Intragenic Suppression of Integration-Defective IS*10* Transposase Mutants

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ABSTRACT

IS*10* transposase mediates excision and integration reactions in Tn*10*/IS*10* transposition. Mutations in IS*10* transposase that specifically block integration have previously been identified; however, the mechanism by which these mutations block integration has not been established. One approach to defining the basis of this block is to identify ways in which the original defect can be corrected. The approach we have taken toward this end has been to isolate and characterize intragenic second site suppressors to two different integration-defective mutants. Of the second site suppressors identified, one, *CY134*, is of particular interest for two reasons. First, it suppresses at least seven different mutations that confer an integration-defective phenotype. Interestingly, these mutations map in two separate segments of transposase, designated patch I and patch II. Second, *CY134* on its own has previously been shown to relax the target DNA sequence requirements for Tn*10* integration. We provide evidence that suppression by *CY134* is not simply a consequence of this mutation conferring a general "transposition up" phenotype, but rather is due to correcting the original defect. Possible mechanisms of suppression for both *CY134* and other second site suppressors are considered.

TN*10* is a composite bacterial transposon made up of two nearly identical copies of the insertion sequence IS*10* and a tetracycline resistance determinant. IS*10* Right encodes a transposase protein which interacts with specialized DNA sequences at IS*10* termini and at target sites to mediate DNA strand cleavage and joining events in Tn*10* or IS*10* transposition (reviewed in KLECKNER 1989; HANIFORD and CHACONAS 1992). During the transposition process transposon sequences are first excised from a donor molecule by a pair of flush double strand breaks made precisely at the transposon-donor junctions (HANIFORD *et al.*, 1991; BENJAMIN and KLECKNER 1992). The free 3′-OH ends of the transposon are then joined to 5′-PO_4 groups spaced 9 residues apart on opposite strands of the target site. This results in the formation of 9-bp overhangs at both ends of the transposon (BENJAMIN and KLECKNER 1989). These overhangs are filled in, presumably by host repair functions, to generate 9-bp direct repeats of the target site DNA. Sequence analysis of many Tn*10* insertions has demonstrated that Tn*10* inserts preferentially into a specific sequence, 5′-NGCTNAGCN-3′ (HALLING and KLECKNER 1982). Mutations in the consensus bases within a particular integration "hotspot" (*his*G1) and in the DNA immediately flanking the hotspot have been shown to drastically reduce the frequency of Tn*10* integrations into this site (BENDER and KLECKNER 1992a).

Although some of the basic steps in Tn*10* transposition have now been defined, structure-function relationships for transposase protein have yet to be firmly established. Most of what we know of the functional organization of transposase comes from genetic studies. Two classes of transposase mutants which influence Tn*10* integration have previously been described. "Exc^+Int^-" mutants promote efficient excision but not integration and thus confer a strong block to transposition (HANIFORD *et al.* 1989), while "ATS" (*A*ltered *T*arget *S*ite specificity) mutants exhibit relaxed target sequence requirements for integration and have only a minor negative effect on transposition frequency (BENDER and KLECKNER 1992b). Whereas Exc^+Int^- mutations constitute roughly 5% of all transposition-defective mutations, mutations that generate an ATS phenotype are extremely rare indicating that only very specific structural/functional changes are compatible with this phenotype.

To date, 16 different Exc^+Int^- mutants have been characterized and 15 of the corresponding mutations map within two segments of transposase, defined as patch I (or PI for short; amino acids 102–167) and patch II (or PII for short; amino acids 243–264). The remaining mutation confers only a minor transposition defect and maps in a region shown to be highly conserved among a number of IS element transposase proteins (MAHILLON *et al.* 1985). The location of the two previously defined ATS mutations are also in PI and PII.

The phenotype of Exc^+Int^- mutants indicates PI and PII play a critical role in integration. The mechanism by which Exc^+Int^- mutations interfere with integration has, however, not been defined. In addition, it is not known if the existing collection of Exc^+Int^- mutants are blocked for integration for the same reason. To learn more about the defects associated with two different Exc^+Int^- mutations, we have used a genetic screen to isolate intragenic second site suppressors to these mutations. The two mutants chosen for this study are

RQ106 (arginine to glutamine change at position 106) and *PS167* (proline to serine change at position 167). These mutants were selected because they exhibit very strong integration defects without affecting earlier steps in transposition. In addition, because these mutations map to opposite ends of PI, they might affect different aspects of the integration reaction.

We report here the initial characterization of several second site suppressors to Exc$^+$Int$^-$ mutants *RQ106* and *PS167*. This analysis has provided a surprising result in that a mutation that relaxes target sequence requirements for Tn*10* integration (*i.e.*, *CY134*) suppresses both *RQ106*, *PS167* and five other Exc$^+$Int$^-$ mutants tested.

MATERIALS AND METHODS

Strains and bacteriophage: NK5830 is *su*$^-$ *rec*A56 *arg*$^-$ *lacpro*XIII *nal*^{R} *rif*^{R}/F′ *lac* i^QL8 *pro*$^+$. NK6641 is *rec*A56 *lacpro*XIII *su*$^-$ *str*^{R}λ^R. NK8044 is NK7378 (*su*$^-$ *rec*A56 *arg*$^-$ *lacpro*XIII *nal*^{R} *rif*^{R} *pOX38*) lysogenized with λNK1276 (Haniford *et al.* 1989). NK8032 is *rec*A56 *arg*$^-$ *lacpro*XIII *nal*^{R} *rif*^{R}. *HMS174 is F*$^-$ *rec*A1 r^-_{k12} m^+_{K12} *su*$^-$ *rif*^{R}. DH5α is *supE*44 Δ*lac*U169 (*ϕ80 lacZ*ΔM15) *hsd*R17 *rec*A1 *end*A1 *gyr*A96 *thi*-1 *rel*A1.

Bacteriophage CE6 (obtained from Novagen) is a λ recombinant which carries the cloned *T7* RNA polymerase gene. λNK1294 carries the *hisG*1 Tn*10* insertion hotspot, 5′-GCTNAGC-3′, upstream of a promoterless *lacZ* gene (Bender and Kleckner 1992b).

Plasmids: Plasmid pDH10 (Figure 1) carries a copy of the IS*10* transposase gene which is expressed from a p*tac* promoter (Haniford *et al.* 1989). Plasmid pDH50 is a pACYC184 derivative containing the mini-Tn*10 KanR* transposon from pNK862 (Morisato and Kleckner 1984). Plasmid pNK2731 carries the transposase gene under the control of the p*tac* promoter and the mini-Tn*10 KanR-plac* transposon (Bender and Kleckner 1992b). Plasmid pDH100 is a derivative of pET11a-*T7* Tag (obtained from Novagen) in which the N terminus of transposase is fused to the 11-amino acid N-terminal peptide of the *T7* gene 10 protein. The resulting gene fusion is expressed from a *T7* promoter (Studier and Moffatt 1986). Details of this construction are as follows. The *Bam*HI site in the 3′-untranslated region of the *T7*-transposase plasmid pNK2853 (Haniford *et al.* 1991) was destroyed by the insertion of a *Sal*I linker to create pDH98. A *Bam*HI linker (5′-TGGGATCCCA-3′) was introduced into the *Nde*I site of pDH98 to produce pDH99. A *Bam*HI-*Pst*I fragment containing the entire transposase gene and a portion of the ampicillin resistance gene was then inserted into pET11a-*T7*-Tag to create pDH100.

Generation of mutants: Versions of pDH10 containing either the *PS167* or the *RQ106* mutation were mutagenized with hydroxylamine as described by Davis *et al.* 1980. At various time points after addition of hydroxylamine, DNA was purified, divided into pools and transformed into a papillation strain (NK8044). A total of 40 separate pools of transformants, 20 for each of the two mutants, were screened for a suppressor phenotype (see below). Each pool generated on average 3,500 transformants. In a separate mutagenesis, pDH10 was treated with nitrous acid for varying lengths of time (Diaz *et al.* 1991) and DNA from different time points was subjected to amplification by polymerase chain reaction (PCR) using primers DH10-P2 (bp 540–556 of IS*10*) and DH30-P1 (bp 1101–1085 of IS*10*). The resulting fragments (560 bp) were cleaved with

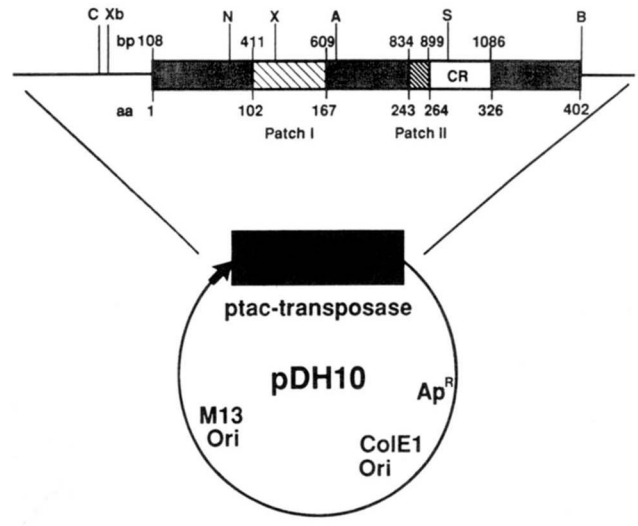

Figure 1.—Structure of the p*tac*-transposase plasmid, pDH10. The black rectangle in pDH10 represents the entire coding sequence of the IS*10* transposase gene and the adjacent arrow represents the p*tac* promoter to which the transposase gene has been fused. The p*tac*-transposase segment is displayed in greater detail in the map presented above pDH10. The primary sequence of IS*10* transposase is drawn to scale. Numbers above and below this map represent base pair and amino acid numbers, respectively. Also indicated are segments "Patch I" and "Patch II," which are parts of transposase defined by the location of Exc$^+$Int$^-$ mutations (see text), and the "conserved region" or CR which is a segment exhibiting significant amino acid homology to portions of other IS element transposase proteins. A, *Avr*II; B, *Bam*HI; C, *Cla*I; N, *Nhe*I; S, *Stu*I; Xb, *Xba*I; X, *Xmn*I.

*Avr*II and *Stu*I (see Figure 1), subcloned into PS167 and RQ106 versions of pDH10 and then transformed into DH5α. Individual libraries of recombinant molecules were then prepared and electroporated into NK8044. In total, 20 separate libraries, each giving rise to approximately 1,500 transformants, were screened for suppressor phenotypes.

Standard DNA manipulations were used to separate first and second site mutations, to generate a variety of double mutants and to randomize codon 134 (details available upon request). In all cases the correct identity of the constructs were verified by DNA sequence analysis.

Screen for suppressors: Potential suppressors for *PS167* and *RQ106* mutations were identified using the papillation assay developed by Huisman and Kleckner (1987). It has previously been shown that the length of time for LacZ$^+$ papillae formation accurately reflects the frequency of transposition events within a colony (Huisman and Kleckner 1987). In the presence of wild-type transposase encoded by pDH10, the levels of transposase expression are sufficient to generate red papillae on greater than 99% of NK8044 transformants after 48 hr of growth on MacConkey agar supplemented with lactose (1% final concentration) and ampicillin (100 μg/ml). However, in the presence of pDH10 encoding *RQ106* and *PS167* mutations, less than 50% of colonies contain red papillae after 96 hr of growth. Potential suppressors of *RQ106* and *PS167* mutations were picked by virtue of increased papillation frequencies relative to either pDH10-*RQ106* or pDH10-*PS167* transformants of NK8044.

For each potential suppressor picked the entire coding sequence of the transposase gene was subcloned into an appropriately prepared pDH10 backbone. Recombinants were retested in the papillation assay to ensure that the mutation

conferring the suppressor phenotype was located within the coding sequence of transposase. In each case the suppressor phenotype was successfully recovered after subcloning. These candidates were then subjected to DNA sequence analysis wherein the DNA sequence of the entire transposase gene was determined.

Conjugational "mating out" assay: The mating out transposition assay was performed according to the procedure of FOSTER *et al.* (1981). Plasmids were transformed into NK5830/pDH50 and transformants were grown on LB plates supplemented with ampicillin (100 μg/ml), chloramphenicol (20 μg/ml) and isopropyl-β-D-thiogalactopyranoside (IPTG) (100 μl of a 0.25 M stock solution of IPTG was spread onto plates). For each sample tested, three separate colonies were grown to saturation in LB supplemented as above. Of each saturated culture 0.05 ml was then inoculated into 1 ml of fresh LB supplemented only with IPTG (final concentration 1 mM) and grown with vigorous aeration at 37° to a concentration of about 1×10^8 cells/ml, and then grown for an additional 60 min with gentle aeration. A 2.5-ml aliquot of recipient strain (NK6641) freshly grown in LB to 4×10^8 cells/ml was then added to the donors and the mixture was incubated at 37° for an additional 60 min with gentle aeration. Each mating mixture was vigorously vortexed, diluted, and plated on LB plates containing streptomycin (150 μg/ml), kanamycin (50 μg/ml) and M9 salts (MILLER 1972). The relative transposition frequencies are average values determined from a single experiment. In all cases mating out experiments were performed at least two times and relative transposition frequencies were not found to differ significantly in these separate experiments. The frequency of transposition for wild-type transposase from pDH10 ranged from 3×10^5 to 1×10^6 $Kan^R Sm^R$ colonies per ml of mating mix in separate experiments.

Immunoblot assay: Mutations isolated in the pDH10 background were subcloned into pDH100 and then transformed into HMS174. Colonies were grown to saturation in LB supplemented with maltose (0.04%) and ampicillin (100 μg/ml). Of each saturated culture, 0.1 ml was inoculated into 10 ml of fresh LB (supplemented as above) and grown to an OD_{600} = 0.3, whereupon glucose was added to a final concentration of 4 mg/ml. After an additional hour of growth when cells were at an OD_{600} = 0.8, $MgSO_4$ was added to a final concentration of 10 mM. Cells were placed on ice for 5 min and then λCE6 was added to a m.o.i. of approximately 5–10 phage particles per cell. After an additional 10 min on ice cells were returned to growth at 37° for 30 min and then 1.0 ml aliquots of cells were pelleted in a microcentrifuge at 14,000 rpm for 30 sec. Cell pellets were resuspended in 100 μl of 25% sucrose, 20 mM Tris-HCl (pH 8.0), mixed with an equal volume of 2 × Laemmli "cracking" mix plus β-mercaptoethanol (final concentration 1%), boiled for 5 min, and then 7.5 μl were loaded onto a 10% Laemmli gel. Immunoblots were then performed using a mouse monoclonal antibody to the *T7*-Tag polypeptide and an anti-mouse IgG alkaline phosphatase conjugate as a secondary antibody. This assay was performed on at least two independent isolates expressing wild-type transposase (pDH100) and each of the indicated transposase mutants. We estimate the sensitivity of the assay to be such that differences in steady-state protein levels of less than 50% could not be distinguished.

***In vivo* product assay:** NK5830 was co-transformed with pDH10 or mutant derivatives and pDH50 which contains mini-Tn*10 KanR*. Transformants were grown to mid-logarithmic phase and then IPTG was added to a final concentration of 1 mM to induce a burst of transposase synthesis. One hour after addition of IPTG, cells were harvested, and DNA was prepared by the method of CLEWELL and HELINSKI (1970). The DNA was subjected to electrophoresis on a 1% agarose gel, in order to resolve transposition products, and transferred to a nylon membrane, and transposition products were identified using a probe generated from mini-Tn*10 KanR* by random priming (see HANIFORD *et al.* 1991).

Target integration assay: The mapping of mini-Tn*10 KanR-plac* integration events was carried out as described by BENDER and KLECKNER (1992b) using versions of pNK2731 encoding wild-type, *RQ106*, *RQ106/CY134*, *PS167*, *PS167/CY134* or *CY134* transposase. These plasmids were transformed into NK8032 (λ1294), which contains the *hisG*1 hotspot for Tn*10* integration upstream of a promoterless *lacZ* gene on the resident λ prophage. Transformants were plated on MacConkey plates supplemented with lactose (1%) and ampicillin (100 μg/ml), and after incubation for up to 72 hr at 37°, $LacZ^+$ papillae formed. Individual papillae were picked with a toothpick and streaked for single $LacZ^+$ colonies on MacConkey-lactose-ampicillin plates. Individual $LacZ^+$ colonies derived from different papillae were inoculated into 4 ml of LB supplemented with ampicillin (100 μg/ml) and grown to saturation. Chromosomal DNA was purified by the method of CAMPBELL and KLECKNER (1990), digested with *Xba*I and *Xho*I and subjected to electrophoresis on a 1% agarose gel. DNA was transferred to a Nytran membrane and probed with a ^{32}P-labeled *hisG* fragment.

RESULTS

Isolation of second site suppressors: Intragenic second site mutations that suppress Exc^+Int^- mutants *RQ106* and *PS167* were identified using the "LacZ turn-on" assay developed by HUISMAN and KLECKNER (1987). In this assay the number of $LacZ^+$ (red) papillae formed on a colony per unit time is roughly proportional to the frequency of transposition of a mini-Tn*10 KanR-lacZY* transposon within that colony (HANIFORD *et al.* 1989). Versions of plasmid pDH10 (Figure 1) encoding Exc^+Int^- mutations *RQ106* or *PS167* were mutagenized with either hydroxylamine or nitrous acid (see MATERIALS AND METHODS), transformed into the "papillation" strain (NK8044) and potential suppressors were identified as transformants exhibiting an increased number of $LacZ^+$ papillae relative to transformants containing unmutagenized *RQ106* and *PS167* plasmids.

For each of the primary mutations approximately 75,000 transformants were screened. Seventeen candidates were chosen and shown to exhibit increased papillation frequencies after subcloning of the entire transposase coding sequence into an unmutagenized plasmid backbone to reconstitute pDH10. DNA sequence analysis revealed that 8 of the 17 candidates contained single amino acid substitutions at different positions in transposase. All of these candidates also contained the original mutation (Table 1). One second site mutation, *CY134*, was found to suppress both *RQ106* and *PS167*, while other second site mutations were specific for either *RQ106* or *PS167*. The *CY134* mutation had previously been identified in a screen for altered target site specificity (ATS) transposase mutants (BENDER and KLECKNER 1992b).

The observation that DN100 and CY134 were each isolated four times against *RQ106* in the hydroxylamine

TABLE 1

Second site suppressors of Exc$^+$Int$^-$ mutations

Suppressors of	No. of independent isolates		Allele	Amino acid change	Base change (position)[b]
	HNO_2[a]	HA[a]			
PS167		2	*CY134*	Cys-134 to Tyr	G to A (508) (PI)[c]
	1		*RS218*	Arg-218 to Ser	G to T (761)
	1		*SP234*	Ser-234 to Pro	T to C (807)
		1	*SL258*	Ser-258 to Leu	C to T (880) (PII)[c]
		1	*GS306*	Gly-306 to Ser	G to A (1023) (CR)[c]
		3	*HY309*	His-309 to Tyr	C to T (1032) (CR)
RQ106		4	*DN100*	Asp-100 to Asn	G to A (405) (PI)
		4	*CY134*	Cys-134 to Tyr	G to A (508) (PI)

[a] Chemical mutagens used to generate suppressor mutations: HNO_2 = nitrous acid, and HA = hydroxylamine.
[b] The first base pair of the coding sequence of the IS*10* transposase gene is at position 108 of the outside end of IS*10*.
[c] Second site mutations that map in patch I, patch II and the conserved region are indicated by PI, PII and CR, respectively.

mutagenesis suggests that the existence of other *RQ106* suppressor mutations generated by CG to TA transitions is unlikely. In contrast, it may be possible to identify additional suppressors of *PS167* by hydroxylamine mutagenesis.

Relative strength of suppression by second site mutations: To accurately quantitate the extent of suppression conferred by second site mutations, the relative transposition frequencies of the suppressors were determined using a conjugal mating out assay (FOSTER *et al.* 1981). In this assay the relative frequency of transposition of mini-Tn*10 KanR* into a resident F′ episome in the presence of either wild-type or mutant transposase plasmids was measured by mating cultures of each donor strain with a suitable recipient strain and determining the relative frequencies of transposon-carrying (Kan^R) exconjugants. The results of this analysis are presented in Figure 2.

We classify second site suppressors using an arbitrary limit of less than 10-fold suppression as being weak and 10-fold suppression or greater as being strong. According to this scheme suppressors *RQ106/DN100*, *RQ106/CY134*, *PS167/CY134*, *PS167/SL258* and *PS167/HY309* exhibit strong suppression, while the suppressor *PS167/RS218* exhibits weak suppression. Suppressors *PS167/SP234* and *PS167/GS306* exhibited papillation frequencies slightly higher than *PS167*, but did not increase transposition frequencies relative to *PS167* in the mating out assay. The basis for the different results with these two assays has not been established.

Suppressors can be further subdivided into two groups based on whether or not the resulting transposition frequency is greater than that observed for either the first or second site mutation; for both *PS167/CY134* and *RQ106/DN100*, the transposition frequencies are greater than those observed for the respective first and second site mutations, while for *PS167/SL258*, *PS167/HY309* and *PS167/RS218* the transposition frequencies are increased relative to *PS167* only.

Also shown in Figure 2 are the transposition frequencies of second site mutations in an otherwise wild-type background. It is significant that none of the second site mutations on their own increase the transposition frequency to a level greater than wild type. This is consistent with the idea that suppression occurs by correcting the defect conferred by the primary mutation rather than by improving some other aspect of transposase function.

***DN100* only suppresses *RQ106* in *cis*:** For suppressor *RQ106/DN100* where both first and second site mutations confer a strong transposition defect in otherwise wild-type backgrounds, we have also looked at the ability of *DN100* to suppress *RQ106* in *trans*. *RQ106* and *DN100* proteins were expressed from compatible plasmids at similar levels in the papillation strain and under these conditions we did not find an increase in papillation frequency relative to that observed for the individual mutants on their own (data not shown). This provides a good indication that suppression in *RQ106/DN100* is a consequence of an intramolecular interaction. The high transposition frequencies of mutations that suppress *PS167* prevented us from doing similar experiments with these mutants and therefore we cannot rule out the possibility that suppression in these cases occurs in *trans*.

Suppression of Exc$^+$Int$^-$ mutations does not involve alterations in protein stability *in vivo*: We have previously shown that for levels of transposase expression used in mating out and papillation assays described here, that the steady state protein levels of *RQ106* and *PS167* do not differ significantly from that of wild-type transposase (HANIFORD *et al.* 1989). Suppression by second site mutations could, however, result from increases in protein stability, relative to wild type, such that residual transposition activities are amplified. To address this possibility we have tagged wild-type and mutant proteins with a 12-amino acid epitope (from the *T7* gene 10 protein) at their N termini and expressed these proteins at a level roughly equivalent to that of p*tac*-transposase on a multicopy plasmid (see MATERIALS AND METHODS). Relative steady state protein levels were then determined using an immunoblot assay. As shown in Figure

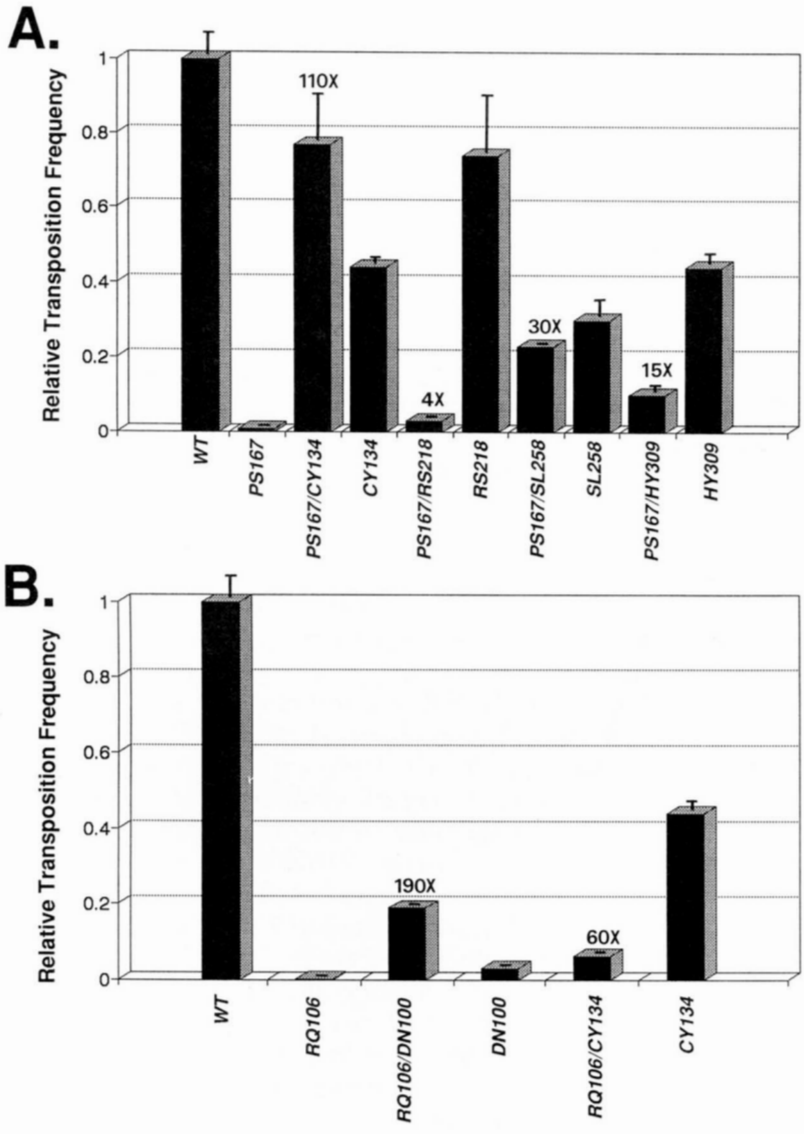

FIGURE 2.—Relative transposition frequencies of suppressors of *PS167* and *RQ106*. Transposition frequencies of (A) suppressors of *PS167* and (B) suppressors of *RQ106*, as determined by a mating out assay, are average values from three independent donor transformants (black bars). Error bars represent the standard deviation associated with the above values. Numbers above bars indicate the "fold" increase in transposition frequency for suppressors relative to the original mutant (*e.g.*, *PS167/CY134* vs. *PS167*). In this experiment a relative level of transposition ≡ 1 corresponds to 3×10^5 transposition events per ml of mating mix.

3A, no significant differences in steady state protein levels were observed for any of the suppressors. It should be noted that the conclusion that second site mutations do not affect protein stability depends on the assumption that the *T7* tag does not itself influence protein stability. We note that the activities of native and tagged proteins do not differ significantly *in vitro* or *in vivo* (data not shown). This is at least consistent with there not being a significant difference in relative steady-state stabilities for native and tagged proteins (also see below).

***CY134* suppresses Exc⁺Int⁻ mutations in both PI and PII:** The observation that *CY134* suppresses two different Exc⁺Int⁻ mutations raised the possibility that mutations that relax integration specificity (*i.e.*, ATS) might be general suppressors of Exc⁺Int⁻ mutations. If this were true, then we would expect to see that a variety of Exc⁺Int⁻ mutations would be suppressed by both *CY134* and the PII ATS mutation, *CY249*. We addressed this possibility by constructing a series of doubly mutant proteins containing different combinations of mutations. The transposition frequencies of these mutants were then determined by the mating out assay (Figure 4). The results indicate that *CY134* is a strong suppressor for two additional PI mutations, *RC102* and *RH102*, and a weak suppressor for three PII mutations, *RH243*, *PL252* and *EK263*. In contrast, *CY249* was found to be a very weak suppressor for only one PII Exc⁺Int⁻ mutation.

The lack of suppression in double mutants containing *CY249* could have been due to a reduced protein stability in these mutants. We have tested this possibility for *RQ106/CY249* and *PS167/CY249* by measuring steady state protein levels as described in Figure 3. The results of this analysis, shown in Figure 3B, indicates that there is no difference in steady state protein levels for these mutants relative to wild-type transposase and therefore the lack of suppression by *CY249* is not a consequence of reduced protein stability.

We have also found that *CY134* does not suppress another class of transposase mutants (A. K. KENNEDY and D. B. HANIFORD, unpublished observations). These

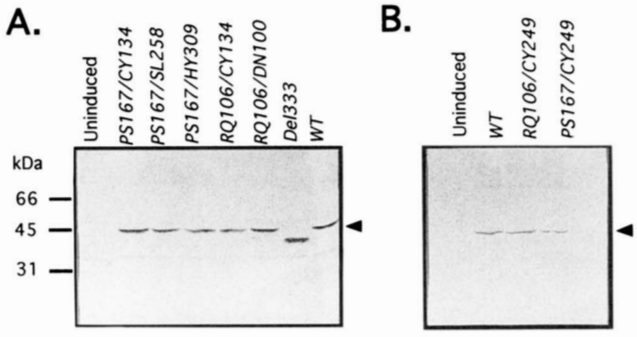

FIGURE 3.—Steady-state levels of wild-type and doubly mutant transposase proteins. (A) Suppressors indicated in Table 1. (B) Double mutants containing *CY249*. Cells containing the indicated *T7*-Tag-transposase genes were infected with a λ phage derivative which expresses the *T7* RNA polymerase gene. After a short period of *T7*-Tag-transposase gene expression, cells were lysed and extracts were subjected to immunoblot analysis using a mouse monoclonal anti-*T7*-tag antibody. The species with an apparent molecular mass just less than 45 kD is full length *T7*-Tag-transposase protein (arrowhead). This species is not detected in the absence of λ phage infection ("Uninduced") and a new species of reduced molecular weight is detected when a version of the *T7*-Tag-transposase gene containing a stop codon at amino acid 333 is expressed ("*Del 333*"). A species with an apparent molecular mass of just over 31 kD cross reacts with the anti-*T7*-Tag antibody and provides a control for the total protein loaded per lane. Based on a comparison of the band intensity of *T7*-Tag-transposase with that of a *T7*-tag protein of predetermined concentration, we estimate from the cell densities used that approximately 1,500 molecules of *T7*-Tag-transposase are synthesized per cell. This estimate assumes that at the m.o.i. of 5–10 λ phage particles per cell, close to 100% of cells are infected and actively synthesizing *T7*-Tag-transposase. This level of expression is very close (±50%) to that estimated for p*tac*-transposase.

mutants retain the ability to bind to and synapse Tn*10* ends, but are greatly reduced for Tn*10* excision. The transposition frequencies of these mutants are comparable to that observed for *PS167* and *RQ106*. Since, these mutants still promote transposition at a low level, the inability of *CY134* to suppress these mutants is further evidence that suppression by *CY134* is not due to an increase in protein stability.

An amino acid substitution at position 134 confers an Exc$^+$Int$^-$ phenotype: It has previously been noted that specific mutations at amino acids 135 and 139 confer an Exc$^+$Int$^-$ phenotype (HANIFORD *et al.* 1989). The close proximity of mutations that confer ATS and Exc$^+$Int$^-$ phenotypes suggests that these phenotypes may be different manifestations of alterations in the same structural domain. If this is true, then it would be expected that some amino acid substitutions at codon 134 would confer an Exc$^+$Int$^-$ phenotype. This possibility was tested by mutagenizing codon 134 with an oligonucleotide made completely degenerate for this codon and then testing the resulting mutants for an Exc$^+$Int$^-$ phenotype. Plasmid DNA was isolated from several transposition defective transformants and co-transformed with a mini-Tn*10* plasmid (pDH50) into NK5830. Transformants were grown up in liquid culture and a synchronous round of transposase synthesis was induced by the addition of IPTG. One hr after the initiation of transposase synthesis, DNA was purified and analyzed for the formation of specific transposition products (see MATERIALS AND METHODS). Under these conditions wild-type transposase produces two distinct products, the transposon circle, which is an intramolecular strand transfer product, and an excision product (Figure 5) (HANIFORD *et al.* 1989). The low level of excision product in a wild-type reaction is a consequence of the conversion of this product to strand transfer products. In the case of an Exc$^+$Int$^-$ mutant, the excision product accumulates and transposon circles are not detected (HANIFORD *et al.* 1991). One amino acid substitution at position 134 (cysteine to tryptophan) was found to confer an Exc$^+$Int$^-$ phenotype (Figure 5).

We have also used this assay to assess the properties of some second site suppressor mutations on their own, including *DN100*, *SL258* and *HY309*. Both *DN100* and *SL258* were found to confer an Exc$^+$Int$^-$ phenotype (data not shown).

***RQ106/CY134* and *PS167/CY134* do not retain an ATS phenotype:** To further characterize the properties of Exc$^+$Int$^-$ mutants that are suppressed by *CY134*, we asked if two such mutants, *RQ106/CY134* and *PS167/CY134*, retain an ATS phenotype. If these mutants retain an ATS phenotype, we expected that integration events directed by these proteins would go into sites other than a consensus target sequence. The target integration assay developed by BENDER and KLECKNER (1992b) was used to test this possibility. Briefly, the transposition of mini-Tn*10 KanR-plac* into a Tn*10* integration hotspot (*hisG*1) positioned in front of a promoterless *lacZ* gene results in LacZ expression. Clones of cells in which the *lacZ* gene is expressed form red (LacZ$^+$) papillae on a background of white (LacZ$^-$) cells in which no such transposition events have occurred. The position of independent integration events upstream of the promoterless *lacZ* gene can then be identified by extracting genomic DNA from individual LacZ$^+$ papillae, digesting this DNA with appropriate restriction enzymes and performing Southern blot analysis as described in Figure 6. A summary of this analysis is presented in Table 2.

For wild-type transposase, 26 of 27 integrations were found to produce a DNA fragment of the size expected for integration into the *hisG*1 hotspot. A similar ratio of integrations into the *hisG*1 hotspot was observed for transposition events directed by *PS167*, *PS167/CY134* and *RQ106/CY134* proteins indicating that none of these mutants produces an ATS phenotype. In contrast, both *CY134* and *RQ106* directed a much higher number of integrations into degenerate target sites located upstream of the *lacZ* gene. The sequence of these degenerate target sites has not yet been determined.

In addition to demonstrating that *PS167/CY134* and *RQ106/CY134* do not retain an ATS phenotype, this

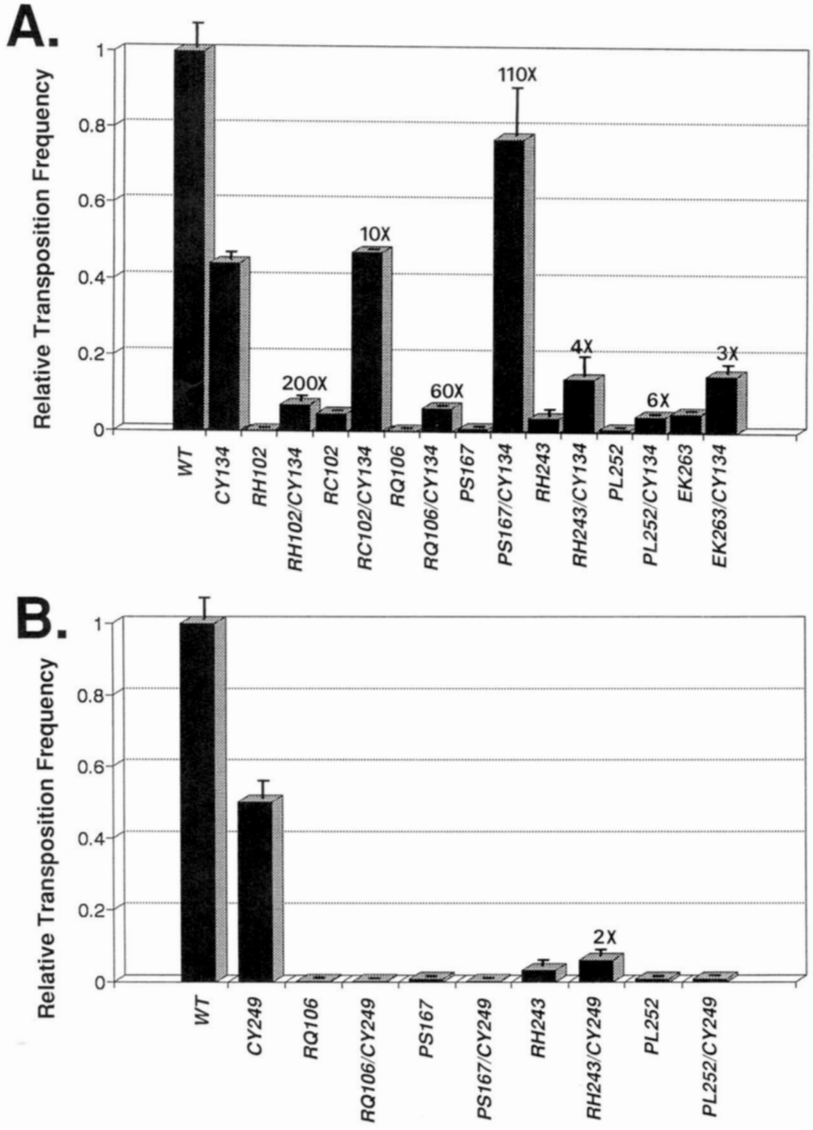

FIGURE 4.—Relative transposition frequencies of mutants containing both Exc⁺Int⁻ and ATS mutations. (A) Double mutants containing the ATS mutation *CY134*. (B) Double mutants containing the ATS mutation *CY249*. Transposition frequencies were determined as described in Figure 2. Numbers above bars indicate the "fold" increase in transposition frequency for suppressors relative to the original Exc⁺Int⁻ mutant (*e.g.*, *RH102/CY134* vs. *RH102*).

experiment has also revealed that the *RQ106* mutation is novel in the sense that it confers a strong integration defect, however, in the rare instances when integration occurs, an ATS pattern of integrations is observed. Given the severity of the transposition defect for *RQ106*, it is possible that only a portion of mini-Tn*10 KanR-plac* has been recombined into the region immediately upstream of the *lacZ* gene by a non-Tn*10*-mediated event. However, using appropriate primers and DNA from *RQ106*-generated LacZ⁺ papillae, we have been able to generate PCR products containing intact segments of mini-Tn*10 KanR-plac* which include both "left" and "right" transposon ends. This is consistent with the region immediately upstream from the *lacZ* gene containing an intact copy of the mini-Tn*10 KanR-plac* element, as would be expected for an authentic transposition event (data not shown).

DISCUSSION

Two segments of IS*10* transposase designated PI and PII have previously been shown to contain amino acid residues that play a critical role in Tn*10* integration; specific amino acid substitutions at these positions block integration but do not interfere with Tn*10* excision (HANIFORD *et al.* 1989). To learn more about the nature of the integration defect for two such mutants we performed an intragenic second site suppressor analysis on these mutants. This approach provides a particularly good way of identifying functionally related amino acid pairs in a protein (NELSON and DOUGLAS 1993). Two different second site mutations were found to suppress a PI proximal mutant, *RQ106*, while four different second site mutations were found to suppress a PI distal mutant, *PS167*. One second site mutation, *CY134*, was initially found to suppress both *RQ106* and *PS167*, and subsequently shown to suppress five other Exc⁺Int⁻ mutations. Suppression by *CY134* is of particular interest because this mutation has previously been shown to relax Tn*10* integration specificity when present in an otherwise wild-type background (BENDER and KLECKNER 1992b).

Suppression of Exc⁺Int⁻ mutations by *CY134* occurs by correcting the initial defect: Intragenic second site suppressors can function by either correcting the initial

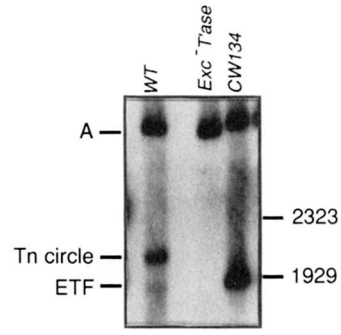

FIGURE 5.—Southern blot assay for transposition products. Transposition products of a mini-Tn*10 KanR* generated by the indicated transposase proteins were detected by Southern blot of small DNA species using a *kan* gene probe. The positions of an intramolecular strand transfer product called a transposon circle (Tn circle) and an excised transposon (ETF) are indicated. The DNA species labeled "A" is supercoiled transposase plasmid which cross-hybridizes with the *kan* probe. Unreacted substrate plasmid is not shown. The mini-Tn*10 KanR* element is 1.8 kb in length. The transposon circle and the excised transposon have almost identical molecular weights; however, these products have different mobilities because the former is a gapped circle and the latter is linear (HANIFORD *et al.* 1991). The lane designated "Exc⁻ T'ase" contains DNA isolated from cells in which a transposase gene containing an undefined deletion is expressed. The corresponding protein does not carry out transposition at a detectable frequency and is defective for Tn*10* excision.

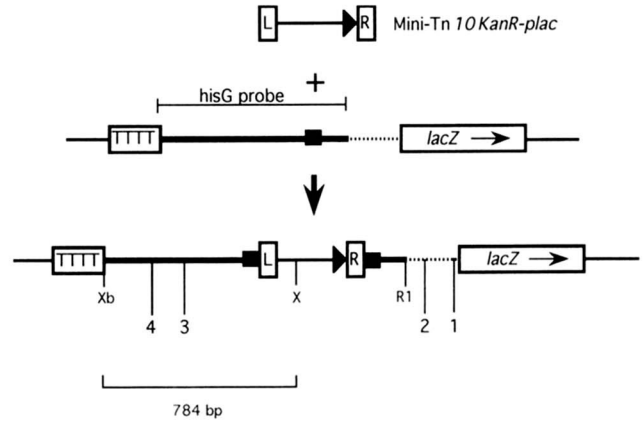

FIGURE 6.—Integration of mini-Tn*10 KanR-plac* into a *hisG*1 hotspot positioned upstream of a promoterless *lacZ* gene. Integrations of mini-Tn*10 KanR-plac* into the region upstream of the promoterless *lacZ* gene were mapped by Southern blot analysis using a *hisG* fragment probe. Integration of this element into the *hisG*1 hotspot results in the formation of a 784-bp *Xba*I-*Xho*I fragment. In contrast, integrations into degenerate target sites (1 and 2) located downstream of the *hisG*1 hotspot will result in larger *Xba*I-*Xho*I fragments while integrations upstream of the *hisG*1 (sites 3 and 4) will result in smaller *Xba*I-*Xho*I fragments. Mini-Tn*10 KanR-plac* is not drawn to scale. Open rectangles = 70 bp IS*10* outside ends designated left (L) and right (R); ▶ = p*lac* promoter; ■ = *hisG*1 hotspot; thick line = *hisG* fragment; dotted line = leader sequence upstream of *lacZ* gene; TTTT = transcription terminators; Xb = *Xba*I; X = *Xho*I; R = *Eco*RI.

defect (NAGATA *et al.* 1989; POTEETE *et al.* 1991) or improving some aspect of protein function that is not directly related to the initial defect (HECHT and SAUER 1985; SHORTLE and LIN 1985; KLIG *et al.* 1988). In the case of Exc⁺Int⁻ IS*10* transposase mutants, a particular step in the Tn*10* integration reaction becomes rate limiting and therefore it is expected that suppression is a consequence of fixing this particular step, as opposed to making some other step, which is not rate limiting, more efficient. For suppression of Exc⁺Int⁻ mutations by *CY134*, this scenario is supported by the fact that both *CY134* and Exc⁺Int⁻ mutations affect target site interactions. In addition, *CY134* does not suppress mutations in transposase that do not influence target interactions. Finally, *CY134* on its own does not increase activity relative to wild-type transposase, as might be expected if *CY134* were acting independently of the initial defect.

The fact that *CY134* suppresses at least seven different Exc⁺Int⁻ mutations indicates that *CY134* is not allele-specific in suppressing Exc⁺Int⁻ mutations. Thus, it is unlikely that the *CY134* effect is a consequence of direct interactions between first and second site positions; a stronger allele specificity is normally associated with suppression by a direct interaction mechanism (SINGER *et al.* 1993). Instead, we predict that *CY134* alters a general aspect of transposase function that specifically affects the integration stage of the reaction. We also predict that this same altered function is responsible for an ATS phenotype when *CY134* is present in an otherwise wild-type background. We suggest two possible features of

TABLE 2

Sites of mini-Tn*10 KanR-plac* integration upstream of a promoterless *lacZ* gene

Transposase	Integration site[a]				
	1	2	3	4	*hisG*1[b]
WT	0	0	0	1	26
CY134	1	4	1	3	11
PS167	0	0	0	0	12
PS167/CY134	0	1	0	0	14
RQ106	2	3	4	4	23
RQ106/CY134	0	0	0	0	12

[a] Sites of integration of mini-Tn*10 KanR-plac* upstream of a promoterless *lacZ* gene.

[b] The target site designated *hisG*1 conforms to the consensus sequence for Tn*10* integration, 5′-NGCTNAGCN-3′.

transposase function that might be altered. (1) The *CY134* mutation could result in a tighter binding of transposase to target DNA so that in an otherwise wild-type background, integration specificity would be relaxed by reducing the contribution of both consensus and flanking base pairs to the formation of a stable transposase-target complex. It follows that if Exc⁺Int⁻ mutations are defective in target binding, then the *CY134* mutation could result in suppression by increasing the affinity of transposase for target DNA. By employing a newly developed *in vitro* assay for target site binding (J. S. SAKAI and N. KLECKNER, manuscript in

preparation), we are currently assessing whether or not Exc⁺Int⁻ mutations are defective in target binding. (2) If binding of target DNA is tightly coupled to subsequent steps in integration (*i.e.*, strand transfer), then relaxed target specificity could reflect a decreased dependence of later steps on the energy made available from target binding. In this case, the *CY134* mutation may predispose transposase to adopting a conformation that is productive for strand transfer in the absence of optimal target interactions. In contrast, Exc⁺Int⁻ mutations might interfere with the ability of transposase to adopt such a conformation. When both Exc⁺Int⁻ and *CY134* mutations are present in the same protein, the ability to adopt a conformation appropriate for strand transfer could be improved resulting in some level of suppression. In light of this possibility, it is of interest to note that Chou-Fasman secondary structure predictions for the central portion of PI suggest that positions 129–136 comprise a "hinge" between two α-helices. It follows that *CY134* might have an important effect on the conformational flexibility of this segment. This possibility could be evaluated by using chemical probes that are sensitive to local changes in protein structure (*e.g.*, ERMACORA *et al.* 1992).

PI and PII are likely involved in the same function: The second site suppressor analysis presented here has provided six examples where first and second site mutations are separated by at least 50 amino acids. Suppression by distantly linked second site mutations is most easily explained if the segments containing the two mutations are involved in a common function (*e.g.*, HARRIS *et al.* 1991; NELSON and DOUGLAS 1993). This raises the possibility that residues in PI and PII might form a functional surface within a domain. Alternatively, residues in PI and PII could be components of two separate domains involved in a common function. In this case we anticipate that the function of these domains would be tightly coordinated. In both scenarios it is likely that residues in PI and PII are either interacting with the target DNA or involved more directly in the catalysis of strand transfer.

Biochemical studies of mutant proteins described here will be required to further determine mechanisms of intragenic suppression. In addition, several other interesting questions remain to be addressed, including defining the basis for the novel phenotype of the *RQ106* mutation, the lack of suppression of Exc⁺Int⁻ mutants by *CY249* and the observation that mutants suppressed by *CY134* do not retain an ATS phenotype.

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