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IS10 transposase mutations that specifically alter target site recognition

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IS10 inserts preferentially into particular hotspots. We describe here mutations of IS10 transposase, called 'ATS' that confer Altered Target Specificity. These mutations yield a general relaxation in target specificity but do not affect other aspects of transposition. Thus, the preference for specific nucleotide sequences at the target site can be cleanly separated from other steps of the transposition reaction. Eleven ATS mutations identified in a genetic screen occur at only two codons in transposase, one in each of two regions of the protein previously implicated in target site interactions (Patch I and Patch II). Genetic analysis suggests that mutations at the two ATS codons affect the same specific function of transposase, thus raising the possibility that Patch I and Patch II interact. For wild-type IS10, insertion specificity is determined in part by a specific 6 bp consensus sequence and in part by the immediately adjacent sequence context of the target DNA. The ATS mutations do not qualitatively alter the hierarchy with which base pairs are recognized in the consensus sequence; instead, sites selected by ATS transposase exhibit a reduction in the degree to which certain base pairs are preferred over others. Models for the basis of this phenotype are discussed.

Key words: insertion specificity/mutational analysis/Tn*10*/transposase binding

Introduction

The bacterial transposon Tn*10* and its component insertion sequence IS10 transpose by a non-replicative mechanism where the transposon is excised from its donor site by double strand breaks at each of its ends and inserted into a new target site (Bender and Kleckner, 1986; Benjamin and Kleckner, 1989). This reaction has been separated by mutation of the IS10 transposase protein into steps that yield the excised transposon and subsequent strand transfer steps that yield insertion in the target site (Haniford *et al.*, 1989, 1991). We report here the isolation and characterization of new IS10 transposase mutants with Altered Target Specificity (ATS) but no other significant defect in transposition. These ATS mutants demonstrate that the preference for specific nucleotide sequences at the target site is not an obligatory feature of the transposition reaction.

Tn*10* and IS10 insertions are flanked by 9 bp direct repeats of the target site DNA (Kleckner, 1979). These repeats arise from a staggered 9 bp cleavage at the target site followed by transposon insertion between the 9 bp overhangs and filling-in of the resulting 9 bp gaps at each end of the transposon (Benjamin and Kleckner, 1989). Sequence analysis of many Tn*10* insertion sites as well as mutational analysis of a particular Tn*10* insertion hotspot have revealed a 6 bp consensus sequence, 5'-NGCTNAGCN-3', embedded in the 9 bp of target site DNA (Halling and Kleckner, 1982; J.Bender and N.Kleckner, submitted). The consensus sequence is an interrupted 3 bp inverted symmetry. This configuration might be recognized and cleaved by symmetrically disposed subunits of a single protein (Halling and Kleckner, 1982), analogously to Type II restriction enzyme cleavage (McClarin *et al.*, 1986).

While some deviation from the perfect consensus can be tolerated for a Tn*10* insertion site, certain 'forbidden' base changes will effectively destroy the site. In particular, a transversion mutation at either the first or the second position of the consensus 3 bp half site 5'-GCT-3' will reduce insertion into a target site by at least three orders of magnitude (J.Bender and N.Kleckner, submitted). The sequences flanking the consensus bases out ~10 bp to each side also influence the efficiency of insertion into a target site over at least three orders of magnitude (J.Bender and N.Kleckner, submitted). However, the nature of the determinants provided by these 'context' sequences is not defined. These sequences probably contribute a certain helix structure rather than sequence-specific DNA contacts to protein bound in the major groove at the consensus bases.

The DNA sequence at the target site is most likely directly recognized by IS10 transposase protein (Halling and Kleckner, 1982). Strong biochemical evidence for this assumption comes from the finding that a highly purified form of transposase can promote Tn*10* and IS10 intramolecular transpositions *in vitro* that display the same patterns of target site preference as *in vivo* transpositions (H.W.Benjamin and N.Kleckner, submitted and personal communication). Thus, particular mutations in transposase should be able to change the insertion specificity of Tn*10* transposition.

ATS mutants were identified in a genetic screen for transposases that increase the frequency of Tn*10* insertion into a small target region lacking a wild-type consensus insertion sequence. This phenotype could result from either a mutant transposase that recognizes a new sequence in the target region as an insertion hotspot, or from a mutant transposase whose insertion specificity is relaxed sufficiently to yield an increased frequency of insertion into secondary target sites.The ATS mutants reported here have the latter phenotype.

A previous mutational analysis of IS10 transposase identified two regions of the protein that are important for insertion of the transposon into a target site. Transposases with mutations in these regions can excise the transposon from its donor site but fail to insert it into a target site (SOS^+ $Tnsp^-$ mutants; Haniford *et al.*, 1989). The regions

are defined as Patch I (amino acids 102–167) and Patch II (amino acids 243–264) of the 402 amino acid transposase protein (Figure 1). The ATS mutations all change one of two codons, codon 134 or codon 249, each of which lies in the middle of one of the patches. However, we show that the ATS mutations have effects that are mechanistically distinct from SOS^+ $Tnsp^-$ mutations. Thus, the ATS mutations surgically alter a particular step in the transposition reaction. Mechanistic models for the relaxed insertion specificity displayed by the ATS mutants are discussed.

Results

Isolation of transposase mutations that alter Tn10 target specificity

Mutations of IS10 transposase that alter Tn*10* target specificity were identified using a genetic assay for Tn*10* insertion into a particular target region. In this assay, a silent *lacZ* gene is activated by transposition of a promoter-bearing Tn*10* construct into a short well-defined upstream target region from the *Salmonella* histidine operon (Figure 2) (J.Bender and N.Kleckner, submitted). In constructs where the IS10 transposase gene and the target region are wild-type, essentially all of the insertions that activate *lacZ* occur at a single well-characterized hot spot *hisG*1 (Materials and methods).

In the insertion specificity assay, the frequency of Lac activation is monitored in single colonies on MacConkey lactose indicator plates. Transposon insertions into the appropriate target region result in formation of red (Lac^+) papillae on otherwise white colonies, and the number of papillae per colony reflects the frequency of insertion in this region. A direct correspondence between the number of papillae and the frequency of Lac activating events has been explicitly demonstrated in another papillation assay for transposition (Huisman and Kleckner, 1987).

The frequency of papillation in the insertion specificity assay decreases dramatically when appropriate deleterious mutations are introduced into the *hisG*1 target site. For example, this frequency decreases ~1000-fold when both 3 bp half sites of the target site are changed from the consensus 5′-*G*CT-3′ to either 5′-*T*CT-3′ (mut-1), 5′-*C*TC-3 (mut-2), 5′-G*G*T-3′ (mut-3) or 5′-G*A*T-3′ (mut-4) (Table I).

We describe here the isolation of mutant transposases that exhibit altered target specificity (ATS). These mutants were identified as transposases that could promote a higher frequency of transposon insertion into one of the symmetrically defective target sites described above. Such mutations could confer on transposase a new sequence specificity such that the new consensus sequence is efficiently recognized. Alternatively, they might confer a relaxation in specificity which permits transposase to insert more efficiently at secondary sites in the target region. We show below that we have identified mutations of the latter type.

The *lacZ* target construct in the assay for altered specificity transposase mutants is located in the *Escherichia coli* chromosome within a lambda prophage, and the transposase gene and the promoter-bearing transposon are carried on a multicopy plasmid (pNK2731, Figure 2). Transposase mutations were isolated in pNK2731 following mutagenesis with hydroxylamine (HA) and transformation into a strain carrying the appropriate target construct. Eleven independent mutants exhibiting the desired phenotype were obtained from a total of 120 000 transformants screened: two of the

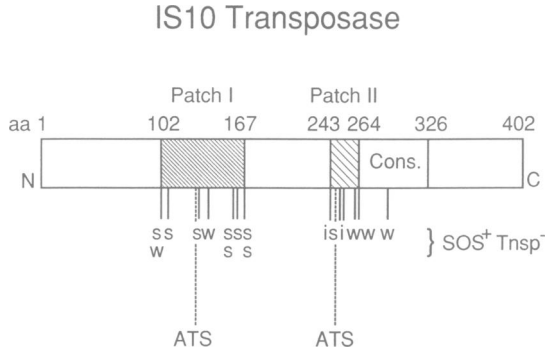

Fig. 1. Positions of SOS^+ $Tnsp^-$ and ATS mutations in the primary sequence of IS10 transposase. The primary sequence of IS10 transposase is shown drawn to scale. Patch I and Patch II are diagonally shaded and a 'conserved region' that shows homology to other bacterial transposases (Cons.) is stippled. The positions and phenotypes of mutations are indicated. s, strong; i, intermediate; and w, weak SOS^+ $Tnsp^-$ phenotypes (Haniford *et al.*, 1989). ATS, altered target specificity.

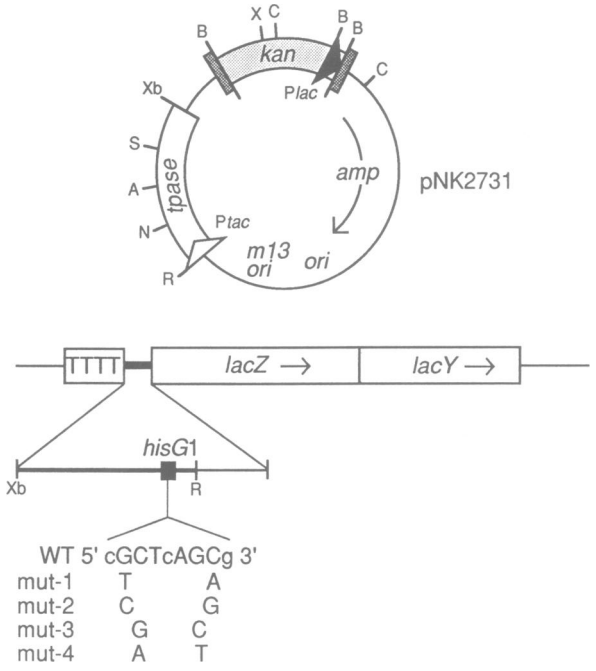

Fig. 2. The papillation assay for ATS mutant transposases. The structure of the P*tac*–transposase/mini-Tn*10* *kan* P*lac* plasmid pNK2731 is shown. The transposase gene on this plasmid (*tpase*) was mutagenized with HA to generate ATS mutants. The target site construction in this assay system is present in single copy in the chromosome of the Δ*lac recA*$^-$ strain NK8032 on a λ prophage. TTTT, four tandem repeats of the *rrnB* operon strong transcriptional terminators. The target fragment between these terminators and the first codon of the downstream promoterless *lacZ* gene is shown in an expanded view. The fragment consists of ~270 bp of *Salmonella his* DNA (indicated by the heavy line) including the strong insertion site *hisG*1 (filled box) plus ~110 bp of leader sequences upstream of the *lac* genes (indicated by the light line). The consensus bases at *hisG*1 in this construction were mutated to make particular altered target sites, shown as WT (λNK1294), mut-1 (λNK1295), mut-2 (λNK1299), mut-3 (λNK1300) and mut-4 (λNK1301). A, *Avr*II; B, *Bam*HI; C, *Cla*I; N, *Nhe*I; R, *Eco*RI; S, *Stu*I; Xb, *Xba*I; X, *Xho*I.

mutants were isolated on a mut-1 target, five were isolated on a mut-3 target, and four were isolated on a mut-4 target. Each of these 11 mutants proved to contain a single relevant altered target specificity or ATS mutation as determined by subcloning and DNA sequence analysis (Materials and methods).

Table I. Papillation frequencies

Target	Transposase	
	Wild-type	ATS
Wild-type	10^4	10^3
Mutant	10	10^2

Papillation frequency is the average number of Lac$^+$ papillae per test strain colony after 4–5 days on MacConkey lactose amp kan medium at 37°C in the dark. For strains with high papillation levels, these numbers were confirmed by determining the frequency of lac$^+$ transpositions selected on minimal lactose medium. In the test system wild-type transposase is supplied from plasmid pNK2731 and ATS mutant transposase is supplied from an isogenic derivative carrying either the CY134 mutant transposase (pNK2792), the CY249 mutant transposase (pNK2854) or the CY134 CY249 double mutant transposase (pNK2858). The wild-type target strain is NK8032(λNK1294). The mutant target strains are mut-1, mut-2, mut-3 or mut-4 = NK8032(λNK1295), (λNK1299), (λNK1300) or (λNK1301). Both ATS mutant transposases and the double mutant transposase yield the same papillation frequency on all four mutant targets.

ATS mutations alter specific codons in Patch I and Patch II

The ATS phenotype is conferred only by changes at very particular positions in transposase protein. The 11 independent mutations represent changes in only two different codons: nine are changes at codon 134 from TGT (Cys) to TAT (Tyr) and two are changes at codon 249 from TGT (Cys) to TAT (Tyr). The CY134 ATS mutant transposase was isolated on all three mutant target sites screened. The CY249 transposase was isolated on both mut-3 and mut-4 target sites.

Each of the ATS codons maps within one of the two general regions of transposase, Patch I and Patch II, previously shown to be involved specifically in target interactions and/or strand transfer (Figure 1; Haniford *et al.*, 1989). These two regions are defined by a particular subclass of transposase mutations, SOS$^+$ Tnsp$^-$, that permit excision of the transposon from the donor site but block strand transfer to the target site. CY134 maps in Patch I and CY249 maps in Patch II.

The limited number of ATS sites identified in this analysis reflects the rarity of mutations that yield the desired phenotype rather than reflecting limited HA mutagenic specificity. HA-generated mutations with more general phenotypes are recovered at many more sites in transposase. For example, 16 HA-induced SOS$^+$ Tnsp$^-$ mutations mapped to 13 different sites throughout Patch I and Patch II (Figure 1; Haniford *et al.*, 1989). The limited number of mutations that confer an ATS phenotype is also reflected in the low frequency with which these mutations are recovered: the frequency of ATS mutations in samples treated with HA under standard conditions is only 10% the frequency of SOS$^+$ Tnsp$^-$ mutations.

In contrast, the fact that only Cys to Tyr changes were isolated at each of the ATS codons is definitely a consequence of HA specificity. Oligonucleotide mutagenesis of these two codons reveals that an ATS phenotype is also conferred by the presence of Asn or Phe at position 134 and by any of 10 other amino acids at position 249 (Materials and methods).

The fact that both ATS mutations alter cysteine residues is not due to the fact that the mutations disrupt a disulfide bond or chelation with a metal ion between these amino acids

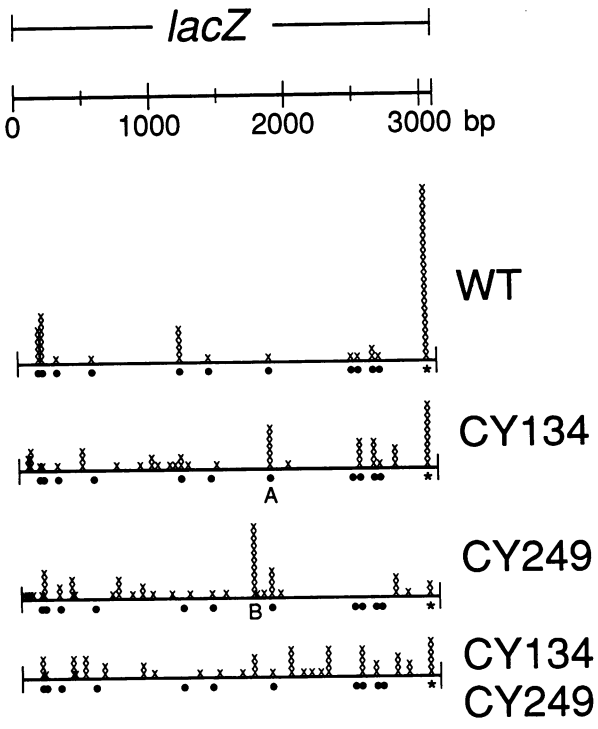

Fig. 3. Tn*10* insertions in the *lacZ* gene generated by wild-type and ATS mutant transposases. Fifty independent mini-Tn*10* Kan^R insertions into the 3.1 kb *lacZ* gene carried on a pGEM-3 plasmid vector were isolated from λ delivery vehicles carrying either wild-type (WT) (λNK1105), CY134 (λNK1298), CY249 (λNK1313) or CY134 CY249 (λNK1315) transposases (see Materials and methods). Approximate positions of insertion are shown: base pair 1 of the scale is at the A of the ATG start codon for *lacZ*. The strong hotspot for wild-type transposase is marked * and the positions of other wild-type insertion sites are indicated by filled circles. The new preferred sites for CY134 and CY249 transposases are marked A and B.

because revertants of CY134 to wild-type insertion specificity will result from either serine or cysteine at this position. Analogous reversion analysis of CY249 yields only cysteines (Materials and methods).

ATS mutations confer a general relaxation of target specificity

The finding that the same ATS mutations were isolated on different mutant target sites suggested that these mutations confer a general relaxation of target specificity rather than a specifically altered interaction with a new target consensus. In fact, both CY134 and CY249 ATS mutant transposases have the same phenotype on all four mutant *hisG*1 target derivatives. Also, the two ATS mutant phenotypes are the same as each other: ~10-fold increased levels of papillation on mutant target sites relative to wild-type transposase (Table I).

Direct evidence that the ATS mutations confer relaxed target specificity is provided by mapping the sites of Tn*10* insertion promoted by ATS mutant transposases in two different target regions. First, mapping analysis reveals that the Lac activation by an ATS transposase in the papillation assay results from insertion of Tn*10* at secondary sites rather than at the mutant *hisG*1 site: 15 of 17 independent Lac-activating insertions promoted by CY134 into the 5′-TGT-3′ target construction map to sites in the 380 bp target region other than *hisG*1 (data not shown; Materials and methods). Second, mapping analysis of Tn*10* insertions into a large reference region, the *lacZ* gene itself, reveals that wild-type

Table II. Sites of wild-type and ATS transposase insertions in *lacZ*

Number of insertions				Position of insertion	Sequence of 9 bp
WT	CY134	CY249	CY134 CY249		
0	0	1	0	16	G A T T C A C T G
0	0	1	0	40	C G T C G T G A C
0	2	1	0	71	A A C T T A A T C
0	3	1	0	87	A G C A C A T C C
5	1	1	3	155	T G C G C A G C C
7	1	4	1	178	C G C T T T G C C
1	1	2	0	286	T A C G A T G C G
0	0	3	3	380	C A T T T A A T G
0	0	1	1	404	T A C A G G A A G
0	3	0	3	472	C G C T G G G T C
1	0	0	0	550	C G C C T C G C G
0	0	0	2	614	G G A T G A G C G
0	0	1	0	681	T G T T G C C A C
0	1	3	0	726	G G C T G A A G T
0	0	1	0	829	G G T G A A A T T
0	1	2	2	902	T G T G G A G C G
0	2	1	1	985	T G C G A T G T C
0	1	0	0	1031	T G C T G A A C G
0	1	1	0	1121	T G C A G G A T A
0	1	0	0	1163	T G C G C T G T T
5	2	0	0	1202	C G C T G T G C G
0	1	1	0	1258	G G C A T G G T G
0	0	0	1	1318	C G C G T A A C G
1	0	1	0	1420	C G C T G G A T C
0	1	0	1	1468	G G C G G A G C C
0	0	1	0	1524	T G A A G A C C A
0	0	0	1	1631	G T A A C A C T C
0	0	10	3	1725	C G C T G A T T A
0	0	1	0	1750	A A C C C G T G G
0	0	1	0	1802	A G T T C T G T A
1	6	4	1	1860	A G C A A A A C A
0	0	1	0	1927	T A C C T G T T C
0	1	0	4	1999	G G T G A A G T G
0	0	0	1	2089	C T C A C A G T A
0	0	0	1	2148	C G C C T G G C A
0	0	0	1	2218	C A T C T G A C C
0	0	0	4	2273	A A T T T A A C C
1	0	0	0	2471	T G C A G T G C A
1	4	0	4	2523	C G C G T G G C A
2	4	0	2	2628	T A C A C C G C A
1	1	0	0	2676	A G C A G A G C G
0	3	3	3	2781	T A C C C C G T A
0	0	1	2	2870	A G T T C A A C A
24	9	2	5	3026	A G C T G A G C G

The exact positions of insertions generated by various transposase alleles in the *lacZ* gene (Figure 2) were determined by sequencing. The sequence of the 9 bp duplicated at each insertion site is shown with consensus bases in bold face. The site of insertion is numbered on a scale where base pair 1 is the A of the ATG start codon for the *lacZ* gene; the base pair number of the first base of the duplicated 9 bp is shown.

and ATS transposases give different insertion spectra (Figure 3). Among 50 independent insertions promoted by wild-type transposase, 24 occur at a single well documented hotspot (*lacZ**, Halling and Kleckner, 1982); the remaining 26 insertions are distributed among 11 other sites and most (19) are in four sites represented by more than one insertion. In contrast, among 50 independent insertions promoted by either mutant transposase CY134 or CY249, fewer insertions occur at *lacZ** (nine and two respectively) and insertions are more broadly distributed among additional target sites. For example, CY134 and CY249 transposases promote insertion into 21 and 25 non-*lacZ** sites respectively as opposed to the 11 non-*lacZ** sites observed for wild-type. The phenotype of the double mutant, CY134 CY249, is similar to that of the single mutants (five insertions at *lacZ** and 22 other sites represented).

The three ATS insertion spectra are roughly similar to each other but display particular differences. For example, while all three ATS spectra have one to four insertions in most sites, sites A (six insertions) and *lacZ** (nine insertions) are strong hotspots in the CY134 spectrum and site B (10 insertions) is a strong hotspot in the CY249 spectrum. However, these data are not sufficient to determine whether the hotspots represent true differences between each ATS allele or whether they arise from statistical variation.

The ATS mutations specifically alter insertion specificity without significantly changing the overall frequency of Tn*10* transposition. In a 'mating-out' assay for transposition,

Table III. Target consensus bases for wild-type and ATS transposases

		G	A	T	C	Hierarcy
WT	1	96	4	0	0	G > A > C, T
	2	0	0	2	98	C > T > A, G
	3	10	19	68	3	T > A > G > C
CY134	1	75	24	0	1	G > A > C > T
	2	0	3	13	84	C > T > A > G
	3	19	23	46	12	T > A > G > C
CY249	1	55	38	3	4	G > A > C > T
	2	3	14	27	56	C > T > A > G
	3	11	18	59	12	T > A > C > G
CY134	1	64	33	3	0	G > A > T > C
CY249	2	0	6	29	65	C > T > A > G
	3	21	8	56	15	T > G > C > A

The symmetric consensus sequence for insertion defines two half sites containing the sequence 5′-GCT-3′ with the half site consensus base positions numbered 1 2 3. The frequency of each of the four bases at each half site position for wild-type, CY134, CY249 or CY134 CY249 transposase-generated insertions in the *lacZ* gene (Figure 2, Table II) and the hierarchies of preferred bases at each position are shown.

CY134, CY249 and the double mutant are reduced from wild-type transposition levels 3-, 2- and 3-fold respectively.

ATS insertion sites deviate more from the consensus sequence than wild-type sites

Detailed analysis of the insertion specificity of the ATS mutants reveals that they are altered in the extent to which the insertion sites they select conform to the the normal 6 bp consensus sequence 5′-GCTNAGC-3′. DNA sequence analysis of the *lacZ*:: Tn*10* insertions promoted by wild-type, CY134, CY249 and CY134 CY249 transposases shows that there is no *qualitative* change in the nature of the target site consensus sequence selected by these different mutant transposases (Table II). All the transposases generate 9 bp direct repeats flanking insertions, and at each position in the 3 bp consensus half-site within this 9 bp sequence the hierarchy of preferred bases is generally the same for the mutant as for wild-type transposase. However, the mutant transposases are *quantitatively* different from wild-type: at each of the three consensus positions, the degree of preference for particular bases is less for the mutant transposases than for wild-type transposase (Table III). Thus, at position 1 of the 3 bp half site, G is the most preferred base, then A, and then C and T for all the transposase alleles, but the preference for G over A is 94:4 for wild-type and 75:25 for CY134, 55:38 for CY249, and 64:33 for CY134 CY249 transposase. At position 2 the C is the most preferred base, then T, and then A and G for all the transposase alleles, but the preference for C over T is 98:2 for wild-type and 84:13 for CY134, 56:27 for CY249, and 65:29 for CY134 CY249 transposase. At position 3, where a high degree of variation occurs even with wild-type transposase, T is the most preferred base with A, G, and C all used at lower frequencies, but the degree of preference is reduced for the ATS mutant cases as compared with wild-type.

ATS mutations are phenotypically distinct from SOS$^+$ Tnsp$^-$ mutations

The ATS mutations map within general regions of the protein where SOS$^+$ Tnsp$^-$ mutations also occur. In fact, the amino acids affected by the two types of mutations lie in very close spatial proximity: both the CY134 and CY249 codons are very close to codons at which SOS$^+$ Tnsp$^-$ mutations have been isolated (SL135, HY139, RH243, PL252 and SL253; Figure 1). The SOS$^+$ Tnsp$^-$ mutations have moderate or severe effects on the overall efficiency of transposition (5- to 10 000-fold; Haniford *et al.*, 1989). The ATS mutations could have been weak SOS$^+$ Tnsp$^-$ alleles because they have only minor effects on transposition (see above). However, the two types of mutations confer completely distinct phenotypes.

SOS$^+$ Tnsp$^-$ mutations do not confer an ATS phenotype. Three SOS$^+$ Tnsp$^-$ mutations, HY139, PL264 and MI289, which still exhibit a significant residual level of transposition (5- to 10-fold reduced from wild-type levels) were

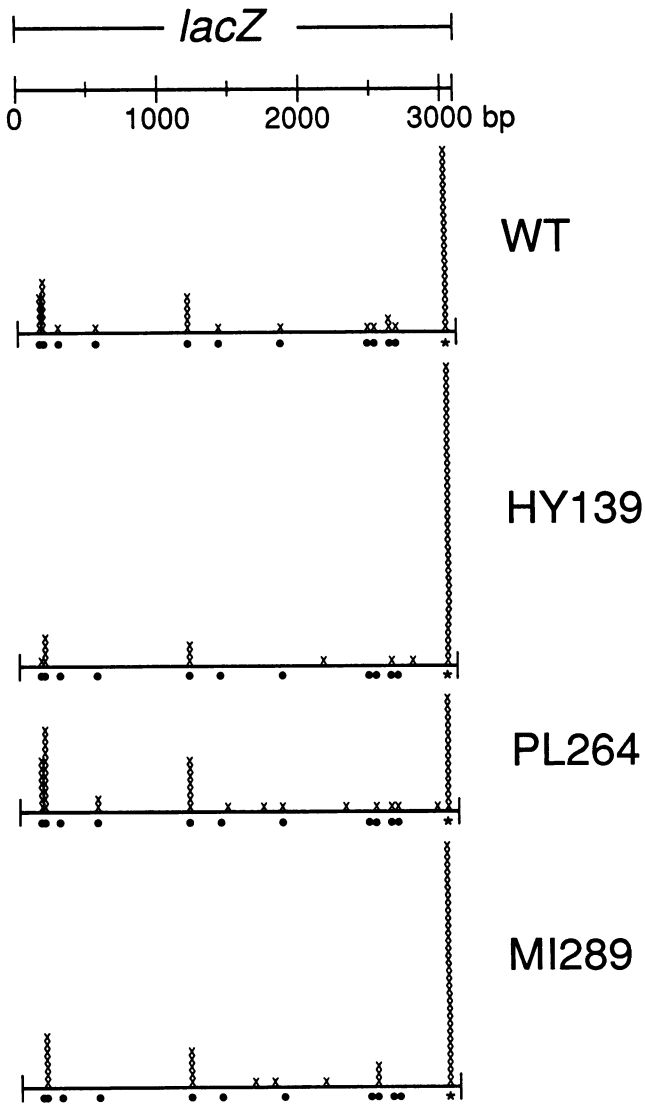

Fig. 4. Tn*10* insertions in the *lacZ* gene generated by wild-type transposase and weak SOS$^+$ Tnsp$^-$ mutant transposases. Fifty independent mini-Tn*10* Kan^R insertions into the 3.1 kb *lacZ* gene carried on a pGEM-3 plasmid vector were isolated from λ delivery vehicles carrying either wild-type (WT) (λNK1105), HY139 (λNIK1320), PL264 (λNK1321) or MI289 (λNK1322) transposases (see Materials and methods). Approximate positions of insertion are shown: base pair 1 of the scale is at the A of the ATG start codon for *lacZ*. The strong hotspot for wild-type transposase is marked * and the positions of other wild-type insertion sites are marked with filled circles.

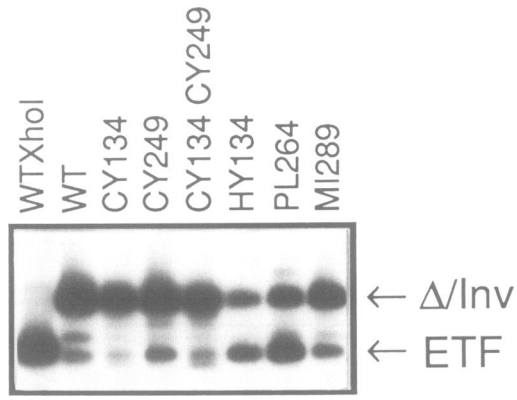

Fig. 5. Southern blot assay for ETFs and strand transfer products. Transposition products of a mini-Tn*10 kan* generated by the indicated mutant transposases were detected by Southern blot of small DNA species using a *kan* gene probe (see Materials and methods). The mini-Tn*10 kan* element is 1.85 kb long, with a unique *Xho*I site ~0.55 kb from one end and 1.2 kb from the other. All samples are native (uncut) DNA except for the leftmost lane labeled WT*Xho*I, which contains WT DNA digested with the enzyme *Xho*I to provide a marker for the position of ETFs. The positions of intramolecular deletion/inversion circles (Δ/inv) and of ETFs are indicated.

analyzed for their spectra of *lacZ* insertions. All three mutations give patterns that are indistinguishable from the wild-type pattern (Figure 4).

Conversely, the ATS mutations do not confer one of the diagnostic phenotypes of the SOS^+ $Tnsp^-$ mutations, increased formation of Excised Transposon Fragments (ETFs). ETFs consist of transposon sequences specifically and precisely excised from the donor DNA without ligation to target DNA and accumulate at a break point in the transposition reaction (Haniford *et al.*, 1989, 1991). ATS mutants are also not significantly affected for levels of a particular transposon strand transfer product, the deletion/inversion circle, whereas SOS^+ $Tnsp^-$ mutations greatly reduce the levels of this product. Deletion/inversion circles result from insertion of the transposon into internal target sites and consist of transposon sequences rearranged so that the sequences between one transposon end and its internal target site are inverted relative to the remaining transposon sequences (Benjamin and Kleckner, 1989). Deletion/inversion circles are the major intra-transposon strand transfer product generated by transposase, but a related strand transfer product, intra-transposon deletion circles, are also generated at a low level.

Specifically, levels of ETFs and deletion/inversion circles were measured for ATS mutants plus wild-type transposase and the representative weak SOS^+ $Tnsp^-$ mutants HY139, PL264 and MI289 by Southern blot analysis of transposase-generated transposon species (Figure 5). For each of the ATS mutants tested, as for wild-type transposase, the proportion of deletion/inversion circles far outweighs the proportion of ETFs. Also, the absolute level of ETFs produced by each ATS mutant does not differ dramatically from the level produced by wild-type transposase: for CY134 the level is ~2-fold lower, for CY249 the level is ~2-fold higher, and for CY134 CY249 the level is about the same as that of wild-type transposase. In contrast, for the representative weak Patch I and Patch II SOS^+ $Tnsp^-$ mutants HY139 and PL264 the proportion of deletion/inversion circles is lower than the proportion of ETFs and the absolute levels of ETFs are ~5- and 10-fold higher than the level produced by wild-type transposase. MI289 is a unique SOS^+ $Tnsp^-$ mutant that maps outside of Patch I and Patch II in a region that shows sequence homology to other bacterial transposases (Figure 1). This mutant has an intermediate ratio of deletion/inversion circles to ETFs that most resembles that of the ATS mutant CY249.

Discussion

ATS mutations specifically alter target site recognition

The phenotypes of ATS mutants demonstrate that the preference for specific nucleotide sequences at the target site is not an obligatory feature of the Tn*10* transposition reaction. These mutations cause a surgical perturbation of transposase function rather than a major disruption, conferring a relaxation in the specificity of insertion without significantly affecting either the overall frequency of transposition or the efficiency of the transition from the excised transposon stage to the strand transfer stage, shown previously to be a sensitive break point in the transposition reaction (Haniford *et al.*, 1989). Target site selection has not yet been placed in the sequence of events leading to transposition. It must precede strand transfer, but might occur either before or after excision of the transposon from its donor site.

ATS mutations alter specific codons in transposase protein

The ATS codons map in regions of transposase protein previously implicated in target site interactions, Patch I and Patch II (Haniford *et al.*, 1989). These patches are defined by SOS^+ $Tnsp^-$ mutations that, in contrast to ATS mutations, disrupt the transition from the excised transposon to the strand transfer stage of the transposition reaction. However, the structural consequences of ATS mutations for transposase protein appear to be as specific as the phenotypic consequences. ATS mutations were recovered at only two codons, one in each patch. At each of these positions, a number of different amino acids will confer an ATS phenotype; a more limited number of amino acids are compatible with the wild-type insertion phenotype. Even more remarkably, the codons where ATS mutations occurred (134 and 249) are immediately adjacent to codons where SOS^+ $Tnsp^-$ mutations occurred (codons 135, 139, 243, 252 and 253), again suggesting a relatively specific change in protein structure.

ATS mutations at codon 134 and codon 249 affect the same function of transposase protein

Two observations suggest that ATS mutations at codon 134 and codon 249 alter the same specific function of transposase protein. First, the CY134 and CY249 single mutants have very similar phenotypes, although there are minor differences in each mutant's insertion site spectrum. Second, the effects of these mutations in combination are neither additive nor synergistic: the CY134 CY249 double mutant phenotype is very similar to that of the two single mutants with respect to residual levels of insertion specificity and the reduction in transposition frequency relative to wild-type transposase. Because ATS mutations at each codon position seem to

affect the same step of the transposition reaction, it is attractive to suppose that the amino acids 134 and 249 are located near one another in the three-dimensional structure of transposase protein.

ATS mutations confer relaxed insertion specificity but do not qualitatively alter recognition of the target site consensus sequence

The ATS phenotype reflects a general relaxation in insertion specificity. In the original construct used for isolation of the mutations, which contained strong mutations in the target site consensus sequence, the increased frequency of insertion by ATS mutant into the target DNA region reflected an increase in insertion into sites other than the mutant hotspot. This relaxation in insertion specificity is further demonstrated by the spectrum of *lacZ* insertion sites selected by ATS transposase.

The relaxation in insertion specificity is accomplished without any qualitative alteration in the way transposase recognizes the target site consensus sequence. Targets selected by ATS transposase exhibit the same hierarchy of base pairs as those selected by wild-type transposase at each position in the consensus sequence half site. The relaxed specificity phenotype is reflected instead in a reduced degree of preference for particular base pairs at each of the three consensus positions. This behavior strongly suggests that the ATS mutations do not specifically affect particular base pair-specific protein–DNA interactions that are involved in sequence-specific recognition of the consensus sequence.

Relaxed specificity of other transposons and other protein–DNA interactions

Wild-type IS10 exhibits the strongest degree of target site preference observed among IS elements and other transposons, excluding certain exceptional cases where target site selection is specifically restricted to a unique site in the bacterial genome (Craig, 1989). The ATS mutations reduce the insertion specificity of IS10 to a level that is more nearly comparable with that observed with IS50 (reviewed in Berg, 1989; Lodge *et al.*, 1988), an element that appears to be relatively closely related to IS10 (as discussed in Huisman *et al.*, 1989; Kleckner, 1989), and to that observed with certain other elements (Halling and Kleckner, 1982), but still more pronounced than the specificity observed for some elements (Mizuuchi and Craigie, 1986).

The IS10 ATS mutations are most closely analogous to relaxed specificity mutations in *Eco*RI restriction endonuclease. The *Eco*RI mutations confer a reduction in specificity that is similar to the 'star' activity observed under suboptimal *in vitro* conditions (Heitman and Model, 1990). Mutations that relax the specificity of a yeast mitochondrial endonuclease have also been reported (Sargueil *et al.*, 1990).

The IS10 ATS mutations contrast with other types of DNA binding protein mutations which alter the specificity of binding in a sequence-specific manner. In these latter cases, the mutant protein binds well to a specific mutant site but not to the wild-type site, while the converse is true for the wild-type protein (Youderian *et al.*, 1983; Ebright *et al.*, 1984a,b; Wharton and Ptashne, 1987; Bass *et al.*, 1988; Danielsen *et al.*, 1989; Gardella *et al.*, 1989; Hanes and Brent, 1989; Mader *et al.*, 1989; Sartorius *et al.*, 1989; Treisman *et al.*, 1989; Umesono and Evans, 1989).

Possible mechanistic bases for relaxed insertion specificity

In the absence of structural data on the IS10 transposase protein the molecular basis for the ATS phenotype cannot be specified. However, it is useful to consider various possible mechanisms for relaxed insertion specificity.

(i) Relationship to known specificity determinants. The frequency of insertion of Tn*10* into any one particular site is determined in part by the target site consensus sequence and in part by the nature of flanking 'context' base pairs, which are presumed to play a role either in binding of transposase and/or in permitting some structural change in the DNA required for the insertion reaction. Both DNA determinants are important for insertion frequency. As discussed above, the ATS mutations presumably do not specifically affect the particular amino acid–DNA interactions with consensus base pairs that confer sequence specificity. The observed relaxation of specificity could arise by reducing the contribution of consensus sequence base pairs (for example, by increasing the distance between the protein and the DNA in the binding pocket), by making the reaction less sensitive to suboptimal context base pairs, or by conferring a general stabilization in binding that would overcome the absence of optimal sequences in either or both regions.

(ii) Relationship to steps in the integration reaction and relevant protein–DNA interactions. The ATS mutations might exert their effects by directly altering the way in which transposase binds to target DNA. In this case, the ATS mutations must confer some alteration to the relevant protein–DNA interface either directly or as the indirect consequence of a change in some other part of the protein. Alternatively, if binding to the target is tightly coupled to subsequent steps of the reaction (for example, cleavage of the target DNA and accompanying strand transfer), the specificity of target site selection might reflect a decreased dependence of later events on the energy provided by target DNA binding. For example, the primary effect of the ATS mutations might be to make transposase protein more likely to undergo a conformational change required for the transition from binding to later steps, and the mutations might alter only interactions within the protein itself and have no effect at all on the protein–DNA interface.

Practical applications of ATS mutations

IS10 and Tn*10* derivatives are widely used for transposon mutagenesis of native or heterologous DNAs in bacteria. Vectors for Tn*10* transposon mutagenesis that contain the ATS transposase are now available (Kleckner *et al.*, 1991; requests must be made by mail or by fax). Such ATS vectors should significantly improve the usefulness of Tn*10* for obtaining a wide variety of different insertions into a limited target region.

Materials and methods

Media, enzymes and chemicals

LB and minimal M9 media were as described by Miller (1972); for solid medium, 1.5% agar was added. λym broth, λ agar, and other media used in propagation of phage λ were as described by Kleckner *et al.* (1978). MacConkey lactose medium was made as 40 g MacConkey agar base and 10 g lactose (1%) per liter (Difco). When used, ampicillin was added to media at a concentration of 100 μg/ml, kanamycin at a concentration of 50 μg/ml and X-Gal at a concentration of 40 μg/ml. Bacteriological supplies

were purchased from Difco, antibiotics and chemicals from Sigma and X-Gal from Bachem, Inc.

Standard cloning techniques were used as described in Maniatis *et al.* (1982). Restriction enzymes, T4 DNA ligase and T4 DNA polymerase were purchased from New England Biolabs.

Plasmid, phage and bacterial strain constructions for the ATS mutant screen

The transposase/mini-Tn*10 kan* P*lac* plasmid pNK2731 mutagenized to isolate ATS transposase mutants was constructed as follows. A progenitor plasmid, pNK862, carries the IS10 transposase gene fused to the strong inducible promoter P*tac* with a mini-Tn*10* marked with the Tn*903 kan* gene *in cis* (Way *et al.*, 1984). A 110 bp *Bam*HI fragment carrying the P*lacUV5* promoter and operator derived from pTR161 (Roberts *et al.*, 1979) was cloned at a *Bam*HI site just inside one end of the mini-Tn*10 kan* on pNK862 oriented to read out across the end. This plasmid was further modified by deleting the inner terminus of IS10 beyond the transposase coding sequence between a *Xho*II site and a *Bgl*II site in downstream sequences (Halling *et al.*, 1982; Way *et al.*, 1984) with an *Xba*I linker placed at the deletion junction to make pNK2718. Finally, the P*tac*–transposase and mini-Tn*10 kan* P*lac* sequences from this plasmid were cloned on an *Eco*RI–*Cla*I fragment into the *Eco*RI–*Cla*I backbone of pGC2 (Myers *et al.*, 1985) which carries both a pBR322 and an m13 origin of replication and can thus be propagated as either double or single stranded DNA, to make pNK2731 (shown in Figure 1).

The target sites for mini-Tn*10 kan* P*lac* in the ATS papillation assay were constructed on lysogenizable λ phages as follows. A 270 bp fragment of *Salmonella his* DNA carrying the *hisG*1 Tn*10* insertion hotspot was cloned from *Bgl*II–*Eco*RV (STHISOP.EMBL # X13464) converted to *Xba*I–*Eco*RI into the *Xba*I–*Eco*RI backbone of pGC1 (Myers *et al.*, 1985) to make pNK2505. The consensus bases at the *hisG*1 hotspot on this plasmid were mutagenized with oligonucleotides by the method of Kunkel (1985) to create a perfect consensus sequence GCTNAGC (WT), the mutant target TCTNAGA (mut-1), the mutant target CCTNAGG (mut-2), the mutant target GGTNACC (mut-3) or the mutant target GATNATC (mut-4). The *Xba*I–*Eco*RI target fragments from the mutagenized plasmids were subcloned into pRS415 (Simons *et al.*, 1987) with the polylinker *Eco*RI site converted to *Xba*I and the *Bam*HI site converted to *Eco*RI to make plasmids with four tandem repeats of the *rrnB* transcriptional terminators upstream and promoterless *lacZ* and *lacY* genes downstream of each of the five different target fragments (pNK2730, pNK2732, pNK2748, pNK2749 and pNK2750 respectively). These plasmids were crossed onto λRS74 (Simons *et al.*, 1987) by screening for pale blue plaques among the parental dark blue plaques on λ X-Gal medium. The resulting phages (λNK1294, 1295, 1299, 1300 and 1301 respectively) are *imm21 nin5 att*$^+$ with the terminator–target site–*lac* gene constructions substituted for the λ sequences between the *Eco*RI sites at 21226 and 26104 bp on the λ map (oriented so that the *lac* genes are closest to the λ *J* gene). These phages were lysogenized into NK8032 (Δ*lacpro*$_{XIII}$ *recA*56 *argE*$_{am}$ Nal^R Rif^R) to create the papillation test strains for ATS phenotypes.

Mutagenesis and ATS mutant isolation

Plasmid pNK2731 was mutagenized by hydroxylamine treatment (Davis *et al.*, 1980). Mutagenized DNA was divided into 180 individual pools, and each pool was transformed into either NK8032(λNK1295) = mut-1 (60 pools), NK8032(λNK1300) = mut-3 (60 pools) or NK8032(λNK1301) = mut-4 (60 pools), and plated on MacConkey lactose medium containing ampicillin (amp) and kanamycin (kan) so that there were ~100 colonies per plate. Each pool yielded ~650 colonies, so for each target strain ~40 000 total colonies were screened.

Transformants were incubated in the dark at 37°C for up to 5 days to allow full growth of papillae. Transformants from the independent mutagenized pools which showed increased papillation relative to wild-type transformant colonies were purified and their plasmid DNA extracted. The putative mutant plasmids were first rescreened by transforming into the appropriate papillation test strain and plating transformants on half of a MacConkey lactose amp kan plate with wild-type pNK2731 transformants into the same strain plated on the other half of the plate for comparison. Various fragments of the transposase gene on the putative mutant plasmids were then subcloned into the wild-type pNK2731 backbone and again screened on 'half plates' to localize the position of the mutation. Eleven independent mutants selected for further analysis mapped to a 680 bp *Nhe*I–*Stu*I fragment in the central region of the transposase gene. (Two other mutants recovered from the original screen failed to subclone within the coding sequence of transposase and were not analyzed further.)

The DNA sequence of the entire subcloned *Nhe*I–*Stu*I region for each mutant was determined by the dideoxy chain termination method of Sanger *et al.* (1977). Transposase on pNK2731 constructions could not be directly sequenced by this method because the inverted repeats present at the ends of the mini-Tn*10* element inhibit replication of single stranded DNA. Instead one end of the mini-Tn*10* was first deleted between the two *Cla*I sites on the plasmid, and then single stranded DNA for sequencing was propagated. Once the mutation CY134 was sequenced it was revealed that this base change creates an *Ssp*I site. Thus, all putative mutants could be screened for this mutation simply by restriction digest.

Mapping insertion sites within papillae

Sites of insertion that activate the *lacZ* gene in the insertion assay strains were determined by restriction digest and Southern blot of chromosomal DNA extracted from the individual papillae. Chromosomal DNA from purified papillae was digested with *Xba*I (which cuts at one end of the target fragment, Figure 1) and *Bam*HI (which cuts inside the mini-Tn*10 kan* P*lac* end, Figure 1) to generate junction fragments between the target DNA and transposon end. Samples were electrophoresed on 5% acrylamide gels, electrotransferred to Nytran filters, and probed with a ^{32}P-kinased fragment that hybridizes to sequences very close to the *Xba*I end of the junction fragments as described in Haniford *et al.* (1991).

This method was used to map sites of insertion for transposition and CY134 transposases on wild-type (λNK1294) and mutant (λNK1295 = mut-1) target constructions. Eight of eight wild-type transposase-generated insertions mapped to the *hisG*1 site in the wild-type target construction and two of nine insertions mapped to this site in the mutant target construction with the other seven insertions distributed among five other secondary sites. In contrast, only five of eight CY134 transposase-generated insertions mapped to the *hisG*1 site in the wild-type target construction with three other insertions in each of three secondary sites and only two of 17 insertions mapped to this site in the mutant target construction while the other 15 insertions were distributed among three other sites. In particular, 11 of the 17 CY134 insertions into this mutant target construction mapped to a secondary site ~40 bp downstream of *hisG*1.

Conjugational 'mating-out' assay

Relative intermolecular transposition frequencies for ATS and wild-type transposases were determined by the conjugational 'mating-out' assay described by Foster *et al.* (1981). In this assay, transpositions into an F episome in a donor strain are recovered as drug-resistant exconjugants into a recipient strain.

Each ATS mutant was tested relative to wild-type transposase in this assay by constructing the various transposases into isogenic plasmids *in cis* to a mini-Tn*10 kan* element. Specifically, each transposase allele was subcloned into a derivative of pNK862 (Way *et al.*, 1984) where the inner terminus of IS10 just beyond the transposase coding sequence has been deleted between a *Xho*II and a *Bgl*II site with an *Xba*I linker at the deletion junction. The double mutant CY134 CY249 was constructed in this context via a unique *Avr*II site between the two mutations in the transposase gene. Purified monomers of the wild-type transposase plasmid (pNK2741), the CY134 mutant plasmid (pNK2794), the CY249 mutant plasmid (pNK2855) and the CY134 CY249 double mutant plasmid (pNK2859) were transformed into the donor strain NK5830 (Δ*lacpro*$_{XIII}$ *recA*56 *argE*$_{am}$ Nal^R Rif^R/F′ *lacpro lacI*^{Q}). Five separate transformant colonies of each plasmid were grown in culture and mated with the recipient strain NK6641 (Δ*lacpro*$_{XIII}$ *recA*56 thy$^-$ Str^{R}λ^R). Transposition frequencies were determined as the ratio of Kan^R exconjugants to total (Pro$^+$) exconjugants.

Isolation, mapping and sequencing of insertions in the lacZ gene

Mini-Tn*10* insertions into the *lacZ* gene generated by various transposase alleles were isolated as transpositions from a non-replicating non-lysogenizing non-killing λ delivery vehicle (a 'hop phage') into a multicopy target plasmid.

The λ hop phages for these experiments were constructed as follows. First, the P*tac*–transposase/mini-Tn*10 kan*-containing *Eco*RI fragment from the plasmids used in the mating-out assays (or isogenic plasmids pNK2866 with HY139 transposase, pNK2867 with PL264 transposase and pNK2868 with MI289 transposase) were cloned into the unique *Eco*RI site (at a deletion between bp 21226 and 26104) of λRP167 (*imm21 nin5 att*$^+$) (Maurer *et al.*, 1980). With the exception of the MI289 mutant fragment, all of these fragments are oriented with the mini-Tn*10 kan* closest to the λ *J* gene. These phages are λNK1297 (CY134), λNK1311 (CY249), λNK1312 (CY134 CY249), λNK1317 (HY139), λNK1318 (PL264) and λNK1319 (MI289). The wild-type transposase version of these phages, λNK1106, was previously constructed by an analogous procedure except that the transposase/mini-Tn*10* fragment was cloned into λRP167 from pNK862 so the transposase gene still has a downstream IS10 inner terminus (W.Raymond, personal communication).

These phages were crossed with λNK780 (*b522 cI857 P*$_{am}$*80 nin5* with *Salmonella his* genes substituted for λ sequences between the *Eco*RI sites

at bp 21226 and 26104) (Foster *et al.*, 1981) to create the non-replicating ($P_{am}80$) non-lysogenizing (*cI857*) hop phage delivery vehicles. Single plaques from the crosses were plated on an *sulI*$^+$ λ*imm21* lysogen at 30°C to counterselect against *imm21* parental phages and then replica plated onto LB kan plates at 30°C where only plaques containing Kan^R lysogens or pseudo-lysogens will yield Kan^R colonies. Kan^R *cI857* $P_{am}80$ phages were purified from the appropriate plaques and checked by genetic tests and restriction digest of phage DNA. Mainly *att*$^+$ plus a few *att*$^-$ (*b522*) phages were recovered from these crosses. The *att*$^+$ phages λNK1105 (wild-type transposase with mini-Tn*10 kan*, previously constructed by W.Raymond) (Way *et al.*, 1984), λNK1298 (CY134), λNK1313 (CY249), λNK1315 (CY134 CY249), λNK1320 (HY139), λNK1321 (PL264) and λNK1322 (MI289), were used in the analyses described below.

A *lacZ* target plasmid pNK2742 was constructed by cloning a *Bam*HI–*Dra*I fragment containing the *lacZ* gene from pRS415 (Simons *et al.*, 1987) into the *Bam*HI–*Hin*cII backbone of pGEM-3 (Promega Biotech). Fifty independent mini-Tn*10 kan* insertions from each of the different hop phages into the *lacZ* gene on pNK2742 were isolated as follows. A culture of NK8032 transformed with purified monomers of pNK2742 was grown to saturation in λym broth and split into 50 0.5 ml aliquots. Approximately 10^8 hop phages were added to each aliquot, allowed to adsorb at room temperature for 20 min, and grown with aeration at 37°C for 1 h, and then 0.3 ml of each aliquot was plated on an LB amp kan plate at 37°C to isolate total Kan^R transpositions. Resulting Kan^R colonies on each plate ($\sim 10^4$ per plate for wild-type and ATS transposases and $\sim 10^3$ per plate for the other mutant transposases) were pooled and plasmid DNA extracted, retransformed into NK8032, and plated on MacConkey lactose amp kan medium to isolate Kan^R *lacZ*$^-$ plasmids. Plasmid DNA was purified from one *lacZ*$^-$ (white) transformant colony from each of the original 50 aliquots and the position of the Kan^R mini-Tn*10* insertion in the *lacZ* gene of each plasmid was determined to within 20 bp by restriction mapping. Sites of insertion were sequenced by subcloning fragments containing one end of the transposon and flanking *lacZ* DNA into pGC1 or pGC2 and priming dideoxy sequencing reactions from a primer in the transposon end out into *lacZ* sequences. A number of insertions generated by CY134, CY249 or CY134 CY249 transposases were sequenced from both ends of the transposon to confirm that, like wild-type, these transposases create 9 bp duplications of the target DNA flanking an insertion. All insertions into the perfect consensus *lacZ** site could be precisely mapped by restriction digest with the enzyme *Esp*I (US Biochemical) which cleaves the perfect consensus; since the consensus bases are duplicated during insertion, the *Esp*I site will be duplicated by an insertion precisely into the *lacZ** site.

Detection of ETFs and strand transfer products by Southern blot

Various transposase alleles (wild-type, CY134, CY249, CY134 CY249, HY139, PL264 and MI289) were tested for ETFs and strand transfer products from the same isogenic plasmid constructions used in the mating-out assays for intermolecular transposition and the construction of λ hop phages in the *lacZ* insertion experiments. These plasmids each carry a P*tac*–transposase fusion with the inner terminus of IS10 deleted and a mini-Tn*10 Kan* element *in cis*. NK8032 transformed with purified monomers of each plasmid was grown to saturation in LB amp broth and plasmid and other small DNA species were extracted by the mini-cleared lysate procedure (Clewell and Helinski, 1970). Native samples were electrophoresed on a horizontal 1.1% agarose gel in TBE buffer, transferred to a Nytran filter, and hybridized with a *kan* gene probe as described by Benjamin and Kleckner (1989). As a control for the position of the ETF band, DNA from the wild-type pNK2741 transformant cultures was digested with *Xho*I, which cuts uniquely in the *kan* gene and thus linearizes the predominant deletion/inversion circle species into linear fragments the length of the transposon (1.85 kb).

Oligonucleotide mutagenesis of codons 134 and 249

Transposase codons 134 and 249 were mutagenized with degenerate oligonucleotides by the method of Kunkel (1985) on plasmids pNK2796, pNK2798 or pNK2857 made by subcloning either wild-type, CY134 or CY249 transposase on an *Eco*RI–*Xba*I fragment from the appropriate derivative of pNK2731 into pGC2. The transposase gene could not be directly mutagenized on a pNK2731-type construction because replication to give the single stranded DNA substrate for mutagenesis is inhibited by the inverted repeats present at the ends of the mini-Tn*10 kan* P*lac* on the construction. After oligonucleotide mutagenesis, plasmids were pooled and the *Eco*RI–*Xba*I transposase fragment was isolated and cloned into a pNK2731 *Eco*RI–*Xba*I backbone to test for papillation phenotypes.

Single stranded DNA from the wild-type transposase plasmid pNK2796 was mutagenized either with an oligonucleotide degenerate for codon 134 or with an oligonucleotide degenerate for codon 249. Pooled mutagenized transposase fragments generated with each oligonucleotide were ligated into the wild-type pNK2731 backbone and transformed into NK8032(λNK1300) (the mut-3 target) on MacConkey lactose amp kan plates to screen for ATS phenotypes. Similarly, single stranded DNA from the CY134 transposase plasmid pNK2798 was mutagenized with the oligonucleotide degenerate for codon 134 and single stranded DNA from the CY249 transposase plasmid pNK2857 was mutagenized with the oligonucleotide degenerate for codon 249. Pooled mutagenized transposase fragments generated with each oligonucleotide were ligated into a backbone purified from the CY134 version of pNK2731 (pNK2792) and transformed into NK8032(λNK1294) (the wild-type target) on MacConkey lactose amp kan plates to screen for wild-type phenotypes.

Candidate mutant transposase plasmids from each screen were first extracted and retested for papillation phenotypes on 'half plates' (see above) versus either wild-type or the appropriate ATS mutant plasmid. Twelve to 15 candidates from each screen were prepared for sequencing by deletion of one of the mini-Tn*10* ends between the two *Cla*I sites on the plasmid (see above). ATS mutant candidates at codon 134 were tested for the TAT tyrosine codon simply by restriction digest with *Ssp*I.

At codon 134, 14 TAT Tyr, one TTT Phe and one ACC Asn mutations with an ATS phenotype were recovered from mutagenesis of the wild-type codon (TGT Cys) and six TGT Cys, five TCT Ser and one AGT Ser mutations with a wild-type phenotype were recovered from mutagenesis of the ATS codon (TAT Tyr). At codon 249, three TAT Tyr, two ATT Asn, two TTA Leu, two AGG Arg, one CGT Arg, one ATT Ile, one ATC Ile, one ATG Met, one GAT Asp, one TTT Phe, one TGG Trp, one AGT Ser and one GGG Gly mutations with an ATS phenotype were recovered from mutagenesis of the wild-type codon (TGT Cys) and 14 TGT Cys and one TGC Cys mutations with a wild-type phenotype were recovered from mutagenesis of the ATS codon (TAT Tyr).

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