The Effect of Attachment Site Mutations on Strand Exchange in Bacteriophage λ Site-Specific Recombination

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ABSTRACT

Recombination of phage λ attachment sites occurs by sequential exchange of the DNA strands at two specific locations. The first exchange produces a Holliday structure, and the second resolves it to recombinant products. Heterology for base substitution mutations in the region between the two strand exchange points (the overlap region) reduces recombination; some mutations inhibit the accumulation of Holliday structures, others inhibit their resolution to recombinant products. To see if heterology also alters the location of the strand exchange points, we determined the segregation pattern of three single and one multiple base pair substitution mutations of the overlap region in crosses with wild type sites. The mutations are known to differ in the severity of their recombination defect and in the stage of strand exchange they affect. The three single mutations behaved similarly: each segregated into both products of recombination,, and the two products of a single crossover were frequently nonreciprocal in the overlap region. In contrast, the multiple mutation preferentially segregated into one of the two recombinant products, and the two products of a single crossover appeared to be fully reciprocal. The simplest explanation of the segregation pattern of the single mutations is that strand exchanges occur at the normal locations to produce recombinants with mismatched base pairs that are frequently repaired. The segregation pattern of the multiple mutation is consistent with the view that both strand exchanges usually occur to one side of the mutant site. We suggest that the segregation pattern of a particular mutation is determined by which stage of strand exchange it inhibits and by the severity of the inhibition.

MANY organisms encode proteins that promote recombination between DNA molecules that carry specific sites (for reviews see WEISBERG and LANDY 1983; SIMON and SILVERMAN 1983; SADOWSKI 1986; CRAIG 1988). One of the better studied site-specific recombination systems promotes integration and excision of the phage λ chromosome into and from the *Escherichia coli* chromosome. Recombinational strand exchange, which occurs at two positions within the 15-bp core region of the phage and bacterial attachment sites, is promoted by the topoisomerase activity of the λ-encoded Int protein: a DNA strand is cut, transferred to Int and then transferred back to a free end from another site (KIKUCHI and NASH 1979; PARGELLIS *et al.* 1988). As shown in Figure 1, the two top strands are exchanged at the position of the left (downward) arrow, and the two bottom strands at that of the right (upward) arrow (MIZUUCHI *et al.* 1981; CRAIG and NASH 1983). The 7-bp region between the two strand exchange points is called the overlap region (WEISBERG *et al.* 1983; WEISBERG and LANDY 1983). Studies with mutant *att* sites have demonstrated that efficient recombination occurs only when the two partners have identical overlap regions (WEISBERG *et al.* 1983; BAUER *et al.* 1984; 1985; KITTS and NASH 1987). It is important to note that many different 7 bp sequences are compatible with efficient recombination provided that the two recombining segments are homologous. The requirement for overlap region homology in site-specific recombination is not confined to λ but has also been found in several distantly related pathways: phage P1 Cre (HOESS, WIERZBICKI and ABREMSKI 1986), *Saccharomyces cerevisiae* Flp (SENECOFF and COX 1986; ANDREWS *et al.* 1986) and, probably, phage P2 Int (SIX 1963; 1966). Since the recombination proteins of all these pathways are members of a larger, structurally related family (ARGOS *et al.* 1986), it is likely that the homology requirement reflects a fundamental feature of the mechanism of strand exchange.

Even though the requirement for overlap region homology is strong, it is not absolute, and Int does promote crossing over within the cores of heterologous sites. (In this article, we shall use the terms

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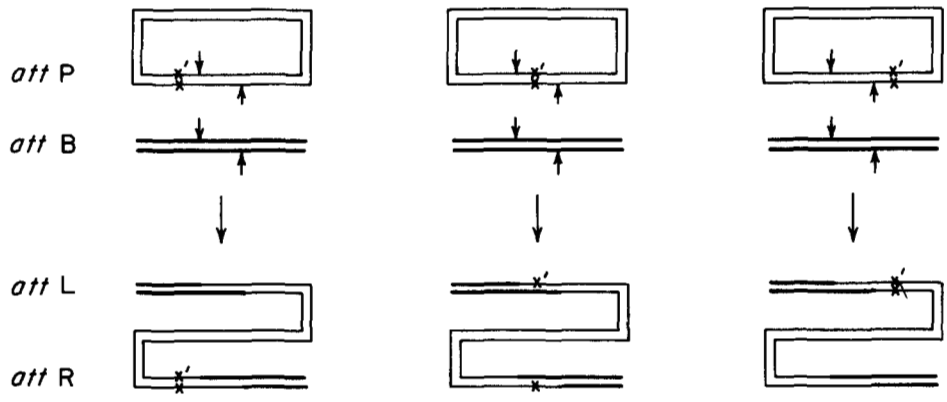

FIGURE 1.—Predicted segregation pattern of *att*P mutations. Left, an *att*P mutation located to the left of the top and bottom strand exchanges would segregate exclusively into *att*R. Middle, an *att*P mutation located between the position of top and bottom strand exchange would form heteroduplex *att*L and *att*R recombinant sites. Right, an *att*P mutation located to the right of both strand exchanges would segregate exclusively into *att*R. Light and heavy lines represent phage and bacterial DNA, respectively, the arrows mark the points of strand exchange and the letters × and ×′ mark the position of the mutations.

homologous and heterologous sites or crosses to signify pairs of sites or crosses between pairs of sites with identical and nonidentical core regions, respectively.) However, previous studies of the segregation of a multiple base substitution mutation in the overlap region suggested that heterology may alter the mechanism of recombination in such a way that strand exchanges occur at an abnormal location (WEISBERG *et al.* 1983). To examine more closely the role of homology during strand exchange, we determined the segregation patterns of several single base substitution mutations of the attachment sites. In contrast to the previous study, we found that the single mutations segregated during heterologous crosses as if strand exchange had occurred at the normal locations. These contrasting results suggest that different heterologies inhibit strand exchange at different stages and to different extents.

MATERIALS AND METHODS

Bacterial and phage strains: *E. coli* and phage λ strains used during this study are described in Table 1. The construction of the −2 and −3 attachment site mutants were described previously (BAUER, GARDNER and GUMPORT 1985). Table 2 shows the location of point mutations within the core that were used in this study.

Segregation pattern of *att*P-3 mutations: The segregation pattern of *att*P-3A and -3G mutations into *att*L and *att*R were followed by isolating λ *gal* (*att*L) *imm*-λ and λ *bio* (*att*R) imm21 recombinant phage from phage crosses between λ *att*P-3 *imm*λ and λ *gal*8 *att*B$^+$ bio936 *imm*21. The crosses were performed and recombinants were isolated as described by WEISBERG *et al.* (1983), using KS302 as the cross host and RW1518 and RW1520 to screen for recombinant phage. The isolated λ *gal* and λ *bio* recombinant phage were then screened for the presence or absence of the mutation by selective hybridization as described subsequently under *Hybridization analysis of att mutations.*

Segregation pattern of the *att*R-3A mutation: The segregation pattern of the *att*R-3A mutation into *att*B and *att*P was determined by isolating λ *gal bio imm*21 and λ *imm*λ recombinants from a phage cross between λ *gal* (*att*L$^+$) *imm*λ and λ bio (*att*R-3A) *imm*21. The cross was performed and the recombinants were isolated as described by WEISBERG *et al.* (1983) and BAUER, GARDNER and GUMPORT (1985). The isolated recombinants were then assayed for the presence or absence of the -3A point mutation by selective hybridization (see below).

Segregation pattern of overlap region mutations during integrative recombination: The segregation patterns of *att*P-2A and *att*P-2G core mutations into *att*L or *att*R were measured by selecting independent lysogens of the mutant phages and then determining the genotypes of the recombinant *att* sites that flank the prophage by three independent assays. One assay was based upon the observation that efficient excisive recombination requires DNA homology between *att*L and *att*R at position −2 (BAUER, GARDNER and GUMPORT 1985). Thus, lysogens conferring a wild-type heteroimmune curing frequency were presumed to contain homologous flanking core sequences, *i.e.*, both *att*L and *att*R were either wild-type or mutant, whereas lysogens conferring a low curing frequency were suspected to contain a mutation in only one flanking *att* site. This assumption was confirmed by the observation that lysogens with wild-type curing frequencies produced cured cells containing either a wild-type or a mutant *att*B phenotype but never a mixture of both. In contrast, lysogens with low curing frequencies gave rise to a mixed culture of cured progeny that possessed either a wild-type or a mutant *att*B at an equal frequency.

The *att*-2 lysogens were constructed by infecting N5813 (an *att*B insertion specificity strain) with λ *att*P-2 *imm*21 at a MOI = 10 followed by the selection and identification of *att*B inserted prophage using techniques previously described for *saf*G (WEISBERG *et al.* 1983). (In N5813, insertion of a prophage into *att*B prevents *gal* operon expression when the operon is derepressed by growth at temperatures greater than 41°. Nonlysogens and cells carrying prophages at other sites are Gal$^+$ in these conditions.) The lysogens were then tested for their heteroimmune curing frequencies by infecting with λ *gal*8 Δ[*gal*T-*att*-*Bio*B] *bio*936 *imm*434 followed by plating the cells on MacConkey-galactose plates. After overnight growth at 30°C, the colonies were assayed for their galactose phenotype by shifting the plates to 42°C for 4–6 hr. Cells that were cured of the prophage regained their Gal$^+$ phenotype at 42°C whereas cells that have retained a prophage inserted in *att*B remain Gal$^-$. The *att*B phenotype in the cured N5813 cells was then determined by the ability of the cured progeny to produce stable lysogens after infection with λ *att*P$^+$ *imm*21$^+$ or λ *att*P-2 *imm*21$^+$ as described by WEISBERG *et al.* (1983) for the *saf*G mutation.

To determine if the *att*-2 mutation was located within *att*L or *att*R in lysogens that had a low curing efficiency, er separated the two sites by isolating λ *gal* and λ *bio* transducing phages from the lysogens and then analyzed each of these phages for the presence of the mutation. Lysogens were induced by UV irradiation and the λ *gal* and λ *bio*

TABLE 1

Bacterial and phage strains

Strains	Relevant genotype	Source and/or reference
A) Bacterial strains		
CA3	*gal*E	CGCS[a]
JRG94	*bio*A2 *gal*31	CGCS
KS302	Δ[*gal-att*B-*bio*]	SHIMADA *et al.* (1973)
N5813	*gal*490 *att*B$^+$ (λ *bio*233 *c*I857)	WEISBERG *et al.* (1983)
N5813 (λ *imm*21$^+$)		This study
N5813 (λ *att*R-2A imm 21$^+$)		This study
N5813 (λ *att*R-2G imm21$^+$)		This study
N5813 (λ *att*L-2A imm21$^+$)		This study
N5813 (λ *att*L-2G imm21$^+$)		This study
MI766	*rec*A, Δ[*gal*K-λA]	HERSKOWITZ and SIGNER (1970)
RW1258	Δ[*att*B]Ω[*att*P]	WEISBERG *et al.* (1983)
RW1518	KS302 (λ *hφ*80 *intφ*80 *imm*21$^+$)	WEISBERG *et al.* (1983)
RW1520	KS302 (λ *hφ*80 *intφ*80 *imm*λ)	WEISBERG *et al.* (1983)
RW1600	N5813 *att*B-*saf*G	WEISBERG *et al.* (1983)
RW1685	N5813 *att*B-*saf*T	DE MASSY *et al.* (1984)
B) Phage λ strains		
λ *att*P$^+$ λ *c*I857		BAUER, GARDNER and GUMPORT (1985)
λ*att*P-3A λ *c*I857		BAUER, GARDNER and GUMPORT (1985)
λ*att*P-3G λ *c*I857		BAUER, GARDNER and GUMPORT (1985)
λ *att*P-2A λ *c*I857		BAUER, GARDNER and GUMPORT (1985)
λ *att*P-2G λ *c*I857		BAUER, GARDNER and GUMPORT (1985)
λ *att*P$^+$ *imm*21$^+$		BAUER, GARDNER and GUMPORT (1985)
λ *att*P-3A *imm*21$^+$		BAUER, GARDNER and GUMPORT (1985)
λ *att*P-3G *imm*21$^+$		BAUER, GARDNER and GUMPORT (1985)
λ *att*P-2A *imm*21$^+$		BAUER, GARDNER and GUMPORT (1985)
λ *att*P-2G *imm*21$^+$		BAUER, GARDNER and GUMPORT (1985)
λ *att*P-*saf*G		WEISBERG *et al.* (1983)
λ *att*P-*saf*T		DE MASSY *et al.* (1984)
λ *bio*936 (*att*R$^+$) *imm*21 *c*Its		WEISBERG *et al.* (1983)
λ *bio*936 (*att*R-3A) *imm*21*c*Its		BAUER, GARDNER and GUMPORT (1985)
λ *bio*936 (*att*R-3G) *imm*21 *c*Its		BAUER, GARDNER and GUMPORT (1985)
λ *bio*936 (*att*R-2A) *imm*21 *c*Its		BAUER, GARDNER and GUMPORT (1985)
λ *bio*936 (*att*R-2G) *imm*21 *c*Its		BAUER, GARDNER and GUMPORT (1985)
λ *bio*936 (*att*R-*saf*T) *imm*21 *c*Its		This work
λ *gal*8 (*att*L$^+$) *imm*λ *c*I857		BAUER, GARDNER and GUMPORT (1985)
λ *gal*8 (*att*L-2A) *imm*λ *c*I857		BAUER, GARDNER and GUMPORT (1985)
λ *gal*8 (*att*L-2G) *imm*λ *c*I857		BAUER, GARDNER and GUMPORT (1985)
λ *gal*8 (*attL-saf*T) *imm*λ *c*I857		This work
λ *gal*8 *bio*936 (*att*B$^+$) *imm*21 *c*Its		BAUER, GARDNER and GUMPORT (1985)
λ *gal*8 *bio*936 (*att*B-2A) *imm*21 *c*Its		BAUER, GARDNER and GUMPORT (1985)
λ *gal*8 *bio*936 (*att*B-2G) *imm*21 *c*Its		BAUER, GARDNER and GUMPORT (1985)
λ *gal*8 *bio*936 Δ(*gal*T-*bio*B) *imm*434		BAUER, GARDNER and GUMPORT (1985)

[a] *E. coli* genetic stock center (CGCS); Yale University School of Medicine.

TABLE 2

The *att* mutations used in this study

att site	Core sequence														
	−7							0							+7
att$^+$	g	c	t	t	t	T	T	T	A	T	A	C	t	a	a
att-3A	·	·	·	·	a	·	·	·	·	·	·	·	·	·	·
att-3G	·	·	·	·	g	·	·	·	·	·	·	·	·	·	·
att-2A	·	·	·	·	·	A	·	·	·	·	·	·	·	·	·
att-2G	·	·	·	·	·	G	·	·	·	·	·	·	·	·	·
*saf*T	·	·	·	·	·	·	·	·	·	·	·	A	·	·	·
*saf*G	·	·	·	·	·	·	·	·	T	C	·	A	·	·	·

The wild-type core sequence is shown in line 1, with the overlap region in upper case letters. The mutant *att* sites contain a base substitution at the positions indicated. The phage and bacterial *att* sites are identical within the core but diverge immediately outside.

transducing phage were isolated by their ability to transduce CA3 to Gal$^+$ or JRG94 to Bio$^+$ respectively as described by WEISBERG *et al.* (1983). The presence or absence of the *att* mutation in the transducing phage was assayed by selective hybridization with an oligonucleotide that is complementary to the mutant core sequence (see below). In addition, λ *gal* transducing phages were also assayed for their *att*L phenotype by measuring their ability to form stable lysogens in MI766, which contains *att*R substituted for *att*B (HERSKOWITZ and SINGER 1970). Finally, we note that individual lysogens displaying a low heteroimmune curing frequency gave rise to λ *gal* and λ *bio* transducing phage which contained the mutation in only one *att* site. In contrast, lysogens displaying a wild-type curing frequency gave rise to λ *gal* and λ *bio* transducing phage that contained either wild-type or mutant *att* sites in both phage.

Repair of *saf*G and *saf*T mutations during integrative recombination: Gal$^-$ lysogens of N5813 (*att*B-*saf*$^+$), RW1600 (*att*B-*saf*G) or RW1685 (*att*B-*saf*T) were selected after infection with λ *att*P$^+$ *imm*21, λ *att*P-*saf*G *imm*21 or λ *att*P-*saf*T *imm*21 as described in WEISBERG *et al.* (1983). (The Gal$^-$ phenotype indicates that the prophage has inserted at *att*B.) Identity or nonidentity of the overlap regions of the two prophage attachment sites in lysogens was determined by one of the two following methods. In the case of lysogens produced by recombination between sites heterologous for *saf*G, we measured the frequency of prophage curing after heteroimmune superinfection. Control experiments with lysogens of known genotype confirmed that curing was 10–100 times more frequent when the two sites were identical (either *saf*$^+$ or *saf*G) than when they differed. Similar control experiments in which the prophage sites differed by the *saf*T mutation revealed that the excision defect resulting from heterology was too small to be a reliable indicator in this case. Therefore, to decide if the overlap regions of the prophage sites were homologous or not, we isolated phage released by the lysogens and cured cells that were produced after heteroimmune superinfection of the same lysogens. If the *att* sites of all phage and cells deriving from a given lysogen were uniformly of one *saf* genotype, we considered that both prophage *att* sites were also of that genotype. These genotypes were determined as follows. Twenty-five to 60 phage released by each lysogen were amplified by plaque formation. Phage from these plaques were used to lysogenize strain N5813, which carries *att*B$^+$, or RW1685, which carries *att*B-*saf*T, and the ability of many independently isolated lysogens to utilize galactose was determined. Control experiments showed that *saf*$^+$/*saf*T site combinations gave similar numbers of Gal$^+$ and Gal$^-$ lysogens, while *saf*$^+$/*saf*$^+$ and *saf*T/*saf*T site combinations gave more than 95% Gal$^-$ lysogens. The *att*B genotype of at least 6 independently isolated cured cells from each lysogen (see above) was analyzed by reinfection with λ *att*P-*saf*$^+$, isolation of lysogens and determination of their ability to utilize galactose.

Segregation pattern of overlap region mutations during excisive recombination: The segregation patterns of *att*L-2 and *att*R-2 mutations into *att*B during excisive recombination were measured by two independent tests. In one assay, recombinant λ *gal-bio* phages were isolated from phage crosses between λ *gal* (*att*L) and λ *bio* (*att*R) (one of which contained the mutation) as described previously (WEISBERG *et al.* 1983). The *att*B transducing phage were then tested for their ability to form stable lysogens in RW1258 a strain that has *att*P substituted for *att*B (WEISBERG *et al.* 1983). Control experiments demonstrated that phage containing *att*B$^+$ can form stable lysogens in RW1258 whereas phage containing *att*B-2 cannot. In another assay, prophage in N5813 that were flanked by mutant *att*L or mutant *att*R sites were isolated and identified as described above for the pattern during integrative recombination and then assayed for the segregation pattern of the mutation into *att*B upon heteroimmune curing as described above. The segregation of *saf*T from *att*L or *att*R into *att*P was followed by isolating *att*P recombinants from the appropriate cross as described by WEISBERG *et al.* (1983) and determining the ability of these recombinants to form lysogens by insertion into the *att*B site of strain N5813 as described in WEISBERG *et al.* (1983) and above for the pattern during integrative recombination. Control experiments showed that nearly all N5813 lysogens formed by infection with phage carrying *att*P-*saf*$^+$ failed to express the gal operon after thermal derepression, while a large fraction (50% or more) of lysogens formed by infection with phage carrying *att*P-*saf*T did express gal.

Hybridization analysis of *att* mutations: Phage λ, λ *bio* and λ *gal bio* were assayed for the presence or absence of the −2 or −3 core mutations by selective hybridization with two families of mixed oligonucleotides d[G-C-T-T-(A,G,C)-T-T-T-A-T-A-C-T-A-A] for *att*-3 and d[G-C-T-T-T-(A,G,C)-T-T-A-T-A-C-T-A-A] for *att*-2 mutations. Under stringent hybridization conditions the mixed oligonucleotide probes display a stronger hybridization signal with the phage DNA containing the perfectly complementary −2 or −3 core mutation, than for the wild-type phage containing a core sequence with which they contain a mismatch (BAUER, GARDNER and GUMPORT 1985). Phage DNA was isolated by the plate lysate procedure (DAVIS *et al.*, 1980), digested with *Ava*I or *Hin*cII restriction endonuclease and the fragments separated by electrophoresis in 1.0 or 1.5% agarose gels. The DNA was denatured and the gel dried as described by PURELLO and BALAZS (1983). The DNA was hybridized at 25° to the ^{32}P-labeled oligonucleotide family using the conditions described by WALLACE *et al.* (1981).

RESULTS

Segregation pattern of *att*-3 mutations: If recombination between heterologous *att* sites occurs by strand exchanges at the same positions as those used in recombination between homologous sites, then mutations located outside and inside of the region of strand exchange should segregate as shown in Figure 1. Thus, mutations in the phage *att* site (*att*P) that are located to the left of the top strand exchange site, such as *att*P-3, should only segregate into *att*R (Figure 1, left), and mutations located to the right of the bottom strand exchange site should only segregate into *att*L (Figure 1, right). The segregation pattern of mutations located between the two points of strand exchange (Figure 1, middle) will be considered below. The segregation patterns of *att*P-3 mutations into *att*L or *att*R during integrative recombination were followed by carrying out phage crosses between λ *att*P-3 and λ *gal bio* (*att*B$^+$) (Figure 2). The resulting recombinant λ *gal* (*att*L) and λ *bio* (*att*R) phage were then isolated and analyzed for the presence or absence of the mutation by selective hybridization of an oligonucleotide probe that is complementary to the mutant core sequence. The results of this analysis (Table 3) are in agreement with expectation: none of the 21

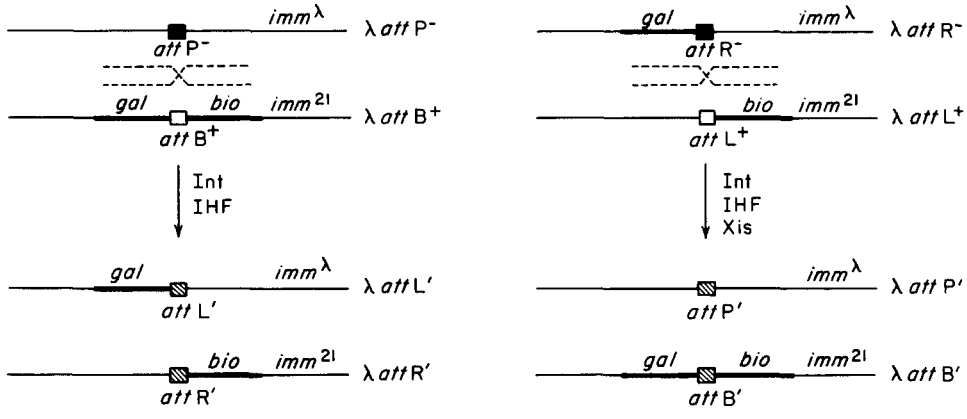

FIGURE 2.—Measurement of the segregation pattern by phage crosses. Left, the segregation patterns of *att*P-3 mutations during integrative recombination were measured by crossing λ *att*P-3 × λ *att*B⁺ and analyzing the recombinant *att*L and *att*R phages for the presence or absence of the mutation by selective hybridization. Right, the segregation patterns of *att*R mutations during excisive recombination were measured by crossing λ *att*R⁻ × λ *att*L⁺ or by crossing *att*R⁺ × *att*L⁻ and analyzing the λ *att*B recombinant for the presence of the mutation by a genetic test or by selective hybridization (see MATERIALS AND METHODS).

TABLE 3

Segregation patterns of *att*-3 mutations

Phage cross	Recombinant genotype			
	*att*P-3	*att*B-3	*att*L-3	*att*R-3
Integration				
λ *att*P-3 × λ *att*B⁺			0/21	11/11
Excision				
λ *att*L⁺ × λ *att*R-3	9/9	0/12		

Phage crosses were performed as described in MATERIALS AND METHODS. The number before the slash indicates the number of recombinant phage with the indicated genotype whereas the number after the slash indicates the total number analyzed. Each phage tested was obtained from an individual phage cross.

independently isolated *att*L recombinants contained the mutation whereas all of the *att*R recombinants did.

The segregation pattern of the *att*-3 mutations during excision were similarly examined. The assay involved a phage cross between λ *gal* (*att*L⁺) and λ *bio* (*att*R-3) which generated λ and λ *gal bio* recombinants (Figure 2) that were assayed for the presence and absence of the mutation as described above. Of 12 independent λ *att*B recombinants assayed none contained the mutation whereas all of the λ *att*P recombinants did (Table 3). These segregation patterns demonstrate that the −3 core mutations are located to the left of strand exchange during both integrative and excisive recombination.

Segregation pattern of *att*-2 mutations: The *att*-2 mutations are located between the two strand exchange points; that is, within the overlap region. If strand exchange in heterologous crosses occurs as diagrammed in Figure 1 (middle) and Figure 3 (right), the recombinant *att*L and *att*R sites will both contain mismatches in the overlap region, with different strands containing the mutation. Subsequent DNA replication through the *att* sites should generate progeny in which one daughter would be flanked by *att*R-mutant and *all*L⁺ and the other by*att*R⁺ and *att*L-mutant. However, if some of the heteroduplexes are corrected by mismatch repair before DNA replication occurs, then it should also be possible to isolate prophages in which both flanking sites are identical, either wild type or mutant.

The segregation patterns for λ *att*P-2 point mutations were measured by integrating λ *att*P-2 into the chromosome and analyzing the genotypes of the recombinant *att*L and *att*R attachment sites that flank the prophage. To do this, we isolated transducing phages that carried either *att*L or *att*R (λ *gal* and λ *bio*, respectively) and determined the presence of the

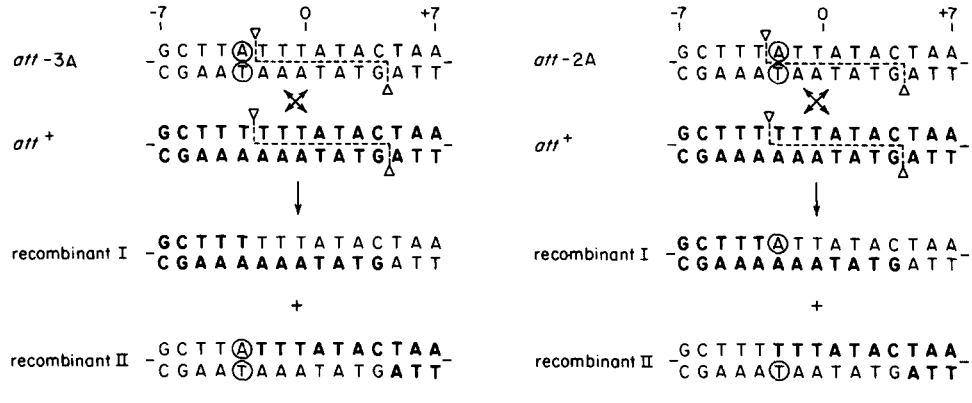

FIGURE 3.—Segregation pattern of *att*-2A and -3A mutations. The predicted segregation pattern of the *att*-3A mutation is diagramed on the left. This mutation (circled bases) is located outside of the positions of strand exchange and thus should exclusively segregate into only one of the recombinant *att* sites (recombinant II). The predicted pattern of the *att*-2A mutation is diagrammed on the right. This mutation, which is located in the overlap region, is predicted to form a pair of heteroduplex recombinants in a cross with a wild type site.

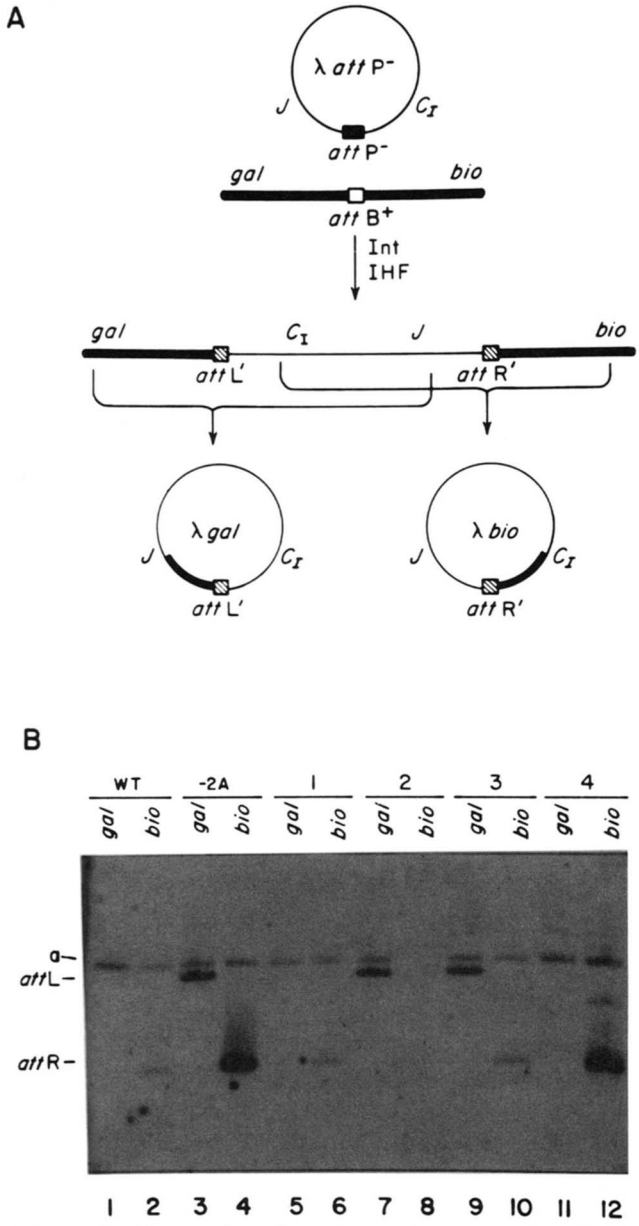

FIGURE 4.—Segregation of *att*P-2 mutations into *att*L and *att*R during lysogenization. A) Phage containing the *att*P-2 mutation were integrated into a wild-type *att*B to form prophages flanked by recombinant *att* sites of unkown genotype. The presence or absence of the mutation in the recombinant *att* sites was analyzed by isolating λ *gal* and λ *bio* transducing phage which were checked for the mutation by selective hybridization using an oligonucleotide as a probe (see B, below). B) λ *gal* and λ *bio* transducing phage were isolated from independent lysogens (A, above) and assayed for the presence or absence of the *att* mutation by selective hybridization with a oligonucleotide that was complementary to the mutant sequence. Under stringent hybridization conditions (high temperature) the mutagenic oligonucleotide will preferentially hybridize to the fully complementary mutant core sequence as opposed to the wild type core sequence with which it has a mismatch. Lanes 1 and 2 contain wild-type λ *gal* and λ *bio* controls respectively. Lanes 3 and 4 contain mutant (-2A) λ *gal* and λ *bio* controls respectively. Lanes 5 through 12 contain λ *gal* and λ *bio* phages isolated from four independent lysogens. The cumulative segregation patterns observed for 16 independent lysogens are shown in Table 4. Band A, which is present in all of the λ *gal* and most of the λ *bio* phage is a region in λ that fortuitously hybridizes to the mixed family of oligonucleotides.

TABLE 4

Segregation patterns of *att*P-2 mutations into *att*L and *att*R during recombination

Genotypes of prophage sites		Frequencies after infection with:		Combined frequencies
*att*L	*att*R	*att*P-2A	*att*P-2G	*att*P-2A + *att*P-2G
+	+	2/10	2/6	4/16
+	−2	4/10	1/6	5/16
−2	+	3/10	2/6	5/16
−2	−2	1/10	1/6	2/16

Lysogens carrying a prophage in the *att*B site of strain N5813 were isolated after infection with phage containing *att*P-2A or *att*P-2G mutations (Figure 4). The segregation patterns of the *att*P mutations into *att*L or *att*R of the prophage were determined by three independent assays as described in MATERIALS AND METHODS. Ten independent λ *att*P-2A lysogens and 6 independent λ *att*P-2G lysogens were assayed.

att-2 mutation by three independent tests (see MATERIALS AND METHODS and Figure 4). The results of this analysis (Table 4) show that the *att*P-2 mutations segregate at an equal frequency into *att*L and *att*R (7 of 16 lysogens contained mutations in *att*L and 7 of 16 lysogens contained mutations in *att*R). In addition, mismatch repair appears to be occurring since it was possible to isolate lysogens containing all four possible combinations of wild-type and mutant *att*L and *att*R sites. These results are in contrast to those observed with *saf*G which showed a markedly biased segregation pattern (WEISBERG *et al.* 1983) (see DISCUSSION).

The segregation patterns of *att*L-2 and *att*R-2 mutations into *att*B during excisive recombination were also followed. In one series of experiments, phage crosses between λ *gal* (*att*L) × *bio* (*att*R) were performed in which one *att* site was wild-type and the other contained an *att*-2 mutation (Figure 2). In another independent assay, heteroimmune curing was performed on lysogens containing mutant (position −2) *att*L or *att*R sites. In both series of experiments the *att*B recombinants were then analyzed for the presence or absence of the mutation. The results of these assays (Table 5) show that *att*-2 mutations in either *att*L or *att*R segregate into *att*B at approximately 50% frequency (38 out of 73 independent recombination events). Again these results are in contrast to the asymmetric pattern observed with *saf*G.

Segregation pattern of the *saf* T mutation: *Saf*G differs from the *att*-2 mutations in the nature, number and location of the sequence changes (Table 2). To see which of these differences is important in determining the asymmetric segregation pattern, we analyzed crosses involving sites carrying the *saf* T mutation, a transversion of the rightmost base of the overlap region (position +4) that is identical to one of the three changes in *saf*G (Table 2).

We found that both *att*L-*saf* T × *att*R$^+$ and *att*L$^+$ ×

TABLE 5

Segregation frequencies of *att*L-2 and *att*R-2 mutations into *att*B during excisive recombination

Crosses	Frequency of *att*B$^+$		
	Phage cross	Curing	Combined[a]
*att*L-2A × *att*R$^+$	4/12	8/16	20/41
*att*L-2G × *att*R$^+$	4/7	4/6	
*att*L$^+$ × *att*R-2A	7/12	4/8	18/32
*att*L$^+$ × *att*R-2G	2/4	5/8	

The frequency of *att*B$^+$ recombinants was measured by isolating *att*B recombinants after a phage cross (column 1) or after heteroimmune curing of a lysogen (column 2), and determining the *att*B phenotype as described in MATERIALS AND METHODS.

[a] The total *att*B$^+$ recombinants from crosses involving an *att*L-2 mutation recombined with a wild-type *att*R (line 2) or from crosses involving wild-type *att*L and an *att*R-2 mutation (line 4).

TABLE 6

Segregation of *saf*T$^+$ into *att*P during excisive recombination

Parental sites		Frequency of *att*P-*saf*$^+$ recombinants
*att*L	*att*R	
saf$^+$	*saf*T	12/24
*saf*T	*saf*$^+$	4/13

Lambda *gal*, which contains *att*L, and λ *bio*, which contains *att*R, were crossed as described in MATERIALS AND METHODS, and recombinants that were unable to transduce either *gal* or *bio*, and which therefore contained *att*P, were isolated as described in WEISBERG *et al.* (1983). These phage were tested for their Saf phenotype as described in MATERIALS AND METHODS. Both homologous crosses yielded *att*P recombinant whose Saf phenotype was always identical to that of their parents (data not shown).

*att*R-*saf*T crosses gave mixtures of *att*P-*saf*T and *att*P$^+$ recombinants (Table 6). The frequencies of the two *att*P recombinants were comparable in both crosses. This contrasts with the results of similar crosses involving *saf*G (WEISBERG *et al.* 1983). If strand exchange between *saf*T and *saf*T$^+$ sites occurs at the normal positions, it will produce heteroduplex intermediates with *saf*T/*saf*T$^+$ mismatches. If repair can convert such mismatches to homoduplex DNA before replication occurs, the two recombinant sites deriving from a single cross will sometimes have identical overlap regions, as described above for crosses involving *att*-2 mutants (see above). To see if such repair occurs, we isolated lysogens resulting from *saf*T × *saf*T$^+$ integrative recombination and characterized the attachment sites of the liberated phage and cured cells that they produced. We found (Table 7) that 7 of 20 such lysogens produced a single type of phage and the identical single type of cured cell. We conclude that the *att*L and *att*R sites of the prophages carried by these 7 lysogens have identical overlap regions, as expected if integration can generate *saf*T/*saf*T$^+$ mismatches that are subject to repair. In contrast, none of 92 lysogens produced by *saf*G × *saf*G$^+$ integration had *att*L and *att*R sites with identical overlap regions (Table 7). This result shows that co-repair of the three *saf*G changes is much rarer than repair of the *saf*-2 or *saf*T changes. Our analysis probably would not have detected repair of a single *saf*G component, but probably would have detected co-repair of two changes (data not shown). These results suggest that the *att*-3, *att*-2 and *saf*T mutants recombine with wild type using the same points of strand exchange as those used in homologous crosses, but that the *saf*G mechanism is different (see DISCUSSION).

DISCUSSION

We have determined the segregation patterns of λ *att* site mutations during *in vivo* Int-promoted recombination between heterologous sites, and compared these patterns to those expected if strand exchange occurs at the normal positions, at each edge of the overlap region. MIZUUCHI *et al.* (1981) and CRAIG and NASH (1983) showed that top strand exchange during *in vitro* integrative recombination between wild-type sites occurs between positions −3 and −2, and bottom strand exchange occurs between positions +4 and +5. We found that single base substitution mutations at the −3 position, to the left of both strand exchange points, cosegregate with the left arm of the attachment site during heterologous crosses, as expected from the *in vitro* results. Three single base substitution mutations of the overlap region, *att*-2A, *att*-2G and *saf*T (at position +4), have a roughly equal frequency of cosegregation with either arm. This is expected if strand exchange in these crosses occurs at the normal positions to produce recombinant sites that contain mismatches, and these mismatched base pairs segregate from each other in an unbiased way. Unbiased segregation could result either from DNA replication through the sites containing the mismatched bases, or from mismatch repair that acts with equal frequency on either strand of the recombinant sites. In fact our results suggest that mismatch repair is frequent, as evidenced by the many prophage recombinants in which information from one strand appears to have been replaced by information from the other. In addition, the segregation patterns reported here confirm that *in vivo* strand exchange during excision appears to occur at the same locations as it does during integration even though these are two distinct recombination events requiring different sets of DNA substrates and proteins (GUARNEROS and ECHOLS 1970; ECHOLS and GUARNEROS 1983; BUSHMAN *et al.* 1985).

In contrast to *att*-2 and *saf*T, we found no evidence of mismatch repair in crosses involving *saf*G, a triple base substitution mutation of the attachment site. This result is consistent with previous results that showed that *saf*G does not segregate during heterologous crosses in the way predicted from strand exchange at the normal positions (WEISBERG *et al.* 1983). Instead,

TABLE 7

Repair of *saf* mutants during integration

Parental sites		Prophage sites			Lysogens analyzed
*att*P	*att*B	Both *saf*$^+$	Both *saf*	Mixed	
*saf*T	*saf*$^+$	1	0	6	7
saf$^+$	*saf*T	3	3	7	13
*saf*G	*saf*$^+$	0	0	60	60
saf$^+$	*saf*G	0	0	34	34

Derivatives of strain N5813 carrying the appropriate *att*B were infected with phage stocks carrying the appropriate *att*P, and independent lysogens carrying a prophage inserted at *att*B were isolated. Lysogens deriving from *saf*T infections (lines 1 and 2) were analyzed by determining the phenotypes of released phage and cured cells. Lysogens deriving from *saf*G infections (lines 3 and 4) were analyzed by determining the frequency of curing after heteroimmune superinfection (see MATERIALS AND METHODS).

*saf*G was found to cosegregate with the right arm of the attachment site about 95% of the time. We argue that such abnormal segregation is not the result of a mismatch repair mechanism that operates in favor of a particular strand, a possibility advanced by WEISBERG *et al.* (1983). This hypothesis can account for the *saf*G segregation pattern during integrative recombination only if the mismatches in both *att*L and *att*R are preferentially corrected in favor of the top strand (see Figure 1, middle panel). Such preferential correction is not likely to be a consequence of the nature of the mismatches since this can be changed without changing the segregation pattern (WEISBERG *et al.* 1983). However, if preferential correction were a consequence of the mechanism of integration or the physiology of λ growth, we would have expected the *att*-2 and *saf*T segregation patterns to be similar to that of *saf*G, which they clearly were not. Therefore, we reject this hypothesis. In addition, the segregation pattern of *saf*G cannot be due solely to the severity of its recombination defect, since the magnitude of the *saf*G defect *in vivo* is between those of *att*-2 and *saf*T.

To explain the abnormal segregation of *saf*G, WEISBERG *et al.* (1983) proposed that *saf*G heterology preferentially inhibits exchange of the bottom strands. Top strand exchange occurs normally, and this leads to a Holliday structure (HOLLIDAY 1964) whose branchpoint is constrained by the unfavorable energy of base mispairing to lie to the left of the heterology. The production of recombinant products requires resolution of this intermediate through exchange of the bottom strands, and this occurs inefficiently and preferentially to the left of the heterology (see below). Since both strand exchanges are to the left of *saf*G, the mutant locus usually cosegregates with the right arm of the attachment site.

Recent *in vitro* studies are consistent with the above proposal about *saf*G and help us to explain why *att*-2 and *saf*T mutations segregate as expected for strand exchange at the normal locations. KITTS and NASH (1988) and NUNES-DÜBY, MATSUMOTO and LANDY (1987) have shown that *in vitro* Int-promoted recombination between attachment sites with identical overlap regions proceeds by two successive strand exchanges. First the two top strands exchange between positions −3 and −2 to produce a Holliday structure, then the two bottom strands exchange between positions +4 and +5 to resolve the intermediate to recombinant products. Heterology involving any of the left three bp of the overlap region (positions −2 through 0) prevents the accumulation of Holliday structures (KITTS and NASH 1987). It is not known if heterology inhibits the first strand exchange or stabilizes its product. Artificial Holliday structures containing the *att*-2A heterology are resolved by Int to give low but detectable numbers of both recombinant and parental molecules (DE MASSY, DORGAI and WEISBERG 1989), suggesting that if the intermediate could accumulate, some level of normal resolution would occur. Heterology involving the right 4 bp of the overlap region, positions +1 through +4, acts differently; it does not inhibit accumulation of the Holliday structure (KITTS and NASH 1987), but inhibits proper resolution. Studies with artificial Holliday structures show that *saf*G (+1, +2 and +4 changes) and *saf*T (+4 change) heterologies inhibit Int-promoted resolution to recombinant products but not resolution to parental molecules (DE MASSY, DORGAI and WEISBERG 1989). To explain such preferential resolution, DE MASSY, DORGAI and WEISBERG (1989) proposed that migration of the branchpoint of the Holliday structure to the second strand exchange point is required for resolution to recombinant products, and heterology impedes such migration.

In view of these *in vitro* studies, we suggest that the rate limiting step in recombination *in vivo* between *att*-2 and *att*$^+$ sites is the formation or stabilization of a Holliday structure in which the top strands have been exchanges at the normal location. Once this structure has formed or has been stabilized, we assume that Int has a limited capacity to promote its resolution to recombinant products by normal bottom strand exchange. *Saf*T and *saf*G heterologies both block resolution of the Holliday structure to recombinant products *in vitro*, but have different segregation patterns *in vivo*. To explain this difference we note that the 3 bp heterology of *saf*G blocks both *in vivo* recombination and Int-promoted resolution of artificial Holliday structures to recombinant products much more effectively than does the 1 bp heterology of *saf*T (DE MASSY *et al.* 1984; DE MASSY, DORGAI and WEISBERG 1989). This presumably reflects the greater barrier to branch migration imposed by the larger heterology. We suggest that in *saf*T × *saf*$^+$ crosses, Holliday structure branchpoints occasionally migrate through the 1-bp heterology and are resolved by Int at the

normal location. In *saf*G × *saf*$^+$ crosses such migration is much less frequent, and therefore the very rare recombinants we find arise by strand exchange at an abnormal location. Such aberrant resolution need not be Int promoted, but could result from the action of a cellular enzyme (such as DNA polymerase or a DNA branch resolvase) on the Holliday structure (WEISBERG *et al.* 1983).

We and previous workers (DE MASSY *et al.* 1984) have noted that heterology for *saf* T depresses the frequency of integrative recombination rather modestly: the magnitude of its defect is about ten-fold, several orders of magnitude less severe than that of *att*-2 even though both mutations are single transversions adjacent to a point of strand exchange. This asymmetry presumably reflects the different stages of recombination blocked by these mutations (KITTS and NASH 1987; see above). It is, however, more difficult to account for the difference between the modest effect of *saf* T heterology on integrative recombination *in vivo* and its much more severe effect on the same reaction *in vitro*; this difference is greater than 100-fold in relative recombination frequencies (KITTS and NASH 1987; M. BRUIST, personal communication). DE MASSY, DORGAI and WEISBERG (1989) report that artificial Holliday structures with *saf* T heterology are resolved to recombinant products *in vitro* with about one-tenth the efficiency of the corresponding homologous structures, a defect quite comparable to that of the overall *in vivo* reaction. This suggests that the *in vitro* reaction is more sensitive than the *in vivo* to disruptions that occur prior to the formation of a resolvable intermediate. For example, some condition or factor missing in the *in vitro* reaction could impede reversal of the initial strand exchange, perhaps by promoting migration of the branchpoint away from the initial strand exchange point.

Although Int promoted recombination between heterologous sites is usually inefficient compared to homologous crosses, its frequency is well above background and probably contributes to the formation of phage variants with altered site affinity and to chromosomal rearrangements of phage and host. Thus, site-specific recombinases acting with low efficiency and reduced specificity could be responsible for some fraction of "illegitimate" recombination. If the rather irregular segregation patterns described here are a general rule, it may not always be straightforward to recognize the activity of a site-specific recombinase from the structure of the recombinants it produces.

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