band were determined by laser scanning densitometry of photographic negatives; negatives corresponding to different exposure times were used to ensure linearity of the response with respect to DNA concentration.

RESULTS

Minimal sequences for β protein-mediated DNA recombination

We have previously shown that two copies of the 447 bp *Ase*I–*Bbr*PI DNA segment from plasmid pSM19035, containing a binding region for the β recombinase, are necessary and sufficient for β protein-mediated DNA recombination; the 447 bp

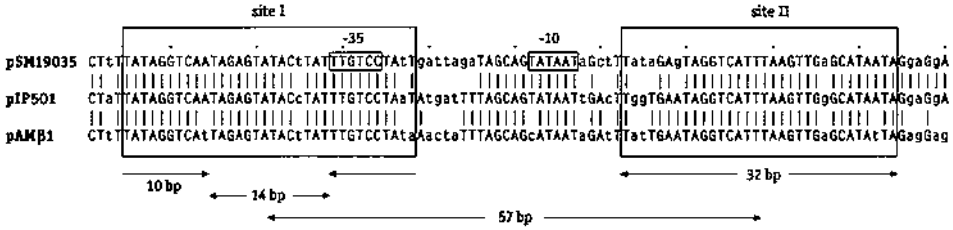

Figure 3. Comparison of the recombinase binding sites of the *inc*18 plasmids pSM19035, pIP501 and pAMβ1. These three plasmids encode similar resolution systems. The β recombinase from pSM19035 is 89% identical to Resβ from plasmid pAMβ1, which is in turn 93% identical to ResIP from plasmid pIP501. Non-identical sequences are shown in lower case. The cognate sites for the β protein (boxed) are conserved among the *inc*18 plasmids. The –35 and –10 boxes for the promoter for the *orfα* gene β in pSM19035 are indicated. The inverted repeat recognized by the β recombinase at pSM19035 site I is shown with arrows at the bottom of the figure; relevant distances are also indicated.

segment was termed the *six* site (2; see Fig. 1). Within this DNA fragment, the sequences specifically recognized by the β recombinase were localized to a 90 bp region (2,13; Fig. 1). It was unknown, however, whether the 90 bp target segment is sufficient to support the recombination reaction or if the rest of the sequence of the 447 bp DNA fragment is also required. Plasmid pCB8 contains two of the 447 bp *six* sites in direct orientation, separated by a DNA region ~2.2 kb in length (Fig. 2A), and is an efficient substrate for β protein-mediated recombination (2). To determine which are the minimal sequences required for recombination, one of the above-mentioned 447 bp DNA segments from plasmid pCB8 was sequentially reduced upstream of site I and downstream of site II.

Sequences upstream of site I were progressively eroded with exoIII on the 447 bp *six* site located on the left in Figure 2A (marked L), leaving the other 447 bp *six* site unaltered (marked R in Fig. 2A). The ability of the resulting plasmids to serve as substrates for β protein-mediated recombination was analyzed *in vitro* (see Fig. 2). In short, the deletions obtained upstream of site I, which eliminated up to 121 bp (plasmid pCB64), had no apparent effect on reaction efficiency. Deletion of site I, however, totally abolished recombination (see plasmids pCB66 and pCB67). The effect of sequences downstream of site II was analyzed by generating a synthetic 91 bp segment lacking all sequences downstream of site II (positions 234–447) and upstream of site I (positions 1–141) but containing sites I and II for β protein binding (positions 142–233). In addition, this synthetic site contained two point mutations in the spacer region between sites I and II that generate a *Sca*I restriction site (see Fig. 2C). This 91 bp DNA fragment was used to replace the left 447 bp *six* site of plasmid pCB8, generating plasmid pCB105. This plasmid was as good a substrate for β-mediated recombination as the parental pCB8; no difference in recombination efficiency could be detected under the conditions used (not shown). Complete deletion of site II, leaving an intact site I (plasmid pCB51), totally abolished β-mediated recombination (Fig. 2C). Substitution of the two 447 bp *six* sites of pCB8 by the 91 bp DNA segment that contains just the β recombinase binding site (positions 142–233, plasmid pCB106) also gave an active recombination substrate (Fig. 2D). Therefore, binding sites I and II are strictly required for efficient recombination, but the sequences upstream of site I or downstream of site II are not and can be substituted by unrelated sequences with no apparent effect on *in vitro* reaction efficiency. These results allow the reduction of the *six* site to the 91 bp segment comprising positions 142–233. Since positions 231–233 are not conserved in the equivalent region of the related plasmid pAMβ1 (Fig. 3), which can also serve as substrate for β-mediated recombination (see below), we can reduce the minimal *six* site to the 89–90 bp segment required for the binding of β protein to DNA.

Strand exchange takes place at site I

The precise position within the 447 bp recombination region at which the crossing over reaction takes place was unknown. To localize it more precisely, we constructed plasmid pBT316A, which contains two similar, but not identical, *six* sites in direct orientation, one of them derived from plasmid pSM19035 and the other from the related plasmid pAMβ1. Plasmids of the *inc*18 incompatibility group (pSM19035, pAMβ1 and pIP501) code for a recombinase (2,17) and recombination regions (13,17,18) that are very homologous (~92%; see Fig 3). The β protein from pSM19035 can bind to the recombination site of plasmid pAMβ1 (2), which suggests that it can recognize it as a recombination substrate. As in pSM19035, the recombination site of plasmid pAMβ1 is composed of two binding sites for the recombinase, named R1-R2 and R3 (6). Site R1-R2 is equivalent to site I of pSM19035, while R3 corresponds to site II for the β protein of pSM19035 (2,6,13). Although sites I and R1-R2 are very similar, some differences are present in the sequences located immediately upstream and the homology decreases considerably downstream of these sites (Fig. 3; 16). On the basis of these differences, we expected that the sequence of a recombination product between the two recombination sites of pBT316A would allow a more precise delimitation of the region of crossing over.

An *in vitro* recombination reaction was carried out with plasmid pBT316A, β recombinase and Hbsu under standard conditions and the reaction products were transformed into *E.coli*. Since recombination should eliminate a DNA region lying between the two recombination sites that includes a marker gene conferring resistance to phleomycin, recombinant plasmids were easily discriminated from parental plasmids on the basis of sensitivity to phleomycin (the plasmid vector confers resistance to ampicillin). Two such plasmids were purified and the nucleotide sequence of their unique *six* site was determined and compared with that of the *six* sites of plasmids pAMβ1 and pSM19035 (Fig. 4A). Sequences immediately upstream of the left arm of the inverted repeat of site I belonged to pSM19035, while the last base of the right arm and those lying downstream of it corresponded to pAMβ1 (Fig. 4A). This indicates that recombination had taken place somewhere in site I. Since pSM19035 site I is almost identical to pAMβ1 site R1-R2, the

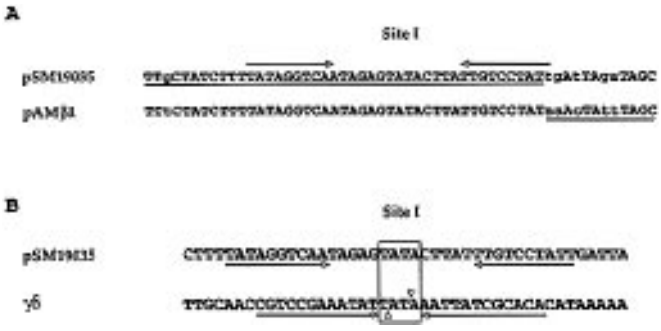

Figure 4. Determination of the site of strand exchange. (**A**) Location of the crossing over site on plasmid pBT316A, which contains two directly oriented *six* sites, one from pSM19035 and the other from the related plasmid pAMβ1. Sites I for recombinase binding of pSM19035 and pAMβ1 are shown. Nucleotides that are different in the two plasmids are indicated in lower case. After β-mediated recombination using pBT316A as substrate, the unique *six* site of the recombinant plasmid obtained was sequenced. The resulting sequence is underlined. (**B**) Comparison of site I for β protein with site I for the γδ resolvase, which is known to include the site of strand exchange. The arrowheads indicate the positions at which the γδ recombinase introduces staggered cuts to mediate the strand exchange (20). The inverted repeated sequences are indicated and the conserved TATA sequence is boxed.

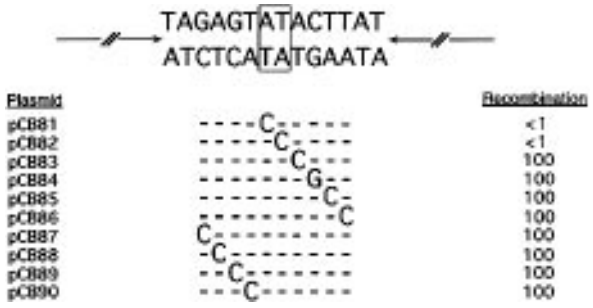

Figure 5. Fine mapping of the nucleotides critical for strand exchange. The nucleotides located in the 14 bp spacer between the arms of the inverted repeat recognized by β protein at site I are indicated on top of the figure. The nucleotides of this spacer region that were sequentially substituted by a different residue are presented in the central part of the figure; dashes indicate unmodified positions. Individual 447 bp *six* segments containing each of the mutated sites I were used to replace one of the wild-type 447 bp recombination segments of plasmid pCB8, generating the plasmids indicated on the left side of the figure. The ability of each of these plasmids to serve as substrate for β protein-mediated recombination was measured *in vitro*; the results are shown on the right of the figure, expressed as percent relative to that observed for the wild-type parental substrate (plasmid pCB8). The two nucleotides whose modification rendered substrates inactive are boxed.

assay cannot indicate the precise crossing over point within site I. The sequences upstream of the left arm of the site I inverted repeat are not required for recombination (see above). In addition, the central 4 nt of the pSM19035 site I are identical to the equivalent regions of site I for the γδ and Tn*3* resolvases. These two enzymes are known to produce staggered cuts at this position (see Fig. 4B). It is likely, therefore, that the β protein produces the strand exchange somewhere in the spacer regions between the two arms of the site I inverted repeat. We further analyzed this possibility.

Positions at site I critical for strand exchange

The above results suggest that the point of crossing over should lie within the short spacer sequence that separates the two half-sites recognized by the β protein. In the case of the γδ and Tn*3* resolvases, the two central nucleotides of the corresponding site I, at which the staggered cuts are introduced, are critical for the recombination reaction and their mutation drastically reduces the formation of recombination products (19). Therefore, determination of the nucleotides essential for β protein-mediated recombination should help to locate the point of crossing over. To this end, every single nucleotide in the center of the spacer separating the two arms of the β protein recognition target in site I was modified by site-directed mutagenesis to cytosine, except at the position where a cytosine was already present, which was modified to guanine (see Fig. 5). In each case, a plasmid was constructed containing two *six* sites in direct orientation, one of them with wild-type sequences and the other with a mutated position (plasmids pCB81–pCB90). The absence of unexpected mutations at the *six* sites of the resulting plasmids was confirmed in all cases by nucleotide sequencing. The efficiency of each of these plasmids as a substrate for β-mediated recombination was analyzed *in vitro* under standard conditions. As shown in Figure 5, all except the two central positions could be modified without impairing the recombination reaction; reaction efficiency was not altered either (data not shown). Modification of the central 5′-AT-3′ nucleotides gave substrates that were totally inactive for recombination (see Fig. 5).

DISCUSSION

Previous studies had indicated that β protein-mediated recombination requires a 447 bp DNA segment from plasmid pSM19035, a segment that contains two adjacent binding sites for the recombinase (2). Chemical footprinting assays delimited the target sequences for the β protein to a 90 bp segment (coordinates 140–229) located within the 447 bp DNA fragment (13). The results presented in this work indicate that the sequences that are necessary and sufficient for the recombination reaction lie in a 89 bp DNA segment (coordinates 142–230) that coincides with the β recombinase binding site. Sequences upstream of binding site I or downstream of binding site II could be modified without significantly affecting reaction efficiency. The first two bases of site I (coordinates 140 and 141) were dispensable for recombination. Though these positions are contacted by the β protein, they are not necessary for protein binding (see 13). Both binding sites I and II were essential for the recombination reaction: elimination of either of them from just one of the two recombination sequences of the substrate plasmid abolished DNA recombination. Therefore, we can define the minimum *six* site as that containing the target sequences for the β recombinase, i.e. the DNA segment including sites I and II. This is consistent with the *six* site suggested for the Resβ recombinase of the pAMβ1 plasmid (6).

These results are particularly interesting in understanding how the accessory protein Hbsu participates in assembly of the recombination complex. About 1–2 Hbsu dimers/DNA molecule are enough to activate the recombination reaction (3), which suggests that it is binding with high affinity to a particular region of the DNA. Since in the presence of the β protein, binding of Hbsu can be competed out by a supercoiled DNA containing the 447 bp DNA segment that includes the *six* site, but not by a DNA lacking these DNA regions, it was hypothesized that Hbsu should bind with high affinity to a site located in this DNA segment (4).

A similar assay with a supercoiled DNA containing just the minimal 91 bp recombination region including sites I and II gave the same result (not shown). Nevertheless, no binding was observed by footprinting techniques when using a linear DNA fragment spanning positions 87–259, irrespective of the absence or presence of the β protein (13). Our results showing that the DNA sequences upstream of site I and downstream of site II can be substituted by unrelated DNA make it very unlikely that Hbsu is recognizing a specific sequence outside the *six* site. Enzymatic (2) and chemical (13) footprinting analyses suggest that between β protein binding sites I and II there is a sequence, 24 bp in length, that is not contacted by the two β protein dimers. It is also unlikely that Hbsu recognizes a specific sequence in this region, for the following reasons: (i) 16 of the 24 bp are not conserved in plasmid pAMβ1 (Fig. 3), whose recombination site is very homologous to that of pSM19035 and supports recombination with the pSM19035 β protein in the presence of Hbsu; (ii) we have shown here that we can modify two of the conserved positions in this 24 bp region (T189 and A190) without affecting β protein-mediated recombination. Therefore, up to 18 of the 24 bp can be modified with no detectable effect on recombination. This means that it is very unlikely that Hbsu can recognize a specific sequence in this DNA region, unless recognition relies more on DNA deformability or structure than on the precise identity of the nucleotide sequence. In agreement with this idea, although the HU and Hbsu proteins are known to bind DNA with little or no sequence specificity, they are known to have a clear binding preference for bent, kinked or distorted DNA sequences, irrespective of their precise nucleotide composition (21,22). The mammalian HMG-1 protein, sharing neither sequence nor structural homology with Hbsu, can substitute for Hbsu in the recombination reaction (4), which argues against a model in which Hbsu is acting as a bridge between β protein dimers bound at the recombination sites. Therefore, our results suggest that Hbsu may bind and stabilize an altered DNA structure that is formed at the recombination site upon assembly of the synaptic complex. In principle, this structure could arise either upstream of site I, downstream of site II or, perhaps, at the small region that lies between both sites. Hbsu would bind efficiently to this target, but recognizing a DNA structure rather than a specific sequence. Indeed, the HU protein has been shown to help to stabilize higher order protein–DNA complexes in a number of cases (23–27) and it has been proposed to do it through its ability to stabilize altered DNA structures.

On the other hand, we present evidence indicating that the strand exchange takes place at the center of site I. Mutation of the two central nucleotides of site I in one of the two directly oriented recombination sequences of the substrate plasmid gave DNA molecules that were unable to generate recombination products. These nucleotides are located at the center of the spacer of the inverted repeat that forms the β protein recognition sequences. Mutations at other positions in this spacer had no effect on recombination. It is likely, therefore, that the two central nucleotides are directly involved in the strand exchange reaction and that the recombination reaction occurs by a mechanism that is probably similar to that described for other recombinases of the Tn*3* family. The mechanism has been most extensively studied for the γδ and Tn*3* DNA resolvases. As stated in the Introduction, both resolvases binds to three adjacent sites (I, II and III) and crossing over takes place at the center of site I (20). Mutations introduced at the central positions of γδ or Tn*3* site I in one of the two directly oriented target sites of a DNA substrate for the resolvases drastically reduced the generation of recombination products (19). It was interpreted that a mismatch at the positions of the two recombination sequences that should hybridize after the strand exchange seriously affected the outcome of the reaction. Therefore, it is very likely that DNA resolution by β recombinase occurs by a mechanism similar to that of γδ and Tn*3* resolvases and, by inference, that the strand exchange reaction occurs at the center of site I. The way of achieving the proper orientation of the two sites I at the synaptic complex would nevertheless differ for the two recombinases, given the differences in their binding sites (three for γδ and two for β recombinase) and the need for Hbsu in the case of β protein.

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