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# The *Int* family of site-specific recombinases: Some thoughts on a general reaction mechanism

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**Abstract.** The FLP recombinase of the yeast 2 micron circle plasmid belongs to the *Int* family of recombinases. Only three amino acid residues are invariant among members of this family. Functional analyses of FLP protein variants mutated at these three residues suggest their involvement at specific steps of the recombination pathway. We propose that these residues play the same functional role in the mechanism of action of all the *Int* family recombinases.

**Keywords.** *Int*-family recombinases; FLP protein variants; site-specific recombination.

## 1. Introduction

Site-specific recombination often provides the key to programmed developmental pathways in prokaryotes and eukaryotes. Examples are integration and excision of phage lambda, flagellar phase variation in *Salmonella*, altered expression of tail-fibre proteins in phage Mu and P1, dimer resolution and stable propagation of the unit copy plasmid/phage P1, resolution of cointegrate intermediates produced during the transposition of Tn*3* and $\gamma\delta$, possible copy control of yeast plasmids by recombinational amplification, and rearrangements of the immunoglobin genes in man and mice (Campbell 1983; Silverman and Simon 1983; Plasterk and van de Putte 1984; Heistand-Nauer and Iida 1983; Austin *et al* 1983; Volkert and Broach 1986; Reynolds *et al* 1987; Tonegawa *et al* 1980). The site-specific recombinase *Int* of phage lambda was the first to be analyzed in detail both genetically and biochemically (Weisberg and Landy 1983). Recently considerable progress has been made in our understanding of a number of site-specific recombinases: the resolvase proteins of Tn*3* and $\gamma\delta$, the *Cre* protein of phage P1, the *Hin* and *Gin* functions of *Salmonella* and phage Mu, respectively, and the FLP recombinase of the 2 micron plasmid of yeast (Sadowski 1986).

The prokaryotic site-specific recombinases can be classified into two broad families: the *Int* family and the *Hin* family (Argos *et al* 1986). The *Hin* family is composed of the highly homologous invertases of *Salmonella* (*Hin*), phage Mu (*Gin*), phage P1 (*Cin*), and *E. coli* (*Pin*). The Tn*3* and $\gamma\delta$ encoded resolvases also belong to this family. The *Int* family is constituted by the site-specific recombinases of phage lambda (*Int*) and the lambda related phage $\phi$80 and P22, the related phage P2 and 186, and the phage P4 and P1. Unlike the *Hin* family, the *Int* family is

highly divergent (Argos *et al* 1986). A moderate degree of homology confined to a 40 amino acid stretch near the carboxy terminal region is the only significant conservation within this family. This homology extends to the FLP protein of the 2 micron circle as well. FLP is therefore considered to be a member of the *Int* family. The most remarkable feature of this family is the invariance of three residues, His396, Arg399, and Tyr433. According to the FLP numbering scheme, these residues are His305, Arg308, and Tyr343. This absolute conservation suggests some critical role for these amino acids in the recombination process. We have begun to address this issue by mutating these residues in the FLP protein and analyzing the properties of the variant proteins (Prasad *et al* 1987; R L Parsons, P V Prasad, R M Harshey and M Jayaram, in preparation). In this report we shall summarize the significance of these results with respect to a general catalytic mechanism for the recombinases of the *Int* family.

## 2. Materials and methods

Mutations were introduced by site-directed mutagenesis on an M13 single-stranded template containing the FLP gene. The mutant FLP genes were placed under the control of the lambda $P_R$ promoter in a plasmid vector and introduced into an *E. coli* host by transformation. The ability of the mutant proteins to catalyze recombination was assayed *in vivo* and *in vitro* (Jayaram 1985; Prasad *et al* 1986). Substrate binding by FLP and FLP variants was determined by gel retardation assays (Fried and Crothers 1981). The capacity of the mutants to effect strand cleavage within the substrate *in vitro* was also assessed. The details of all the above experimental procedures have been described earlier (Jayaram 1985; Prasad *et al* 1987).

## 3. Results

### 3.1 Recombination

The various mutants of FLP studied by us so far are listed in table 1. All of the mutants were tested in recombination assays *in vivo* and *in vitro*. The substrate in

**Table 1.** Mutants of FLP with altered amino acids at the conserved family positions.

| FLP Variant | FLP residue number | Family residue number | Conserved family residue |
|---|---|---|---|
| FLP (Tyr343→Phe)<br>FLP (Tyr343→Ser) | 343 | 433 | Tyr |
| FLP (His305→Gln)<br>FLP (His305→Pro)<br>FLP (His305→Leu) | 305 | 396 | His |
| FLP (Arg308→Gly) | 308 | 399 | Arg |

The indicated mutations at the conserved family positions were introduced by site-directed mutagenesis as described earlier (Prasad *et al* 1987).

the *in vivo* assay was a plasmid that contained the Tn*5* derived kanamycin resistance gene between two directly repeated FLP recombination sites (figure 1A). Recombination between the sites would exclude the kanamycin resistance gene on a circular piece of DNA that lacks a replication origin. Loss of kanamycin resistance, therefore, gives a measure of FLP mediated recombination. *In vitro* recombination assays were carried out using partially pure preparations of FLP or of FLP mutants obtained from the *E. coli* host strains expressing them. The substrates for *in vitro* recombination were two linear molecules of identical length, each of which contained one FLP site (figure 1B). The relative location of each of these sites with respect to the ends of the molecule was different for the two substrates. An intermolecular recombination event between the two types of parental DNA molecules would result in two recombinant products which differ in length from each other and from the parents. These were visualized by electrophoretic fractionation in agarose gels and ethidium bromide staining. The

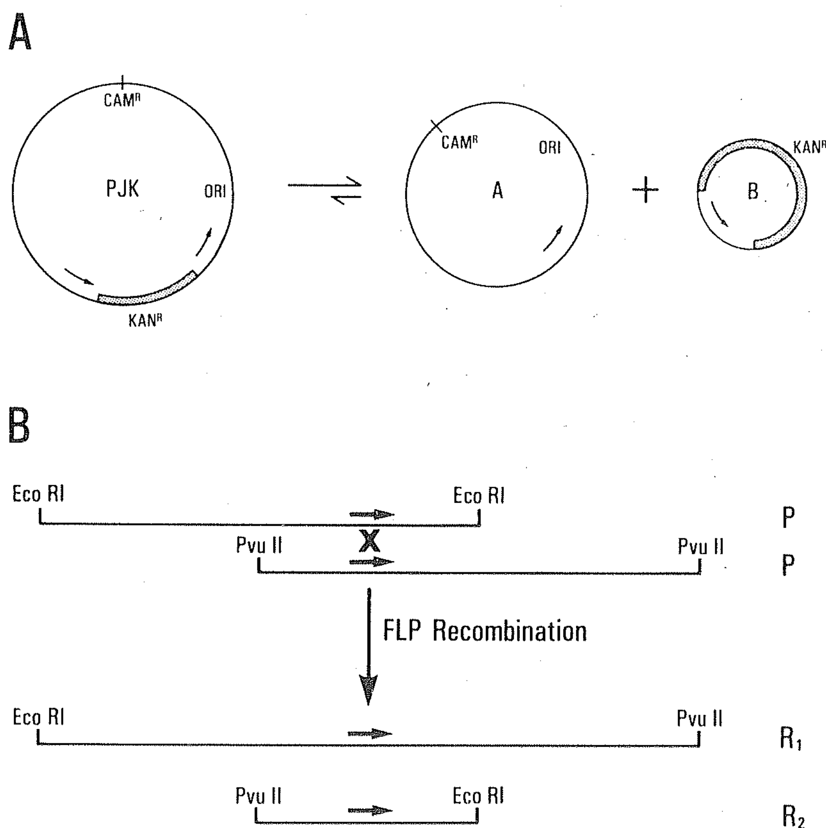


**Figure 1.** FLP recombination *in vivo* and *in vitro*. (**A**) Plasmid pJK contains the kanamycin-resistance marker ($KAN^R$) between two directly oriented FLP recombination sites (→). FLP mediated combination results in a plasmid that contains the chloromphenicol resistance marker ($CAM^R$) and a circular DNA molecule that contains the $KAN^R$ gene but lacks a replication origin. (**B**) The substrates for *in vitro* recombination are derived from a plasmid containing the FLP recombination site (→) by cutting it with *Eco*RI or with *Pvu*II. These fragments are labeled P. Recombination between them results in fragments R1 (larger than P) and $R_2$ (smaller than P).

results of the recombination assays are given in table 2. Substitution of Tyr343 by Phe or by Ser, of Arg308 by Gly, and of His305 by Pro or by Leu abolished recombination. The FLP variant containing a Gln instead of His at 305 was severely depressed in recombination *in vivo*. Extracts of this mutant were either inactive or very poorly active in recombination assays *in vitro*. These results indicated that alterations of the invariant trio of His305, Arg308, and Tyr343 of FLP almost invariably results in loss of catalytic activity.

### 3.2 *Substrate recognition*

The failure of the FLP mutants to catalyze recombination could conceivably be due to defects in substrate recognition. To check this, gel retardation assays were done for all of them using an approximately 100 bp DNA fragment (that includes the FLP substrate), end labeled one on one of the DNA strands at the 3' end ( Prasad *et al* 1987). Binding of FLP to its substrate has been shown to generate three protein DNA complexes, CI, CII, CIII, which correspond to association of one, two and three protomers, respectively, of the FLP protein (figure 2) (Andrews *et al* 1987; Prasad *et al* 1987). The results of these assays are summarized in table 3. All mutants gave the expected three protein complexes. However, all of them except FLP (His305→Gln) bound substrate with lower affinity than the wild type. To detect qualitative alterations in binding, DNase footprinting of the mutants was carried out on the same DNA fragment that was used for gel retardation experiments. The footprints of the Tyr343 mutants and of the His305 mutants were in general similar to the wild type footprints (Prasad *et al* 1987; R L Parsons, P V Prasad, R M Harshey and M Jayaram, in preparation). While the band corresponding to strand cleavage (see below) was enhanced in the footprints of the His mutants, this band was conspicuously absent in the footprints of the Tyr343 mutants (table 3; Prasad *et al* 1987). No significant DNase protection was seen with FLP (Arg308→Gly).

### 3.3 *Strand cleavage*

The FLP protein can cause specific single-stranded nicks within its substrate to generate a free 5′ hydroxyl and a 3′ phosphate linked to the protein through a Tyr

**Table 2.** Recombination by FLP mutants *in vivo* and *in vitro*.

| | Recombination | |
|---|---|---|
| | *in vivo* | *in vitro* |
| FLP | +++ | +++ |
| FLP (Tyr343→Phe) | – | – |
| FLP (Tyr343→Ser) | – | – |
| FLP (His305→Gln) | ± | ± |
| FLP (His305→Pro) | – | – |
| FLP (His305→Leu) | – | – |
| FLP (Arg308→Gly) | – | – |

The rationale of the recombination assays is diagrammed in figure 1 and described in the text. FLP (His305→Gln) showed a low level of recombination. All other mutants were inactive in recombination.

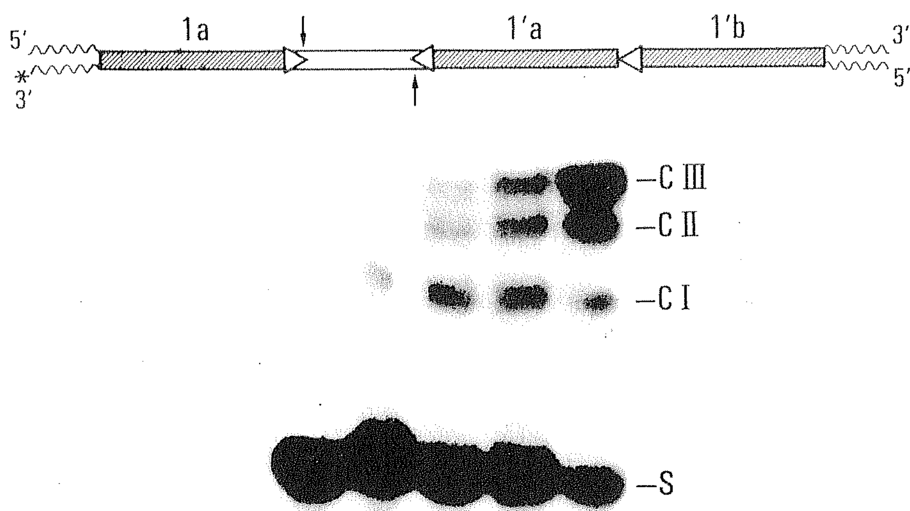


**Figure 2.** Substrate binding by FLP. The DNA fragment (approximately 100 bp long; Prasad *et al* 1987) used for the assays is schematically shown at the top. The FLP substrate, contained within this fragment, consists of the three 13 bp symmetry elements (1a, 1'a, and 1'b) plus the 8 bp spacer of the dyad 1a-1'a. FLP can cleave this substrate at the junction of the 1a, 1'a symmetry elements and the spacer (indicated by the vertical arrows). The vector sequences are shown by the wavy lines. The fragment is labeled with $\alpha$-$^{32}$P dCTP at the 3' end on the bottom strand (*). The FLP-DNA complexes formed with increasing concentrations of FLP can be visualized by electrophoresis in non-denaturing polyacrylamide gels and autoradiography. The three complexes are labeled CI, CII, and CIII. The substrate is labeled 'S'.

residue (Gronostajski and Sadowski 1985; Andrews *et al* 1985). This cleavage and the associated conservation of the energy of the phosphodiester bond in the form of a phosphoprotein linkage represents a key intermediate step in the recombination reaction. The footprinting results indicated that the mutants may be differentially affected in the strand cleavage step. To verify this, cleavage assays were done with the FLP variants using the same end-labeled substrate used for binding and footprinting assays. The two mutants of Tyr343, FLP(Tyr343→Phe) and FLP(Tyr343→Ser), and the mutant of Arg308, FLP(Arg308→Gly), gave no detectable cleavage (table 4). Mutants of His305 were active in cleavage and accumulated the cleavage product in excess of that obtained with wild type FLP.

## 4. Discussion

The results of the mutational analysis on the invariant family residues of FLP permits a genetic dissection of the steps involved in the recombination reaction. We

**Table 3.** Substrate recognition by the FLP mutants.

| | Gel retardation | DNase footprints |
|---|---|---|
| Protein | CI CII CIII | |
| FLP | All three complexes | |
| FLP (Tyr343→Phe)<br>FLP (Tyr343→Ser) | Same as FLP | Same as FLP, but no FLP cleavage band |
| FLP (His305→Gln)<br>FLP (His305→Pro)<br>FLP (His305→Leu) | Same as FLP | Same as FLP; FLP cleavage band more intense than wild type |
| FLP (Arg308→Gly) | Same as FLP | No significant DNase protection |

All mutants gave rise to complexes CI, CII and CIII with the FLP substrate (see figure 2). However, higher concentrations of mutant proteins [except FLP (His308→Gln)] relative to the wild type were required to obtain comparable amounts of CII and CIII. With FLP (Arg308→Gly) no DNase footprints were observed. All other mutants gave gel retardation patterns and DNase footprints similar to those of the wild type FLP. The DNase footprints of the Tyr343 mutants lacked the band corresponding to the FLP induced DNA cleavage, while the His305 mutants contained an excess of this band.

call these mutants step-arrest mutants. First, the mutants of Tyr343 recognize the substrate normally but fail to execute strand cleavage. Hence they fail to complete recombination. We suggest that this Tyr is directly involved in DNA breakage and covalent attachment. For two members of the *Int* family, FLP, and *Int* , the protein DNA bridge has been shown to be a phosphotyrosine (Gronostajski and Sadowski 1985; C A Pargellis, S E Nunes-Duby, L M Vargas and A Landy, personal communication). The precise Tyr involved in this step has not been identified for

**Table 4.** Substrate cleavage by the FLP mutants.

| | DNA cleavage |
|---|---|
| FLP | + |
| FLP (Tyr343→Phe) | − |
| FLP (Tyr343→Ser) | − |
| FLP (His305→Gln) | ++ |
| FLP (His305→Pro) | +++ |
| FLP (His305→Leu) | +++ |
| FLP (Arg308→Gly) | − |

Cleavage assays were done using the FLP substrate (see figure 2) end-labeled, on one of the strands at the 3′ end. Since FLP cleavage produces a free 5′-OH, the labeled fragment resulting from cleavage is not protein bound and requires no proteolysis for detection. The reaction mixtures were evaporated under vacuum and run on denaturing polyacrylamide gels to visualize the cleavage fragment.

either of these proteins. Since Tyr433 (family number; corresponds to Tyr343 of FLP) is the only invariant Tyr in the family, our assumption appears to be a valid one. This Tyr may initiate a nucleophilic attack on the phosphodiester bond of the DNA backbone, forming the phosphotyrosine bond and generating a free 5′-hydroxyl. It is conceivable that this reaction could be facilitated by an acid or a base catalyst, by making the phosphate a better target for nucleophilic attack or by making the Tyr a stronger nucleophile. An Arg would fit this role nicely. Could Arg308 then fulfill this function? Our results do not allow us to answer this question, since the one mutant of FLP at this position, FLP(Arg308→Gly) forms a significantly weaker complex with the substrate than the normal recombination complex. More mutations are being made at this position to address this issue. Mutations of His305 do not affect substrate recognition or strand cleavage. But these FLP mutants are defective in the subsequent steps of recombination, namely exchange and reunion of strands between partner substrates. The strand reunion step is mechanistically the reverse of the strand cleavage step, except that for recombination to occur, this union must take place between like strands of two substrate molecules. Here the nucleophilic attack would be initiated by the free 5′-hydroxyl of DNA on the 3′-phosphotyrosine. The reaction would reform the phosphodiester bond, heal the nick of the DNA backbone, and liberate the protein from its covalent DNA attachment. His305 could potentially facilitate this step, possibly by functioning as an acid catalyst and making the tyrosylphosphate more receptive for nucleophilic attack. One cannot rule out the less likely possibility that His305 mutants are in some manner affected in the proper synapsis of the partners of recombination, so that exchange of strands between them is aborted. We propose that their invariance within the *Int* family implies a common role for these amino acids in reactions catalyzed by all members of the family.

Recently several 2 micron circle-like plasmids have been isolated from strains of *Zygosaccharomyces* and *Kluyveromyces*. All these plasmids encode recombinases analogous to the FLP protein. However, there is no significant amino acid homology among these proteins (Utatsu *et al* 1987). The FLP recombination site is also quite distinct from the putative recombination sites of the FLP protein analogs of these plasmids. Yet they all share the conserved trio of His305, Arg308, and Tyr343. There is an interesting region of conservation surrounding His305 and Arg308 among these proteins. In addition, approximately 70 to 150 amino acids upstream of these two residues, regions of homology can be detected. It is hoped that mutational analysis of these portions of the FLP protein, by quasi-random as well as directed mutagenesis, will provide more information on the nature of substrate recognition by FLP and on the catalytic mechanism of FLP recombination. Experiments along these lines are currently being done.

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