

## Identifying Determinants of Recombination Specificity: Construction and Characterization of Mutant Bacteriophage Integrases

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The Integrases of bacteriophages  $\lambda$  and HK022 promote recombination between DNA molecules that carry attachment sites. The two integrases are about 70 % identical in sequence and catalyze nearly identical reactions, but recognize different sets of sites. To identify the amino acids that determine this difference in specificity, we selected mutants of  $\lambda$  integrase with increased ability to recombine HK022 sites. This selection yielded eleven different amino acid substitutions at eight different positions. Three of the positions belong to a larger set that were identified as important for the  $\lambda$ /HK022 specificity difference by analysis of chimeric integrases. Substitution of the HK022 for the corresponding  $\lambda$  residue at each of these three positions increased recombination of HK022 sites, and one double substitution, N99D-E319R, increased recombination to nearly wild-type HK022 levels. Mutations at the other five positions changed residues that are identical in the wild-type proteins or are at positions identified by chimera analysis as unimportant for the  $\lambda$ /HK022 specificity difference. All of the mutants isolated by selection for increased recombination of HK022 sites retained considerable ability to recombine  $\lambda$  sites. However, we found that substitution of HK022 for  $\lambda$  residues at three additional positions, S282P, G283K, and R287K, specifically reduced recombination of  $\lambda$  sites. These three substitutions when combined with N99D and E319R were sufficient to change the specificity of  $\lambda$  to that of HK022 integrase. The first three substitutions act principally to prevent recombination of  $\lambda$  sites, and the second two to remove a barrier to recombination of HK022 sites. We suggest that many natural alterations in the specificity of protein-DNA interactions occur by multi-step changes that first relax and then restrict specificity.

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### Introduction

The integrases (Ints) encoded by bacteriophages  $\lambda$  and HK022 are recombinases that promote prophage integration and excision (see Yagil *et al.*, 1995, accompanying article). They are members of a large family of related enzymes that catalyze reciprocal double-strand exchange between DNA molecules with specific sites. Both integrases recognize sets of sequences, called arm and core binding sites, within their cognate phage and bacterial attachment sites. They use the same accessory proteins, Xis, Fis, and IHF, to increase the efficiency and alter the directionality of recombination. Although these two

proteins appear to use the same biochemical mechanism and are 70% identical in sequence, neither recombines the attachment sites of the other phage. Heterospecific recombination is prevented by the inability of either protein to recognize the heterologous core binding sites (Yagil *et al.*, 1989; Nagaraja & Weisberg, 1990). The two proteins recognize similar sets of arm binding sites and have identical arm-binding domains. In the accompanying article (Yagil *et al.*, 1995), we showed that substitution *en bloc* of 13 HK022-specific residues for their  $\lambda$  counterparts is sufficient to confer full HK022 specificity on  $\lambda$  integrase. In this report we describe the isolation and characterization of individual mutations and combinations of mutations on Int specificity. We first isolated and characterized mutants of  $\lambda$  Int that promote recombination of HK022 sites. All of these mutants retained

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considerable ability to recombine  $\lambda$  sites. We then identified HK022 residues that specifically prevented recognition of  $\lambda$  sites. A quintuple mutant containing changes of both types had nearly complete HK022 specificity.

## Results

### Tests for recombination proficiency

To facilitate the isolation and characterization of mutants synthesizing altered Int proteins, we constructed recombination reporter strains in which  $\lambda$  and HK022-specific integrative and excisive recombination are signalled by a change in gene expression. The strains used to measure integrative recombination contain the phage and bacterial attachment sites (*attP* and *attB*, respectively), and those used to measure excisive recombination contain the left and right prophage attachment sites (*attL* and *attR*, respectively). Int and Xis were provided by phage infection. The construction of these strains and the methods for using them are described in Materials and Methods and in the accompanying paper (Yagil *et al.*, 1995). Briefly, excisive recombination of either  $\lambda$  or HK022 attachment sites deletes a fragment that interrupts the *lacZ* gene, and recombination frequency was measured by assaying  $\beta$ -galactosidase or by estimating the intensity of blue color on plates containing the indicator dye Xgal. Integrative recombination of  $\lambda$  attachment sites deletes the *gal* operon and was measured by counting the fraction of Gal<sup>-</sup> colonies. Integrative recombination of HK022 attachment sites disrupts the *lac* operon and was measured by determining the fraction of Lac<sup>-</sup> colonies or, since high level expression of the *lac* operon is lethal in our conditions, by the fraction of IPTG-resistant colonies.

### Point mutants with relaxed specificity

Mutants of  $\lambda$  Int that have an increased ability to promote HK022 excisive recombination were isolated by plating mutagenized phage cultures on a lawn of an indicator strain that detects HK022 excisive recombination (LD70). Wild-type  $\lambda$  produces colorless plaques and wild-type HK022 and the selected mutants form blue plaques in the presence of Xgal. We note that LD70, unlike LD205, the strain routinely used to assay HK022 excisive recombination, does not have a wild-type HK022 B' site (see Materials and Methods), and was used because  $\lambda$  forms very pale blue plaques on lawns of LD205. The increased stringency of LD70 may have prevented us from isolating certain classes of mutants.

The frequency of blue plaque forming mutants in different mutagenized stocks varied between  $10^{-5}$  and  $10^{-6}$  per phage, about three orders of magnitude less than the frequency at which clear plaque forming mutants arose. The *int* coding regions of 45 mutants, each isolated from an independently

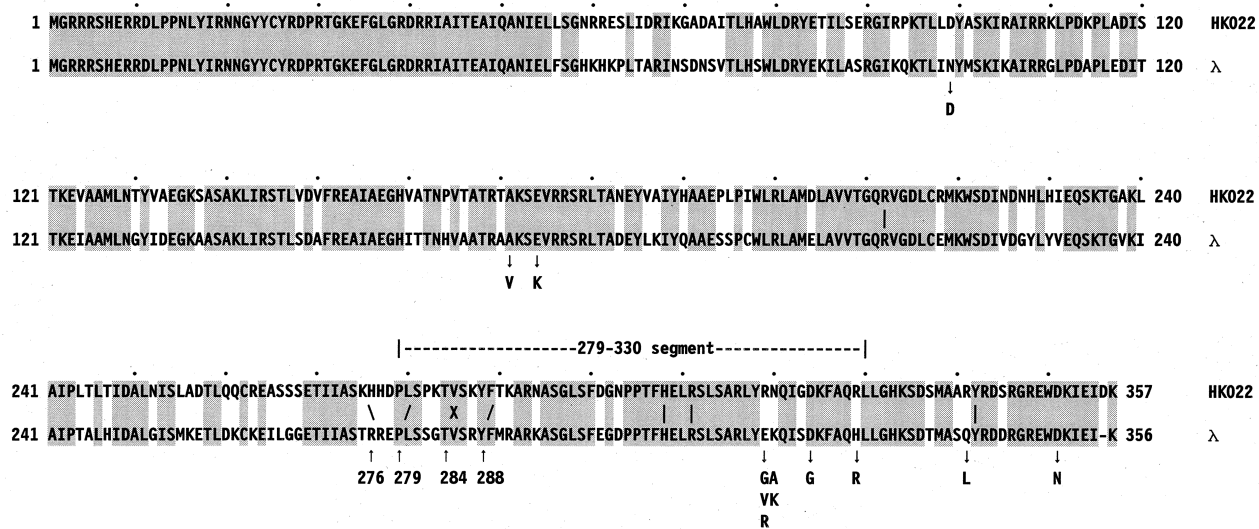
**Table 1.** Lambda *int* mutations that increase recombination of HK022 sites

Amino acid change	Codon change	Number of isolates
N99D	<u>A</u> AT → <u>G</u> AT	1
A171V	<u>G</u> CA → <u>G</u> TA	4
E174K	<u>G</u> AG → <u>A</u> AG	15
E319G	<u>G</u> AG → <u>G</u> GG	15
	<u>G</u> AG → <u>G</u> GT	1
E319A	<u>G</u> AG → <u>G</u> CG	1
E319V	<u>G</u> AG → <u>G</u> TG	1
E319K	<u>G</u> AG → <u>A</u> AG	1
D324G	<u>G</u> AT → <u>G</u> GT	4
H329R	<u>C</u> AT → <u>C</u> GT	1
Q341L	<u>C</u> AG → <u>C</u> TG	1
D351N	<u>G</u> AC → <u>A</u> AC	1

The base change is underlined for each mutation. All of the mutations were induced by growth of  $\lambda$  on a *mutD* host except E319A, which is a spontaneous mutation.

mutagenized phage stock, were sequenced. All had a single mutation that altered one of eight amino acids in three regions of Int (see Table 1 and Figure 1). Quantitative measurements of recombination (Table 2) showed that the mutants promoted modest to substantial levels of HK022 excisive recombination, and low to modest levels of HK022 integrative recombination (the most active had about 2% of wild-type HK022 activity). The frequency of excisive recombination relative to wild-type always exceeded that of integrative, as if the specificity barrier were lower for the former than for the latter (see Yagil *et al.*, 1995). All retained the ability to recombine  $\lambda$  sites efficiently, although they differed quantitatively from the wild-type  $\lambda$  parent. Most increased the frequency of  $\lambda$  excisive recombination 1.5 to twofold and decreased the frequency of  $\lambda$  integrative recombination to 0.3 to 0.5 that of wild-type (see Discussion). We first consider mutations affecting positions 99, 319, and 329, since these are among the residues identified as determinants of the  $\lambda$ /HK022 specificity difference in the accompanying article (Yagil *et al.*, 1995).

We found that substitution of Gly, Ala, Val, or Lys for Glu319 increased the activity of  $\lambda$  Int toward HK022 sites (Table 2). The increase was most substantial for the first three. The different sizes and chemical natures of the side-chains of the four substituted amino acids suggest that the mutant phenotype arises from removal of the negative charge of Glu319 rather than substitution of a specific amino acid, and are consistent with the hypothesis that the substituted residue is not involved in a base-specific interaction (see Discussion). HK022 Int has Arg at this position, and substitution of Arg for Glu319 by site-directed mutagenesis gave a phenotype similar to that of the four original mutants (Table 2). The Arg substitution requires a multiple base change, and we assume this is why it was not isolated in the original selection. The two other possible single base substitution mutations, E319D and E319Q, were not found in our collection, possibly because Asp is negatively charged, and Gln carries a partial negative charge.



**Figure 1.** Alignment of HK022 and  $\lambda$  Ints. The amino acid substitutions in  $\lambda$  Int that increase recombination of HK022 sites are shown below the  $\lambda$  sequence by downwards-pointing arrowheads. The diagonal lines between the two sequences give the location of the chimera joints of the chimeras referred to in Table 3. The orientation of each line indicates the origin of the carboxyl and amino termini of that chimera, and the upwards-pointing arrowheads indicate the reference numbers of the chimeras. The shaded blocks indicate sequence identities, and the four most highly conserved amino acids of the integrase family are marked by vertical lines. The 279-330 segment includes HK022 residues that confer HK022 excision specificity on  $\lambda$  integrase (see the text and Yagil *et al.*, 1995).

Mutations N99D and H329R also occur at positions that we previously identified as important for specificity (Yagil *et al.*, 1995). Both mutations are directional changes, i.e. they substitute the HK022 for the corresponding  $\lambda$  amino acid. Neither is as

active in promoting recombination of HK022 sites as is the most active of the position 319 mutants. N99D reduced integrative recombination of  $\lambda$  sites to about 14% of wild-type, but had no significant effect on excisive recombination. In these respects it differs

**Table 2.** Relative activities of Int mutants and combinations of mutants selected for increased HK022 excisive recombination

Int	$\lambda$ -Specific recombination		HK022-Specific recombination	
	Excisive	Integrative	Excisive	Integrative
$\lambda$ wt	100 (892 $\pm$ 119) <sup>a</sup>	100 (83.2 $\pm$ 2) <sup>a</sup>	0.8 $\pm$ 0.19	0.0015 $\pm$ 0.0008
HK022 wt	2.7 $\pm$ 0.5	$\leq 0.4$	100 (4727 $\pm$ 263) <sup>a</sup>	100 (64.8 $\pm$ 2.2) <sup>a</sup>
$\lambda$ Mutants:				
N99D	108 $\pm$ 7.4	14 $\pm$ 2.8	8.2 $\pm$ 1.8	0.0072 $\pm$ 0.0024
A171V	210 $\pm$ 10	40 $\pm$ 9	7.2 $\pm$ 0.4	0.029 $\pm$ 0.004
E174K	190 $\pm$ 11	34 $\pm$ 9	17 $\pm$ 0.3	0.040 $\pm$ 0.004
E319G	210 $\pm$ 18	49 $\pm$ 10	44 $\pm$ 2	0.15 $\pm$ 0.03
E319A	184 $\pm$ 10	35 $\pm$ 4	38 $\pm$ 2	2.3 $\pm$ 0.2
E319V	201 $\pm$ 16	43 $\pm$ 6	44 $\pm$ 4	0.32 $\pm$ 0.03
E319K	214 $\pm$ 16	40 $\pm$ 4	3.6 $\pm$ 0.06	0.0021 $\pm$ 0.0002
E319R	224 $\pm$ 18	47 $\pm$ 6	33 $\pm$ 4	0.072 $\pm$ 0.016
D324G	188 $\pm$ 10	48 $\pm$ 8	8.9 $\pm$ 0.2	0.0068 $\pm$ 0.0007
H329R	148 $\pm$ 11	31 $\pm$ 8	9.3 $\pm$ 1.1	0.0078 $\pm$ 0.003
Q341L	197 $\pm$ 14	45 $\pm$ 10	7.7 $\pm$ 0.03	0.0034 $\pm$ 0.0003
D351N	169 $\pm$ 21	35 $\pm$ 1	6.2 $\pm$ 0.2	0.006 $\pm$ 0.0005
N99D-H329R	91 $\pm$ 9	15 $\pm$ 3	17 $\pm$ 1	0.1 $\pm$ 0.03
E319R-H329R	164 $\pm$ 21	41 $\pm$ 4	36 $\pm$ 5	0.11 $\pm$ 0.02
N99D-E319R	226 $\pm$ 37	73 $\pm$ 3	69 $\pm$ 4	25 $\pm$ 5
N99D-E319A	201 $\pm$ 19	83 $\pm$ 6	102 $\pm$ 5	50 $\pm$ 3
N99D-E319R-H329R	214 $\pm$ 36	38 $\pm$ 8	56 $\pm$ 2	19 $\pm$ 4

The figures to the right of the  $\pm$  symbol are standard errors of the mean.

<sup>a</sup> The numbers within parentheses are the absolute activities of the homologous wild-type combinations. These are  $\beta$ -galactosidase specific activities for both types of excisive recombination, the frequency in per cent of IPTG-resistant, Lac<sup>-</sup> colonies for HK022 integrative recombination, and the frequency of Gal<sup>-</sup> colonies for  $\lambda$  integrative recombination.

**Table 3.** Recombination proficiency of Int chimeras relative to wild-type homospecific controls

No.	Int	Additional HK022 residues <sup>a</sup>	$\lambda$ -Specific recombination		HK022-Specific recombination	
			Excisive	Integrative	Excisive	Integrative
1	h276 $\lambda$	N/A <sup>b</sup>	7	$\leq 0.1$	9	$\leq 0.2$
2	h284 $\lambda$	H277, D278, P282, K283	$\leq 0.1$	$\leq 0.1$	21	$\leq 0.2$
3	$\lambda$ 288h	N/A	13	20	29	$\leq 0.2$
4	$\lambda$ 284h	K287	11	1.5	64	$\leq 0.4$
5	$\lambda$ 279h	P282, K283	1	$\leq 0.1$	105	$\leq 0.4$

The locations of the chimera joints of these five chimeras are shown in Figure 1. Data taken from Yagil *et al.* (1995).  
<sup>a</sup> Additional HK022-specific residues relative to the chimera listed on the immediately preceding line.  
<sup>b</sup> N/A: not applicable.

from all of the other mutants of this collection, which decreased  $\lambda$  integrative recombination more modestly and increased  $\lambda$  excisive recombination as much as twofold.

The following five mutations occur at positions that were not previously identified as important for the difference in specificity of the two integrases. Q341L, which changes a residue that corresponds to Arg in HK022 Int, is located in a region that is quite unlikely to be involved in the HK022/ $\lambda$  specificity difference (Yagil *et al.*, 1995). A171V, E174K, D324G, and D351N change residues that are identical in the two proteins, and these residues, therefore, cannot be determinants of the natural specificity difference. All of these mutants have high levels of activity for  $\lambda$  attachment sites. E174K, formerly called Int-h, has been isolated in previous selections. In addition to promoting recombination between pairs of variant attachment sites (Miller *et al.*, 1980; Enquist *et al.*, 1979; C. Gritzmacher & R.A.W., unpublished experiments), it has a reduced requirement for the accessory recombination proteins IHF (Miller *et al.*, 1980; Lange-Gustafson & Nash, 1984) and Xis (C. Gritzmacher & R.A.W. unpublished experiments; Bear *et al.*, 1987). This suggests that IntE174K has increased catalytic activity rather than (or in addition to) altered DNA recognition. Conceivably, the other four mutations are similar (see Discussion).

### Multiple substitutions in $\lambda$ Int

The residues at positions 99, 319, and 329 are likely candidates for determinants of the HK022/ $\lambda$  specificity difference because substitutions of the HK022 for the  $\lambda$  residue relax specificity, and because they are a subset of the 13 specificity-determining residues identified by analysis of chimeric Ints (Yagil *et al.*, 1995). The relaxation of specificity conferred by each of these three changes individually is small, and we therefore constructed and characterized all possible double and triple combinations (Table 2). The multiple mutants exhibited a further increase both in HK022-specific excision and integration, although the effect of combining E319R with H329R was small. The effect of combining N99D with E319R and, to a lesser extent, with H329R was larger than expected from simple additivity. This is particularly clear for HK022 integrative recombination: N99D and E319R are about five and 50-fold more active,

respectively, than wild-type  $\lambda$  Int, but the double mutant is about 17,000-fold more active. This suggests that Asp99 cooperates with Arg39 in overcoming the block to recognition of HK022 sites. This huge increase in HK022 activity does not specifically require Arg at position 319, since we have also seen it with the double mutant N99D-E319A (Table 2). This is consistent with our earlier conclusion that the presence or absence of a negative charge is the major specificity determinant at this position. Addition of H329R to the double mutant N99D-E319R did not result in any further significant increase in HK022 activity.

### Requirements for a switch in specificity

It is noteworthy that both Int-N99D-E319R and Int-N99D-E319R-H329R promoted efficient  $\lambda$  integrative and excisive recombination. We conclude that additional mutations are needed for a specificity switch, and that these mutations act principally to reduce recombination of  $\lambda$  sites. Comparison of the phenotypes of chimeric Int proteins to each other helped to reveal the identity of the residues in question. Chimera  $\lambda$ 279h has three more HK022-specific residues than does chimera  $\lambda$ 288h and one more than does chimera  $\lambda$ 284h (Table 3). A triple substitution of Pro282, Lys283, and Lys287, for their  $\lambda$  counterparts reduced  $\lambda$  excisive recombination more than tenfold and  $\lambda$  integrative recombination more than 100-fold while increasing HK022 excisive recombination only three to fourfold (Table 3, lines 3 and 5; Yagil *et al.*, 1995). A single substitution of Lys287 for its  $\lambda$  counterpart had an intermediate effect (Table 3, lines 3 and 4). Chimera h284 $\lambda$  has four more HK022-specific residues than does chimera h276 $\lambda$ , His277, Asp278, Pro282, and Lys283, and substitution of these residues for their  $\lambda$  counterparts depressed  $\lambda$  excisive recombination more than 70-fold while increasing HK022 excisive recombination only two to threefold (Table 3, lines 1 and 2). These comparisons directed our attention to the residues at positions 282, 283, and 287, since we had previously shown that His277 and Asp278 are not required for full HK022 specificity (Yagil *et al.*, 1995).

To see if substitution of HK022-specific residues at just these three positions reduces the capacity of  $\lambda$  Int to recombine  $\lambda$  sites, we made and characterized the triple mutant S282P-G283K-R287K. These substi-

**Table 4.** Relative recombinational activities of Int mutants

No.	Int	$\lambda$ -Specific recombination		HK022-Specific recombination	
		Excisive	Integrative	Excisive	Integrative
1	$\lambda$ wt	100	100	$0.8 \pm 0.2$	$0.0015 \pm 0.0008$
2	HK022 wt	$2.7 \pm 0.5$	$\leq 0.4$	100	100
3	S282P	$132 \pm 23$	$34 \pm 7$	$0.89 \pm 0.05$	$0.00061 \pm 0.0001$
4	S282P-G283K	$12 \pm 0.4$	$\leq 0.4 \pm 0.03$	$4.7 \pm 0.2$	$0.0012 \pm 0.0001$
5	S282P-G283K-R287K	$2.5 \pm 0.8$	$\leq 0.5 \pm 0.1$	$22 \pm 1$	$0.034 \pm 0.006$
6	N99D-S282P-E319R	$74 \pm 5$	$56 \pm 10$	$39 \pm 1$	$25 \pm 3$
7	N99D-S282P-G283K-E319R	$117 \pm 12$	$2.6 \pm 0.4$	$87 \pm 8$	$35 \pm 1$
8	N99D-S282P-G283K-R287K E319R	$11 \pm 0.7$	$\leq 0.3 \pm 0.05$	$90 \pm 3$	$56 \pm 4$
9	N99D-S282P-E319R-H329R	$106 \pm 6$	$50 \pm 12$	$60 \pm 2$	$13 \pm 3$
10	N99D-S282P-G283K-E319R-H329R	$111 \pm 7$	$0.49 \pm 0.08$	$132 \pm 9$	$70 \pm 7$
11	N99D-S282P-G283K-R287K-E319R-H329R	$16 \pm 0.8$	$\leq 0.3 \pm 0.08$	$93 \pm 4$	$68 \pm 3$

For more information, see legend to Table 2.

tutions, as predicted, strongly reduced  $\lambda$ -specific recombination (Table 4, line 5). They also increased HK022-specific recombination, but to considerably less than wild-type HK022 levels. The decrease was greatest for  $\lambda$  integrative recombination, and the absolute increase was greatest for HK022 excisive recombination, a differential effect that is reminiscent of the phenotypes of many of the single mutants that relax specificity. The phenotype of the double mutant S282P-G283K was less severe than that of the triple mutant, and that of the single mutant S282P was still less severe (Table 4, lines 3 and 4). This suggests that the change at position 282 is not essential for the phenotype, or that it has an effect only in the presence of the other two substitutions. We have not constructed the double mutant G283K-R287K, which would have allowed us to distinguish between these hypotheses.

Int-S282P-G283K-R287K recombines both  $\lambda$  and HK022 sites poorly. We predict that combining N99D and E319R with these three substitutions will specifically restore Int activity on HK022 sites. Indeed, we found that the quintuple mutant recombined the HK022 substrates very well and the  $\lambda$  substrates poorly (Table 4, compare line 8 to line 5). The full phenotype required R287K (Table 4, line 7), and a triple mutant that lacked G283K and R287K was relaxed (Table 4, line 6). Addition of H329R to the triple, quadruple, or quintuple mutant had only small effects, indicating that the minimal set of HK022 amino acids that confers HK022 specificity on  $\lambda$  Int does not include Arg329 (Table 4, lines 9, 10, and 11). These results show that the S282P-G283K-R287K triple substitution prevents  $\lambda$  Int from recombining  $\lambda$  sites, and they argue that the primary role of Lys283 and Lys287 (perhaps together with Pro282) is to moderate the specificity-broadening effect of N99D and E319R.

## Discussion

Substitution of HK022 for  $\lambda$  residues at five positions confers almost complete HK022 recombinational specificity on  $\lambda$  integrase. Two of these HK022 residues, Asp99 and Arg319, act mainly to broaden specificity; that is, they allow  $\lambda$  integrase to recombine HK022 sites without substantially reducing its activity on  $\lambda$  sites. The other three, Lys283, Lys287 and possibly Pro282, act mainly to prevent the quintuple mutant from recombining  $\lambda$  sites. These five are a subset of the 13 residues shown to be sufficient to confer HK022 recombinational specificity on  $\lambda$ /HK022 chimeras (Yagil *et al.*, 1995). It is likely that some of the eight additional residues implicated by analysis of chimeras are not determinants of specificity and were included in the set of 13 only because they are neighbors of real specificity determinants. However, it remains possible that additional sets of positive/negative or other types of context-dependent specificity determinants remain to be identified.

How do these substitutions alter specificity? We have shown that the nucleotide sequences that are differentially recognized by the two integrases are located in the core binding sites of the two sets of attachment sites, and that differences at positions 1, 3, and 4 of the 7 bp consensus sequence are important for the HK022/ $\lambda$  specificity difference (Nagaraja & Weisberg, 1990; L.D. & R.A.W., unpublished experiments). Amino acid substitutions can switch specificity directly, by altering base-specific interactions between the substituted amino acid and nucleotides of the core sites, or indirectly, by altering the conformation of the DNA binding surface of the protein or its orientation relative to the DNA (see below). Amino acid substitutions can broaden specificity by preventing an unfavorable

contact of the protein with the non-cognate sites, by increasing catalysis of a step that limits the rate of heterospecific recombination, or by increasing catalysis of a step that limits the rate of both heterospecific and homospecific recombination. Although the third class of mutation should increase recombination of the cognate sites, this increase could be masked by conditions that are difficult to control *in vivo*, such as occurrence of the reverse reaction or an inadequate supply of substrate or accessory factors. The data presented in this article do not allow us to decide unambiguously among these possibilities for each substitution, but they do argue against some of the possibilities.

It is unlikely that the E319R substitution affects residues that directly contact DNA. First, substitutions of widely varied character at position 319 increased recombination of HK022 sites, suggesting that the phenotype is the result of removal of the negative charge of Glu319 rather than introduction of a specific side-chain. Second, all of these substitutions had rather small and discrepant effects on recombination of  $\lambda$  sites: the relative frequency of excisive recombination increased about twofold and that of integrative recombination decreased to about 40%. We speculate that the increase in excisive recombination causes an apparent decrease in integrative recombination by reducing the accumulation of products. Third,  $\lambda$  insertion into many different secondary bacterial attachment sites (reviewed by Weisberg & Landy, 1983) was substantially and uniformly increased by E319R (L.D. & R.A.W., unpublished experiments). We suggest that E319R increases catalysis of a step that limits the rate of recombination promoted by  $\lambda$  Int between wild-type HK022 sites as well as that between  $\lambda$  *attP* and many secondary bacterial attachment sites. Increased catalysis would partially suppress inefficient enzyme-substrate interaction. By contrast, N99D is unlikely to increase catalytic activity. This substitution increased recombination of HK022 sites, decreased  $\lambda$  integrative recombination, and did not substantially change  $\lambda$  excisive recombination (Table 2 and other data not shown). This substitution also decreased  $\lambda$  insertion into some but not all secondary attachment sites (L.D. & R.A.W., unpublished experiments). We suggest that N99D alters a base-specific protein-DNA interaction that impedes recognition of HK022 sites by  $\lambda$  Int. The triple substitution S282P-G283K-R287K reduced recombination of  $\lambda$  sites even more severely than did N99D, and it selectively reduced recombination of  $\lambda$  sites in the presence of Asp99 and Arg319. Therefore, it may also alter base-specific interactions between integrase and attachment sites.

Recognition of core sites by Int occurs within a protein-DNA complex that consists of several Int protomers and two attachment sites (Kim & Landy, 1992; Kim *et al.*, 1990). Binding of Int to isolated core sites is weak, and *attB* is known to acquire Int from an Int-*attP* complex, not from solution, during integrative recombination (Richet *et al.*, 1988). Each set of two attachment sites has four similar but

non-identical core binding sites. One of the four core sites, C', is identical in HK022 and  $\lambda$ , and therefore the DNA binding surfaces of the two proteins are probably similar. The other three core sites differ between the two phages, and swaps show that each of the three is important for specificity (Nagaraja & Weisberg, 1990; L.D. & R.A.W., unpublished experiments). However, inspection of the differences does not reveal a sequence motif that could be used for recognition by each integrase, and analysis of site mutants suggests that the effect of a base substitution at a given core position depends on which core site contains the mutation (L.D. & R.A.W., unpublished experiments). These considerations argue that the sequence elements important for recognition of a particular core binding site by Int depend in some way on the location of that site within the recombination complex. Therefore, specificity-altering alterations need not change the conformation of the DNA binding surface of the protein, but can act by modifying the architecture of the complex in such a way as to change the orientation of the binding surface and particular core sites. This type of specificity-altering mutation is already known or suspected to exist in other systems (Mondragon & Harrison, 1991; Harrison & Aggarwal, 1990).

The locations of the determinants of the HK022/ $\lambda$  specificity difference suggest that the Integrase family of proteins does not have a single, compact core binding domain or module that is comparable to the Int arm binding domain or to the helix-turn-helix DNA binding motif of the resolvase family of site-specific recombinases (Grindley, 1993; Abdel-Meguid *et al.*, 1984). We have argued that one of the specificity-broadening substitutions, E319R, alters the catalytic activity of Int. The location of this substitution, within a segment that includes the catalytic residues His308, Arg311, and Tyr342, lends additional support to this argument. It has recently been shown that an Int protomer acting as part of a multimeric DNA-protein complex preferentially cleaves the site to which it is bound (Nunes-Düby *et al.*, 1994), a result that is anticipated if site recognition is coupled to catalysis. If this view is correct, Int joins a group of enzymes in which recognition of specific sequences is coupled to catalysis of DNA cleavage or modification (Klimasauskas & Roberts, 1995; Kumar *et al.*, 1994; Vipond & Halford, 1993; Vermote *et al.*, 1992).

Our division of the five specificity-determining residues into positively and negatively acting classes has evolutionary implications. It also has a precedent, as specificity determination by the joint action of positive and negative elements has also been found in the nuclear receptor family of ligand-inducible transcription factors (Zilliagus *et al.*, 1994) and in a translational repressor of RNA bacteriophages (Lim *et al.*, 1994). We expect that more instances will be found as more cases are examined. Such separation of function can facilitate evolutionary change by DNA binding proteins. A change in specificity that occurs by mutations that simultaneously increase recognition of a new

**Table 5.** Bacteria, phage, and some of the plasmid strains used in this work

A. Bacteria			
Strain	Genotype and/or use	Source or reference	
GM2163	<i>dam13::Tn9 dcm-6</i>	Woodcock <i>et al.</i> (1989)	
LD70	MC1000/pLD70; used to select <i>int</i> mutants with HK022 specificity	This work	
LD177	MC1000/pLD177; used to measure $\lambda$ -specific excision	This work	
LD205	MC1000/pLD205; used to measure HK022-specific excision	This work	
LD300	TAP114/pLD300; used to measure HK022-specific integration	This work	
LE30	<i>mutD5</i> ; used for mutagenesis of phage	Silhavy <i>et al.</i> (1984)	
LE392	Used for propagation of phage	Silhavy <i>et al.</i> (1984)	
MC1000	$\Delta(lac)X74$ ; Host for plasmids pLD70, pLD177, and pLD205	Silhavy <i>et al.</i> (1984)	
RW1817	Carries $\lambda$ <i>gal8</i> $\Delta 10(int)$ <i>imm21</i> prophage inserted into chromosomal <i>attR</i> site; used to measure $\lambda$ -specific integration	This work; construction similar to that of RW1709 of Enquist & Weisberg (1984)	
TAP114	$\Delta(lacZ)M15$ ; Host for plasmid pLD300	D. Court	
B. Phage			
Strain	<i>int</i> (plasmid) <sup>a</sup>	Strain	<i>int</i> (plasmid) <sup>a</sup>
Y1	$\lambda$ wild-type	Y1219	N99D-H329R
Y1096	HK022 wild-type	Y1220	E319R-H3294 (pLD295)
Y1206	N99D (pLD310)	Y1221	N99D-E319R (pLD326)
Y1207	A171V	Y1222	N99D-E319A (pLD347)
Y1208	E174K	Y1223	N99D-E319R-H329R (pLD330)
Y1209	E319G	Y1224	S282P-G283K (pLD339)
Y1210	E319A	Y1225	S282P-G283K-R287K (pLD335)
Y1211	E319V	Y1226	N99D-S282P-E319R (pLD320)
Y1212	E319K	Y1227	N99D-S282P-G283K-E319R (pLD343)
Y1213	E319R (pLD292)	Y1228	N99D-S282P-G283K-R287K-E319R (pLD341)
Y1214	D324G	Y1229	N99D-S282P-E319R-H329R (pLD324)
Y1215	H329R	Y1230	N99D-S282P-G283K-E319R-H329R (pLD338)
Y1216	QE41L	Y1231	N99D-S282P-G283K-R287K-E319R-H329R (pLD337)
Y1217	D351N	Y1232	$\Delta(MscI/NcoI)$ (pLD257)
Y1218	S282P (pLD260)		

All phage are derivatives of Y1. The construction of other plasmids is described in Materials and Methods.

<sup>a</sup> The names of the plasmids used to construct the indicated phage *int* mutants (if any) are given in parentheses.

site and decrease recognition of the old will cause a functional mismatch between an altered DNA binding protein and its original site, and such a mismatch might be disadvantageous. A multi-step change, in which protein specificity is first broadened and then narrowed, allows a window of opportunity for co-evolution of the target site.

## Materials and Methods

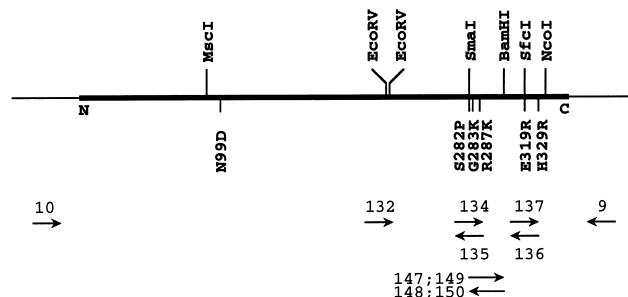
### Strains and media

Bacterial and phage strains and plasmids used in this work are listed in Table 5. Tryptone or LB broth, supplemented with 0.2% (w/v) maltose and 10 mM MgSO<sub>4</sub> where needed to promote  $\lambda$  adsorption, were used to propagate bacteria and phages. Methods for growing and assaying phage and bacteria have been described (Oberto *et al.*, 1989; Yagil *et al.*, 1989; Silhavy *et al.*, 1984). Ampicillin was added to a concentration of 100  $\mu$ g/ml when required. 100  $\mu$ l of 2% Xgal and 100  $\mu$ l of 40 mM IPTG were added per plate for estimation of  $\beta$ -galactosidase production.

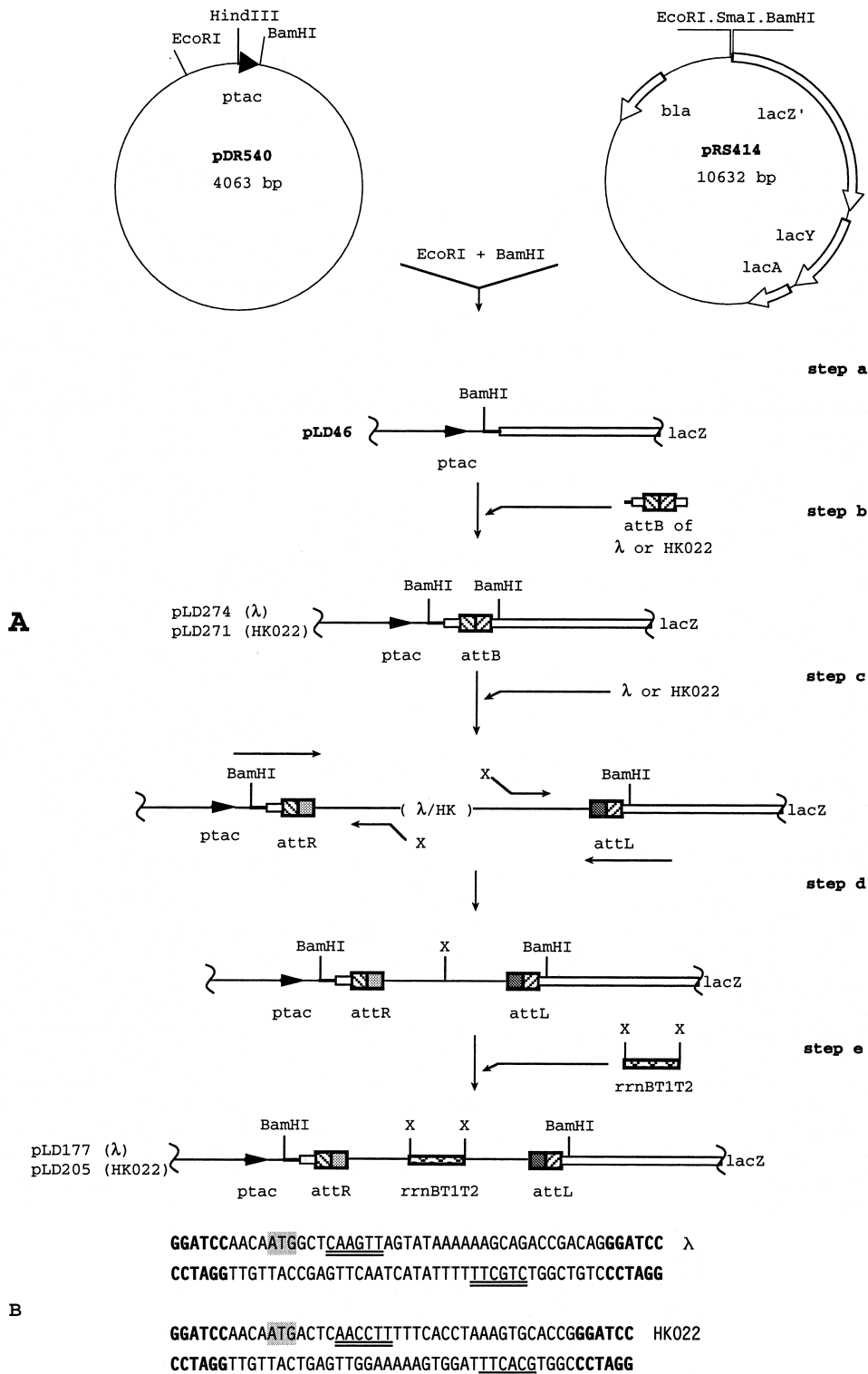
### Mutagenesis

Lambda was mutagenized by growing phage Y1 on a *mutD5* mutator host (LE30). Mutations S282P, G283K, R287K and E319R were introduced *in vitro* by oligonucleotide-directed mutagenesis on plasmid pNR169. The experimental strategy for mutagenesis is schematically

shown in Figure 2. For example primer pairs 132/135 and 134/136 were used to amplify segments of *Int* in order to introduce S282P. Oligomers 134 and 135 contained the appropriate mutation to change codon 282 and they also carried a same-sense mutation to create a *Sma*I restriction site. The PCR products were cut with *Sma*I and ligated together. The ligation product was then cut with *Eco*RV and *Bam*HI, gel-purified and inserted into *Eco*RV and *Bam*HI cut pNR169 resulting in plasmid pLD260.  $\lambda$  *Int* has two adjacent *Eco*RV sites. During this work we utilized the N-proximal one; the C-proximal site was eliminated by a silent mutation in oligomer 132. E319R was introduced in



**Figure 2.** Introduction of mutations into  $\lambda$  *int*. The *int* coding region is represented by a heavy line. The positions of the oligonucleotides relative to the mutations and the restriction sites that were utilized, introduced or eliminated during mutagenesis are indicated (for details, see Materials and Methods). Primer pair 9/10 was also used to amplify the entire coding region for sequencing.



**Figure 3.** A, Construction of indicator plasmids for excisive recombination. Details are explained in the text. The restriction site indicated by the letter X in step (d) was *PstI* for HK022 and *BglII* for  $\lambda$ . B, Sequence of the HK022 and  $\lambda$  *attB* regions present in the excision test plasmids after excision. The ATG initiation codon of *lacZ* is shaded, the *BamHI* sites are bold, and the core *Int* binding sites are doubly underlined.

a similar way (primer pairs 132/136 and 137/9), except this time an *SfiI* site was created by choosing the “right” Arg codon and the unique *BamHI* and *NcoI* sites were used for cloning. The new *SmaI* and *SfiI* sites also allowed us to verify the presence of S282P and E319R, respectively, without sequencing. Mutation H329R in combination with

S282P or E319R was introduced by using H329R mutant phage DNA for template in PCR reactions, as described above. In order to introduce G283K or G283K-R287K into *Int* already carrying mutation S282P two pairs of complementary oligonucleotides (149/150 and 147/148, respectively) were synthesized overlapping the entire

region between codons 283 and 302. The annealed oligomers had N-proximal blunt and *Bam*HI compatible C-proximal ends and were cloned into *Sma*I and *Bam*HI cut pLD260. This procedure also eliminated the *Sma*I site allowing a quick screen for the desired insert. Mutation N99D was crossed from phage into plasmid pLD257 (see below) by homologous recombination. Mutant combinations not mentioned so far were made by exchanging restriction fragments between mutant Int plasmids.

### Crossing *int* mutations from plasmid to phage

*Int* mutations were moved from plasmid to phage by homologous recombination. We constructed a phage that carries a 749 bp deletion within *int* that overlaps all the mutations we worked with (Y1232, see below). After growing this phage on a host carrying the appropriate plasmid, phage-plasmid co-integrates were isolated by selecting transductants that inherited the Ap<sup>R</sup> marker of the *int* plasmid. Among phages released by those lysogens we screened for those that regained Int activity by searching for blue plaques on  $\lambda$  and/or HK022-specific blue-plaque test strains (LD177 and LD205, respectively). The deletion in Y1232 was made as follows: plasmid pNR169, isolated from methylation deficient host GM2163, was digested with *Msc*I and *Nco*I, and the *Nco*I ends were filled in with Klenow polymerase. The large fragment was then isolated from a gel, recircularized by ligation and transformed into MC1000. Transformants carrying plasmids with the desired deletion were screened for. One of them (pLD257) was used to cross the deletion to phage Y1 as described above except this time we screened for phages that were inactive in  $\lambda$  excisive recombination. To verify the presence of the deletion, the *int* region of several isolates was amplified by PCR, and the size of the product determined. One of these phages (Y1232) was chosen to work with. Finally, the entire *int* coding region of Y1221, 1222, 1224, 1225, 1227, 1228, 1230 and 1231 was sequenced to verify the presence of the introduced mutations and the absence of any unrelated changes that might have been generated by PCR.

### Construction of indicator plasmids for HK022 and $\lambda$ excisive recombination (Figure 3)

Plasmids for signalling excisive recombination are derivatives of pRS414 (Simons *et al.*, 1987) with insertion of a *tac* promoter to drive transcription of *lacZ*. The *lacZ* gene is interrupted by a segment containing *attR* and *attL* of either  $\lambda$  (pLD177) or HK022 (pLD205) specificity that was inserted just downstream of the start of translation. The *attL* sites extend from coordinate +229 to -9 for  $\lambda$  and +255 to -13 for HK022 (Yagil *et al.*, 1989). The *attR* sites extend from +14 to -159 for  $\lambda$  and from +16 to -382 for HK022. The substrates were made as shown in Figure 3 using strain JM109 as a host. A 391 bp *Eco*RI/*Bam*HI fragment was isolated from pDR540 (Pharmacia) and ligated into *Eco*RI/*Bam*HI cut pRS414 resulting in plasmid pLD46 (step a). This insert provides the *tac* promoter and a ribosome binding site upstream of an amino-terminal truncated *lacZ* gene. Two complementary synthetic oligo-nucleotides (for the sequences see Figure 3B) carrying *attB* of  $\lambda$  or HK022 specificity were annealed, and ligated into pLD46 at the unique *Bam*HI site located between the ribosome binding site and the *lacZ* gene (step b) resulting in plasmids pLD271 with HK022 and pLD274 with  $\lambda$  *attB*. This insert also provides a translation initiation site that is upstream of *attB* and in frame with *lacZ* when in the right orientation. Colonies formed by chromosomally *lacZ*-deleted cells that

harbor such a plasmid are blue on plates containing Xgal. To convert the *attB* site to *attR* + *attL* with retention of the translation start site we inserted HK022 or  $\lambda$  at the cognate *attB* site by infecting cells harboring pLD271 or pLD274 at high multiplicity (step c), allowing them to grow in liquid and isolating single colonies on a plate containing Xgal. Some of these colonies were white because *lacZ* was disrupted by prophage insertion at *attB*. This was confirmed by restriction analysis of the phage-plasmid co-integrates. To construct plasmids that lacked unwanted portions of the prophage, we amplified the *attL* and *attR* regions of an HK022 and a  $\lambda$  phage-plasmid co-integrate (primer pairs 54/6 and 55/8 for HK022 and 54/34 and 55/35 for  $\lambda$ ). The amplified *attL* and *attR* products were gel-purified, cut at one end with a restriction enzyme (*Pst*I for HK022 and *Bgl*III for  $\lambda$  substrates) and ligated together. The ligation products were separated on an agarose gel, and the band having the expected size for *attL* + *attR* was isolated, cut with *Bam*HI and ligated back to *Bam*HI-cut and phosphatase-treated plasmid pLD46 (step d). Although the ATG initiation codon is not in frame with the remainder of *lacZ* in these plasmids, nevertheless strains carrying them formed a lawn of pale blue color on Xgal indicator plates. To eliminate this residual expression of  $\beta$ -galactosidase, we inserted the strong T1 and T2 transcription terminators from the *rrnB* gene (Sarmientos *et al.*, 1983) between *attL* and *attR*. *Pst*I and *Bgl*III fragments containing these terminators were isolated from plasmid pBM5 (C. M. Cashel, unpublished) and inserted into the *Pst*I site of the HK022-specific or the *Bgl*III site of the  $\lambda$ -specific plasmids to form pLD205 and pLD177, respectively (step e). Cells harboring these plasmids formed white colonies on plates containing Xgal. To confirm the structure, we sequenced the *attL* and *attR* joints and the functionally important P and P' arm regions using primers 54 and 55 flanking the insert.

Plasmid pLD70, which carries an altered HK022 B' core binding site, was used to select mutants of  $\lambda$  Int. It differs from the wild-type HK022 excisive recombination indicator plasmid (pLD205) by a 6 bp deletion that replaces the first two nucleotides of B' and the two nucleotides immediately flanking it (acaaTGCCTT instead of actcAACCTT, where the capital letters indicate the bases at the canonical B' core site positions). This deletion reduced excisive recombination promoted by  $\lambda$  Int to undetectable levels and measurably reduced excisive recombination promoted by HK022 Int as judged by plaque color on plates containing Xgal. pLD70 was constructed essentially in the same way as pLD205 except that different primers were used.

### Construction of an indicator plasmid for HK022 integrative recombination

Plasmid pLD300 was used to measure HK022-specific integrative recombination. It is similar to pLD271 (Figure 3A, step b) except that *attP* of HK022 has been inserted upstream of the *tac* promoter. Recombination between *attP* and *attB* removes the promoter and the translation start of *lacZ*. The *attP* region of HK022 was amplified from phage using the same oligonucleotides we used above to amplify HK022 *attL* and *attR*. The PCR product was cloned directly into plasmid pCR<sup>tmk</sup> II (Invitrogen) and recovered from it as an *Eco*RI fragment. This was cloned into the unique *Eco*RI site of pLD271, which is located upstream of the *tac* promoter, to give plasmid pLD300. pLD300 confers a Lac<sup>+</sup> phenotype in the presence of Lac repressor, but is lethal in the absence of repressor, perhaps because of overproduction of Lac permease. The product of integrative recom-

bination no longer confers a Lac<sup>+</sup> phenotype, and is no longer lethal in the presence of the *lac* operon inducer IPTG. These properties allow measurement of moderate to high levels of integrative recombination by screening for Lac<sup>-</sup> colonies in the absence of IPTG, and allow direct selection for cells carrying recombined plasmids in the presence of IPTG.

### Measuring recombination

To measure HK022 integrative recombination we grew cells carrying plasmid pLD300 until late logarithmic phase ( $A_{600} = 1$ ) in LB broth supplemented with maltose and magnesium. The cells were collected by centrifugation, resuspended in 10 mM MgSO<sub>4</sub> and starved for 30 to 60 minutes at 32°C. After starvation the cell density was adjusted to  $A_{600} = 1 (\pm 0.05)$ ,  $1 \times 10^8$  to  $2 \times 10^8$  cells (100  $\mu$ l) were infected with 100  $\mu$ l phage ( $2 \times 10^9$ /ml) resulting in a multiplicity between 1 and 2. The infected cells were incubated at 32°C for ten minutes without shaking, then 0.8 ml LB broth supplemented with 10 mM MgSO<sub>4</sub> and 0.2% maltose, prewarmed to 32°C was added and incubated 20 minutes further with gentle agitation. Appropriate dilutions were plated on LB-Ap plates to measure cells surviving phage infection and on LB-Ap-Xgal-IPTG plates to measure cells that carry plasmids rearranged by integrative recombination. Integrative recombination is expressed as the fraction of Lac<sup>-</sup>, IPTG<sup>R</sup> colonies among cells that survived infection normalized to wild-type level after infection by a phage carrying the cognate wild-type *int* gene. The relatively low multiplicity was chosen because at higher multiplicities the response was no longer linear with respect to infecting phage (data not shown). The background of this test in the absence of Int is 10<sup>-4</sup> or less, which allows measurement of extremely low activities, and the frequency of recombination promoted by HK022 Int is  $6 \times 10^4$ -fold greater than that promoted by  $\lambda$  Int (data not shown).

Lambda integrative recombination was measured as described (Yagil *et al.*, 1995) by monitoring the loss of a *gal* transducing prophage bordered by *attP* and *attB*. Int was supplied *in trans* by infecting strain RW1817 with the given phage at a multiplicity of ten phage/cell.

HK022 and  $\lambda$  excisive recombination was measured by determination of  $\beta$ -galactosidase activity produced by the

indicator plasmids described above after excision (strains LD205 and LD177, respectively). The cells were grown and treated as described above and infected by about ten phage/cell at 37°C. After ten minutes incubation at 37°C, prewarmed supplemented LB broth was added and incubation was continued for 50 minutes more. The cells were permeabilised by SDS/chloroform treatment and the enzyme activity was determined as described using a microtiter plate reader (Menzel, 1989). The numbers reported are specific activities in arbitrary units. The amount of  $\beta$ -galactosidase produced is proportional to the number of infecting phage up to a multiplicity of 20 phage/cell, and also increases with time after infection (data not shown). To compare wild-type and mutant Ints we chose non-saturating conditions for quantitative measurements.

### Reagents and procedures for DNA manipulation

Restriction enzymes, the Klenow fragment of DNA polymerase I and T4 DNA ligase were purchased from BRL, Boehringer and New England Biolabs. The GeneAmp PCR Core Reagent and AmpliTaq Cycle Sequencing kits were purchased from Perkin Elmer. Oligonucleotides were synthesized on a BioSearch 8750 DNA synthesizer or purchased from BioServe Biotechnologies. Plasmids were purified using Quiagen mini or midi kits. Sequencing primers were end-labelled with [ $\gamma$ -<sup>32</sup>P]ATP (Amersham) and T4 polynucleotide kinase using the 5' end-labelling kit from Boehringer. All kits and enzymes were used as recommended by the manufacturer. For sequencing we amplified the entire *int* coding region from phage using primers flanking *int* (9 and 10). The PCR products were purified on Centricon-100 (Amicon) and sequenced by *Taq* polymerase in a 30 cycle reaction (one minute at 94, 55 and 72°C, each) using a set of primers (9 through 23) within *int*.

### List of oligonucleotides

The numbers and sequences of oligonucleotides used in this work are listed. Newly created or eliminated restriction sites are underlined, and the mutation(s) are doubly underlined.

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6:  ACGACTGCAGTCACGCAATAATTAACGGG
8:  ACGACTGCAGATTTGATGAAACCCGCCAA
9:  CAGTGAATTCCAATTTGTTGCAACGAACAGG
10: CGGTAAGCTTACACCAAGCCGCTGATGGC
17: ATCCATCTACGATATCAGAC
18: GGCCTTCAGCTATTGCCTCTC
19: GAGAGGCAATAGCTGAAGGCC
20: TTCATGGCTTGATCGCTACG
22: CAAGGTATTTTATGCGCGCACG
23: GAACTGGCTGTTGTTACCGGGC
34: GACTAGATCTGCGCTAATGCTCTGTTACAG
35: CAGAAGATCTACGAGTTGCGCAGTTTGTCT
54: GTGTGGAATTGTGAGCGGATAAC
55: GGGTAACGCCAGGTTTTC
132: GGTCTGATATCGTAGATGGATACCCTTTATGTCGAGC
134: GAACCGCTTTCACCCGGGACAGTATCAAGG
135: CCTTGATACTGTCCCGGGTGAAAGCGGTTT
136: GCTTATCTGCTTTCTATAGAGTCTTGC
137: GCAAGACTCTATAGAAAGCAGATAAGC
147: AAAACAGTATCAAAGTATTTTATGCGCGCACGAAAAGCATCAGGCTTTTCCTCGAAGGG
148: GATCCCTTTCGAAGGAAAGACCTGATGCTTTTCGTGCGCGCATAAAATACTTTGATACTGTTT
149: AAAACAGTATCAAGGATTTTATGCGCGCACGAAAAGCATCAGGCTTTTCCTCGAAGGG
150: GATCCCTTTCGAAGGAAAGACCTGATGCTTTTCGTGCGCGCATAAAATACCTTGATACTGTTT

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