

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

Ezra Yagil<sup>2</sup>, László Dorgai<sup>1</sup> and Robert A. Weisberg<sup>1\*</sup>

<sup>1</sup>Section on Microbial Genetics, Laboratory of Molecular Genetics, National Institute of Child Health and Human Development, NIH Bethesda, MD 20892, USA

<sup>2</sup>Department of Biochemistry The George S. Wise Center for Life Sciences, Tel Aviv University, Tel Aviv 69978 Israel

Bacteriophage integrases are members of a family of structurally related enzymes that promote recombination between DNA molecules that carry specific sites. Phages  $\lambda$  and HK022 encode closely related integrases that recognize different sets of sequences within the core regions of their respective attachment sites. To locate the amino acid residues that determine this difference in specificity, we isolated recombinant phages that produce chimeric integrases and measured the ability of these chimeras to promote recombination of  $\lambda$  and HK022 sites *in vivo*. A chimera that is of  $\lambda$  origin except for one HK022 residue at position 99 and 12 HK022 residues located between positions 279 and 329 had wild-type HK022 specificity and activity for both integrative and excisive recombination. Chimeras containing certain subsets of these 13 residues had incomplete specificity. The region around position 99 is not well-conserved in other members of the integrase family, but the 279-329 segment includes residues that are highly conserved and believed to be directly involved in catalysis. Many chimeras were inactive in recombining either HK022 or  $\lambda$  sites. Selection for mutants that restored activity to these chimeras revealed sets of residues that are likely to interact with each other.

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\*Corresponding author

### Introduction

Bacteriophage integrases are members of a family of enzymes that promote recombination between DNA molecules that carry specific sites. The mechanism of recombination, which has been characterized for several integrase family members, includes cleavage of a particular internucleotide bond in each strand of the site with formation of a phosphotyrosine bond between the 3'-end of the cleaved DNA and the protein. Strand exchange and religation occurs by attack of a 5'-hydroxyl end of another cleaved strand on the phosphotyrosine bond (Evans *et al.*, 1990; Pargellis *et al.*, 1988; Gronostajski & Sadowski, 1985). The prototypical family member is phage  $\lambda$  integrase (Int), which promotes prophage integration after infection and prophage excision after lysogenic induction. Integration occurs by two pairs of strand exchanges between the bacterial attachment site, *attB*, and the phage attachment site,

*attP* (reviewed by Landy, 1989). *attB* contains two Int binding sites, called core sites, that are adjacent to the points of strand exchanges and are separated by a short spacer. *attP* contains two similarly oriented core sites, and also a distinct group of Int binding sites, called arm sites, that flank the region of strand exchange. Arm and core sites are recognized by different domains of Int (see below). *attP* also contains binding sites for the  $\lambda$ -encoded Xis protein and the host-encoded IHF and Fis proteins. These three proteins are important for the efficiency and directionality of recombination but do not directly catalyze strand exchange. An integrated prophage is flanked by hybrid sites, called *attL* and *attR*, and Int-promoted recombination of these sites excises the prophage from the bacterial chromosome and regenerates *attP* and *attB*. IHF stimulates both integrative and excisive recombination, but Xis and Fis specifically stimulate excisive recombination.

Although little is known about the three-dimensional structure of  $\lambda$  Int, its functional architecture has been investigated by characterization of the products of partial proteolysis, sequence comparison to related integrases, and the isolation of

Present address: L. Dorgai, Zoltán Bay Institute for Biotechnology, H6726 Szeged, Derkovits Faszor 2, Hungary.

*int* mutants that have retained partial function. Limited proteolysis cleaves integrase into a ca 65-residue amino-terminal and a ca 290-residue carboxyl-terminal domain. The former binds specifically to arm sites and the latter to core sites (Moitoso de Vargas *et al.*, 1988). This localization of function is corroborated by the properties of a closely related integrase encoded by lambdoid phage HK022 (Yagil *et al.*, 1989). The 55 amino-terminal residues of HK022 and  $\lambda$  Ints are identical, and both proteins recognize the same set of arm sites (Lee *et al.*, 1990; Nagaraja & Weisberg, 1990). Of the remaining 301 residues, 91 are different, and the two proteins recognize different sets of core sites (Nagaraja & Weisberg, 1990). Lambda *int* mutations that reduce core binding are found in the carboxyl-terminal segment (Han *et al.*, 1994). The carboxyl-terminal domain of Int also contains the most highly conserved amino acids of the integrase family: Arg212, His308, Arg311, and Tyr342 (Abremski & Hoess, 1992; Argos *et al.*, 1986). During recombination the hydroxyl group of Tyr342 is transiently joined to a 3' phosphate group of a specific internucleotide bond in each strand of the substrate (Pargellis *et al.*, 1988). This finding together with the results of additional studies of  $\lambda$  Int and other members of the family suggest that this quartet of residues is part of the catalytic site (Han *et al.*, 1993; Pan *et al.*, 1993; Abremski & Hoess, 1992; Chen *et al.*, 1992; Evans *et al.*, 1990; Wierzbicki *et al.*, 1987).

Although HK022 and  $\lambda$  Ints are structurally and functionally similar, neither recombines the attachment sites of the other phage (Yagil *et al.*, 1989). Apart from this specificity difference, the mechanism of recombination and its dependence on Xis, Fis and IHF appear identical for the two proteins (Nunes-Düby *et al.*, 1994; Dorgai *et al.*, 1993; Nagaraja & Weisberg, 1990; Kolot & Yagil, 1994). The *attP* sites of the two phages are related, especially in the regions containing the Int arm and the IHF, Fis, and Xis binding sites. In addition these regions are functionally interchangeable (Nagaraja & Weisberg, 1990). The two *attB* sites, however, are not closely related. The determinants of the specificity differ-

ence have been localized to the core binding sites of *attP* and *attB* (Nagaraja & Weisberg, 1990). Lambda *attP* and *attB* have four core sites that are similar but not identical. HK022 *attP* and *attB* also have four non-identical core sites. One of the HK022 core sites is identical to its  $\lambda$  counterpart, but the other three are different. Previous work suggests that two of these three are especially important for differential recognition by the two integrases (Nagaraja & Weisberg, 1990).

In this and the accompanying article (Dorgai *et al.*, 1995) we describe the identification of integrase residues that are important for the  $\lambda$ /HK022 specificity difference. This article reports the construction and characterization of chimeric proteins that contain sequences of  $\lambda$  and HK022 origin. The accompanying article reports the isolation and analysis of integrase mutants with altered specificity.

## Results

### Tests for recombinational specificity and proficiency

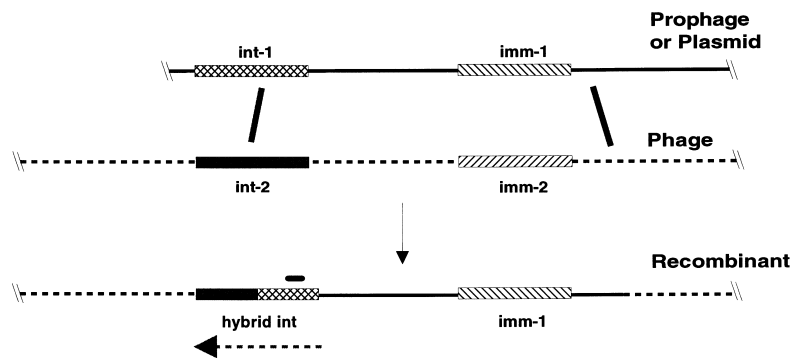
We have constructed strains in which site-specific recombination is signalled by a change in the expression of a reporter gene (see Materials and Methods and Dorgai *et al.*, 1995). The tests for excisive recombination measure the extent to which integrase supplied by phage infection restores activity to *lacZ* genes that are disrupted by a segment containing *attL* and *attR*. The tests for integrative recombination measure the extent to which integrase diminishes expression of reporter genes that are disrupted or destabilized by recombination of *attP* with *attB*. We used these tests to confirm our previous finding that  $\lambda$  and HK022 integrases prefer to recombine their cognate attachment sites (Yagil *et al.*, 1989; Nagaraja & Weisberg, 1990). The preference of each integrase for cognate over non-cognate *attL* and *attR* sites (excisive recombination) is about 100-fold, and preference for cognate over non-cognate *attP* and *attB* sites (integrative

**Table 1.** Mean recombination activities  $\pm$  (standard errors of the mean) after infection of strains carrying  $\lambda$  or HK022 excision or integration sites

Type of recombination (host)	Measurement	Specificity of Int	
		$\lambda^a$	HK022
$\lambda$ Excisive (LD177)	$\beta$ -galactosidase	1875 $\pm$ 837	22 $\pm$ 12 (1.2%)
HK022 Excisive (LD205)	$\beta$ -galactosidase	107 $\pm$ 97 (1.1%)	9623 $\pm$ 2405
$\lambda$ Integrative (RW1817)	% Gal <sup>-</sup> colonies	84 $\pm$ 4	$\leq$ 0.13 ( $\leq$ 0.2%)
HK022 Integrative (LD300)	% Lac <sup>-</sup> colonies	$\leq$ 0.2 ( $\leq$ 0.3%)	83 $\pm$ 9

The infecting phages were Y1096 (HK022-specific Int) or Y1097 ( $\lambda$ -specific Int).  $\beta$ -Galactosidase values are proportional to specific activities. The numbers in parentheses give per cent recombination relative to the relevant homospecific combination of Int and attachment sites. The excisive recombination activities reflect integrase specificity because Xis protein, which is also required for this reaction, is functionally interchangeable and nearly identical in sequence between  $\lambda$  and HK022 (Yagil *et al.*, 1989). The difference between  $\lambda$  and HK022 excisive recombination activity appears to be due to different extents of *attB*-region sequence in pLD177 and pLD205 (L.D. & R.A.W., unpublished experiments).

<sup>a</sup> The  $\lambda$ -specific Int was not wild-type (see the text).



that of the phage insert in the plasmid is located about 390 bp to the left of this point. The *int* genes and *imm* regions, indicated by hatched rectangles, differed in specificity between the two parents. Recombinants with the immunity specificity of the plasmid or defective prophage were selected by plating the phage progeny on hosts that were immune to the infecting phage, and those with crossovers within the 3'-terminal 900 bp of *int* were identified by hybridizing plaques to an oligonucleotide, indicated by a black bar, identical to the prophage or plasmid parent in the region just 3' to bp 164 of *int*. The broken arrow indicates the direction of *int* transcription.

recombination) is greater than 100-fold (Table 1). The phage strains used to supply Int in these experiments were Y1096, a phage hybrid that encodes wild-type HK022 integrase, and Y1097, a hybrid that encodes a chimeric integrase that differs from that of wild-type  $\lambda$  by insertion of an Asp residue at the penultimate carboxyl-terminal position. We used these two phages as standards for subsequent experiments because they are more closely related to some of the hybrids under study than are wild-type  $\lambda$  or HK022, and because the activity of the integrase encoded by Y1097 was indistinguishable from that of wild-type  $\lambda$  (see Figure 5, chimera  $\lambda$ 346h).

### Construction of single-joint chimeras

The chromosomes of phage that encode single-joint chimeras have a single crossover within the segment of the *int* gene that has diverged between HK022 and  $\lambda$ . These phage were recombinants issuing from crosses between a phage and a plasmid that carries a segment of the phage chromosome, or between a phage and a defective prophage (Figure 1; see Materials and Methods). The *int* gene of one parent was from HK022 and that of the other from  $\lambda$ . Hybridization with several phage and *int*-specific oligonucleotide probes localized the crossover, and its exact position was subsequently determined by sequencing the appropriate DNA segment. It is likely that the sequenced crossover is the only one within *int*. Our screening procedure, which uses oligonucleotide probes located approximately 150 bp apart, failed to detect any additional crossovers, and therefore, if any existed, they would have to be even-numbered exchanges that encompass rather short segments. Of 38 sequenced recombinants, none had changes from the two parental sequences in the vicinity of the joint. All joints were located within a segment of nucleotide sequence identity, the shortest segment being two consecutive identical base-pairs (positions 502-503 and 828-829; Figure 2), and crossovers appeared to be clustered in regions with

**Figure 1.** Diagram of crosses used to produce phage hybrids. Phage were crossed with a defective prophage or a plasmid containing an insertion of phage DNA to generate phage progeny with recombinant *int* genes as described in Materials and Methods. The relevant segment of the phage chromosome is indicated by a broken line, and that of the prophage or plasmid by a continuous line. The left end of the defective prophage is located at the crossover point within the attachment site, and

greater than average fractional local identity (Figure 3; see Discussion).

The 38 recombinants had 27 different recombination joints and encoded 21 different chimeric proteins. Figure 4 shows an alignment of the two parental integrases with the locations of the predicted chimera joints. Nine chimeras had HK022 amino and  $\lambda$  carboxyl termini, and 12 had the reciprocal arrangement. The names of the chimeras reflect the type and position of the chimera joint, and can be understood by referring to the following two examples and to Figure 4. Chimera  $\lambda$ 134h has a joint within the block of identical amino acids extending from residue 134 to residue 136, and has  $\lambda$  sequences amino-terminal and HK022 sequences carboxyl-terminal to the joint. Chimera h276 $\lambda$  has a joint between residues 276 and 277, with HK022 sequences amino-terminal and  $\lambda$  sequences carboxyl-terminal to the joint.

### Analysis of chimeras

The amino-terminal 55 residues of the two integrases are identical, and we therefore screened only for hybrids with crossovers 3' of codon 55. Figure 5 shows the activities of these hybrids in recombination assays. Activities are normalized to that of wild-type HK022 Int carried by phage Y1096 for recombination of HK022 sites, and to that of chimera  $\lambda$ 346h carried by phage Y1097 for  $\lambda$  sites (see above). Although the hybrids fall into two sets with respect to their gene content outside of *int* (one set has the immunity region of  $\lambda$  and the *attP* site of HK022, and the other set has the reciprocal arrangement) control experiments suggest that these differences do not have large effects on recombination frequencies (data not shown). Moreover, many of our conclusions are based on comparisons within rather than between sets.

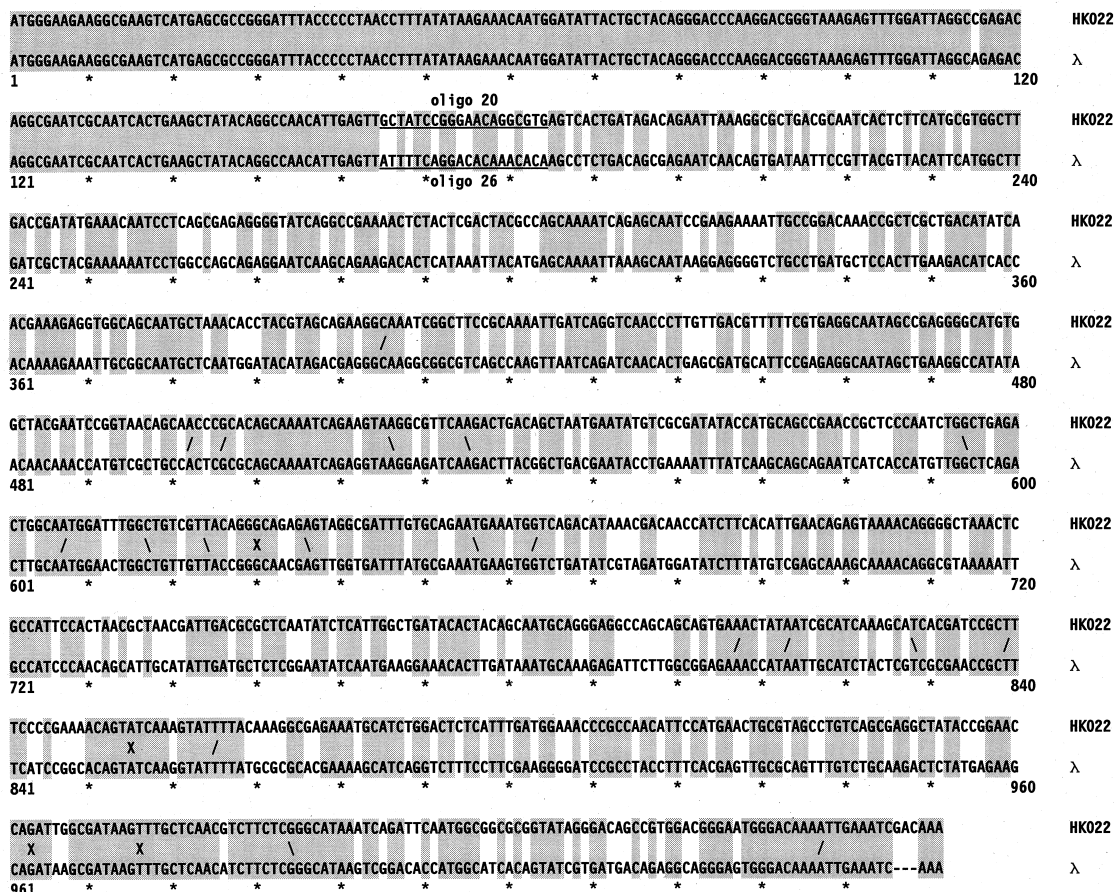
The most striking finding to emerge from the data presented in Figure 5 is that only two single-joint chimeras, both of whose joints are close to the

carboxyl terminus, retained full parental specificity for both integrative and excisive recombination. These are  $\lambda$ 346h and h330 $\lambda$ , which have  $\lambda$  and HK022 specificity, respectively. This result argues that the segment extending from the h330 $\lambda$  joint to the carboxyl terminus, which contains five residues that differ between the two integrases, does not contain determinants of the HK022/ $\lambda$  specificity difference. The remaining single-joint chimeras can be classified as relaxed, partially specific, and inactive. (1) Relaxed chimeras, such as  $\lambda$ 321h and h198 $\lambda$ , promoted  $\lambda$  integrative and excisive and HK022 excisive recombination with at least 10% of the wild-type efficiency. None of these chimeras promoted appreciable levels of HK022 integrative recombination. (2) Partially specific chimeras, such as  $\lambda$ 269h and  $\lambda$ 279h, promoted HK022 excisive but not integrative recombination, and were unable to recombine  $\lambda$  sites. We found no chimeras that efficiently promoted only  $\lambda$  excisive recombination. The existence of relaxed and partially specific chimeras that promoted HK022 excisive but not integrative recombination shows that the requirements for the former are less stringent than those for the latter (see Discussion). By contrast, the activity of chimeras for  $\lambda$  integrative and excisive recombination usually varied in parallel. (3) Inactive chimeras, such as  $\lambda$ 167h and  $\lambda$ 219h, had little or no activity on any

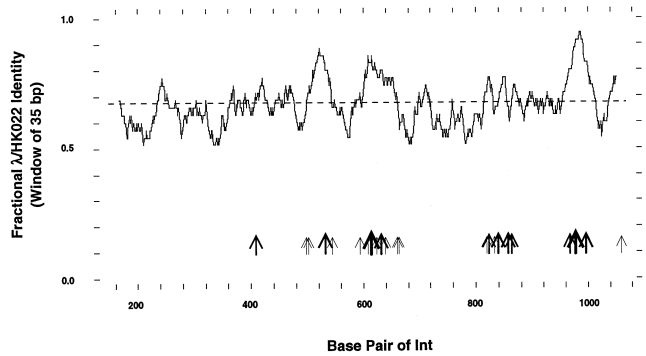
of the substrates. Inactivity can result from protein misfolding or instability, and we therefore used only the first two classes of chimeras to guide our initial search for determinants of specificity.

The relaxed phenotype can be accounted for by assuming that several residues are important for the  $\lambda$ /HK022 specificity difference. One possibility is that discrimination between HK022 and  $\lambda$  sites requires proper interaction between two or more residues, and chimeras in which one of these residues has been replaced promote recombination of both types of site. Another possibility is that several individual residues contribute incrementally and independently to site discrimination, and that relaxed chimeras carry residues that enable recognition of both types of sites and lack residues that cause rejection of either type of site. Since all of our chimeras were formed by exchanges between corresponding positions in the two genes, neither the enabling nor the rejecting residues can occupy corresponding positions in the two protein sequences. We consider these possibilities in more detail in the Discussion and in the accompanying article. The following analysis confirms our initial assumption that several residues are important for the  $\lambda$ /HK022 specificity difference and identifies some of these residues.

We argued above that residues located carboxy-



**Figure 2.** Alignment of the coding regions of *int-λ* and *int*-HK022. Shaded blocks show sequence identity. The sequence identity blocks containing crossover points are indicated by the diagonal lines, and the orientation of these lines indicates the origin of the 5' and 3' regions of the hybrids.



**Figure 3.** Relation between crossover position and local sequence similarity. The graph shows the fractional sequence identity between the *int* genes of  $\lambda$  and HK022 from bp 144 to the end of the gene. Fractional identity was plotted as a running average with a window size of 35 bp using the “Plotsimilarity” program of the University of Wisconsin sequence analysis package (Devereux *et al.*, 1984). The broken line shows the average identity over the whole region. The arrowheads show the locations of sequenced HK022/ $\lambda$  crossovers, and the width of the arrowhead shaft is proportional to the number of crossovers, with the thinnest equal to one and the thickest equal to five. Not included in this Figure are approximately nine unsequenced hybrids in the 570-662 segment and 92 unsequenced hybrids 3' to bp 911. The approximate location of these recombination joints was determined by hybridizing plaques to different oligonucleotide probes.

terminal to position 330 are not determinants of the  $\lambda$ /HK022 specificity difference. Chimera  $\lambda$ 324h, whose recombination joint is amino-terminal to this position, falls into the relaxed class since it has full activity on  $\lambda$  sites and weak but significant activity in HK022 excisive recombination. This suggests that the His/Arg difference between  $\lambda$  and HK022 at position 329 (Figure 4) contributes to specificity. The phenotype of the reciprocal chimera, h324 $\lambda$ , is

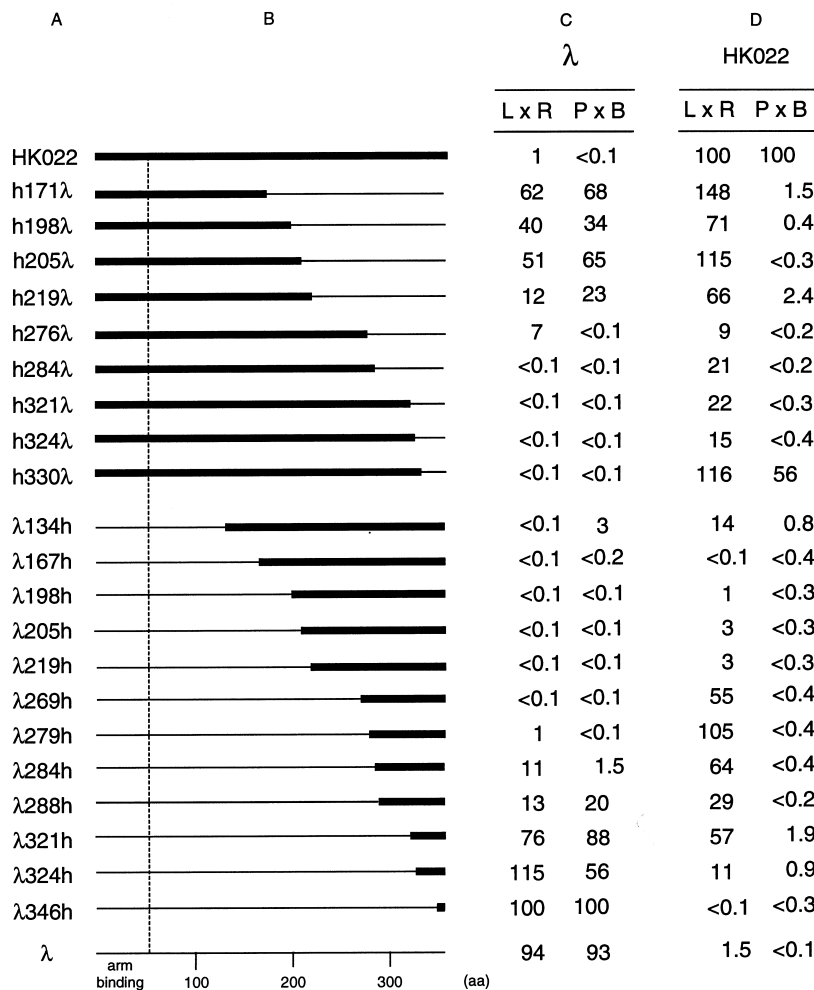
consistent with this suggestion but its low activity precludes us from drawing a strong conclusion. Chimera  $\lambda$ 279h is HK022-specific but promotes only excisive recombination, suggesting that the HK022-specific amino acid residues located amino-terminal to position 279 are not important for enabling recognition of HK022 *attL* and *attR*, or for preventing recognition of  $\lambda$  *attL* and *attR*. These arguments are consistent with the properties of a double-joint chimera,  $\lambda$ 279h-h330 $\lambda$ , which has only 12 HK022-specific residues, all from the 279-330 segment. The phenotype of this chimera, constructed by crossing phages encoding the single joint chimeras  $\lambda$ 279h and h330 $\lambda$ , was HK022-specific for excisive recombination and inactive for integrative recombination, as expected (Figure 6, line a). Similar double-joint chimeras lacking the two most amino-proximal HK022 residues, Ser282 and Gly283, or the most carboxyl-proximal HK022 residue, His329, were relaxed or inactive, respectively (Figure 6, lines b and c). We assume that additional HK022-specific residue(s) located amino-terminal to position 279 are needed for HK022 integrative recombination (see below) because chimera h330 $\lambda$  is fully HK022-specific.

**A mutation that restores HK022-specific activity to a group of inactive chimeras**

To locate the additional residue(s) needed for full HK022-specificity, we used the following strategy. A group of five sequential chimeras,  $\lambda$ 134h,  $\lambda$ 167h,  $\lambda$ 198h,  $\lambda$ 205h, and  $\lambda$ 219h, was inactive or only weakly active even though all contain the HK022 279-330 segment that confers HK022-specific excisive recombination on other chimeras (e.g.  $\lambda$ 279h and  $\lambda$ 279h-h330 $\lambda$ ; Figures 5 and 6). We selected a variant that restored activity to one of these chimeras in the hope that its structure would disclose additional



**Figure 4.** Alignment of  $\lambda$  and HK022 integrases. The shaded blocks indicate amino acid identities, the diagonal lines indicate the deduced location of chimera joints, and the vertical lines show the quartet of most highly conserved integrase family residues. The arrowheads and bold-faced numbers give the amino-terminal position of identity blocks containing chimera joints, except for position 99, which is the location of a mutation described in the text. The most amino-terminal portion of the two integrases is believed to form a domain that recognizes arm binding sites (see Introduction), and the 279-330 segment is described in the text.



**Figure 5.** Activities of single-joint Int chimeras. Column A lists the names of the chimeras, column B is a diagram showing the approximate extent of HK022 and  $\lambda$  sequences (thick and thin lines, respectively), columns C give frequencies of  $\lambda$ -specific excisive (L x R) and integrative (P x B) recombination relative to that of chimera  $\lambda$ 346h, and columns D give the frequencies of HK022-specific excisive and integrative recombination relative to that of HK022 Int. The bottom line shows the activity of wild-type  $\lambda$  Int relative to that of chimera  $\lambda$ 346h, and also shows an approximate amino acid scale from the amino to the carboxyl terminus. The vertical broken line shows the carboxyl-terminal limit of the arm-binding domain.

specificity determinants. To isolate such a variant we infected cells with phage encoding inactive chimera  $\lambda$ 198h, selected the rare lysogens that arose, and collected the phage they released after induction (see Materials and Methods). One among several thousand phage tested formed a blue plaque on a lawn of the host used to test for HK022 excision. This variant was purified and proved to have HK022 specificity both for excisive and integrative recombination (see below). Sequencing revealed two changes in the *int* gene. One is a substitution of Asp for Asn at position 99 (N99D). This is an informative mutation for two reasons: (1) HK022 Int has Asp at position 99 and (2) we have independently isolated the same change in a selection for  $\lambda$  variants with altered specificity (see Figure 6 and accompanying paper). The second change is a shift in the position of the chimera joint from 198 to 134. We surmise that this shift was a result of recombination of chimera  $\lambda$ 198h with a cryptic *Escherichia coli* lambdoid prophage whose *int* gene resembles that of HK022. The existence of a cryptic HK022-like *int* gene or *int* fragment in *E. coli* K12 was demonstrated by Southern blots of a non-lysogenic host (strain MC1000) with an HK022 probe and PCR amplification with primers located within or flanking HK022 *int* (data not shown). Whatever the origin of the

shifted chimera joint, the following experiments show that the N99D mutation restores at least partial activity to all members of this group of inactive chimeras. We crossed  $\lambda$  *int*N99D (see accompanying paper) with inactive chimeras  $\lambda$ 134h,  $\lambda$ 167h,  $\lambda$ 198h,  $\lambda$ 205h, and  $\lambda$ 219h, and isolated recombinants containing N99D together with each of the five hybrid joints (Figure 6). Two recombinants, N99D- $\lambda$ 134h and N99D- $\lambda$ 205h, had full HK022 specificity for integrative and excisive recombination. Three, N99D- $\lambda$ 167h, N99D- $\lambda$ 198h, and N99D- $\lambda$ 219h, were specific for HK022 excisive recombination but inactive or partially active for HK022 integrative recombination. The changes in the HK022 integrative recombination phenotype as the chimera joint is moved rightwards in this set of recombinants (i.e. as  $\lambda$  residues are substituted for HK022 residues) are hard to interpret, and suggest that local interactions between  $\lambda$  and HK022 residues on each side of the chimera joint affect activity in ways that may not be informative about the role of these residues in the wild-type proteins. Nevertheless, the importance of Asp99 is supported by the observation that the N99D substitution conferred full HK022 specificity on the partially specific double-joint chimera  $\lambda$ 279h-h330λ (Figure 6). The last result shows that Asp99 together with the 12 additional of HK022-specific residues of

the 279-330 segment are sufficient to confer full HK022 specificity on a chimera that is otherwise of  $\lambda$  origin. Moreover, this chimera was indistinguishable from wild-type HK022 Int in promoting recombination of a battery of mutant attachment sites (L.D. & R.A.W., unpublished results).

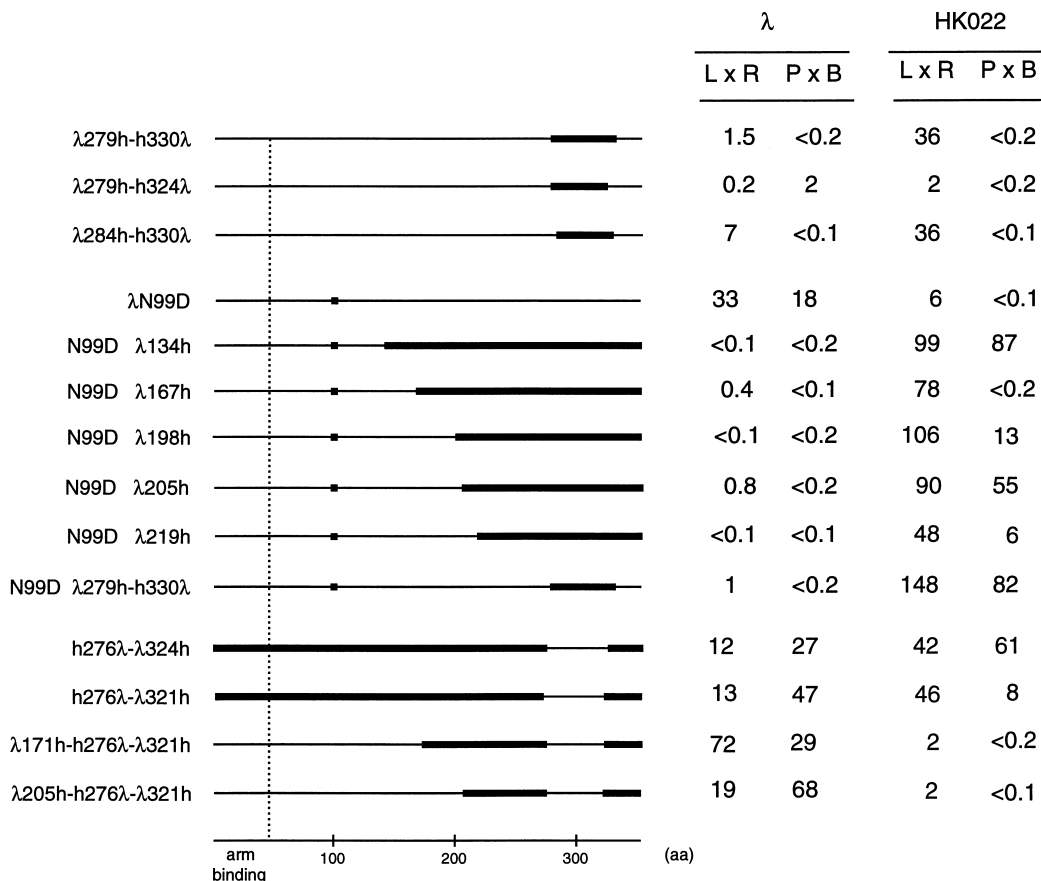
**Chimeras with joints in the 279-330 segment**

The work reported above shows that specificity determinants are clustered in the 279-330 segment. The following analysis has allowed us to draw limited conclusions about the importance of residues and groups of residues within this region. The HK022 residues Pro282 and/or Lys283 appear important for preventing recognition of  $\lambda$  sites. This can be seen by comparing the phenotypes of chimeras  $\lambda$ 279h and  $\lambda$ 284h (Figure 5) or chimeras  $\lambda$ 279h-h330 $\lambda$  and  $\lambda$ 284h-h330 $\lambda$  (Figure 6). In both cases, the S282  $\rightarrow$  P, G283  $\rightarrow$  K substitution reduced activity on  $\lambda$  sites and was neutral or had a slightly positive effect on recombination of HK022 sites. This suggestion is supported by examination of the phenotypes of chimeras h276 $\lambda$  and h284 $\lambda$ , which differ at positions 277 and 278 as well as at 282 and 283 (Figure 5). We provide additional support by characterization of the S282  $\rightarrow$  P G283  $\rightarrow$  K mutations in an otherwise  $\lambda$  background (Dorgai *et al.*, 1995). Comparison of chimeras  $\lambda$ 284h and  $\lambda$ 288h

suggests that substitution of Lys287 of HK022 for Arg287 of  $\lambda$  reduces  $\lambda$  integrative recombination, a suggestion that is supported by the results reported by Dorgai *et al.* (1995). The importance of the amino acid differences between the two integrases at positions 290, 291, 294, 319, 320 and 323 is unclear. The changes in phenotype are incremental as the position of the chimera joint changes, and reciprocal groups of chimeras do not always change in parallel (compare the difference between chimeras h284 $\lambda$  and h324 $\lambda$  on the one hand, and between chimeras  $\lambda$ 284h and  $\lambda$ 324h on the other). However, analysis of  $\lambda$  mutants reveals that Glu319 of  $\lambda$  helps to prevent recombination of HK022 sites (Dorgai *et al.*, 1995). Chimeras  $\lambda$ 324h and  $\lambda$ 346h differ in their ability to promote recombination of HK022 but not  $\lambda$  sites, and are changed at positions 329, 337, 340 and 341. Our analysis shows that the last three residues are unimportant for specificity, and we therefore suggest that His329 of  $\lambda$  helps to prevent recognition of HK022 sites. This suggestion is supported by the relaxed phenotype of  $\lambda$  carrying an *int*H329R mutation (Dorgai *et al.*, 1995).

**Residues required for  $\lambda$  specificity**

Since 13 HK022-specific residues are sufficient to confer HK022 specificity on  $\lambda$  integrase, it is tempting to speculate that the reciprocal substitution of the



**Figure 6.** Activities of other Int chimeras. The conventions are described in the legend to Figure 5.

corresponding 13  $\lambda$  residues, or some subset of them, will confer  $\lambda$  specificity on HK022 integrase. We have not critically tested this speculation because we have not constructed the 13-residue substitution, but our attempts to do so have revealed unanticipated complexities in the determination of recombination specificity. Chimeras h171 $\lambda$ , h198 $\lambda$ , h205 $\lambda$  and h219 $\lambda$  have 12 of the 13  $\lambda$ -specific residues, lacking only Asn99. They recombined both  $\lambda$  and HK022 sites with high efficiency (Figure 5). Therefore, if the speculation is correct, the primary role of Asn99 must be to prevent recombination of HK022 sites. Consistent with this prediction, we have shown that the substitution of Asp for Asn99 increases the ability of  $\lambda$  Int to recognize HK022 sites and only modestly reduces its ability to recombine  $\lambda$  sites (Dorgai *et al.*, 1995; Figure 6).

We have constructed two triple-joint hybrids that contain different members of the set of 13  $\lambda$  residues and have  $\lambda$  specificity:  $\lambda$ 171h-h276 $\lambda$ - $\lambda$ 321h and  $\lambda$ 205h-h276 $\lambda$ - $\lambda$ 321h (Figure 6). Both chimeras contain Asn99 but lack the  $\lambda$ -specific residues Ser323 and His329. Related chimeras, which lack Asn99 and His329 or Asn99, Ser323, and His329, had a relaxed phenotype (Figure 6). These results again support the idea that Asn99 is essential for preventing recognition of HK022 sites, but they also argue that Ser323 and His329 are not required to prevent recombination of HK022 sites. However, the latter argument is difficult to reconcile with the observation that chimera  $\lambda$ 321h is relaxed (Figure 5). This protein, like the two  $\lambda$ -specific triple-joint chimeras, contains Asn99 and lacks Ser323 and His329. Indeed, comparison of  $\lambda$ 321h to the triple-joint chimeras reveals that substitution of HK022 for  $\lambda$  residues in the 205-276 segment has had the paradoxical effect of reducing HK022 excisive recombination from 57% to 2%. We draw two conclusions from these observations. First, the effects of substitution of a particular residue on specificity can depend on other residues. Second, the entire set of 13  $\lambda$ -specific residues is not required for  $\lambda$  specificity; a subset is sufficient in certain contexts. We consider these points in greater detail in the accompanying article (Dorgai *et al.*, 1995).

## Discussion

### The action of residues involved in site-discrimination

To locate amino acid residues required for recognition of the core region of the attachment sites, we constructed and characterized chimeras between the closely related integrases of phages  $\lambda$  and HK022. We found that many chimeras retained activity, and some of these discriminated between the parental attachment sites. A chimera with only 13 HK022-specific residues, one located at position 99 and the other 12 within the 279-330 segment, was indistinguishable from wild-type HK022 integrase in recombinational specificity. Mutational studies reported in the accompanying article show that a

subset of these 13 residues is sufficient for most of the differential recognition. Our results are consistent with but do not critically test the hypothesis that the corresponding set of 13  $\lambda$  residues confers  $\lambda$  specificity on HK022 integrase.

Many chimeras recombined both  $\lambda$  and HK022 sites, consistent with the conclusion that site discrimination is determined by multiple residues. One possibility is that each specificity-determining residue both promotes recombination of the cognate sites and impedes recombination of the non-cognate sites, and that complete specificity requires the cumulative effect provided by several such residues. However, the existence of chimeras that promote both types of excisive recombination at near-wild-type levels suggests that this explanation of relaxed specificity is incomplete at best. A second possibility is that site-discrimination requires interaction between a set of specific residues in each integrase, and that chimeras lacking a complete set may be undiscriminating. A third possibility is that some specificity-determining residues act primarily by enabling recombination of the cognate sites, while others act primarily by rejecting the non-cognate sites. If the enabling and rejecting residues do not occupy corresponding positions in each protein, recombination could generate chimeras with both types of enabling and neither type of rejecting residues, and these would be unable to discriminate. Mutational studies reported in the accompanying article (Dorgai *et al.*, 1995) confirm the existence of enabling and rejecting residues, and therefore support the third possibility.

Lambda site-specific recombination requires formation of a complex that probably contains one integrase protomer for each of the four core recognition sites (Kim *et al.*, 1990). The four  $\lambda$  sites differ from each other, and three of them differ from the corresponding HK022 core sites. These three, C, B, and B', are responsible for the HK022/ $\lambda$  specificity difference (Nagaraja & Weisberg, 1990; L.D. & R.A.W., unpublished experiments). The fourth core site, C', is conserved between two phages, which suggests that the two integrases have overlapping rather than completely different DNA recognition specificities. The specificity-determining integrase residues could interact directly with core site nucleotides that differ between the phages, could influence the conformation of the DNA binding surface of the proteins in a less direct way, could alter protein-protein interactions in the recombination complex in such a way as to change the orientation or alignment of the core-binding surface to the DNA, or could improve catalysis of a step that limits the rate of the heterospecific reaction. We discuss these possibilities more fully in the accompanying article (Dorgai *et al.*, 1995).

Nunes-Düby *et al.* (1994) have shown that the  $\lambda$ /HK022 Int specificity difference is manifested in partial reactions. They found that efficient Int-promoted cleavage of *att* sites that contained either  $\lambda$  or HK022 B' core sites, required integrase of the corresponding specificity. This was also true

for cleavage and resolution of Holliday structures. When the substrates contained core sites of both specificities, both types of integrase were required for optimal cleavage. This suggests that cleavage is promoted by interactions between integrase protomers (Kho & Landy, 1994; Franz & Landy, 1990), and that these interactions can occur between protomers of different specificity.

### Differences between integrative and excisive recombination

Previous studies of  $\lambda$  show that integrative and excisive recombination have different requirements for accessory factors, arm binding sites, and Int protein, and that these differences allow control of the directionality of recombination (reviewed by Landy, 1989; Thompson & Landy, 1989). We found that many chimeras were proficient for HK022 excisive but deficient for HK022 integrative recombination. Some of these chimeras were proficient in both types of  $\lambda$  recombination (e.g. chimera h171 $\lambda$ ; Figure 5), and in general, chimerization did not have a differential effect on  $\lambda$  excisive and integrative recombination. Since the directionality of recombination appears to be controlled in the same way in the two phages, and their recombination mechanisms are similar or identical (Dorgai *et al.*, 1993; Nagaraja & Weisberg, 1990), we were surprised to find chimeras that affected the integration-excision balance in one phage but not the other. This difference does not appear to be a consequence of low stringency of the  $\lambda$  integrative recombination assay (which uses a single copy substrate) because we have isolated  $\lambda$  mutants that have the reciprocal phenotype to chimera h171 $\lambda$ : they were defective solely in  $\lambda$  integrative recombination (Dorgai *et al.*, 1995). We suggest that the requirements for core recognition differ for integrative and excisive recombination, and that the differences among the four core sites within each phage affects the directionality of recombination. The greater sensitivity of integrative recombination to changes in the structure or level of integrase has previously been seen in studies of  $\lambda$  recombination (Weisberg & Enquist, 1977; Enquist *et al.*, 1979; Bushman *et al.*, 1985).

### Previous mutational and evolutionary studies of integrase

Han *et al.* (1994) have recently isolated and characterized a set of  $\lambda$  *int* mutants that no longer promote excisive recombination but have retained some level of integrase binding to arm sites. Eight recombination-defective mutations lie within the 279-330 segment, which contains determinants of the  $\lambda$ /HK022 specificity difference. These fall into several different phenotypic classes, and it is not clear that any of them produce proteins that are defective solely in interaction with core sites. However M290I, which is a relatively conservative change at a position where HK022 integrase contains

Thr, is a candidate for such a mutant. IntM290I interacts poorly with *attL* (Han *et al.*, 1994), and this is probably a result of poor binding to core sites, since the mutant protein binds normally to arm sites. Interestingly, six other mutations located elsewhere have a similar phenotype: K93E, T96I, T120I, R144K, V175E, G214D. The first two are close to position 99, the location of another determinant of the HK022- $\lambda$  specificity difference.

There is evidence that the residues that are important for core recognition in other members of the integrase family are also widely distributed in the protein sequence. The Cre protein of phage P1 and the Flp protein of *Sacharomyces cerevisiae* are site-specific recombinases that are distantly related to  $\lambda$  integrase (Argos *et al.*, 1986), and recombine sites that are analogous to  $\lambda$  and HK022 *attB*; i.e. they consist of two core-type sites separated by a short spacer (Senecoff *et al.*, 1985; Hoess & Abremski, 1984). Some proteolytic fragments of Cre and Flp retain the ability to bind specifically to their respective sites, and it appears that most of them contain the conserved H-X-X-R motif of the integrase family, which is located near the carboxyl terminus (Pan & Sadowski, 1993; Chen *et al.*, 1991; Pan *et al.*, 1991; Hoess *et al.*, 1990). However, Flp fragment 2-123, which lacks the H-X-X-R motif, has recently been found to protect specifically a portion of the binding site (Panigrahi & Sadowski, 1994). It is not known if a comparable amino-terminal fragment of Cre can bind specifically to DNA, but mutants that decrease affinity for the recombination site are located close to the amino terminus (T87I and H91Y) as well as to the H-X-X-R motif (Wierzbicki *et al.*, 1987). Although most of these Cre mutants are defective in recombination under all conditions tested, CreH91Y promoted efficient recombination *in vitro* but not *in vivo*. This result was explained by assuming that the amino acid substitution depressed specific but not non-specific DNA binding, and that the relative concentration of non-specific sequences is low in *in vitro* recombination conditions (Wierzbicki *et al.*, 1987).

Encouraged by these reports, we looked for similarities in other integrases to the two regions implicated in the HK022/ $\lambda$  specificity (see Materials and Methods). We found seven among a collection of 33 integrases that contained segments with some similarity to the segment around residue 99 of HK022 integrase (Figure 7A). All were located well upstream of the H-X-X-R motifs of their respective proteins. The statistical significance of the individual pairwise alignments is far from overwhelming, especially considering the probable evolutionary relatedness of many of these proteins, and it is quite clear that the constraints to evolutionary divergence are weaker here than in other regions of integrase family members. Nevertheless, the conservation of residues identified as important by mutational studies in Cre and  $\lambda$  integrase is intriguing and suggests that the multiple sequence alignment has biological significance. The residue that corresponds to His91 of Cre in this alignment is the conserved

A.

	Int	Similarity to HK022
<sup>83</sup> YETILS. ERGIRPKTLL. D. YASK. IR. AIR	HK022	N/A
<sup>83</sup> YEKILA. SRGIKQKTLI. N. YMSK. IK. AIR	$\lambda$	13-15
<sup>108</sup> YDYLMN. ERGISEKTAK. D. YINA. ISKPYK	SSV1	7-8
<sup>11</sup> YLRVLSVERQLSPITLL. N. YQRQ. LE. AII	XerC	4-5
<sup>74</sup> YLLYLQ. ARGLAVKTIQ. Q. HLGQ. LN. MLH	Cre	3-4
<sup>128</sup> YRTYLT. KRGVRIITTT. . N. Y. . K. IT. ANQ	Tn554B	3-4
<sup>35</sup> YH. I. K. TSGFKVTTLT. N. LKTR. IK. NIK	L54A	3-4
<sup>93</sup> YISIQE. DR. LQHNELRPNSYRQK. GK. PIR	21	2-3
Y*-!!--!R!!!-!T!!-!.Y--!!--!*		

B.

<sup>279</sup> PLSPKTVSKYFTKARNASGLSFD. GNPPTFHELRSLSARLYRNQIGDKFAQR	HK022
<sup>279</sup> PLSSGTVSRVFMRAKASGLSFE. GNPPTFHELRSLSARLYEKQISDKFAQH	$\lambda$
<sup>302</sup> QVSANTLTTAFKKAKEKCGIKWEQGTAPTFFHEQRSLSERLYREQGLDT. . QK	21

**Figure 7. A.** Alignment of amino-proximal regions of integrases. The HK022 integrase segment extending from position 75 to 125 was used to search for similar regions in a collection of 36 integrase sequences by pairwise alignment as described by Kanehisa (1982). Those pairwise alignments that fell in the amino-terminal half of the integrases and whose similarity scores exceeded those of scrambled sequences by at least four standard deviations (plus less significant alignments to Cre and Flp) were multiply aligned by eye, using Asn99 of  $\lambda$  and Asp99 of HK022 as an anchor. The flanks of the aligned sequences were shortened to produce the Figure. Similarity to HK022 is measured by the difference in units of standard deviations between the similarity score of the alignment between HK022 and the given sequence and the mean similarity score between 20 pairs of randomly scrambled sequences of the same lengths and compositions as the two test sequences. The shaded residues have been implicated in DNA binding by mutational studies (see the text). The degree of conservation is indicated below the alignments and was judged by eye. An amino acid code indicates high conservation of a particular residue; '!', indicates good conservation; '\*', indicates modest conservation, and '.', indicates little or no conservation. B Alignment of the carboxyl-proximal regions of HK022,  $\lambda$ , and phage 21 integrases.

Tyr100 of HK022, supporting the argument that the phenotype of CreH91Y is caused by relaxed or altered specificity rather than defective binding. Several other residues are also conserved; notably, Tyr83, Arg90, and Thr96, which is changed in the Thr96  $\rightarrow$  Ile mutant that appears to decrease  $\lambda$  Int core binding (see above). Asn99 is also conserved, but since we used this position as a guide for alignment, it cannot be taken as evidence of biological significance.

The 50 bp 279-329 Int segment contains the H-X-X-R motif that is believed to be part of the integrase family active site (Han *et al.*, 1993; Chen *et al.*, 1992; Serre & Jayaram, 1992; Parsons *et al.*, 1990). Although the segment located carboxyl-terminal to the highly conserved His308 is one of the better conserved regions of the integrase family, the amino-terminal segment is very weakly conserved, and it is necessary to introduce short gaps of variable size upstream of His308 to obtain any reasonable alignments to the corresponding segment of HK022 (data not shown). The positions C-terminal to position 308 that differ between HK022 and  $\lambda$  (positions 319, 320, 323, and 329) contain the less strongly conserved amino acids, as might have been expected for residues that play a role in site recognition. We show only the alignment of the closely related phage 21 integrase to that of  $\lambda$  and

HK022 (Figure 7B). Of the 12 residues that differ between  $\lambda$  and HK022 in the 279-329 segment, phage 21 integrase differs from both at eight positions.

#### Evidence for interaction between integrase residues

Five chimeras having joints in the 134-219 segment, and  $\lambda$  sequences upstream and HK022 sequences downstream of the joint ( $\lambda$ 134h,  $\lambda$ 167h,  $\lambda$ 198h,  $\lambda$ 205h, and  $\lambda$ 219h) were inactive or only weakly active on sites of either specificity (Figure 4). A similar phenomenon (inactivity of a group of chimeras whose joints are located in a particular region) was noted by Ogawa *et al.* (1992) in an analysis of chimeric RecA proteins. These workers suggested that an unacceptable interaction (or loss of a favorable interaction) between amino acid residues on each side of the region containing the joints of the inactive chimeras is responsible for their lack of activity. The similarity between our results and theirs is striking. According to their model, the inactivity of the chimeric integrases results from an unacceptable interaction (or loss of a favorable interaction) between a  $\lambda$  residue(s) in the 55-134 segment and a HK022 residue(s) in the 224-269 segment (note that chimera  $\lambda$ 269h is active). A genetic selection for restoration of activity indicates that the residue in the

amino-terminal segment is Asn99. We do not know how the putative interaction between Asn99 and the segment 224-269 HK022 residue inactivates the proteins. Since the inactive chimeras have a  $\lambda$  specificity-determining residue upstream of the joint and HK022 specificity-determining residues from the 279-330 segment, they may be unable to recognize either  $\lambda$  or HK022 sites. Although this hypothesis is attractive, additional assumptions are required to account for the activity of chimeras  $\lambda$ 269h and  $\lambda$ 279h, which should suffer from the same problem.

### Generation of chimeras by recombination between partially identical sequences

We assume that the homologous recombination enzymes of the phage and/or host are involved in generating hybrid *int* genes. Recombination between the two *ints* must be responsive to sequence identity because all of the sequenced recombinants had joined the two parental sequences in register, and crossovers were clustered in regions with greater than average local identity (Figure 3). The graph of Figure 3 does not include an apparent excess of unsequenced crossovers that occurred within the 3' end of *int* or further downstream (see Figure 3 legend). These crossovers could have occurred by Int-promoted recombination between the two *attP* sites, or within regions of homology located to the left and right of the *attPs*. The appearance of clustering shown in this Figure depends on the size of the segment compared within each *int* gene. We calculated mean local sequence identity within a 35 bp "window" around each nucleotide. Attempts to relate recombination frequency to fractional identity using sampling windows larger than 65 bp or smaller than 20 bp did not reveal clustering: the larger windows levelled the peaks and valleys, and the smaller windows increased the background noise (analysis not shown). We suggest that the optimum window size reflects the size of the DNA segment used for sampling the extent of sequence identity during recombination. Interestingly, a segment of ca 30 bp long has also been identified as the minimal length of perfect homology required for efficient recombination in the *E. coli* RecBCD pathway (Shen & Huang, 1986, 1989), and it is tempting to speculate that the similar segment size reflects a similarity in the recombination mechanism.

In-register recombination between sequences that are 70% identical has also been found in a different system (Ogawa *et al.*, 1992), and has even been detected between regions that are only 50% identical (Miyazawa *et al.*, 1994) under conditions in which a lambdaoid phage-encoded recombinase was active. Recombination between regions with partial sequence identity almost certainly contributed to the genesis of the wild-type HK022 *int* gene (Yagil *et al.*, 1989) and to the diversity of phages of the lambdaoid family (Casjens *et al.*, 1992; Highton *et al.*, 1990; Campbell & Botstein, 1983). It can be argued that the capacity to promote such recombination is a

selectable trait, and that phage recombination pathways are less sensitive to the barriers that impede recombination than are the analogous bacterial pathways (Radman & Wagner, 1993; Rayssiguier *et al.*, 1989; Shen & Huang, 1989).

## Methods and Materials

### Strains

Bacteria, phage and plasmids used are listed in Table 2. 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 and to assay bacteria and bacteriophages. 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 was added per plate for estimation of  $\beta$ -galactosidase production.

### Construction of Int chimeras

Single-joint hybrid *int* genes with amino-terminal coding segments derived from HK022 and carboxyl-terminal coding segments from  $\lambda$  were constructed by a phage-plasmid cross in which the parents were pJR177 (strain RW3581) and  $\lambda$  *imm*21 (strain B265; Figure 1). Plasmid pJR177 contains an insert of a 20 kbp *Pst*I fragment from HK022 whose left end is located about 240 bp to the left of *attP* and which includes the *int* gene and the immunity region (*int-htt* and *imm-htt*, respectively; Yagil *et al.*, 1989; Oberto *et al.*, 1989). Phage B265 contains the immunity region of phage 21 (*imm*21) and *int*- $\lambda$ . Phage recombinants that had rescued *imm-htt* from the plasmid were selected by plating the progeny on strain RW3297, which is immune to  $\lambda$  *imm*21. To screen for recombinants that had crossed over within *int*, we hybridized the plaques to oligomer 20, which contains HK022 sequences just downstream of bp 165 of *int*, which is the point where the two genes diverge (see Figure 2). Phage from plaques hybridizing to this oligomer were hybridized to other downstream *int* oligomers to determine the region where the crossover is located, and the precise location of the hybrid joint was determined by sequencing *int* within 30 bp or more of the joints. In two cases (h205 $\lambda$  and h171 $\lambda$ ), the phage parent used in the above cross contained a point mutation that inactivates *int*, and we screened for *imm-htt* recombinants that had rescued a functional *int* gene that promotes  $\lambda$  excision, as judged by the red plaque test (Enquist & Weisberg, 1976). The same host, RW3297, was used to select these recombinants, and the location of the crossover within *int* was determined as described above.

Single-joint hybrid *int* genes with amino-terminal coding segments from  $\lambda$  and carboxyl-terminal coding segments from HK022 were isolated by infecting a strain carrying the defective prophage  $\lambda$   $\Delta$ [*J-attR*] *int*- $\lambda$  *imm* $\lambda$  (strain SA430) with  $\lambda$  *int-htt imm-htt* (strain O254) as previously described for the construction of phage strains Y1096 and Y1097 (Yagil *et al.*, 1989; Figure 1). The resulting *imm* $\lambda$  recombinants were screened by plaque hybridization to oligomer 26 (Figure 2), and those that hybridized were further analyzed as described above. Double-joint hybrids were constructed by crossing single-joint hybrids and selecting recombinants for the outside markers *h* (for host range) and *imm*. The crossover joints in such hybrids were always verified by DNA sequencing. Mutation *int*N99D was crossed into the various single and double-joint

hybrids by constructing parents with the proper combination of the outside markers *imm* and *h*, selecting recombinants for these markers by plating on selective host strains, and screening these recombinants for the ability to form blue plaques on host LD205 (see Results). The presence of the N99D mutation and the hybrid joint(s) were verified by DNA sequencing.

The frequency of recombinants carrying the immunity region of the plasmid or defective prophage ranged between  $10^{-6}$  and  $10^{-8}$  per progeny phage particle. The low recombination frequency is presumably a consequence of limited sequence identity between  $\lambda$  and HK022. Ten to 30% of these recombinants had crossed over within the carboxyl-terminal 300 codons of *int* or further to the left. Presumably, the phage with crossovers to the right of bp 165 had recombined within one of the intervals that is highly conserved between the  $\lambda$  and HK022 *int-imm* segment. This region has not been completely sequenced,

but we do know that it contains four segments totalling about 780 bp in length that are 97% identical to corresponding regions of the  $\lambda$  chromosome. The segments range in size from about 35 to 390 bp and are divided by blocks of non-homologous DNA (Yagil *et al.*, 1989; Oberto *et al.*, 1989). If we assume that all of the unsequenced HK022 DNA in the *int-imm* interval is non-homologous to  $\lambda$ , and the probability of recombination in non-homologous regions is negligible, then 70 to 90% of the crossovers have occurred within a region that contains a total of about 780 bp of near perfect sequence identity, and 10 to 30% have occurred within a 990 bp region (in the phage-prophage crosses) of 67% sequence identity. This suggests that reduction in sequence identity from 97% to 67% reduces recombination frequency per nucleotide about 4- to 12-fold. This is an underestimate of the effect of reduction in sequence identity if division of the highly conserved sequences into non-contiguous blocks reduces

**Table 2.** Bacteria, phage and plasmids

Strain	Relevant genotype and/or use	Origin/reference
<b>A. Bacterial strains:</b>		
DC7	Defective lysogen carrying $\lambda$ <i>imm21</i> $\Delta$ 443 [ <i>Q-chIA</i> ]; host for introducing <i>imm21</i> by recombination with infecting phage.	Feiss <i>et al.</i> (1972)
EY1248	$\lambda^R$ ( $\lambda$ $\Delta$ BAM <i>rex::kan cI857</i> $\Delta$ [ <i>cro-bio</i> ]); host for selecting against phage recombinants with the host range and immunity of $\lambda$ .	Lambda-resistant derivative of TAP117 (Patterson <i>et al.</i> , 1993)
EY1257	<i>recA</i> $\lambda^R$ /pJO35; host for selecting against phage recombinants with the host range of $\lambda$ and the immunity of HK022.	Oberto <i>et al.</i> (1989)
LD177	$\Delta$ X74[ <i>lacZ</i> ]/pLD177; host for testing $\lambda$ excisive recombination.	Accompanying paper
LD205	$\Delta$ X74[ <i>lacZ</i> ]/pLD205; host for testing HK022 excisive recombination.	Accompanying paper
LD300	<i>lacI</i> <sup>R</sup> $\Delta$ M15[ <i>lacZ</i> ]/pLD300; host for testing HK022 integrative recombination.	Accompanying paper
LE392	<i>supE44 supF58</i> ; host for phage growth.	Silhavy <i>et al.</i> (1984)
MC1000	$\Delta$ X74[ <i>lacIPOZY</i> ]	Silhavy <i>et al.</i> (1984)
RW1817	$\Delta$ [ <i>gal-<math>\lambda</math>J</i> ] ( $\lambda$ <i>gal8</i> $\Delta$ 10[ <i>int-red</i> ] <i>imm21</i> ); host for measuring $\lambda$ integrative recombination.	This work; Construction similar to that of RW1709 of (Enquist & Weisberg, 1984).
RW3297	Multiple lysogen containing $\lambda$ <i>h-<math>\phi</math>80 int-<math>\phi</math>80 imm21</i> and $\lambda$ 842 (a defective prophage inserted within <i>galT</i> ).	Weisberg <i>et al.</i> (1983)
RW3261	Host carrying plasmid pJO57, which expresses the HK022 <i>nun</i> gene; used for selecting against phage carrying <i>imm<math>\lambda</math></i> .	This work
RW3302	<i>recA tonA</i> /pJO35; host for selection against phage recombinants with host range and immunity of HK022.	Oberto <i>et al.</i> (1989)
RW3532	<i>tonA</i> ( $\lambda$ $\Delta$ BAM <i>rex::kan cI857</i> $\Delta$ [ <i>cro-bio</i> ]); host for selection against phage recombinants with the host range of HK022 and the immunity of $\lambda$ .	T5-resistant derivative of TAP117 (Patterson <i>et al.</i> , 1993)
RW3581	LE392/pJR177; host for crosses to generate chimeras.	Oberto <i>et al.</i> (1989)
SA430	( $\lambda$ <i>cI857</i> $\Delta$ 430[ <i>J-uvrB</i> ]); host for crosses to generate chimeras and for selection against phage recombinants with $\lambda$ immunity.	Feiss <i>et al.</i> (1972)
<b>B. Phage strains:</b>		
B265	$\lambda$ <i>imm21</i>	NIH collection
B363	$\lambda$ <i>int4 imm21</i> ; Parent of chimera h205 $\lambda$	Enquist & Weisberg (1977)
B346	$\lambda$ <i>int2038 imm21</i> ; Parent of chimera h171 $\lambda$	Enquist & Weisberg (1977)
EY211	$\lambda$ <i>h-htt</i>	Y1 $\times$ O228
EY212	$\lambda$ <i>intN99D imm21</i>	Y1026 $\times$ DC7
EY213	$\lambda$ <i>h-htt intN99D imm21</i>	EY211 $\times$ EY212
O228	HK022	Dhillon (1981)
O254	<i>h-<math>\lambda</math> attP-htt int-htt imm-htt</i>	Yagil <i>et al.</i> (1989)
W248	$\lambda$ <i>h-<math>\phi</math>80 <math>\Delta</math>9[<i>int</i>] imm<math>\lambda</math> c</i> ; Selection for $\lambda$ lysogens.	NIH collection
Y1	$\lambda$ <i>cI857</i>	NIH collection
Y1096	$\lambda$ <i>attP-htt int-htt cI857</i>	Yagil <i>et al.</i> (1989)
Y1097	$\lambda$ <i>attP-htt int-<math>\lambda</math>346h cI857</i>	Yagil <i>et al.</i> (1989)
Y1206	$\lambda$ <i>cI857 intN99D</i>	Dorgai <i>et al.</i> (accompanying paper)
Phage hybrids with a single crossover within <i>int</i> :		
5'-HK022-/3'- $\lambda$ hybrids	This set of chimeras is <i>h-<math>\lambda</math> attP-<math>\lambda</math> imm-htt</i>	This work; B265 $\times$ RW3581
5'- $\lambda$ /3'-HK022 hybrids	This set of chimeras is <i>h-<math>\lambda</math> attP-htt cI857</i>	This work; O254 $\times$ SA430
5'- $\lambda$ /3'-HK022 hybrids with <i>h-htt</i>	The <i>h-htt</i> (host range of HK022) marker was introduced into certain hybrids to select recombinants in crosses with other hybrids	This work; relevant chimera $\times$ O228

*continued*

Table 2. (continued)

Strain	Relevant genotype and/or use	Origin/reference
5'-λ/3'-HK022 hybrids with <i>intN99D</i>	The <i>intN99D</i> mutation was crossed into certain hybrids. These hybrids contain <i>h-λ attP-λ imm21</i>	This work; relevant chimera × EY213 with selection for excision proficiency (see the text)
<i>intN99D</i> λ219h	<i>h-λ attP-λ imm21</i>	This work; λ219h <i>h-htt</i> × EY212
Phage hybrids with two or three crossovers within <i>int</i> (see Figure 6):		
h276λ-λ321h	<i>h-htt attP-htt imm-htt</i>	This work, h276λ × λ321h
h276λ-λ324h	<i>h-htt attP-htt imm-htt</i>	This work, h276λ × λ324h
λ279h-h324λ	<i>h-λ attP-λ immλ cI857</i>	This work, λ279h <i>h-htt</i> × h234λ
λ279h-h330λ	<i>h-λ attP-λ immλ cI857</i>	This work, λ279h <i>h-htt</i> × h330λ
λ284h-h330λ	<i>h-λ attP-λ cI857</i>	This work, λ284h <i>h-htt</i> × h330λ
<i>intN99D</i> λ279h-h330λ	<i>h-λ attP-λ imm21</i>	This work; λ279h-h330λ × EY212
λ171h-h276λ-λ321h	<i>h-htt attP-htt cI857</i>	This work; h276λ-λ321h × Y1 with screening for λ excision specificity
λ205h-h276λ-λ321h	<i>h-htt attP-htt cI857</i>	This work; h276λ-λ321h × Y1 with screening for λ excision specificity
<b>C. Plasmids</b>		
pJO35	pBR322:: <i>cI-htt</i>	Oberto <i>et al.</i> (1989)
pJO57	pAR1206:: <i>nun</i>	J. Oberto (unpublished work)
pJR177	pBR322 with an insert containing <i>attP</i> through <i>cI</i> of HK022	Yagil <i>et al.</i> (1989)
pLD177	Derivative of pRS414 with λ <i>attL</i> and <i>attR</i> sites inserted within <i>lacZ</i>	Accompanying paper
pLD205	Derivative of pRS414 with HK022 <i>attL</i> and <i>attR</i> sites inserted within <i>lacZ</i>	Accompanying paper
pLD300	Derivative of pRS414 with HK022 <i>attP</i> and <i>attB</i> sites upstream of and within <i>lacZ</i> , respectively	Accompanying paper
pRS414	Lac fusion vector used in strain construction	Simons <i>et al.</i> (1987)

recombination frequency within these intervals, and an overestimate if there are undiscovered blocks of highly conserved sequence.

#### Selection of an integration-proficient revertant from an inactive chimera

Cells of strain LE392 were infected with chimera λ198h, lysogens were selected by spreading infected cells at 32°C on a plate seeded with about 10<sup>9</sup> particles of phage strain W248, which kills non-immune cells. Colonies formed by several thousand independently arising lysogens were resuspended by flooding the plates with diluent and scraping the surface with a glass rod, washed by repeated centrifugation, grown in LB medium at 32°C, and induced to produce phage by shifting the temperature to 42°C. These phage were plated on a tester strain for HK022 excisive recombination (similar to LD205) in the presence of Xgal, a rare blue plaque was isolated and purified, and the *int* gene of this phage was sequenced.

#### DNA manipulations

Plaque hybridization was done with NEN Research NEF-978 membranes according to the manufacturer's instructions. The oligomer used as probe was end-labelled with [ $\gamma$ -<sup>32</sup>P]ATP (Sambrook *et al.*, 1989). DNA was sequenced by the dideoxy chain termination technique (Sanger *et al.*, 1980). The template for DNA sequencing was either purified phage DNA or polymerase chain reaction (PCR) fragments. In the former case we used the USB Sequenase kit, different oligomers as primers and [ $\alpha$ -<sup>35</sup>S]ATP. In the latter case we used a Perkin-Elmer AmpliTaq Cycle Sequencing kit with <sup>32</sup>P-end-labelled oligomers as primers.

#### Strains for measuring excisive and integrative recombination

We have constructed plasmids that allow signalling of excisive recombination by synthesis of β-galactosidase.

One plasmid (pLD177) contains attachment sites of λ and the other (pLD205) sites of HK022 origin. The details of the construction are given in the accompanying article. Briefly, a segment containing *attR*-T1-T2-*attL* was inserted within the early coding region of *lacZ* so that most transcription stopped at the T1-T2 transcription terminators, and translation of the residual message occurred in an incorrect frame. Excisive recombination deletes the terminators and restores the correct translational frame. The *lacZ* gene of the recombinant plasmid has the *attB* site that results from recombination of *attR* with *attL*, but this short insertion does not prevent synthesis of active β-galactosidase. We have constructed a third plasmid (pLD300) that allows signalling of HK022 integrative recombination by cessation of β-galactosidase synthesis (details in accompanying article). Briefly, *attP-htt* was inserted upstream of the promoter in the recombinant plasmid that carries *attB-htt* within the coding region of *lacZ* (see above). Integrative recombination deletes the promoter and the early coding region of *lacZ*. Recombination was detected by the formation of Lac<sup>-</sup> colonies. The strain used to measure λ integrative recombination is deleted for the *gal* operon, but is Gal<sup>+</sup> because it carries a λ *gal* transducing prophage inserted into its chromosome. This prophage is deleted for *int*, carries the immunity region of phage 21, and is flanked by *attB* and *attP* (strain RW1817). Its construction was as described by Enquist & Weisberg (1984) for strain RW1714. Integrative recombination causes loss of the prophage *gal*<sup>+</sup> gene, and is signalled by the formation of Gal<sup>-</sup> colonies after infection.

#### Measuring excisive and integrative recombination

For a qualitative estimate of excision activity, we plated phage on lawns of the appropriate indicator hosts (LD177 or LD205) on plates containing Xgal, and estimated the intensity of blue plaque color. For a quantitative estimate of excision activity these strains were grown to a concentration of 5 × 10<sup>8</sup> cells/ml in LB medium supplemented with 0.2% maltose and 10 mM MgSO<sub>4</sub>, and infected with phage at a multiplicity of 20. After 20 minutes

adsorption at 37°C they were diluted tenfold with prewarmed LB medium, shaken for an additional 40 minutes at 37°C, treated with chloroform and assayed for  $\beta$ -galactosidase activity as described by Miller (1972) except that the cells were diluted into rather than centrifuged and resuspended in Z-buffer. We found in control experiments that there was a linear increase in enzyme activity from 20 to 80 minutes after infection (data not shown). Each assay was done at least twice, and an estimate of variability is given in Table 1. Our estimates of HK022 excision were generally three to fivefold higher than those of  $\lambda$  excision (Table 1). Part of this difference appears to be due to the different extents of *attB*-region sequence in plasmids pLD177 and pLD205 (L.D. & R.A.W., unpublished experiments).

To measure HK022 integrative recombination we infected strain LD300 at a multiplicity of 10. After ten minutes adsorption at 32°C the cells were diluted as described above, shaken for an additional 30 minutes, diluted further and plated on plates containing ampicillin and X-gal. The ratio of white (Lac<sup>-</sup>) to blue (Lac<sup>+</sup>) colonies indicated the degree of integrative recombination. To measure  $\lambda$  integrative recombination we grew and infected cells of strain RW1817 as described previously (Yagil *et al.*, 1989) and plated the cells on galactose-tetrazolium plates (Silhavy *et al.*, 1984). The ratio of red (Gal<sup>-</sup>) to white (Gal<sup>+</sup>) colonies indicated the degree of integrative recombination.

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