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Preincubation without *att*B DNA inhibits *In Vitro* Integrative Recombination of P'1 Mutant *att*P DNA of Bacteriophage Lambda

YOO, SEUNG-KU*

Department of Food and Biotechnology, College of Engineering and Bioproducts Research Center, Yonsei University, Sudaemun-ku, Seoul, 120-749, Korea

The lambda integrase (Int) is believed to bind to several arm and core sites of *att*P DNA in order to facilitate intasome formation. We have done systematic mutagenic analysis on all 5 arm sites and found that P1 is absolutely required for integration while P2 is not. We also found that all 3 P' arm sites(P'1, P'2, and P'3) are required for efficient integrative recombination. P'1, which is an important binding site for excision, also seems to be crucial for integration when preincubation of *att*P DNA with Int and IHF is performed before recombination. Preincubation assay revealed that preincubation with Int and IHF improved the efficiency of recombination of wild type *att*P DNA and demolished recombinations of P'1 mutant *att*P DNAs.

The integration of bacteriophage lambda DNA into its *Escherchia coli* host chromosome depends on site-specific recombination between two specific sequences called attachment sites (for reviews see references 7, 12, 20). The viral attachment site, *att*P, is approximately 240 bp in length, including 160 bp to the left (P arm) and 82 bp to the right (P' arm) of the center of the common core (3, 8). The *att*P has a complex arrangement of binding sites for recombination proteins which are integrase (Int) and integration host factor (IHF)(22). In *att*P, there are seven Int-binding sites, which can be categorized into two distinct classes of sequences based on sequence comparison: core-type sites that are adjacent to the point of strand exchange (17), and arm-type sites that are located on P and P' arm (16) (Fig. 1).

*att*B, in contrast to *att*P, is quite simple (Fig. 1). Two inverted core-type Int binding sites separated by a 7 bp overlap region comprise the 25 bp *att*B site (9, 17). *att*B has neither IHF binding sites nor arm-type Int binding sites. *att*B, however, contains the identical 15 bp sequence (including the 7 bp overlap) that is found at the core of *att*P.

During recombination, strand exchange is performed by the phage encoded protein, Int. Int with type I topoisomerase activities can cleave the DNA accurately at the positions of strand exchange and form a covalent protein-DNA complex which is an intermediate of the strand exchange (1, 4).

The ligating activity of Int completes the recombination reaction. It has been reported that the 40 kDa lambda Int protein contains two DNA binding domains with different sequence specificities (11). Proteolytic cleavage of Int and footprinting analysis of the resulting two major peptides led us to the conclusion that an amino-terminal peptide interacts with arm-type sequences and a carboxy-terminal peptide binds to core type sequences. These observations suggest that the two domains of lambda Int are able to bind DNA simultaneously. Thus, the binding of lambda Int to *att*P will contribute to the formation of higher order, protein-DNA complexes. Several experiments actually revealed that, in the presence of supercoiling, *att*P, Int and IHF formed an ordered structure, which is now called intasome (2, 14). It has been suggested that during recombination reactions, *att*B obtains its Int by collison with the intasome and that synapsis between *att*B and *att*P is governed solely by protein-protein and protein-DNA interactions (15). It is obvious that, within the intasome or synaptic complex, Int plays the role of "coordinator" for complicated interactions. However, the nature of those interactions is not well known. Determining the properties of those interactions orchestrated by Int is critical to unterstanding of the mechanism of lambda integration because it will help us gain an insight into higher order protein-DNA complexes formed during recombination process. In this

*Corresponding author
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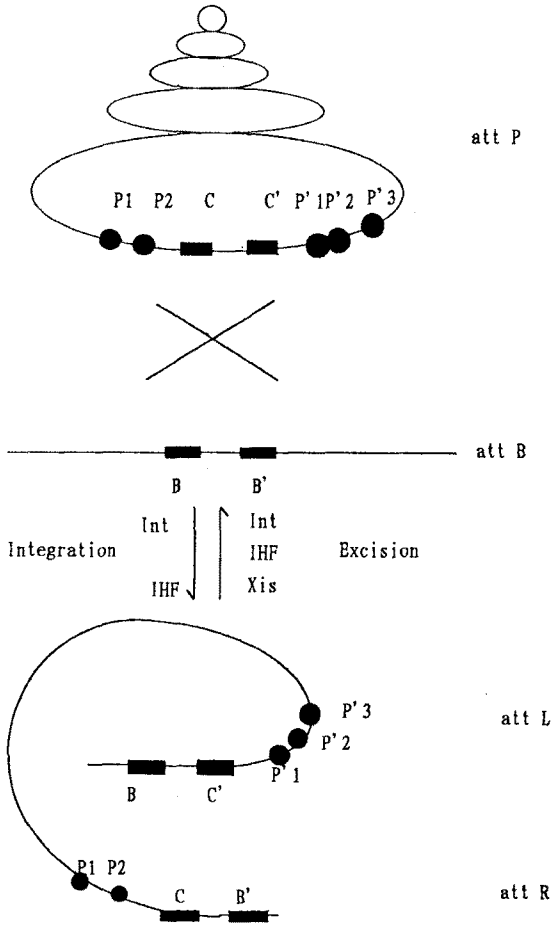

Fig. 1. Integrase binding sites in *att*P and *att*B.

paper, our results show that a simple preincubation of P'1 mutant *att*P DNAs with Int and IHF completly inhibits their integrative recombination with *att*B DNAs, suggesting the importance of P'1 arm-binding site for efficient integration. The involvement of P'1 in integration process togather with P1, P'2, and P'3 implies that 4 Int molecules participate in intasome formation.

MATERIALS AND METHODS

Plasmid Constructions

The construction of plasmid pLV8 has been described (10). pSKY11 (a $P2^-$ *att*P containing plasmid) was made by ligating a gel-purified 1349-bp *Sph*I-*Pst*I fragment from pLV8 with a gel-purified 941-bp *Pst*I-*Sph*I fragment from pSKY6 (a $P2^-$ and $P'1^-$ *att*P containing plasmid)(S. Yoo, unpublished data). Site-specific mutagenesis by polymerase chain reaction (PCR) was used to construct pSKY12, 13, 14, 15, 16, 17, 18, 19, 21, 22 and 23. Various mutagentic primers containing mismatch(s) to the λ core or arm binding sequences and pBR322 *Pst*I site primers (clockwise or counter-clockwise, New England Biolabs) were used for PCR reaction. Site-specific mutagenesis resulted from fixation of mutagenic oligonucleotide primer sequences into the amplification product DNAs off the plasmid pSKY11. The amplification product DNAs were treated with two kinds of restriction enzymes and ligated to the proper backbone fragment obtained from pSKY11. After each mutant was isolated, the entire attP region was sequenced using the dideoxy method (18).

The PCR reaction was carried out in a total volume of 100 μl containing 1 ng of template DNA (pSKY11), 2.5 units Taq DNA polymerase, 100 pmol of each primer, 200 mM dNTP, 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$ and 0.01% gelatin. The amplification was performed for 25 cycles with an annealing temperature of 37℃.

Preparation of *att*P and *att*B Substrates

All *att*P containing plasmids were prepared with the Qiagen kit as recommended by Qiagen (Studio City, CA). Oligodeoxynucleotides for construction of attB substrates were synthesized in a DNA synthesizer using beta-amidite synthesis kits purchased from Biosearch Company (San Rafael, Ca). Normally 100-300 pmol each of the two appropriate oligodeoxynucletide pairs were mixed in TE (10 mM Tris-HCl, pH 7.6/1 mM EDTA), heated to 75℃ for 10 min and cooled slowly to room temperature. The 5' end of an oligonucleotide was labeled by T4 polynucleotide kinase in the presence of $[\gamma-^{32}P]$ ATP.

Integrative Recombination *In Vitro*

Standard recombination assays were carried out in 20 μl of 65 mM NaCl/25 mM MOPS, pH 7.9/6 mM spermidine/5 mM EDTA/2.5 mM dithioerythritol/0.5 mg of bovine serum albumin per ml/0.1 pmol *att*P DNA/2 pmol ^{32}P-labeled *att*B DNA. *att*P plasmids were incubated together with excess *att*B substrates with 2 units of IHF and Int. After 4 hours at 25℃, the reactions were stopped by the addition of 5 μl of a solution containing 20% (w/v) ficoll-400, 5% sodium dodesyl sulfate, and 0.5% bromophenol blue. The samples were loaded onto a 1.2% agarose gel and the DNAs seperated by electrophoresis. Each gel was dried onto Whatman Diethylaminoethyl Cellulose (DE81) paper using a vacuum drier and exposed to X-ray film. The resulting autoradiographs were analyzed on a LKB Ultroscan XL laser densitometer.

Preincubation Assay

Preincubation assay was done *in vitro* in a manner similar to that used for integrative recombination assay except for 15 min preincubation of *att*P DNAs with 2 unit IHF and Int in the absence of *att*B substrates. Normally, 0.1 pmol *att*P DNAs were mixed with 2 unit IHF and Int and incubated for 15 min under the standard recombination assay conditions as described above. The excess *att*B substrates (2 pmol) were then added to

preincubation reaction mixture and further incubated for 5 min. The samples were loaded onto a 1.2% agarose gel for densitometric analysis of the recombinant DNA bands.

RESULTS

Preincubation inhibits Integrative Recombination of P'1⁻ *att*P DNA

The site-specific recombination reactions of the λ virus involve four proteins and 15 protein binding sites (7). Previous studies (13, 20) suggest that 2 proteins (Int, IHF) and 10-11 protein binding sites are required for integrative recombination between the phage and bacterial *att* sites (*att*P and *att*B). It is believed that the Int and IHF proteins bind to several protein binding sites of *att*P DNA and facilitate formation of the *att*P intasome which is a DNA-protein complex wrapped in a nucleosome-like structure.

We were interested in studying the order on pathway(s) of intasome formation. Therefore we introduced several mutations to all arm-type and core-type sites and observed the relationship between the degree of perturbation by mutation and the efficiency of recombination (Table 1). First, very similar changes (the third and seventh bases of each arm-type sites were changed) were used to compare all arm-type sites. Under the standard conditions, the double-base changes in P1 or P'3 demolished the recombinogenic property of *att*P DNA, while the same changes in the P'2 site allowed about 80% efficiency of recombination compared to wild-type *att*P DNA. Any changes in the P2 site did not affect the efficiency of integrative recombination indicating that P2 is not needed for integration. Interestingly, the doublebase changes in P'1 reduces efficiency to 50% suggesting that the P'1 site is somehow involved in integrative recombination. Numrych et al. (13) reported that the P1, P'2 and p'3 sites are required for integration. They also observed that triple-base changes in P'1 reduced *in vitro* integration 3 fold and the effect *in vitro* was partially overcome by increasing Int concentrations. We have examined the properties of the P'1 site further through mutagenic analysis and come up with a couple of strange observations. First, drastic mutations in P'1 did not completely demolish *in vitro* integration. Second, as previously reported, P'1 mutation responded to the increase of Int concentrations faster than any other arm-type mutations. Our first impression was that the function(s) of P'1 during integration may be different from those of other arm-type binding sites (P1, P'2, and P'3). The peculiarities of P'1 led us to perform preincubation assay.

As shown in Fig. 2, the efficiency of integration of the control *att*P DNA(P2⁻ only) was slightly improved by preincubation. We originally thought that the preincubation of *att*P DNA with IHF and Int improved the overall intasome formation, which in turn contributed to efficient recombination. However, all of the arm-type mutant *att*P DNAs suffered a decrease in efficiency of integration through preincubation, even though the degree of decrease varied depending on arm-type mutations. The preincubation inhibited integrative recombination 1.2 to 2-fold in the cases of P1⁻, P'2⁻, and P'3⁻. Surprisingly, the preincubation completly prevented the P'1⁻ mutant

Table 1. Effects of mutations in Int-binding sequences on *in vitro* integrative recombination.

Plasmid	Location of mutation	Sequence of mutation	Efficiency of plasmid DNA in integrative recombination
pWR1	Wild type	Wild type	++++
pSKY11	P2 only	TCGACCATAT(P2⁻)	++++
pSKY14	P1 + P2	AGCTCAGTA(P1⁻)+P2⁻	+/−
pSKY18		AGGTCAGTA(P1⁻)+P2⁻	+
pSKY17		AGCTCACTA(P1⁻)+P2⁻	+++
pSKY6	P'1 + P2	AGCTGGCAAT(P1'⁻)+P2⁻	+
pSKY22		AGGGTCCTAT(P1'⁻)+P2⁻	+
pSKY23		AGCTCAGTAT(P1'⁻)P2⁻	++
pSKY15	P'2 + P2	CACTCAGAAT(P2'⁻)+P2⁻	+++
pSKY16	P'3 + P2	AACTCAGTAT(P3'⁻)P2⁻	+/−
pSKY19		AACTCATTAT(P3'⁻)P2⁻	+++
pSKY21		AAATCAGTAT(P3'⁻)P2⁻	+++
pSKY12	C + P2	CACCTTTTT(C⁻)+P2⁻	+++
pSKY13	C' + p2	CAACTTAGT(C'⁻)+P2⁻	+++

Note. (1) All DNAs listed except for pWR1 contain a common P2 mutation (P2⁻). Here the actual sequence of mutated P2 site is shown for only pSKY11 for simplicity. (2) Mutated bases in Int binding sites are indicated as underlined (3) ++++ : 81~100% recombination, +++ : 61~80% recombination, ++ : 41~60% recombination, + : 21~40% recombination, +/− : 0~20% recombination.

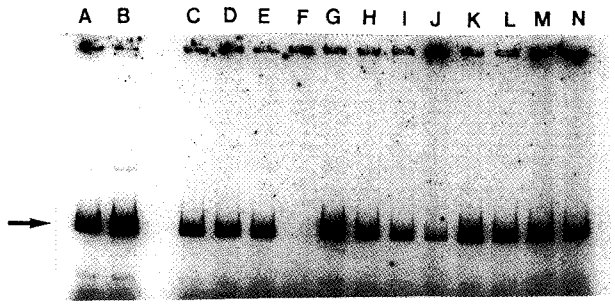

Fig. 2. Preincubation assay for Int binding site mutant DNAs.
A. Wild type (pSKY11) : no preincubation, B. Wild type (pSKY11) : preincubation, C. P1 mutant (pSKY17) : no preincubation, D. P1 mutant (pSKY17) : preincubation, E. P'1 mutant (pSKY23) : no preincubation, F. P'1 mutant (pSKY23) : preincubation, G. P'2 mutant (pSKY15) : no preincubation, H. P'2 mutant (pSKY15) : preincubation, I. P'3 mutant (pSKY21) : no preincubation, J. P'3 mutant (pSKY21) : preincubation, K. C mutant (pSKY12) : no preincubation, L. C mutant (pSKY12) : preincubation, M. C' mutant (pSKY13) : no preincubation, N. C' mutant (pSKY13) : preincubation. Note. (1) All DNAs tested contain a common P2 mutation (see Table 1). (2) Arrow indicates recombination products. (3) *att*B DNA is not shown.

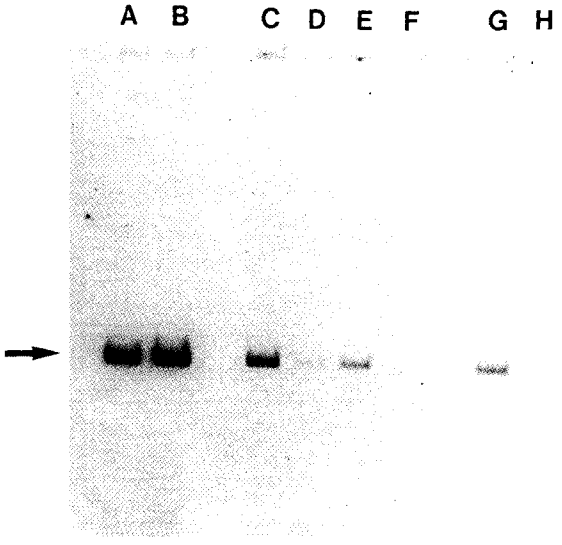

Fig. 3. Preincubation assay for different P'1 mutant DNAs.
A. Wild type (pSKY11) : no preincubation, B. Wild type (pSKY11) : preincubation, C. P'1 mutant (pSKY23) : no preincubation, D. P'1 mutant (pSKY23) : preincubation, E. P'1 mutant (pSKY6) : no preincubation, F. P'1 mutant (pSKY6) : preincubation, G. P'1 mutant (pSKY22) : no preincubation, H. P'1 mutant (pSKY22) : preincubation. Note. (1) Arrow indicates recombination products. (2) *att*B DNA is not shown. (3) Sequences of P'1 mutations (pSKY23, 6, and 22) are shown in Table 1.

from recombining. This evidence again suggests that the role of P'1 during integration is important. The strong inhibition effect of preincubation on the P'1 mutant could not be overcome by increasing Int concentrations. The preincubation inhibited integrative recombination of core-type mutants (C^- or C'^-) down to 70~80%.

The drastic inhibition of the P'1 mutant by simple preincubation with IHF and Int was totally unexpected.

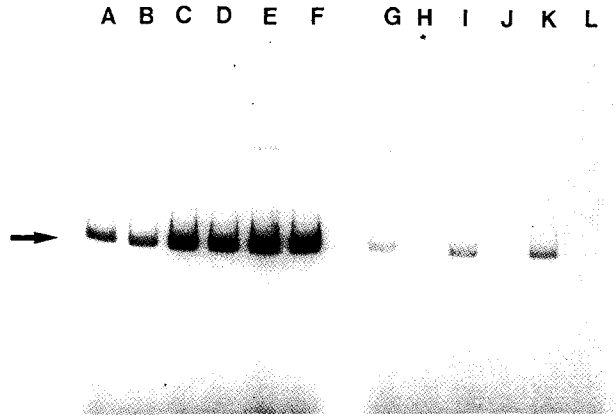

Fig. 4. Preincubation assay for P'1 mutant with different incubation time.
A. Wild type (pSKY11) : no preincubation+5 min incubation, B. Wild type (pSKY11) : preincubation+5 min incubation, C. Wild type (pSKY11) : no preincubation+30 min incubation, D. Wild type (pSKY11) : preincubation+30 min incubation, E. Wild type (pSKY11) : no preincubation+60 min incubation, F. Wild type (pSKY11) : preincubation+60 min incubation, G. P'1 mutant (pSKY23) : no preincubation+5 min incubation, H. P'1 mutant (pSKY23) : preincubation+5 min incubation, I. P'1 mutant (pSKY23) : no preincubation+30 min incubation, J. P'1 mutant (pSKY23) : preincubation+30 min incubation, K. P'1 mutant (pSKY23) : no preincubation+60 min incubation, L. P'1 mutant (pSKY23) : preincubation+60 min incubation.
Note. (1) Arrow indicates recombination products. (2) *att*B DNA is not shown.

Therefore, we tested a few more $P'1^-$ mutants for the same preincubation assay in order to ascertain whether the strong inhibition through preincubation is arm-type binding site ($P'1^-$) specific or just DNA sequence specific. Fig. 3 shows that the drastic inhibition of recombination by preincubation is $P'1^-$ specific as three different $P'1^-$ mutants exhibited the same phenomenon. The recombinations of $P'1^-$ mutants were depressed approximately 10~50 fold after preincubation regardless of the changes in the P'1 site.

Inhibition on $P'1^-$ *att*P DNA by Preincubation is Irreversible

It was tempting to postulate that the $P'1^-$ mutants allow a certain aberrant structure formation which is not recombinogenic with incoming *att*B substrates. To examine the persistency of inhibition caused by preincubation, different lengths of incubation time (5, 30, and 60 min) were tested for preincubation assay, while the length of preincubation time (15 min) was fixed. The extended incubation time (30 and 60 min) stimulated the overall recombination 1.5 fold as compared to recombination without preincubation. By contrast, the longer incubation time did not help preincubated $P'1^-$ mutant produce recombinant DNAs (Fig. 4). These findings suggest that the inhibition of $P'1^-$ mutants during preincubation is irreversible for at least 60 min so that

incoming *att*B substrates can not recombine with *att*P DNAs. It is known that our Int protein is active for at least 1~2 hours under the standard conditions mentioned in Materials and Methods. Therefore, we concluded that the inability of P'1⁻ mutant DNAs to recombine after preincubation is not due to inactivation of Int, but due to inhibition resulting from preincubation.

DISCUSSION

One of the most fascinating features of λ phage site-specific recombination is its strict directionality. It is true that the normal products of integration are the normal substrates of excision, and vice versa. However, integration and excision are two distinct pathways in terms of molecular biological level. There are some evidences to show that integration is not a reversible reaction of excision. First of all, of a total of 5 arm-binding sites (P1, P2, P'1, P'2, and P'3), P1 and P'3 are required only for integrative recombination while P2 is uniquely required for excisive recombination (7). Second, *att*P has all five arm-binding sites and its partner (*att*B) for integration has no arm-binding site. By contrast, *att*L and *att*R (partners for excision) share arm-binding sites (See Fig. 1). Therefore, it is fairly logical to think that integration should be somewhat different from excision.

Several pathways by which attL and attR assemble have been proposed by Stark et al. (19). Biochemical and genetic studies (5, 6) suggested a possible model for the Int-mediated synaptic interactions between the arm and core sites of *att*L and *att*R during excisive recombination. In this paper, we have used a genetic approach to study Int-Int binding site interactions in order to achieve a better understanding of the integration pathways. We have done systematic mutagenetic analysis on all 5 arm-binding sites and found that P1 is absolutely required for integration while P2 is not. We also realized that all 3 P' arm binding sites (P'1, P'2, and P'3) are required for efficient integrative recombination. P'1, which is a very important binding site for excision, turned out to be crucial for integration as well when preincubation of *att*P DNA with Int and IHF was performed before the recombination reaction. It is striking that a simple preincubation improves the efficiency of recombination of wild type and demolishes recombination of P'1 mutants. One possible explanation for this contrast is that binding of Int molecules to arm-binding site is sequencial so that poor binding of Int to mutated P'1 site results in either pretermination of intasome formation or abnormal intasome formation. The irreversibility of the inhibition on P'1⁻ attP DNAs by preincubation favors the possibility of aberrant intasome formation. Another explanation for inhibition by preincubation is that P'1⁻ *att*P DNAs form stable, intermolecular dimers via unusual Int bridges induced by mutation on P'1. *att*P dimers formed through preincibation could not be identified by electrophoresis analysis (S. Yoo, unpublished data). The observation of strong inhibition on P'1⁻ mutant DNA by preincubation does not explain the properties of the interactions led by Int. However, the degree of inhibition during preincubation gives us a clue to how many Int molecules are involved in integration. It has been reported that the approximate binding affinities for P'1, P1 and P2 indicates a hierarchy of P'1=P1>P2 (16, 21). P'2 and P'3 have not been tested individually and all three of the P' sites are subject to cooperative binding effects amongst themselves (L. Moitoso de Vargas, and A. Landy, unpublished data). We can easily imagine that the affinity of P'2 and P'3 would be decreased when Int binding to P'1 is poor. If there are vacancies on P' arm-binding sites, the formation of functional intasome seems to be impossible because Int binding to both P and P' arm are necessary for recombination. If P'1 is occupied by an Int, P'2 and P'3 should be quickly occupied by cooperative binding. Considering the fact that P1 is indispensable and P2 is not during integration, we guess that 4 Int molecules on P1, P'1, P'2, and P'3 are involved in integrative recombination. This assumption is consistent with the finding that *att*B obtains its Int by collision with the intasome (15). It is obvious that protein-protein interactions contribute to the stable higher order protein-DNA complexes. Crosslinking should be used to define precise Int-protein bridges. A big question about site-specific recombination is "Where does the specificity come from?" Here, we can only speculate that precise, sequencial interactions among the recombination proteins and *att*P DNA lead to the formation of a unique protein-DNA complex which acts on *att*B DNA specifically, just like an enzyme acts on its substrates.

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