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Integrative recombination of bacteriophage λ: Extent of the DNA sequence involved in attachment site function

(*in vitro* recombination/trimming of DNA fragment/cloning)

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ABSTRACT **We have investigated the minimum extent of DNA sequence required for the attachment site of bacteriophage λ to function in integrative recombination. A DNA fragment carrying the phage attachment site (*attP*) of bacteriophage λ was trimmed, recloned, and tested for recombination proficiency. In order to recombine with the bacterial attachment site (*attB*), the phage attachment site must retain about 250 base pairs of its original sequence. On the left side, the essential sequence extends beyond 106 base pairs from the center of the 15-base-pair common core sequence but not beyond 152 base pairs. On the right side the required sequence extends beyond 68 base pairs but not beyond 99 base pairs from the center of the core. A trimmed site that has lost part of the sequence mentioned above cannot function as the phage attachment site. However, depending on which part of the sequence is present, such a site can still act in reactions normally requiring one of the prophage attachment sites or the bacterial attachment site. The results also suggest that the essential sequence of the bacterial attachment site consists only of the sequence common to the phage and bacterial attachment sites.**

Stable lysogeny of bacteriophage λ is established by integration of the phage genome into the bacterial chromosome. This integration takes place through a reciprocal crossover reaction involving two specific sites (attachment sites), *attP* (POP′) and *attB* (BOB′), which are located on the phage and bacterial chromosome, respectively. As the result of the reaction, two hybrid sites, *attL* (BOP′) and *attR* (POB′), are produced on the left and right side of the prophage genome. These four types of attachment sites are functionally distinct from each other, judged by their ability to recombine with other types of attachment sites.

This recombination reaction has been extensively studied by making use of both *in vivo* genetic analysis and an *in vitro* reaction system (1, 2). Important information has become available recently from the determination of the nucleotide sequence around the attachment sites. *attP* and *attB* have been found to share a common core sequence spanning 15 base pairs (bp), and therefore so do the recombinant forms *attL* (BOP′) and *attR* (POB′) (3). However, it has not been known how much of the sequence outside of the core is required for the function of each attachment site.

A first step to approach this question is to define the minimum size of the DNA fragment that contains the essential functional sequence. We have carried out such an analysis on the *attP* site.

Restriction endonuclease fragments containing the core were isolated from plasmids carrying an active *attP* site and were further shortened. Such trimmed fragments were recloned onto plasmids, with pBR322 (4) as the cloning vehicle. We made use of plasmid DNAs carrying a cloned fragment with one of four attachment sites, *attP*, *attB*, *attL*, or *attR*, and tested them against the newly isolated attachment site plasmids in an *in vitro* intermolecular integrative recombination system (5).

For an attachment site to be functional as *attP*, we find that the plasmid has to carry more than 175 bp of the original *attP* sequence, but that a stretch of 252 bp is sufficient. Those plasmids that have lost parts of the required *attP* sequence still can retain the capacity to act as *attL*, *attR*, or *attB*, depending on which portion of the *attP* sequence has been lost. When most of the original *attP* sequence outside of the core is deleted, the site becomes functionally indistinguishable from *attB*.

MATERIALS AND METHODS

Materials. The bacterial factor for integrative recombination was prepared from *Escherichia coli* K-12 strain NH356, as described by Kikuchi and Nash (6). A crude extract of WCi22642 (7) was a gift of Y. Kikuchi and was used as the source of Int protein without further purification (8). Endonuclease *Eco*RI was a gift of J. McGhee. Endonuclease *Tha* I was obtained from Bethesda Research Laboratories (Rockville, MD). Other restriction enzymes and T4 RNA ligase were obtained from New England BioLabs. S1 nuclease and T4 polynucleotide kinase were obtained from P-L Biochemicals. DNA polymerase I (enzyme A of Klenow) and calf intestine alkaline phosphatase were obtained from Boehringer Mannheim. Micrococcal nuclease was obtained from Worthington. Pronase (nuclease free) was obtained from Calbiochem. Nicking-closing extract from duck reticulocytes was a gift from R. D. Camerini-Otero. T4 DNA ligase was prepared according to Panet *et al.* (9). The decanucleotide C-C-G-G-A-T-C-C-G-G (*Bam*HI linker) was obtained from Collaborative Research (Waltham, MA). [γ-^{32}P]ATP (≈3000 Ci/mmol; 1 Ci = 3.7×10^{10} becquerels) was obtained from New England Nuclear.

Trimming and Recloning of *att* Fragments. This was carried out either by partial digestion with micrococcal nuclease followed by S1 nuclease or by a treatment with S1 nuclease alone. S1 nuclease first digests any single-stranded portion of the DNA and then digests the double-stranded region from the ends at a slower rate (10). The reaction mixture with micrococcal nuclease contained 5 mM Tris·HCl, pH 7.5, 1 mM $CaCl_2$, and 0.8 μg of the enzyme per ml; the mixture was incubated at 37°C for 10 min. The reaction mixture with S1 nuclease contained 0.1 M NaCl, 50 mM sodium acetate at pH 4.95, 1 mM $ZnSO_4$, and 200 units of the enzyme per ml. To trim off single-stranded ends from a DNA fragment, the mixture was incubated at 37°C for 2 min. To generate further shortened fragments of DNA, the incubation was carried out at 37°C for 30 min. The trimmed fragment was ligated overnight at 15°C with *Bam*HI linker. The reaction mixture (50 μl) contained 50 mM Tris·HCl at pH 8.0, 10 mM $MgCl_2$, 1 mM ATP, 0.2 mM

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Abbreviation: bp, base pair(s).

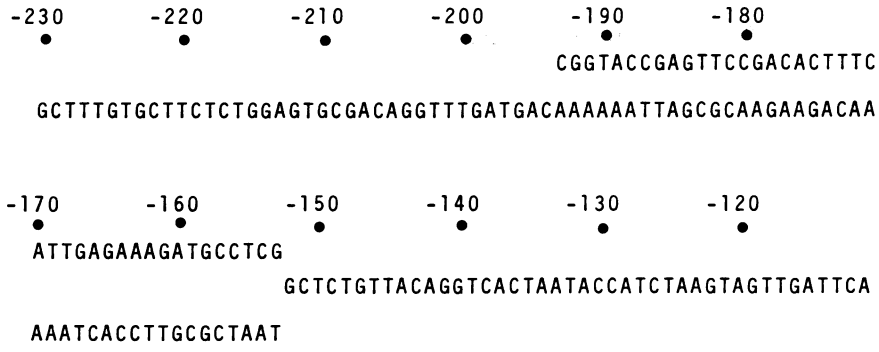

FIG. 1. Comparison of the P arm sequence of pPA214 and wild-type *attP*. The two sequences agree up to position −152 and diverge beyond this point. The upper sequence to the left of position −152 is that carried by pPA214. Position −193 is the 5′ end of the fragment. The terminal sequence C-G-G is derived from the *Msp* I-cleaved *Bam*HI linker. The lower sequence is that of wild-type *attP*. This sequence agrees with that determined independently by Hsu *et al.* (15).

of each four deoxynucleotide triphosphates, 3.5 units of large fragment of DNA polymerase 1, 1.5 μM *Bam*HI linker labeled with ^{32}P at the 5′ ends, 0.75 μg of T4 DNA ligase, 6.5 units of T4 RNA ligase, and about 1 μg of the trimmed fragment. The reaction was stopped by heating at 65°C for 5 min and the DNA was digested with *Bam*HI to generate *Bam*HI cohesive ends. Then the sample was passed through a Sephadex G-100 column to remove excess linker that was not attached to the trimmed fragment. The fragment with *Bam*HI linker was ligated with the cloning vehicle (pBR322, previously digested with *Bam*HI and treated with calf intestine alkaline phosphatase at 8 units/ml for 15 min at 37°C) by T4 ligase at 15°C overnight. The reaction mixture (50 μl) contained 50 mM Tris·HCl at pH 7.5, 10 mM $MgCl_2$, 20 mM dithiothreitol, 1 mM ATP, 5 μg of bovine serum albumin, 0.15 μg of T4 DNA ligase, 5 μg of pBR322, and about 1 μg of fragment. Transformation was carried out according to Cohen *et al.* (11).

***In Vivo* Test for Attachment Site Plasmids.** The procedure for screening clones with attachment site-carrying plasmids was described previously (5). The screening method is based on the ability of active attachment site plasmids to form transducing phages for drug resistance markers upon infection with λ carrying *attP*, through recombination between the phage genome and the plasmids. This test does not discriminate among the four standard attachment sites. For a test specific for functional *attP* plasmids, λ*gal*49*bio*958 was used in place of λb515b519 as the transducing vehicle. This phase was constructed by R. Weisberg and carries an active *int* gene and the bacterial attachment site *attB*.

***In Vitro* Integrative Recombination.** The condition for *in vitro* intermolecular recombination reaction previously described (5) was modified for this study. A reaction mixture (20 μl) contained 50 mM Tris·HCl at pH 7.5, 40 mM KCl, 6 mM EDTA, 12.5 mM spermidine, 0.5 μl of the crude extract containing Int, and 4 μl of the bacterial factor preparation. To each mixture were added 0.25 μg of a plasmid DNA carrying a standard attachment site and 0.25 μg of a second plasmid DNA with an attachment site to be tested. When *attP* was the standard partner, it was added in a supercoiled form together with the *Eco*RI-treated linear form of the plasmid DNA to be tested. When *attB* was the standard partner, it was added in an *Eco*RI-treated linear form together with the supercoiled form of the plasmid DNA to be tested. For the reaction with standard *attR* or *attL*, 40 mM KCl was replaced with 18 mM NaCl. Then 0.25 μg of a plasmid DNA carrying *attR* or *attL*, which had been relaxed by treatment with duck reticulocyte nicking-closing extract, was incubated with 0.25 μg of the *Eco*RI-treated plasmid DNA to be tested. All reactions were carried out at 25°C for 1 hr, followed by the addition of 20 μg of Pronase and 30-min incubation at the same temperature. Samples were shaken with chloroform-isoamyl alcohol and analyzed by gel electrophoresis (12).

The nucleotide sequences of DNA fragments were determined according to the chemical modification method of Maxam and Gilbert, using G, A+G, T+C, and C reactions (13).

RESULTS

Size of the Phage Attachment Site. We have previously shown that the *Hha* I fragment derived from the *attP* region of λ *attB-attP* contains all the necessary sequence for *attP* function. Plasmids that carry this fragment in a pBR322 cloning vehicle can recombine with *attB* both *in vivo* and *in vitro* in an Int-promoted recombination (5). It should be noted here that the *Hha* I fragment from λ *attB-attP* that contains the *attP* region is different from the corresponding fragment from wild-type λ. The *Hha* I *attP* fragment from λ *attB-attP* is about 600 bp long, whereas the fragment expected from wild-type λ is about 380 bp long (3). This is presumably due to the substitution of a part of the λ sequence at the left end of this fragment by the bacterial sequence of the *bio* region. Because further digestion with *Hin*fI produces the same 317-bp-long fragment as from wild-type λ, the *bio-attP* junction in λ *attB-attP* resides between the *Hha* I site at position −158 and the *Hin*fI site at position −115 (3).* This is consistent with the result of an electron microscopic heteroduplex analysis of λ *attB-attP* (14).

The 600-bp *Hha* I *attP* fragment was further digested with *Mbo* II and S1 nuclease, inserted with a *Bam*HI linker into pBR322, and cloned. Some of the plasmids obtained retained the activity of *attP* by both *in vivo* and *in vitro* tests. One such plasmid, pPA214, was further characterized by sequence analysis and shown to contain the sequence from −190 to +120 of the *Hha* I *attP* fragment (see Figs. 1 and 3). Because we expected a part of the P arm sequence that lies to the left of the core in this plasmid to be different from that of wild-type λ as described above, we compared the sequence beyond the *Hin*fI site at −115 between pPA214 and an *attP* plasmid carrying the *Hin*dIII *attP Bam*HI fragment from wild-type λ. [The DNA of this plasmid pYK100 (6) was a gift of Y. Kikuchi.] As shown in Fig. 1, the two sequences agree up to position −152 and diverge beyond this point. Thus the original POP′ sequence in pPA214 extends from −152 to +120.

The 315-bp *Msp* I fragment extending from −193 to +121 containing the POP′ sequence was purified from pPA214, further trimmed with S1 nuclease, inserted with a *Bam*HI linker into pBR322, and recloned. (*Msp* I has the same target sequence as *Hpa* II and cuts at C-C-G-G in the *Bam*HI linker

* The numbering system proposed by Landy and Ross (3) for positions around the attachment site is used in this paper. The center position of the core is designated as 0, with positive numbers assigned for the right side and negative numbers for the left side.

Table 1. Relative efficiency of recombination between plasmid DNAs carrying standard attachment sites.

	attB	*attP*	*attL*	*attR*
*attB**	−	+++	−	−
attP†	+++	−	±	++
attL‡	−	±	+	++
attR‡	−	+	++	−

* *Eco*RI-treated linear form of *attB* plasmid was recombined with supercoiled closed circular form of each one of four types of *att* plasmids.

† Supercoiled closed circular form of *attP* plasmid was recombined with *Eco*RI-treated linear form of each one of four types of *att* plasmids.

‡ Relaxed closed circular form of *attR* or *attL* plasmid was recombined with *Eco*RI-treated linear form of each one of four types of *att* plasmids.

used.) Clones carrying plasmids with functional *attP* were screened *in vivo* and the plasmids were characterized for the size of the *attP*-carrying fragments. One plasmid, pPA259, carried the sequence from −184 to +99, of which −152 to +99 is the original POP′ sequence. Thus all the necessary sequence for *attP* function is contained between positions −152 and +99.

When the 317-bp *Hin*fI *attP* fragment extending from −115 to +201 was purified and recloned, we could not find any clone that retained POP′ activity. Instead, we found clones with altered attachment site activity, which will be described later. Because the necessary POP′ sequence is contained within the stretch from −152 to +99, the *Hin*fI fragment should contain all the necessary sequence for the P′ arm. Thus the sequence required for function of the P arm of POP′ must extend beyond the *Hin*fI site at −115 but not beyond −152. Because, as described later, the functional P′ arm requires sequences beyond position +68, the end of the essential sequence in the P′ arm resides between +68 and +99.

Relative Efficiency of *in Vitro* Recombination Between Two Attachment Sites. Because we obtained plasmids with modified attachment site activity, it was necessary to ask whether they function as one of the four standard attachment sites or act differently from any known attachment site. To answer this question a series of four *in vitro* recombination reactions was used. In each reaction, the plasmid to be tested was incubated with a plasmid carrying one of the four types of attachment sites (*attP*, *attB*, *attL*, or *attR*) along with the necessary protein factors. Each of the four standard attachment sites behaves in a characteristic manner in this series of four test reaction (Table 1).

As shown in Fig. 2 *A–D*, lanes a, a plasmid carrying standard *attB* recombined well only with the plasmid carrying *attP* and not with the plasmids carrying the other three types of attachment site. A plasmid carrying standard *attP*, on the other hand, gave efficient recombination with a plasmid carrying *attB* and less efficient recombination with plasmid carrying *attR* or *attL* in that order (Fig. 2 *A–D*, lanes b).† A plasmid carrying standard *attL* recombined efficiently with a plasmid carrying *attR* and less efficiently with a plasmid carrying *attL* or *attP* (Fig. 2 A–D, lanes c). A plasmid carrying standard *attR* showed efficient reaction with plasmid carrying *attL* or *attP* but showed no detectable reaction with a plasmid carrying *attB* or *attR* (Fig. 2 A–D, lanes d). These results are summarized in Table 1. As is clear from Table 1, each standard attachment site has a unique pattern of relative reaction efficiencies among the set of four reactions. Thus with this series of four test reactions we were able to make a functional classification of a plasmid with an unknown attachment site. It should be noted here that relative efficiency indicated in the table does not necessarily reflect that of the same reactions carried out *in vivo*. In fact, we have observed that changes in molecular form of the substrate or the concentration of the enzymes involved can affect each reaction differently.

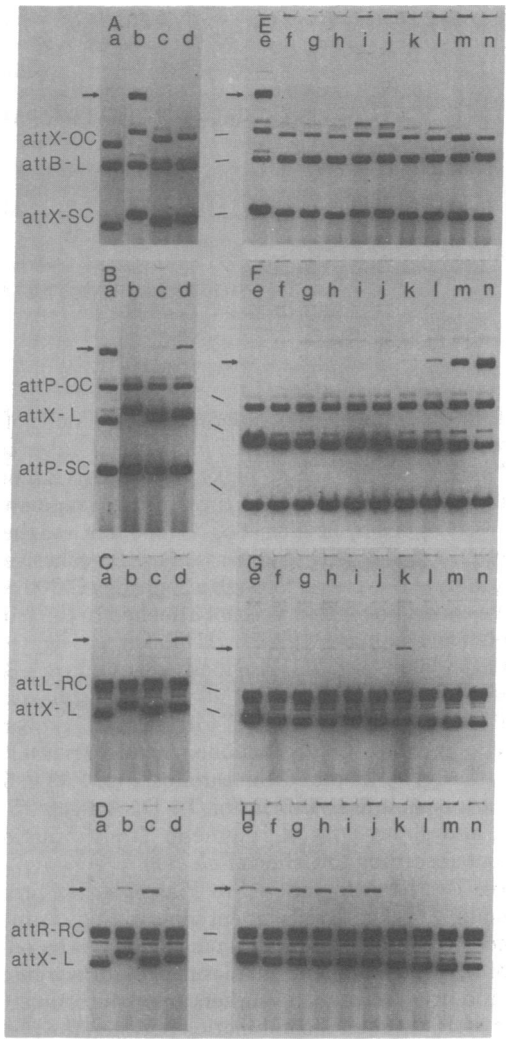

FIG. 2. Intermolecular recombination *in vitro* between two plasmids. One of the substrates was: an *attB* plasmid (*A* and *E*), an *attP* plasmid (*B* and *F*), an *attL* plasmid (*C* and *G*), or an *attR* plasmid (*D* and *H*). The other substrate (indicated by *attX* in the figure) was: an *attB* plasmid (a), an *attP* plasmid (b), an *attL* plasmid (c), an *attR* plasmid (d), pPA214 (e), pPA173 (f), pPA182 (g), pPA176 (h), pPA203 (i), pPA206 (j), pPA 175 (k), pPA171 (l), pPA177 (m), or pPA150 (n). The position of the recombination product is indicated by the arrow. The position of each substrate was indicated with its molecular form (OC, open circles; L, linear form; SC, supercoiled circles; RC, relaxed circles).

Characterization of Trimmed *Hin*fI *attP* Clones. As mentioned above, when the 317-bp *Hin*fI *attP* fragment was recloned after minimum treatment with S1 nuclease to remove

† In the previous paper we have shown that supercoiled *attP* can recombine with both linear *attB* and *attP* at similar efficiencies (5). For the experiments presented in this paper, it was desirable to adjust reaction conditions so that each type of attachment site behaved distinctively. Recently, it was found that, by using higher concentrations of Int protein and host factor, the reaction between *attB* and *attP* becomes more efficient, whereas the reaction between two *attP* sites becomes less efficient. These new conditions were used for the experiments in this study to obtain better discrimination among the various types of attachment sites.

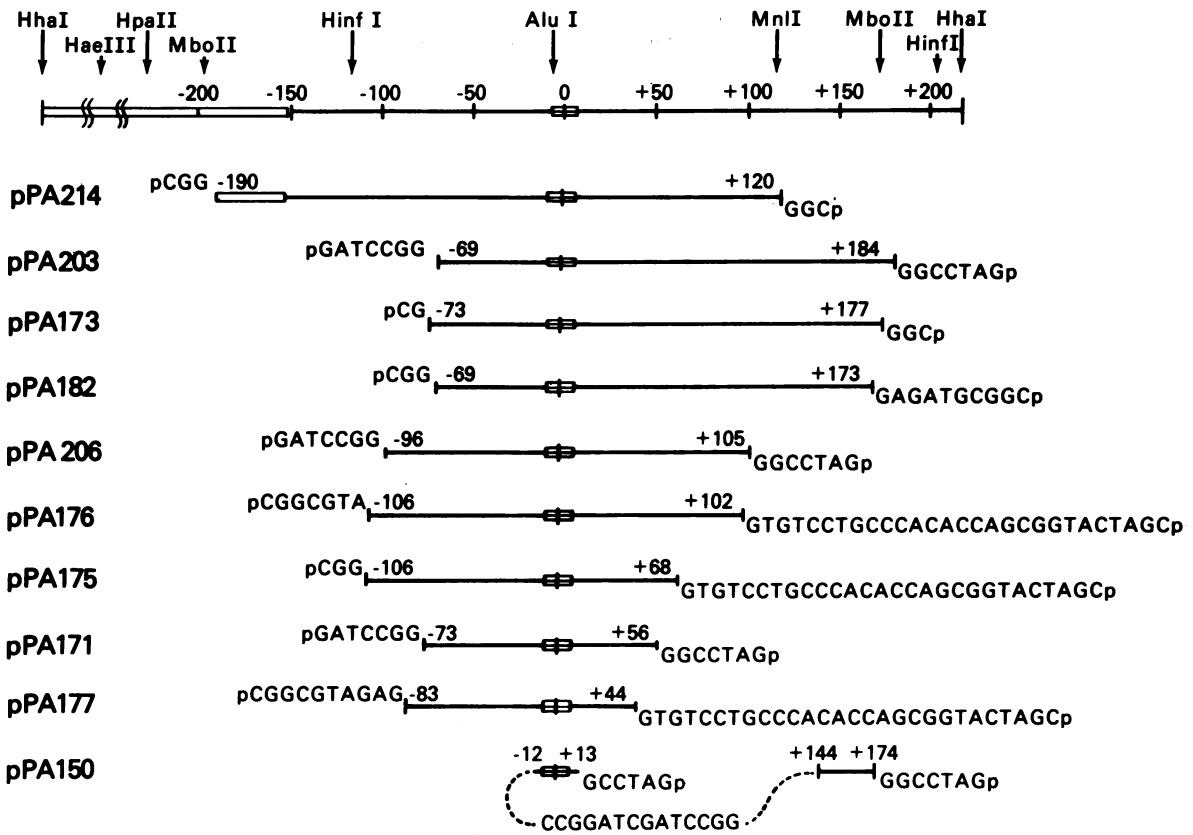

FIG. 3. The extent of original sequence carried by recloned *attP* sites. The extent of original sequence carried by each plasmid is shown by the line, and the position of each end of the original sequence is given. The following enzymes were used to generate fragments whose sequences were to be determined; *Bam*HI, *Msp* I, and *Tha* I. Fragments were purified, labeled with ^{32}P at 5′ ends, and cleaved with *Alu* I to separate the two ends. The 5′ end sequences outside of the original sequence shown in the figure are derived from either the *Bam*HI linker or the insertion site on pBR322 and indicate the extent of the fragments used for sequencing. Plasmid pPA214 acts like *attP*. Plasmids pPA203, pPA173, pPA182, pPA206, and pPA176 act like *attL*. Plasmid pPA175 acts like *attR*. Plasmids pPA171, pPA177, and pPA150 act like *attB*.

single-strand ends, the resulting plasmids did not retain *attP* activity. This was confirmed by both *in vivo* and *in vitro* tests for *attP* function. However, these plasmids did recombine with phage DNA carrying *attP* in an *in vivo* reaction.

We further trimmed the *Hin*fI fragment either by a partial digestion with micrococcal nuclease (for pPA150) or by a treatment with S1 nuclease (for others reported here) to generate shorter fragments and recloned such fragments with a *Bam*HI linker into pBR322. Clones with active attachment sites were screened and plasmids from such clones were further characterized *in vitro*.

Fig. 2, lanes e–n, shows the results of *in vitro att* test for some of these recloned attachment site-carrying plasmids. pPA214 (lanes e, *E–H*), which has been described earlier and did not derive from the *Hin*fI fragment, reacted efficiently with a standard *attB* and also produced the same response in every one of the four reactions as a standard *attP* plasmid (lanes b, *A–D*). None of the plasmids derived from the *Hin*fI fragment could recombine with a standard *attB*. However, plasmids pPA203, pPA206, pPA173, pPA182, and pPA176 (lanes f–j) were indistinguishable from a standard *attL* plasmid (lanes c, *A–D*) in this test. The plasmid pPA175 (lanes k) showed a pattern of recombination ability similar to a standard *attR* plasmid (lanes d). Plasmids pPA177 (lanes m) and pPA150 (lanes n) behaved like a standard *attB* plasmid (lanes a), with pPA177 being slightly less active in its recombination with *attP* DNA (*F*). Plasmid pPA171 (lanes l) also behaved like an *attB* plasmid but at much lower efficiency.

The sequences of the cloned fragments in these plasmids were determined and compared with the POP′ sequence published by Landy and Ross (3) to find the end points of the original POP′ sequence retained in these plasmids. The results are shown in Fig. 3.

Those plasmids that are missing the sequence beyond −106 on the P arm but retain the sequence at least up to +102 on the P′ arm act like *attL*. One plasmid that acts like an *attR* plasmid has the POP′ sequence from −106 to +68 (pPA175). For the plasmids to act like *attB*, more POP′ sequence has to be removed. pPA177, which carries the sequence from −83 to +44, shows good *attB* activity but is still slightly less active than an authentic *attB* plasmid. On the other hand, pPA150, which carries two portions of the POP′ sequence (−12 to +13 and +144 to +174) is indistinguishable from an authentic *attB* plasmid. Because the existence or absence of the POP′ sequence +144 to +174 does not seem to affect the behavior of the plasmids in other cases (compare, for example, pPA203 and pPA206), it is likely that the sequence from −12 to +13 is solely responsible for the attachment site activity of pPA150. To confirm this point, we generated a *Msp* I digest of a purified 81-bp *Bam*HI fragment of pPA150 and tested its ability to recombine with an *attP* plasmid. *Msp* I cuts this fragment at four sites. Two of these sites are located between the core-containing segment and the other P′ arm segment, and cuts at these sites will separate the two segments. After *Msp* I digestion the fragment retained the ability to recombine with *attP*. Because the fragments were not purified for this experiment after digestion with *Msp* I, we still cannot rule out the possibility of the participation of a fragment carrying the sequence from +144 to +174. However, at least the continuity of this sequence with the core-carrying fragment of −12 to +13 is not required for the reaction.

DISCUSSION

We have demonstrated that for a plasmid to carry functional *attP* it has to retain a fragment about 250 bp long containing the core from the original *attP* sequence. The essential sequence on the P arm of POP′ extends farther than 106 bp from the center of the core but not beyond 152 bp.

This is fully consistent with the recent results of Hsu *et al.* (15), which indicate that there is a specific binding site for Int protein around position −129 to −148. Likewise the essential sequence of the P′ arm extends farther than 68 bp from the center of the core but not beyond 99 bp. This again agrees well with the findings of Ross *et al.* (16), which indicate that there is a strong Int binding site at position +50 to +86. Thus the sequence of about 250 bp required for *attP* function contains four Int binding sites, including those at the positions around the core and −98 to −116 (15, 16). The question of exactly how this long stretch of the sequence interacts with the protein system and how it is involved in the mechanism of the recombination reaction remains to be unveiled by further study. Of the DNA sequences involved in specific recognition by protein systems, this is among the largest stretches revealed so far.

The attachment sites that have a shortened P arm but retain the P′ arm sequence up to +102 or more act like *attL* (BOP′). This suggests that the essential sequence of the B arm of *attL* consists only of the sequence common to the B arm and P arm sequences. One plasmid, pPA175, behaves like *attR* (POB′). This was somewhat unexpected. This plasmid lacks the sequence in the P′ arm beyond position +68. The P arm of this plasmid ends at position −106, which is the same as pPA176. Because we attributed the loss of *attP* activity in pPA176 to the defective P arm, the P arm of pPA175 should also be defective. Nevertheless, this plasmid can recombine with a DNA carrying authentic BOP′. This means in this particular reaction (i.e., for *attR* function) no sequence beyond position −106 on the P arm is required. In other words, the sequence required for the P arm of *attP* is different from that of *attR*. Further quantitative kinetic study is required to clarify the basis for the difference in P arm sequence requirement.

It should be emphasized that the reactions used here to define *attL* and *attR* function took place in the absence of any source of Xis protein. We assume that we are seeing the true reverse reaction of the Int-promoted recombination in the kinetic sense. The functional classification of *attL* and *attR* in this test can be different from that of the conventional sense, in which excisive recombination in the presence of Xis function is used.

When the sequences from both the P arm and P′ arm become shorter, the attachment site can act only as *attB*. Because pPA150, which carries the POP′ sequence from position −12 to +13, is as active for recombination with *attP* as authentic *attB*, it is likely that all the essential structure of *attB* is confined within this sequence. This result strongly suggests that the functional sequence of *attB* is composed only of the sequence common with *attP*, namely the 15-bp core sequence (−7 to +7), cytosine at position −9, and the sequence T-T-G at position +9 to +11 (3). This possibility was suggested by Shulman *et al.* (17) and was supported by the sequence analysis of Hoess and Landy (18). Our result completely agrees with such earlier suggestions. In more recent studies, we have been able to extend the results described above by studying plasmids with trimmed *attB* fragments. A preliminary result shows that the 15-bp common core alone does have the function of *attB* but that the additional common sequences mentioned above make the function of *attB* more efficient.

A large portion of the original POP′ sequence outside of the core has to be removed for the attachment site to recombine efficiently with a functional *attP*. We might be able to interpret this by the following scheme. In the forward reaction, the protein factor(s) recognizing the POP′ sequence binds to the *attP* site first. This *attP*–protein complex will contact the other attachment site and recombine efficiently only if the second attachment site is not in a complex with the same protein factor(s). This scheme is supported by the observation that the reaction between two *attP* plasmids becomes less efficient when too high a concentration of host factor is used for the reaction, whereas the reaction between *attB* and *attP* becomes more efficient with the same high concentration of the host factor.†

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