# A comparison of the effects of single-base and triple-base changes in the integrase arm-type binding sites on the site-specific recombination of bacteriophage lambda

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## ABSTRACT

**Triple-base changes were made in each of the five Integrase (Int) arm-type binding sites of bacteriophage lambda. These triple changes, called *ten* mutants, were compared with single-base changes (*hen* mutants) for their effects on integrative and excisive recombination. The presence of *ten* or *hen* mutations in the P1, P'2, or P'3 sites inhibited integration, but the *ten* P'3 mutant was 10-fold more defective than the analagous *hen* mutant. The results with these mutants suggest that the P1, P'2, P'3, and possibly the P'1 sites are required for integration. In wild-type *E. coli*, the *ten* P'1 mutant reduced the frequency of excision 5-fold, whereas the *hen* P'1 mutant had no effect. The presence of *ten* mutations in the P2, P'1, or P'2 sites inhibited λ excision in an *E. coli* strain deficient in the production of FIS, while *hen* mutations in the P2 and P'2 sites had little or no effect. The results with the *ten* mutants suggest that the P2, P'1, and P'2 sites are required for excision. The differences in the severity of the effects between the *ten* and *hen* mutations may be due to the inability of cooperative interactions among Int, IHF, Xis, and FIS to overcome the disruption of Int binding to sites with triple-base changes compared to sites with single-base changes.**

## INTRODUCTION

Site-specific recombination occurs widely, being involved in processes as diverse as controlling phase variation in *Salmonella* and providing antibody diversity in mammals. Bacteriophage λ integration into and excision from the *E. coli* chromosome serves as a prototypic example (for reviews, see 1 and 2). When λ infects *E. coli*, it follows either of two pathways. In the lytic pathway, λ replicates and matures into progeny phage. However, in the lysogenic pathway, λ integrates its DNA into the chromosome to form a prophage. Under appropriate conditions, this process can be reversed and the prophage excises its genome from the chromosome and initiates lytic development. Both integration and excision occur by reciprocal, site-specific recombination reactions.

Integration and excision of λ occur at specific regions of the interacting DNA molecules called attachment (*att*) sites (Figure 1). The phage *att* site (*att*P) and the bacterial *att* site (*att*B) contain a common core sequence (O) of 15 base pairs, flanked by non-homologous DNA sequences called arms that are designated as P and P' for *att*P or B and B' for *att*B. Deletion experiments have shown the *att*P and *att*B sites to be approximately 234 base pairs and 25 base pairs, respectively (3,4). Integration of λ into the bacterial genome forms two recombinant prophage sites called *att*L (BOP') and *att*R (POB') which themselves react to reform *att*P and *att*B during excision (5).

Integrative recombination between the *att*P and *att*B sites requires the lambda-encoded protein integrase (Int) and integration host factor (IHF) of *E. coli* (6,7). Excisive recombination between the *att*L and *att*R sites requires these proteins and, in addition, the lambda-encoded protein excisionase (Xis) (8). The *E. coli* protein FIS (*F*actor for *I*nversion *S*timulation) enhances excision *in vitro* when Xis is limiting (9) and *in vivo* (R. Johnson and C. Ball, personal communication). Footprinting experiments show that each of these proteins binds to specific regions within the *att* sites (Figure 1: [5,9,10,11]). For example, Int binds to two sites in the core and to five locations in the P and P' arms of *att*P (Figure 1). Analysis of these sites revealed separate consensus sequences for the core-type sites versus the arm-type sites (12,13,14). Subsequent studies (15) revealed that Int has two different binding domains that associate with the two different consensus sequences. The sequences of the five Int arm-type sites and the consensus sequence for the Int core-type sites are shown in Table 1.

The goal of this study is to define more thoroughly the roles of Int and the Int arm-type binding sites in both recombination events. Previously, single nucleotide changes, called *hen* mutations, were made in each Int arm-type site (Table 1: [16]). These studies suggested that λ does not require the P2 or P'1 sites for integration, and that the mutations had little effect on excision unless more than one arm-type site was mutated. Footprint studies of different combinations of *hen* mutations suggested that Int interaction with an altered site was abolished. One difficulty in the interpretation of these results was that cooperative interactions among Int, Xis, and IHF may have

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suppressed the disruptive effect of a single-nucleotide change by promoting Int binding to the mutated site. For example, Int binds cooperatively to the P′ sites (15,16,17,18) and binding to the P2 site is stimulated by Xis (19). In addition, IHF is thought to enhance Int binding by bending the DNA at the H′ site, which brings the Int core and arm-type sites closer together, thereby stabilizing Int binding simultaneously to these sites (20). We reasoned that a triple mutation should better prevent recognition of an arm-type site by Int and thus more completely disrupt potential Int binding due to cooperative interactions.

In this study, triple mutations, called *ten* mutations, were made in each Int arm-type site. The original *hen* mutant was isolated *in vivo* as a C to T transition in the P′3 site which reduced λ integrative recombination. The *ten* mutations change the three completely conserved TCA nucleotides in each arm-type sequence to GTC (Table 1), and thereby contain two additional transversions in addition to the original *hen* mutation. The effects on integrative and excisive recombination of such alterations at each site were determined by both *in vivo* and *in vitro* assays. In addition, excision was also analyzed in an *E. coli* strain deficient in FIS production (a *fis* mutant). The results suggest that the *hen* mutations were not sufficiently disruptive to completely prevent Int binding at the P2, P′1, P′2, and P′3 sites,

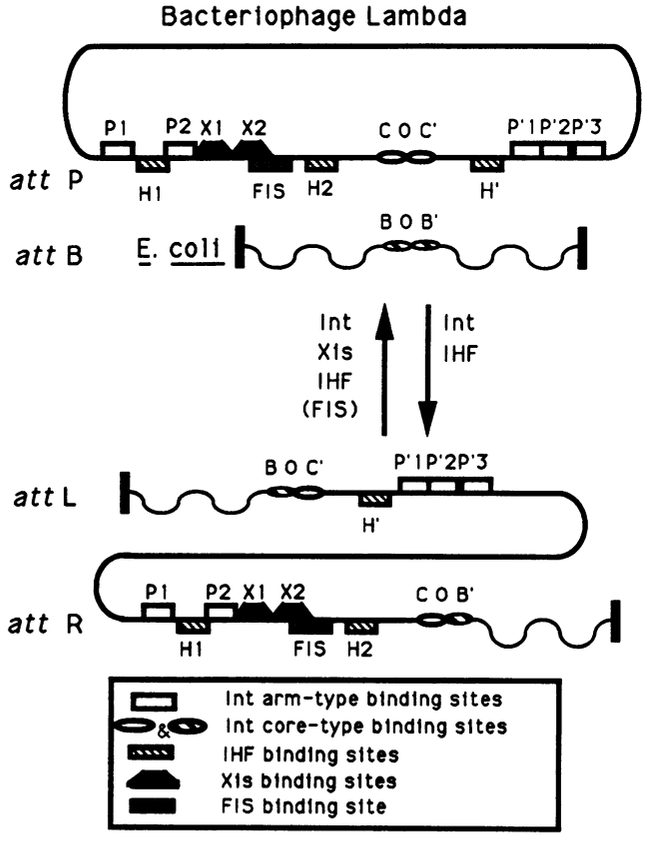


**Figure 1.** Integration and Excision. Bacteriophage λ integrates into and excises out of specific sites on the DNA called attachment (*att*) sites. The four attachment sites are: *att*P (POP′) the λ site; *att*B (BOB′), the *E. coli* site; and *att*L (BOP′) and *att*R (POB′), the prophage sites. Integration requires Int and IHF. Excision requires Int, IHF, and Xis, and is stimulated by FIS when Xis is limiting. Each site contains a subset of protein binding sites. Int binds to five arm-type sites in the P (P1 and P2) and P′ (P′1, P′2, and P′3) arms and to two sites in the core (C and C′ in *att*P or B and B′ in *att*B). IHF binds to three sites (H1, H2, and H′). Xis binds to the two adjacent sites X1 and X2. FIS binds to one site overlapping the X2 site. (5,9,10,11)

and in addition, show that a *fis* mutation in the host dramatically modulated the effects on excision of *ten* mutations at the P2, P′1, and P′2 sites.

## MATERIALS AND METHODS

### Construction of *att*P-*ten* Mutations

Triple alterations, which we call *ten* mutations, were constructed in each of the int arm-type binding sites (Table 1) using oligonucleotide-directed mutagenesis on bacteriophage M13mp9:*att*P6 DNA containing the *att*P site (16). The *att*P-*ten* mutations were made using an amber enrichment procedure and identified by colony hybridization on nitrocellulose (21). The sequence of the oligonucleotides used were the following: *ten* P1, d(TATTAGGACCCTGTA); *ten* P2, d(TTATTTGACC-TGATA); *ten* P′1, d(TGATAGGACCCTGTT); *ten* P′2, d(TTA-TTTGACCTGATA); and *ten* P′3, d(CAAATAAGACTT-TTAT). After each mutant was isolated, the entire *att*P region was sequenced using the dideoxy method (22). The *att*P-*ten* mutations were subcloned into the *Bam*HI/*Hin*dIII sites of pBR322, producing a vector suitable for isolating duplex DNA for the *in vitro* assays.

### Recombining the *att*P-*ten* Mutations from M13 onto λ

The *att*P mutations were transferred from M13 to λ as described previously (21). After plaque purification, the presence of the appropriate mutation in each recombinant was verified by plaque hybridization to the appropriate labelled oligonucleotide on nitrocellulose. The final products of the crosses were λ*cI*857 derivatives containing each of the *ten* mutations.

### Integrative Recombination *in vivo*

The bacteriophage 21 immunity region (*imm*21) was crossed onto each of the λ*cI*857*att*P derivatives and strain N5813 was used to determine the efficiency of integration of each phage as described previously (5,16). Strain N5813 contains an insertion placing the *gal* genes under control of the $\lambda P_L O_L$ promoter/operator and the λ*cI*857 temperature sensitive repressor. Thus the strain is $Gal^+$ at 42°C and $Gal^-$ at 30°C. In addition, the *att*B site is located between the promoter $P_L$ and the *gal* genes. If λ inserts into *att*B, the strain is $Gal^-$ at either temperature. For determining integration frequencies, N5813 was infected with each of the λ*imm*21*att*P *ten* mutants at a multiplicity of infection of 10, plated in duplicate on MacConkey-galactose plates, and incubated overnight at 30°C to allow colony formation. The plates were then shifted to 42°C for 6 hours for color development. The frequency of integration was determined

**Table 1.** Int Core and Arm-Type Recognition Sequences

| Site | Wild-Type[a] | *hen* Mutation | *ten* Mutation |
|---|---|---|---|
| Core-type consensus[b] | CaacTTnnT | ---------- | ---------- |
| Arm-type | | | |
| P′1 | aggTCActAt | aggT*T*ActAt | agg*GTC*ctAt |
| P′2 | cagTCAaaAt | cagT*T*AaaAt | cag*GTC*aaAt |
| P′3 | aaaTCAttAt | aaaT*T*AttAt | aaa*GTC*ttAt |
| P1 | aggTCActAa | aggT*T*ActAa | agg*GTC*ctAa |
| P2 | cagTCActAt | cagT*T*ActAt | cag*GTC*ctAt |

a) Capital letters represent completely conserved bases in either core or arm-type sites.
b) This sequence represents the consensus sequence from the core-type sites in *att*B and *att*P.

by dividing the number of white colonies on a plate seeded with phage B274 by the total number of colonies on a duplicate plate without B274 and multiplying by 100. B274 is a virulent phage, which kills cells not lysogenic for *gimm*21, thereby allowing only lysogens to grow.

### Excisive Recombination *in vivo*

The frequency of excision was measured by the heat-pulse-curing assay (23) using λ*cI*857 lysogens that were resistant to infection by λ*vir* (λ*vir*$^R$). The assays were performed as follows. The λ*cI*857 lysogens were grown in LB media (1% tryptone, 1% NaCl, and 0.5% yeast extract) overnight at 30°C, and 0.1 ml of each culture was subcultured into 5 ml LB media and grown for 2 hours at 30°C. Aliquots (50 μl) were further subcultured and incubated for 6 minutes in 5 ml LB media pre-equilibrated at 42°C. The samples were then further diluted 1:100 at 30°C and grown for 3 hours. Appropriate dilutions of the cultures were spread on duplicate T-plates (1% tryptone, 1% NaCl, and 1.5% agar) and incubated overnight at 30°C or 42°C. Only cells that have excised the prophage grow at 42°C, whereas both λ lysogens and cured lysogens can grow at 30°C. The frequency of excision was calculated by dividing the number of colonies at 42°C by the number of colonies at 30°C and multiplying by 100.

### Construction of *fis* Mutants

A *fis* mutation was transduced into lysogens by P1-mediated generalized transduction. Strain RJ1529 contained an insertion of a gene encoding kanamycin resistance within its *fis* gene that inactivates *fis* function (R. Johnson, personal communication). A $P1_{vir}$ lysate was made on strain RJ1529 (24) and used to infect each lysogen. The infected cells were spread onto LB plates containing 50 μg/ml kanamycin and the transductants were saved for further use.

### Integrative Recombination *in vitro*

Integration was measured *in vitro* by comparing the frequency of recombination by a plasmid containing a mutant *att*P with a larger plasmid containing a wild-type *att*P. All five *att*P-*ten* mutations and wild-type *att*P were cloned into pBR322 (see above) to generate 4.5 kilobase (kb) plasmids. The wild-type *att*P plasmid for use as a competitor in the experiments was made by cloning a 2 kb kanamycin resistance cassette into the *Eco*RI site of pBR322:*att*P$^+$. The 5.7 kb *att*B fragment was made by linearizing the plasmid pBB105 (25) with *Eco*RI, dephosphorylating the 5′ ends with bacterial alkaline phosphatase, labeling with [γ-$^{32}$P]ATP and T4 polynucleotide kinase, and passing the DNA through a Sephadex G-50 spin column to remove unincorporated label.

Both *att*P plasmids were incubated together with a linear *att*B fragment with 1 unit of IHF and Int. Each reaction (20 ul) contained equimolar amounts of the *att*P plasmids (250 ng small plasmid and 360 ng large plasmid) and 250 ng [$^{32}$P]-*att*B DNA, in a buffer of 30 mM Tris-HCl (pH 7.4), 5 mM NaCl, 90 mM KCl, 4 mM spermidine, 5 mM $Na_2EDTA$, 1% glycerol, and 1 mg/ml BSA. After one hour at 25°C, the reactions were stopped by the addition of 5 ul of a solution containing 25%(w/v) ficoll-400, 5% sodium dodecyl sulfate, 0.4% bromphenol blue, and 0.4% xylene cyanol. The samples were loaded onto a 1% agarose gel and the DNA separated by electrophoresis for 16 hours at 100 V in TBE buffer. Each gel was dried onto Whatman 3MM paper using a vacuum dryer and exposed to preflashed X-ray film (26). The resulting autoradiographs were analyzed on a LKB Ultroscan XL laser densitometer. In addition to the recombination assays, each gel contained serial dilutions of unreacted [$^{32}$P]-*att*B DNA. Densitometric analyses of these control lanes verified that the intensities of the recombinant bands were within the linear range for each gel. The relative frequencies of recombination are given as ratios obtained by dividing the density of the mutant recombinant band by the density of the wild-type recombinant for each reaction, and then multiplying by 100.

### Excisive Recombination *in vitro*

Excision was analyzed *in vitro* in a manner similar to that used for measuring integration. Each reaction contained a small plasmid with a mutant *att* site (*att*L or *att*R), a larger plasmid with the corresponding wild-type *att* site, and a [$^{32}$P]-labeled DNA fragment containing the wild-type partner *att* site. Excision was measured by comparing the relative amount of recombination (or density of the recombinant band) in the small plasmid and the larger one.

Plasmids containing the *ten* mutations in *att*L and *att*R were made by integration *in vitro* of the pBR322:*att*P *ten* mutant plasmids into the *att*B-bearing plasmid pHN861 as described previously (27). The recombinant plasmid contained both *att*L and *att*R. A *Bam*HI cleavage/religation separated the sites onto independent plasmids containing either *att*L and kanamycin resistance or *att*R and ampicillin resistance. The attachment site of each of the *ten* mutants in the respective *att*L or *att*R plasmids was sequenced to verify the sequence of the mutant and the remaining *att* site. The larger 'competitor' plasmids were made by cloning a 1.8 kb DNA fragment containing a gene for chloramphenicol resistance into the unique *Hin*dIII or *Pst*I sites of the *att*L$^+$ and the *att*R$^+$ plasmids, respectively.

Linear DNA (containing *att*L or *att*R) was isolated by *Bam*HI digestion of the respective *att*L or *att*R plasmid, and labeled in a manner analagous to that used for the *att*B DNA used in the *in vitro* integration assay. Likewise, the reaction conditions and the treatment of the reaction mixtures were performed identically to those in the *in vitro* integration assay, with the exception being that Xis and, sometimes, FIS were included. The proteins Int, IHF, and Xis were adjusted to maximize recombination. The frequencies of excision were represented as ratios by dividing the density of the mutant recombinant band divided by the density of the wild-type recombinant for each reaction.

### Recombination Proteins

IHF was isolated and purified from strain HN356 as described (28). Int was isolated and purified from strain HN695 as described (25). Xis was purified from strain HN804 by a modification of the procedure of Abremski and Gottesman (29). FIS was a gift from Dr. Reid Johnson (U.C.L.A.).

The IHF was diluted in 50 mM Tris-Cl (pH 7.4),10% glycerol, 2 mg/ml BSA, and 200 mM KCl. Int was diluted in 50 mM Tris-Cl (pH 7.4), 10% glycerol, 2 mg/ml BSA, 600 mM KCl, and 1 mM EDTA. Xis was diluted in 50 mM Tris-Cl (pH 7.4), 5% glycerol, 2 mg/ml BSA, 250 mM NaCl, 4mM DTT, and 1 mM EDTA. FIS was diluted in 20 mM Tris-Cl (pH 7.4), 50% glycerol, 1 M NaCl, and 0.1 mM EDTA. One unit of each protein is defined as the amount required to produce maximal recombination in the presence of optimal amounts of the other proteins.

## RESULTS

### Mutagenesis of *att*P, *att*L, and *att*R

In order to study the role of each Int arm-type site in integrative and excisive recombination, we made and verified by sequencing triple mutations called *ten* mutations, in each of the sites. Three completely conserved base pairs in the consensus sequence of the Int arm-type sites were changed from TCA to GTC (Table 1) using oligonucleotide-directed mutagenesis on M13 vectors containing *att*P. Each *ten* mutation was then crossed onto λ by homologous recombination for the *in vivo* assays and cloned into pBR322 for the *in vitro* assays as described in Materials and Methods. The construction of lysogens and plasmids bearing mutated *att*L and *att*R sites is also described in the Materials and Methods.

### Integrative Recombination *in vivo*

We compared the effects of single- and triple-base pair substitutions in the Int arm-type binding sites by testing both the *hen* and *ten* mutants for their ability to integrate into the genome of *E. coli*. The integration assay measured the percentage of N5813 cells that became lysogens when infected by different λ mutants. The results are shown in Table 2. The *ten* mutations affected integrative recombination in a manner similar to the respective *hen* mutations. The P1, P'2, and P'3 mutations inhibited integrative recombination 3 to 100-fold, while the P2 and P'1 mutations had no effect. Both the *hen* and *ten* P2 mutants consistently integrated as well as or better than the wild-type site. However, the assay was too imprecise to allow quantitation of this effect. The only dramatic difference between the two types of mutations in this assay appeared in the P'3 site, where the *ten* mutation inhibited integration more severely than did the analogous *hen* mutation.

**Table 2.** In Vivo Integrative Recombination[a]

| Phage | Frequency of Integration | Standard Deviation |
|---|---|---|
| λ*att*P-*ten*P1 | 4.9 | 4.5 |
| λ*att*P-*ten*P2 | 90 | 11 |
| λ*att*P-*ten*P'1 | 65 | 11 |
| λ*att*P-*ten*P'2 | 21 | 13 |
| λ*att*P-*ten*P'3 | 0.6 | 0.6 |
| λ*att*P-*hen*P1 | 2.8 | 1.3 |
| λ*att*P-*hen*P2 | 85 | 26 |
| λ*att*P-*hen*P'1 | 70 | 26 |
| λ*att*P-*hen*P'2 | 26 | 15 |
| λ*att*P-*hen*P'3 | 5.4 | 3.9 |
| λ*att*P$^+$ | 72 | 19 |

a) The values represent the averages of six independent assays. The frequency of integration was calculated by dividing the number of N5813 λ lysogens by the number of surviving N5813 cells following infection, and then multiplying by 100.

### Integrative Recombination *in vitro*

Integrative recombination was also measured for the *ten* mutants *in vitro* by comparing the amount of recombination with a small plasmid containing each *ten* mutation to that with a larger plasmid containing a wild-type *att*P site. An autoradiograph of one such assay is shown in Figure 2 and the results of six independent assays are summarized in Table 3. In general, the *in vitro* results corroborated the *in vivo* results; a *ten* mutation in the P1 or P'3 sites completely inhibited integration, whereas a P2 mutation slightly increased integrative recombination. A *ten*-P'1 mutation reduced *in vitro* integration 3-fold whereas this mutation had no effect *in vivo*; however, the effect *in vitro* was partially overcome by increasing Int concentrations (data not shown). Lastly, a *hen*-P'3 mutation integrated at least 10-fold better than the respective *ten* mutation, consistent with the results obtained *in vivo* (Table 2). In summary, the results with the *ten* mutants suggest the P1, P'2, P'3 and possibly the P'1 sites are required for integration.

### Excisive Recombination *in vivo*

The heat-pulse-curing assay (23) was used to examine the effect of these mutations on excisive recombination. The results are presented in Table 4. As observed for integrative recombination,

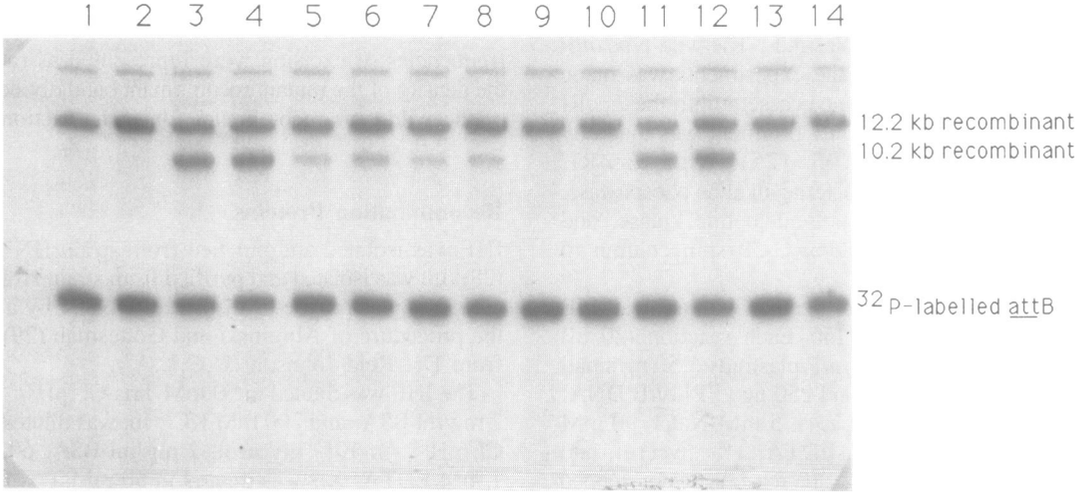


**Figure 2.** *In vitro* integration assays. Reactions displayed in lanes 1−12 contained linear $^{32}$P-labeled *att*B, two supercoiled *att*P substrates, one unit of IHF, and one or two units of Int. Reactions displayed in odd and even lanes contained 1 and 2 units of Int respectively. Each reaction in lanes 1−12 contained the wild-type *att*P on a 6.5 kb plasmid and a mutant or wild-type *att*P on a 4.5 kb plasmid. In addition to a wild-type *att*P substrate, lanes 1 and 2 contained an *att*P-*ten*P1 substrate, lanes 3 and 4 contained an *att*P-*ten*P2 substrate, lanes 5 and 6 contained an *att*P-*ten*P'1 substrate, lanes 7 and 8 contained an *att*P-*ten*P'2 substrate, lanes 9 and 10 contained an *att*P-*ten*P'3 substrate, and lanes 11 and 12 contained wild-type *att*P substrates. Lanes 13 and 14 contained only one *att*P substrate, the larger wild-type *att*P.

the *hen* and *ten* mutations displayed similar *in vivo* excision frequencies, with the exception of the *ten* P'1 mutant. The P'1 *ten* mutation is the only mutation that markedly inhibits excisive recombination in this assay, reducing it five-fold. A P1 or P'3 mutation increases excision three-fold, and a P2 or P'2 mutation has no effect.

A previous study (16) used a phage-cross assay that measures excisive recombination between co-infecting phages bearing *att*L and *att*R sites. They found that the *hen* P'1 mutant exhibited a three-fold decrease in excisive recombination, which in this study displayed no difference from wild-type in the heat-pulse-curing assay. However, we note that the *ten* P'1 mutant showed a five-fold reduction in excisive recombination in the heat-pulse-curing assay which is similar to the defect observed for the *hen* P'1 mutation in the phage cross assay. We have no specific explanation for the differences observed, but it could be due to the different types of assays.

Recently, the *E. coli* protein FIS was reported to enhance λ excisive recombination *in vitro* when Xis concentrations were limiting (9). It was subsequently shown that FIS can bind to the P arm of *att*P and *att*R at a site overlapping the X2 site for Xis (Figure 1). Xis cannot bind to the X2 site when FIS is bound (9). Because FIS has been implicated in excision, we assayed the *ten* mutants in a strain deficient in FIS. The mutants displayed more dramatic effects on recombination in the *fis* strain than in the wild-type strain (Table 4). The frequency of wild-type λ excision is reduced two-fold in a *fis* strain relative to a wild-type strain. A *ten* mutation in the P1 site had no effect on excision from a *fis* strain and the *ten* P'3 mutation enhanced excision three-fold compared to a wild-type site. In contrast, a *ten* mutation in either the P2 or the P'2 sites had little effect on λ excision in a wild-type *E. coli* strain, but reduced excision 15-fold and five-fold respectively relative to a wild-type site in the *fis* strain. The *ten* mutation in the P'1 site reduced excision five-fold relative to the wild-type site in a normal strain, but 15-fold with respect to the wild-type site in a *fis* strain. The *hen* mutants with mutations in the P2 or P'2 sites may show slightly reduced excision in a *fis* strain relative to a wild-type strain, but the defects were not as severe as those of the respective *ten* mutants.

**Table 3.** *In Vitro* Integrative Recombination[a]

| Plasmid | Integration relative to *wild-type* | Standard Deviation |
|---|---|---|
| *att*P-*ten*P1 | ≤0.3[b] | – |
| *att*P-*ten*P2 | 114 | 8.7 |
| *att*P-*ten*P'1 | 31 | 7.5 |
| *att*P-*ten*P'2 | 25 | 2.3 |
| *att*P-*ten*P'3 | ≤0.3[b] | – |
| *att*P-*hen*P'3 | 8.7 | 3.3 |
| *att*P[+] | 99 | 4.9 |

a)The ratio of mutant to wild-type integration was calculated by dividing the amount of the recombination product with the mutant by the amount of the recombination product with the wild-type and multiplying by 100. The values represent the averages of six independent assays with 1 unit each of Int and IHF.
b)represents the lower limitation of the assay

**Table 4.** *In Vivo* Excisive Recombination[a]

| λ*cI*857 Lysogens | Wild-Type Strain Frequency[b] of Excision | Wild-Type Strain Standard Deviation | *fis* Strain Frequency[c] of Excision | *fis* Strain Standard Deviation |
|---|---|---|---|---|
| *att*R-*ten*P1 | 86 | 7.2 | 20 | 7.0 |
| *att*R-*ten*P2 | 40 | 1.9 | 1.3 | 1.2 |
| *att*L-*ten*P'1 | 6.5 | 2.5 | 1.1 | 1.1 |
| *att*L-*ten*P'2 | 43 | 7.4 | 3.4 | 1.1 |
| *att*L-*ten*P'3 | 95 | 8.6 | 69 | 8.4 |
| *att*R-*hen*P1 | 88 | 9.2 | – | – |
| *att*R-*hen*P2 | 37 | 6.1 | 11 | 5.2 |
| *att*L-*hen*P'1 | 31 | 4.4 | – | – |
| *att*L-*hen*P'2 | 51 | 4.7 | 13 | 6.3 |
| *att*L-*hen*P'3 | 91 | 7.6 | – | – |
| *att*[+] | 30 | 4.8 | 16 | 4.9 |

a) The frequency of excision was determined using the heat-pulse-curing method (as described in Materials and Methods) and calculated by dividing the number of cured colonies which grow at 42°C by the total number of colonies which grow at 30°C and multiplying by 100.
b) The results represent the average of 7 independent assays.
c) The results represent the average of 5 independent assays.

## Excisive Recombination *in vitro*

The effects of the mutations on excision were also analyzed by competition experiments *in vitro*. Excision was measured by comparing the amount of recombination with a small plasmid containing a mutant *att*L or *att*R site, relative to that of a larger plasmid containing the wild-type sequence of the same site. An autoradiograph of one assay is shown in Figure 3 and the results of five independent assays are summarized in Table 5. In contrast to the integration *in vitro* assays, the smaller plasmids recombined more efficiently than the larger ones. In general, the *in vitro* results were similar to the *in vivo* results when *fis* strains were used. The *ten* P1 and P'3 mutations had little or no effect on excisive recombination. The *ten* mutations in the P'2, P'1, and P2 sites reduced excision by 15-, 30-, and 3-fold, respectively.

FIS protein was added to recombination reactions to test whether it could enhance recombination of any of the *ten* mutations. When 1 unit of Xis was present, there was no difference in recombination with wild-type *att* sites. However, when Xis was limiting (1/4 unit), the addition of 25 ng of FIS

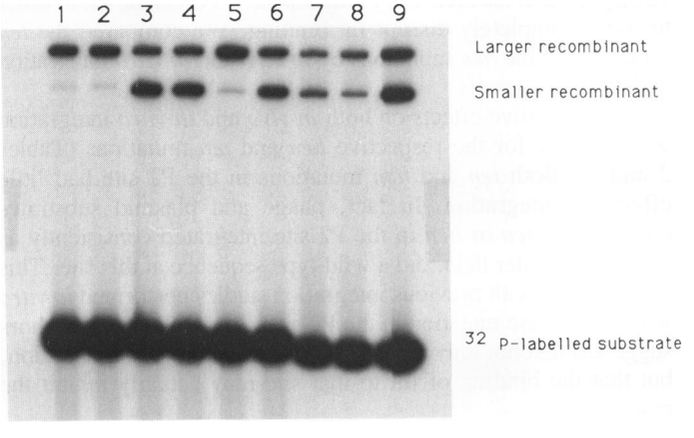


**Figure 3.** *In vitro* excision assays. Lanes 1–6 represent recombination reactions between a linear *att*L substrate ($^{32}$P-labeled) and two supercoiled plasmid substrates: a small plasmid containing an *att*R *ten* mutation and a larger plasmid containing the wild-type *att*R site. Lanes 7–9 show recombination between a linear *att*R substrate ($^{32}$P-labeled) and two supercoiled plasmid substrates: a small plasmid containing the respective *att*L mutation, and a larger plasmid containing the wild-type *att*L site. The reactions contained the following sites on the small plasmids: lane 1, *att*L-*ten*P'1; lane 2, *att*L-*ten*P'2; lane 3, *att*L-*ten*P'3; lane 4, wild-type *att*L; lane 5, *att*L-*hen*P'1; lane 6, *att*L-*hen*P'2; lane 7, *att*R-*ten*P1; lane 8, *att*R-*ten*P2; and lane 9, wild-type *att*R. Each reaction contained 1 unit of Int, IHF, and Xis.

**Table 5.** In Vitro Excisive Recombination[a]

| Site on Plasmid | Linear DNA Partner | Competitor DNA | Excision Relative to wild-type | Standard Deviation |
|---|---|---|---|---|
| *attR-ten*P1 | *attL*$^+$ | *attR*$^+$ | 111 | 0.5 |
| *attR-ten*P2 | *attL*$^+$ | *attR*$^+$ | 47 | 14 |
| *attR*$^+$ | *attL*$^+$ | *attR*$^+$ | 137 | 6.5 |
| *attL-ten*P'1 | *attR*$^+$ | *attL*$^+$ | 4.9 | 0.5 |
| *attL-ten*P'2 | *attR*$^+$ | *attL*$^+$ | 9.4 | 3.5 |
| *attL-ten*P'3 | *attR*$^+$ | *attL*$^+$ | 185 | 16 |
| *attL-hen*P'1 | *attR*$^+$ | *attL*$^+$ | 12 | 1.2 |
| *attL-hen*P'2 | *attR*$^+$ | *attL*$^+$ | 126 | 3.8 |
| *attL*$^+$ | *attR*$^+$ | *attL*$^+$ | 154 | 8.3 |

a) The values are an average of five independent assays performed as described in Materials and Methods. Each assay contained 1 unit of Int, Xis, and IHF. Excision was calculated by dividing the amount of the recombinant product with the mutant by the amount of the recombinant product with the wild-type and multiplying by 100.

restored full recombination activity (data not shown). This result agrees with previous *in vitro* studies (9). There was a slight increase in the frequency of excisive recombination upon the addition of FIS to the reactions containing the *attR-ten*P2 or *attL-ten*P'2 mutants, but the effects were less than the differences observed between the same mutants in the wild-type and *fis* strains *in vivo*.

## DISCUSSION

In a previous study (16), we constructed *hen* mutations in each of the Int arm-type binding sites by changing the conserved C to a T (Table 1), and analyzed them for integration, excision, and Int binding. It is possible that a single base change was not sufficient to disrupt Int binding at a mutant site, because cooperative interactions among Int, Xis, IHF, and FIS may have promoted Int binding to a mutated site. In this report, triple mutations (*ten*) were made in each of the Int arm-type sites, changing the conserved TCA sequence to GTC (Table 1), in order to more completely disrupt Int binding. We compared the *ten* mutations to the *hen* mutations by *in vivo* and *in vitro* integration and excision assays.

The qualitative effects on both *in vivo* and *in vitro* integration were similar for the respective *hen* and *ten* mutations (Tables 2 and 3). Both *ten* and *hen* mutations in the P2 site had little effect on integration. In fact, phage and plasmid substrates containing a *ten* or *hen* in the P2 site integrated consistently as well as, or better than, did a wild-type sequence at this site. This result agrees with previous integration studies performed *in vitro* with a five-base mutation in the P2 site (30) in which the authors suggested that not only is the P2 site unnecessary for integration, but that the binding of Int to that site may actually inhibit the reaction.

Integrative recombination was most severely inhibited when the P1 and P'3 sites carried *ten* mutations. In fact, the P'3 *ten* mutation inhibited integration more severely than the P'3 *hen* mutation in both *in vivo* and *in vitro* assays. One explanation for this difference is that the P'3 *hen* mutation may be partially overcome by cooperative interactions whereas a P'3 *ten* mutation cannot. We note that the *ten* P'1 mutant reduced integration in the *in vitro* assay, but appeared normal in the *in vivo* assay. The reason for this discrepancy is not known, but occupancy of the P'1 site by Int may actually be required for integration. This possibility is strengthened by the observation that Int can partially cover a *ten* P'1 site in a nuclease protection assay with *att*P DNA when Int is present in high concentrations along with IHF (data not shown). Thus for integration, the sites of greatest importance are those farthest from the core (P1, P'2, and P'3).

The effects of the *ten* mutations on excision suggest that Int binding to the P1 and P'3 sites is unnecessary for excision. The P'3 *ten* mutation increased the frequency of *in vivo* excision three- to four-fold in both wild-type and in *fis* hosts. The P1 *ten* mutation increased the frequency of excision three-fold in a wild-type strain, but had little or no effect in a *fis* strain. Neither of these *ten* mutations (P1 or P'3) affected *in vitro* excision in our assay. These results are consistent with studies in which the P1 site was deleted (30) or mutated (16) without affecting excision. It has been proposed (30) that Int bound to the P1 site may augment the inhibition of excision by promoting IHF binding to the H1 site. Another factor that may contribute to the high, heat-pulse-curing frequencies exhibited by the P1 and P'3 mutants is that the phage chromosome may be able to reintegrate into the host chromosome after excision under these conditions. If some reintegration takes place normally with wild-type λ after excision, then mutations which inhibit integration, such as those in the P1 or P'3 sites, may exhibit a higher frequency of excision with respect to a wild-type strain.

In wild-type *E. coli*, the P'1 *ten* mutation was the only construct that inhibited excisive recombination, reducing it 5-fold. However, in a *fis* strain, *ten* mutations in the P2 and P'2 sites, as well as those in the P'1 site, reduced the frequency of excision. *In vitro* excision assays showed that mutations in the P'1 or P'2 sites dramatically decreased the frequency of recombination. Taken together, these results demonstrate that the P2 and P'1 are important for excision as suggested previously (16,31) and provide the first direct evidence that the P'2 site is required.

The effects of the *ten* mutations on excision *in vivo* depended, in some cases, on the presence of FIS in the *E. coli* host. The decrease in excision of a λ lysogen containing a *ten* P2 mutation in a *fis* strain may be explained by the possible consequences of Int binding to the P2 site. According to one model, Int bound to the P2 site is required for excision, but recombination may occur with *hen* or *ten* mutations at this site, because they are overcome by the strong cooperative binding between Int and Xis (19,30). Cooperative interactions between Int and Xis have been observed to allow Int bindng of a mutant P2 site (31). If Xis is limiting under the heat-pulse-curing assay conditions, FIS may play an active role in stimulating excision by promoting Xis binding X1, which in turn may help Int bind to P2. Thus the severe defect in excision of a *ten* P2 mutation in a *fis* lysogen could be caused by incomplete occupancy of the X1-X2 or X1-FIS sites, leading to a decrease in the cooperativity that promotes Int binding to the mutant P2 site.

According to another model, Int binding to the P2 site is not required for excision, but may enhance it by facilitating the binding of Xis to the P arm in order to bend *att*R and, consequently, form a recombinogenic substrate. Other studies have also shown that P2 mutations and deletions have little effect on excision (31,32). In this model, Xis requires Int bound to the P2 site or FIS bound to the FIS site in order to efficiently bind to one or both of the Xis sites. If either Int or FIS binding is hindered, the presence of the other protein could continue to promote Xis binding. However, with both a P2 mutation in λ and a deficiency of FIS, the defect may be so marked that Xis cannot bind well enough to promote excision.

The efficiency of excision in a *fis* strain was also decreased in a λ lysogen containing a P′1 or P′2 *ten* mutation in *att*L. If, as described above, occupancy of X1-X2 and X1-FIS in *att*R is incomplete in a *fis* strain, this could lead to a decrease in the population of reactive *att*R structures. Nuclease-protection assays performed on *att*P DNA suggested that Int may bind to the P′1 or P′2 sites containing a *ten* mutation (data not shown), possibly through cooperative interactions. However, these sites may not be as fully occupied as in a wild-type *att*L *in vivo*, which may lead to a decrease in the population of active *att*L structures. Therefore, in a *fis* host, the *ten* P′1 and P′2 mutants may be defective in excision because the population of active *att*R and *att*L structures is reduced.

The results with the *ten* mutations demonstrate that, in some cases, the *hen* mutations were not always sufficiently disruptive to completely prevent Int binding to the P2, P′1, P′2, or P′3 sites. The effect of the P′3 *ten* mutation was worse than the analogous *hen* mutation in integration *in vivo* and *in vitro*; the P′1 *ten* mutation inhibited excision *in vivo* more than the respective *hen* mutation; and the *ten* P2 and P′2 mutations inhibited excision in a *fis* host more than the same *hen* mutations. Thus, a single-nucleotide mutation changing the highly conserved C to a T (*hen* mutation) did not disrupt the function of the Int arm-type binding site as much as the triple-nucleotide change. The differences between the *hen* and *ten* mutations may be due to the ability of Int to bind better to single-base mutations compared to triple-base mutations because of cooperative interactions between Int and Int, Int and IHF, and Int and Xis. Furthermore, these cooperative interactions may, for some sites, be strong enough to promote Int binding to a site with a triple-base change. For example, nuclease protection studies suggested that Int may be able to bind to mutated *ten* P′1 or *ten* P′2 sites (data not shown). Also, Int bound to the unaltered P′ sites at lower concentrations when IHF was present (data not shown). Moitoso de Vargas *et al.* (20) has suggested that IHF bends the DNA at the H′ site bringing the Int core and arm-type sites closer together thereby enhancing Int binding to both sites. Thus, the cooperative effect arising from IHF-induced bending of the DNA may have promoted Int binding to a mutant site. In addition, cooperativity between Int monomers has been reported in the P′ arm (15,16,17,18) which could also have promoted the binding of Int to a mutated P′1, P′2, or P′3 site. Collectively, the results with these triple mutations strengthen and extend the previous conclusions drawn from studies using single mutations with regard to the role of Int arm-type sites in recombination; the P1, P′2, P′3, and possibly the P′1 sites are required for efficient integration, and the P2, P′1, and P′2 sites for excision (16).

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