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Characterization of Two Hypertransposing Tn*5* Mutants

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Transposition of Tn*5* in *Escherichia coli* is regulated by two transposon-encoded proteins: transposase (Tnp), promoting transposition preferentially in *cis*, and the *trans*-acting inhibitor (Inh). Two separate transposase mutants were isolated that replace glutamate with lysine at position 110 (EK110) and at position 345 (EK345). The EK transposase proteins increase the Tn*5* transposition frequency 6- to 16-fold in *cis* and enhance the ability of transposase to act in *trans*. The purified mutant transposase proteins interact with transposon outside end DNA differently from the wild-type protein, resulting in the formation of a novel complex in gel retardation assays. During characterization of the transposase proteins in the absence of inhibitor, we found that wild-type transposase itself has a transposition-inhibiting function and that this inhibition is reduced for the mutant proteins. We present a model for the regulation of Tn*5* transposition, which proposes the existence of two transposase species, one *cis*-activating and the other *trans*-inhibiting. The phenotype of the EK transposase mutants can be explained by a shift in the ratio of these two species.

Prokaryotic transposable elements can be viewed as existing in a symbiotic interaction with the bacterial host cell. The cell provides the environment necessary for replication and transposition of the element, and it benefits from the antibiotic resistance genes many transposons carry. Furthermore, a low level of random DNA rearrangements caused by transposition might help cells to adapt to environmental changes and increase the survival rate (23). The transposition process, however, must be tightly regulated to prevent detrimental effects of an excessive level of DNA rearrangements.

Tn*5* is a prokaryotic composite transposon consisting of two inverted insertion sequences, IS*50*R and IS*50*L, flanking several antibiotic resistance genes (1). Two features of Tn*5* are essential for transposition: the peripheral 19 bp of the insertion sequences, called outside ends (16), and the 476-amino-acid transposase protein (Tnp) encoded on IS*50*R (11). The second IS*50*R protein, inhibitor (Inh), is expressed in the same reading frame as transposase but from independent transcriptional and translational start sites, resulting in a 421-amino-acid polypeptide lacking the first 55 residues of transposase (13).

Tn*5* transposition is controlled by a complex array of regulatory mechanisms, some inherent to the element and others involving host proteins. This regulation can be separated into three levels of control: limitation of transposase expression, regulation mediated by host proteins, and regulation of the transposase activity. Transposase expression is limited due to inefficient transcription initiation from a weak promoter (13). The promoter activity is further reduced through DNA methylation by host-encoded Dam methylase (29). As a consequence, the concentration of transposase in the cell is fourfold lower than the inhibitor protein concentration (12). Transcripts initiated from spurious promoters outside the element cannot increase the level of transposase expression since these transcripts sequester the translational start site of transposase in a secondary structure and are not translated (22). The host-encoded DnaA protein regulates transposition through direct contact with the transposon DNA at specific binding sites (30), perhaps resulting in the formation of a transposase-host protein-DNA complex. Integration host factor was found to affect Tn*5* transposition (17) and might be involved in the formation of this complex. The regulatory mechanisms of other host proteins involved in transposition, GyrA (8), PolA (20, 24), LexA (26), RecA (26), and SulA (21), are less well understood and may act through more indirect mechanisms.

Posttranslational regulation of the transposase activity is caused by the inhibitor protein (31). Details of this inhibition remain unclear, but mounting evidence suggests that it is due to direct protein contact with transposase. The transposase protein seems to be able to form oligomers in vitro (4), and evidence for the existence of transposase-inhibitor complexes in vivo was presented (5). These observations led to the current hypothesis that Tn*5* transposition is inhibited by the formation of inactive transposase-inhibitor heterooligomers. While the inhibitor protein inhibits transposition in *trans*, transposase is a *cis*-acting protein. It activates transposition efficiently only when expressed from the same replicon on which the outside end DNA sequences are located (12). It is not understood what prevents transposase from diffusing to outside ends in *trans* to promote their transposition. The *cis* preference of transposase is apparently not caused by the interaction with the inhibitor protein (6). A possible explanation for this phenomenon is that transposase is physically or functionally unstable and does not reach donor sites located on a different replicon.

In this paper, we describe the characterization of two transposase mutants that increase the Tn*5* transposition frequency and partially overcome the transposase *cis* preference. On the basis of the observation that the transposase protein itself has an inhibiting function, we propose the existence of two transposase species, one that is *cis* activating and one which is *trans* inhibiting. The transposase *cis* preference may be due to rapid conversion of the activating protein into the inhibiting protein. The phenotype of the transposase mutants, i.e., increased transposition and higher *trans* activity, is consistent with a shift in the ratio of these two transposase species.

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TABLE 1. Plasmids, phage, and bacterial strains

Strain, plasmid, or phage	Description	Reference or source
Strains		
RZ211	Δ(*lac-pro*) *ara str recA56 srl thi*	12
RZ212	RZ211 pOX38-Gen	11
RZ221	*polA* Δ(*lac-pro*) *ara str nal*	12
CJ236	*dut ung thi relA*; pCJ105	15
DH5α	Δ(*lac*)*U169 endA1 gyrA96 hsdR17 recA1 relA1 supE44 thi1* ϕ80d*lacZ*Δ*M15*	7
MC1061	Δ(*lac*)*X74* Δ(*ara-leu*)*7696 araD139 galU galK hsdR2 mcrB1 rpsL*	27
Plasmids		
pBR322	Medium-copy-number vector	New England Biolabs
pBKS-	High-copy-number vector	Stratagene
pRZ620	Papillation vector; Tn*5* ORF*lac* on ColE1	14
pRZ620 EK110	pRZ620 with EK110 mutation	This study
pRZ620 EK345	pRZ620 with EK345 mutation	This study
pRZ620 EK110/EK345	pRZ620 with EK110 and EK345 mutations	This study
pRZ7015	Tnp overproducer on pBKS-	This study
pRZ7015 EK110	pRZ7015 with EK110 mutation	This study
pRZ7015 EK345	pRZ7015 with EK345 mutation	This study
pRZ7013	IS*50*R on pBKS-	This study
pRZ7013 EK110	pRZ7013 with EK110 mutation	This study
pRZ7013 EK345	pRZ7013 with EK345 mutation	This study
pRZ7016	IS*50*R MA56 on pBKS-	This study
pRZ7016 EK110	pRZ7016 with EK110 mutation	This study
pRZ7016 EK345	pRZ7016 with EK345 mutation	This study
pRZ7017	Tn*5* on pTZ19U	This study
pRZ7017 EK110	pRZ7017 with EK110 mutation	This study
pRZ7017 EK345	pRZ7017 with EK345 mutation	This study
pRZ7018	Tn*5* with IS*50*R MA56 on pTZ19U	This study
pRZ7018 EK110	pRZ7018 with EK110 mutaiton	This study
pRZ7018 EK345	pRZ7018 with EK345 mutation	This study
pRZ7024	IS*50*R on pBR322	This study
pRZ7024 EK110	pRZ7024 with EK110 mutation	This study
pRZ7024 EK345	pRZ7024 with EK345 mutation	This study
pRZ7029	IS*50*R MA56 on pBR322	This study
pRZ7029 EK110	pRZ7029 with EK110 mutation	This study
pRZ7029 EK345	pRZ7029 with EK345 mutation	This study
pRZ4711	Kanamycin resistance gene flanked by Tn*5* outside end on pACYC184	Michael Weinreich
Phage λ NK467	*b*221 *c*I857 *O*am29 *P*am80 *rex*::Tn*5*	Nancy Kleckner

MATERIALS AND METHODS

Media and reagents. Bacterial strains were grown in LB or 2× YT (19). Antibiotics were purchased from Sigma and used in the following concentrations: kanamycin, 40 μg/ml; ampicillin, 100 μg/ml; tetracycline, 15 μg/ml; nalidixic acid, 20 μg/ml; gentamycin, 5 μg/ml; and chloramphenicol, 30 μg/ml. Restriction endonucleases were obtained from New England Biolabs. T7 DNA polymerase and T4 DNA ligase were from United States Biochemical Corp. Calf intestinal alkaline phosphatase was purchased from Boehringer Mannheim. Rabbit antitransposase antibodies were a gift from Mark Krebs. Goat anti-rabbit alkaline phosphatase was obtained from Sigma. Radioactive isotopes were purchased from Amersham.

Strains, phage, and plasmids. All bacterial strains, phage, and plasmids used in this study are described in Table 1. The structures of IS*50*R carrying plasmids are shown in Fig. 1B. DNA manipulations were performed essentially as previously described (19).

pRZ620 carries Tn*5* ORF*lac* inserted into ColEI (14) and was used to screen for hypertransposing Tn*5* mutants. pRZ620 EK110/EK345 combines the two EK mutations and was constructed by substituting the wild-type *Nhe*I-*Bgl*II DNA fragment of pRZ620 EK110 with the corresponding mutant fragment of pRZ620 EK345.

pRZ7013 contains IS*50*R on the pBKS- vector and resulted from the ligation of the *Eco*RI-*Bam*HI fragment from pRZ860 (28), which carries IS*50*R, to the *Eco*RI-*Bam*HI vector backbone of pBKS-. The EK110 and EK345 mutant derivatives were obtained by replacement of wild-type fragments with the *Hpa*I-*Nhe*I fragment from pRZ620 EK110 and the *Nhe*I-*Bgl*II fragment from pRZ620 EK345, respectively. Site-directed mutagenesis, following the method of Kunkel et al. (15), was used to insert the MA56 mutation into these plasmids, resulting in pRZ7016, pRZ7016 EK110, and pRZ7016 EK345. The MA56 oligonucleotide (5′ GGTAGTG AAGCCGCCCAGGAAGGCGC 3′) was synthesized on an Applied Biosystems (Foster City, Calif.) model 391 DNA oligonucleotide synthesizer, and its incorporation was confirmed by DNA sequence analysis. The GCC sequence introduces the MA mutation.

Tn*5* plasmids containing mutant alleles in IS*50*R were constructed in the following way: pRZ7009, an insertion of IS*50*L and the left half of the unique internal Tn*5* sequence into pTZ19U, was digested with *Bam*HI and *Sca*I. The resulting large DNA fragment was ligated with *Sca*I-*Bam*HI fragments from pRZ7013, pRZ7016, and their EK derivatives to yield pRZ7017, pRZ7017 EK110, pRZ7017 EK345, pRZ7018, pRZ7018 EK110, and pRZ7018 EK345.

pRZ7024 consisting of IS*50*R on pBR322 was constructed by first inserting the *Eco*RI fragment from pRZ7017, carrying Tn*5*, into the *Eco*RI site of pBR322 (resulting in pRZ7027)

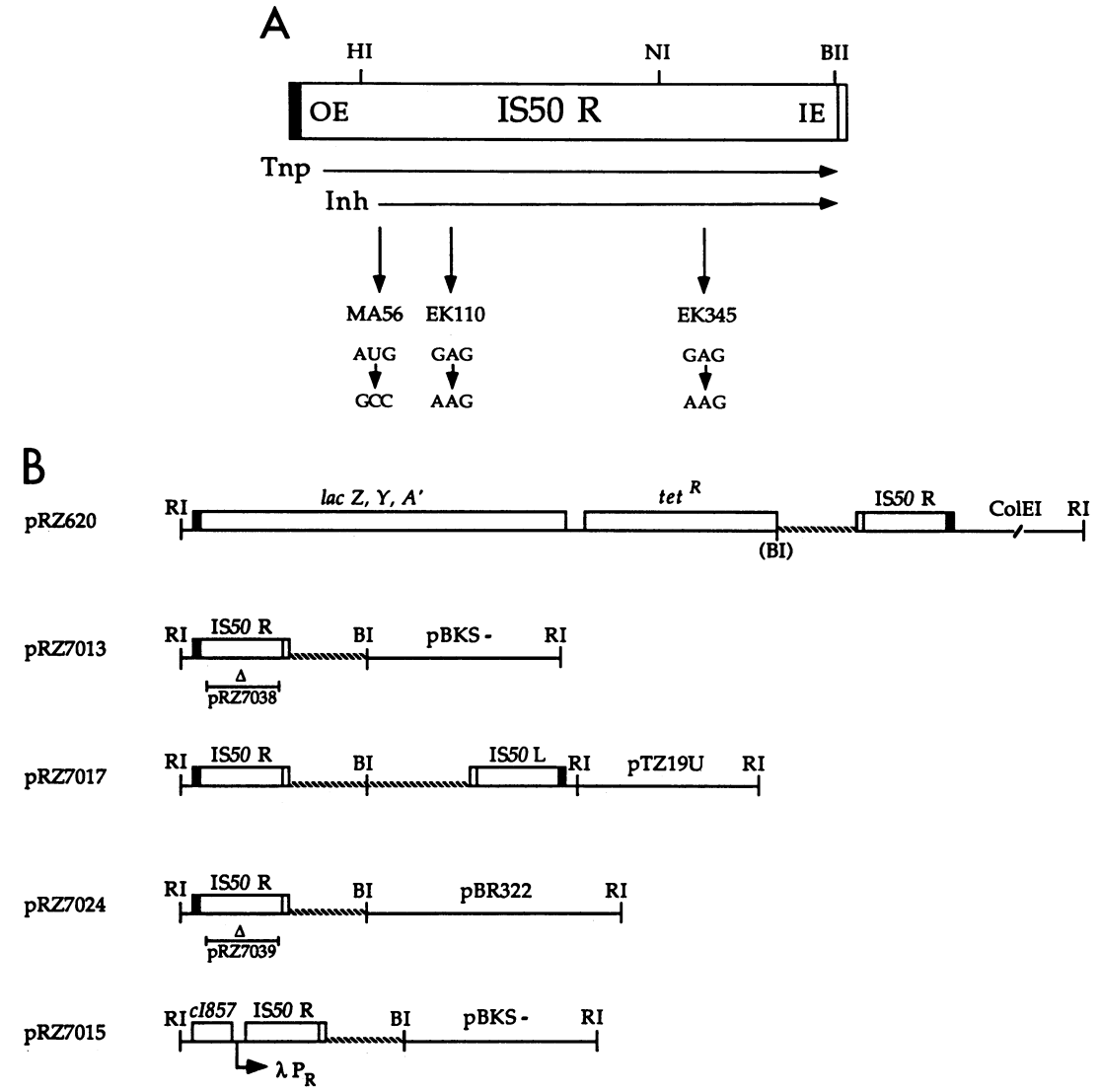


FIG. 1. Structure of IS*50*R and IS*50*R containing plasmids. (A) The 1,534-bp IS*50*R encodes two proteins involved in transposition: transposase (Tnp), 476 amino acids in length, and inhibitor (Inh), which is 55 residues shorter. Transposase and inhibitor share the same reading frame but use independent transcriptional and translational start sites. Nineteen base pairs of the outside end (OE) and inside end (IE) of IS*50*R are necessary and sufficient DNA requirements for the transposition process. Utilization of two outside ends results in transposition of the whole Tn*5* element. The location and sequence changes of the two hypertransposing EK mutations and the inhibitor-abolishing MA56 mutation are shown. Restriction sites used during construction of various IS*50*R plasmids are *Hpa*I (HI), *Nhe*I (NI), and *Bgl*II (BII). (B) The schematic structures of the IS*50*R plasmids used in this paper are shown. IS*50* outside ends are represented by closed boxes, inside ends are represented by open boxes, and internal Tn*5* DNA is indicated by hatched bars. The extent of the deletions in pRZ7038 and pRZ7039 is shown beneath their respective parent plasmids. The relevant restriction sites are *Eco*RI (RI) and *Bam*HI (BI, destroyed in pRZ620).

and then deleting a *Bam*HI fragment that includes IS*50*L and part of the vector. pRZ7024 EK110, pRZ7024 EK345, and the MA56 versions pRZ7029, pRZ7029 EK110, and pRZ7029 EK345 were obtained the same way, except that the *Eco*RI Tn*5* fragments were derived from the appropriate pRZ7017 and pRZ7018 plasmids.

pRZ7015, pRZ7015 EK110, and pRZ7015 EK345 are transposase-overproducing pBKS-based plasmids. They were constructed by replacement of the *Eco*RI-*Hpa*I fragments of pRZ7013, pRZ7013 EK110, and pRZ7013 EK345 with the *Eco*RI-*Hpa*I fragment of pRZ986 (4). This fragment carries the bacteriophage λ *c*I857 gene, the P_R promoter, and *cro* Shine-Dalgarno sequence fused to the first 32 codons of the transposase gene.

pRZ7038 and pRZ7039 are derivatives of pRZ7013 and pRZ7024, respectively, which delete most of the transposase gene but retain the outside and inside ends of IS*50*. They were constructed by digestion of pRZ7013 and pRZ7024 with *Hpa*I and *Bgl*II and religation of the large fragment after filling in the *Bgl*II overhang.

Isolation of hypertransposing Tn*5* mutants. To obtain random mutations in IS*50*R, pRZ620 (14) was subjected to in vivo diethyl sulfate mutagenesis. Strain RZ211, containing pRZ620, was grown in LB to saturation, harvested, and resuspended in M9 buffer. Diethyl sulfate was added to 0.4% final concentration. The cells were incubated at 37°C for 10 h, resuspended in LB medium, and grown overnight. The plasmid was extracted, purified, and transformed into fresh

RZ211 cells. Approximately 5,000 colonies were screened on lactose-MacConkey agar for an increase in papillation. Quantitative mating-out assays of eight candidates revealed three mutants with significantly increased transposition phenotypes, two of which were found to be identical by DNA sequencing. The two unique mutants are analyzed in this paper.

Sequencing of EK mutations. Subcloning of IS*50*R fragments from mutagenized into unmutagenized pRZ620 localized the position of the hypertransposing mutations to a 748-bp *Pst*I fragment for EK110 and a 519-bp *Nae*I-*Nhe*I fragment for EK345. These fragments were cloned into single-stranded DNA producing vectors pTZ18U and pTZ19U for DNA sequence analysis. By using the Sequenase kit (United States Biochemical Corp.) with slight modifications (22), these fragments were sequenced entirely, and both mutations turned out to be single-base changes.

Transposition assays. The mating-out transposition assay was performed essentially as described previously (29). The assay measures the movement of a transposable element from a plasmid to the F factor in the cell. After transfer of the F factor into recipient cells, the total number of exconjugants is compared with the number of exconjugants which received the transposon.

Ten microliters of donor RZ212- and 30 μl of recipient RZ221-saturated cultures were mixed with 1 ml of LB and incubated at 37°C in a roller drum shaker for 6 to 10 h. The cells were then diluted and plated on appropriate medium. Exconjugants were selected on LB agar plates containing 20 μg of nalidixic acid per ml and 5 μg of gentamycin per ml. Cells containing transposition products were identified on similar agar plates to which 40 μg of kanamycin per ml was added.

The bacteriophage λ infection assay has been described previously (12). The assay measures transposition of Tn*5* from an infecting λ phage to the chromosome of the host cell. λ NK467 is conditionally defective for DNA replication and is unable to lysogenize the host. Recovery of the Tn*5* kanamycin resistance, therefore, indicates a transposition event. Aliquots of stationary-phase cultures of MC1061 containing various plasmid derivatives of IS*50*R were infected with λ NK467 at a multiplicity of infection of 0.2. The mixture was incubated at 37°C for 15 min for phage absorption, LB medium was added, and incubation was continued for 30 min to allow the expression of kanamycin resistance. The cells were diluted and plated on LB plates with 40 μg of kanamycin per ml.

In both assays, dilutions were used that resulted in approximately 50 to 500 colonies per plate. Assays were performed in quintuplicate, and the standard deviations are indicated in the results (see Fig. 2, 4, and 5). The variation within one set of assays was usually less than twofold.

Transposase purification. The purification of Tn*5* transposase will be described in detail elsewhere (4). In summary, transposase and the two EK mutant derivatives were overproduced in strain DH5α from pRZ7015, pRZ7015 EK110, and pRZ7015 EK345 by heat induction. Exponentially growing cultures were shifted from 32 to 42°C for 30 min, which causes denaturation of the bacteriophage λ CI857 repressor and activates the λ P_R promoter. All following steps were performed at 4°C. One-liter batches of cells were harvested, resuspended in 0.1 M NaCl-TEG (20 mM Tris, 2 mM EDTA, 10% glycerol, 0.1% Triton X-100), and passed through a French pressure cell twice at 16,000 lb/in^2. The supernatant from successive 26,000 × *g* and 100,000 × *g* spins was precipitated by addition of ammonium sulfate to 47% saturation. The pellets were resuspended in 0.4 M NaCl-TEG and dialyzed overnight. Crude transposase fractions were loaded onto a 10-ml heparin-agarose column and purified by elution with a 0.4 to 1.2 M NaCl gradient on a fast protein liquid chromatography system (Pharmacia LKB Biotechnology, Uppsala, Sweden). The protein peak fractions were adjusted to 20% glycerol–10 mM $MgCl_2$ and quick frozen in liquid N_2. A modified Bradford assay (2) was used to determine the protein concentrations, which were routinely about 0.5 mg/ml. Wild-type and mutant transposase species showed very similar purification profiles (data not shown).

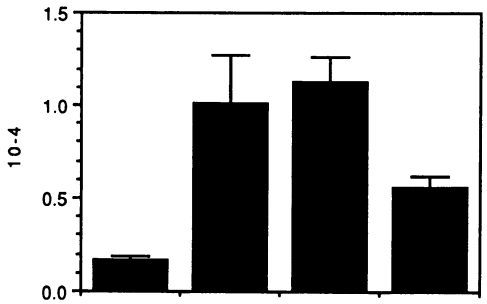
Immunoblotting of IS*50*-encoded proteins. MC1061 cells carrying IS*50*R plasmids were grown in Luria broth with 100 μg of ampicillin per ml at 37°C to an A_{600} of approximately 0.5. The cells were pelleted and resuspended in protein gel loading buffer to contain 0.1 A_{600} equivalent of cells per 20 μl (pRZ7013 and pRZ7016) or a 10-fold higher cell concentration (pRZ7024). The samples were incubated at 100°C for 5 min and sonicated for 30 s, and 20-μl aliquots were loaded onto a 7% polyacrylamide gel. After electrophoretic separation, the proteins were electroblotted to nitrocellulose. The blot was incubated with rabbit antitransposase antibody and then with goat anti-rabbit immunoglobulin G–alkaline phosphatase conjugate. Antibody complexes were visualized with 5-bromo-4-chloro-3-indolylphosphate and nitroblue tetrazolium.

Gel retardation assay. A total of 0.5 to 2 μg (0.1 to 0.4 pmol) of purified transposase proteins was incubated with 1.5 ng (8×10^{-3} pmol) of ^{32}P-labelled 266-bp fragment of DNA, containing the outside end of Tn*5*, in 20-μl reactions at the final concentration of 20 mM PO_4 buffer (pH 7.5)–1 mM dithiothreitol–0.5 mM EDTA–5% bovine serum albumin–100 mM potassium glutamate–0.1% Triton X-100–240-fold weight excess of unlabeled nonspecific competitor DNA. The reactions were incubated at 30°C for 30 min, glycerol was added to 4%, and 10-μl aliquots were analyzed by gel electrophoresis. Then, 1.2-mm-thick 5% (39:1) polyacrylamide gels were run in 0.5× Tris-borate-EDTA (TBE) at 8 W for 90 min at 4°C. Gel electrophoresis at room temperature resulted in a smearing of the retarded complexes (data not shown). Gels were dried, quantitated with a Betascope 603 blot analyzer (Betagen Corporation, Waltham, Mass.), and exposed to X-ray film overnight.

RESULTS

IS*50*R EK mutations result in increased transposition frequencies. In order to screen for mutations in IS*50*R that increase transposition frequencies, the papillation assay (14) was used. In this transposition assay, Tn*5*-mediated DNA rearrangements are visualized by the activation of a silent β-galactosidase gene embedded in the transposon (Tn*5* ORF*lac*). Eight hypertransposing IS*50*R mutants were isolated after diethyl sulfate mutagenesis of the papillation vector pRZ620 (Tn*5* ORF*lac* on ColE1, Fig. 1B) by screening for colonies with an increased number of papillae on lactose-MacConkey indicator plates. Three candidates increased papillation drastically, while the other five mutants showed only a modest effect.

Subcloning of IS*50*R restriction fragments from the three candidates with strong phenotypes followed by sequence analysis identified two separate mutations. Both mutations were found in the reading frame shared by transposase and inhibitor and are G to A transitions, resulting in glutamate to lysine substitutions. The mutations are located at amino



Trp. Freq.	1.68±0.23 x10^{-5}	1.01±0.26 x10^{-4}	1.13±0.13 x10^{-4}	5.56±0.57 x10^{-5}
Relative Trp. Freq.	1	6.0	6.7	3.3

FIG. 2. Transposition frequencies of Tn*5* and EK mutant derivatives. The plasmid used in this assay is pRZ620, a ColEI vector carrying Tn*5* ORF*lac*. Transposition frequencies were determined by mating donor RZ212 cells containing pRZ620 (column 1), pRZ620 EK110 (column 2), pRZ620 EK345 (column 3), or pRZ620 EK110/345 (column 4) with recipient RZ211 cells. The bars indicate the average of the transposition frequencies measured in quintuplicate, and the error bars represent the standard deviations. The numerical averages of transposition frequencies and standard deviations are listed below each column. The bottom row shows the increase in transposition frequencies of the EK mutants over that of wild-type transposase.

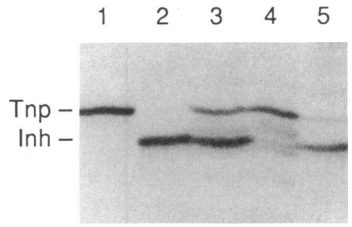


FIG. 3. Immunoblotting of proteins encoded on IS*50*R and IS*50*R MA56. MC1061 cells containing IS*50*R plasmids were heated in sample buffer, electrophoresed on a polyacrylamide gel (0.1% SDS, 3% stacker, 7% running gel), and electrotransferred to nitrocellulose paper. IS*50*R-encoded proteins were detected by using rabbit antitransposase as first antibody and goat anti-rabbit alkaline phosphatase as second antibody. Lanes 1 and 2 contain 0.25 μg of purified transposase and inhibitor protein, respectively. Lanes 3 and 4 show IS*50*R proteins from 0.1 A_{600} equivalent of cells carrying pRZ7013 (IS*50*R on pBKS-) and pRZ7016 (IS*50*R MA56 on pBKS-). Lane 5 contains IS*50*R proteins from a 1.0 A_{600} equivalent of cells carrying pRZ7024 (IS*50*R on pBR322). Transposase (Tnp) and inhibitor (Inh) bands are indicated. Note that 10-fold more cells were used in lane 5 than in lanes 3 and 4.

acids 110 (EK110) and 345 (EK345) of the transposase protein (Fig. 1A).

Transposition frequencies of the mutants were determined by the mating-out assay in which transposition of Tn*5* from a plasmid to the F factor is scored. Figure 2 shows that the transposition frequencies of both EK mutations are increased by about sixfold compared with those of the wild type. Throughout this study, the EK345 mutant consistently displays a stronger phenotype than does EK110, although the difference in transposition frequencies in this experiment falls within the observed error.

To address the question of whether the two mutations work independently of each other, we constructed the double mutant pRZ620 EK110/345. If the mutations act independently during transposition and no other step becomes rate limiting, the transposition frequency of the double mutant should be higher than the frequencies of the single mutants. Figure 2, column 4, however, shows that the increase in transposition of the EK110/345 double mutant is smaller than the increase of either individual mutant. This result indicates that the two EK mutations do not act independently of each other, even though they are quite separated on the linear amino acid sequence.

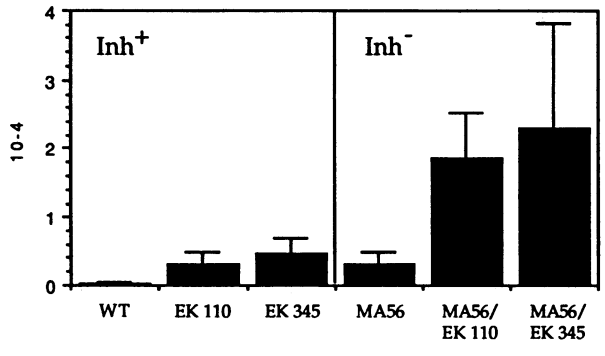
Increased transposition frequencies of EK mutations are caused by changes in transposase. Both EK mutations are located in the part of the reading frame that is shared by transposase and inhibitor. Therefore, each mutation results in the alteration of two proteins that are involved in transposition. The observed increase of transposition frequency could be caused either by the change in the transposase molecule, increasing its stability or activity, or by the alteration in the inhibitor, resulting in a decrease of its inhibitory function. To distinguish between these possibilities, we constructed an IS*50*R derivative that does not express the inhibitor. If the phenotype of the EK mutations is caused by the amino acid substitution in transposase, then the hypertransposing phenotype should not depend on the presence of inhibitor.

We used site-directed mutagenesis to abolish expression of the inhibitor protein. The AUG start codon of inhibitor-encoding methionine was changed to GCC, the codon for alanine. Unavoidably, this MA56 mutation also changes the amino acid sequence of transposase, substituting methionine with alanine at position 56 (Fig. 1A).

To test whether MA56 constructs indeed fail to express the inhibitor protein, we used immunoblotting analysis with antibodies that were raised against transposase and cross-react with the inhibitor. Figure 3 shows a Western blot (immunoblot) of IS*50*R proteins expressed from high-copy-number plasmids with the wild-type inhibitor start codon (lane 3) or the MA56 mutation (lane 4). Comparison of lanes 3 and 4 confirms that no inhibitor is expressed from MA56 constructs and that transposase stability is not affected by the missense mutation. Lane 5 contains IS*50*R proteins expressed from a medium-copy-number plasmid and was loaded with a 10-fold higher concentration of cells. This lane indicates that the amounts of transposase and inhibitor in the cell are dependent on the copy number of the IS*50*R plasmid (see below). The shift in the ratio of transposase to inhibitor could be due to a lower methylation state of the high-copy-number plasmid which would favor transposase expression.

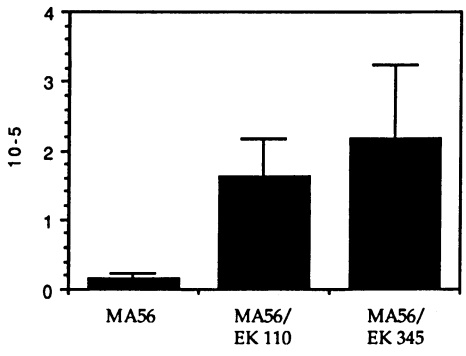
To compare transposition frequencies in the presence and absence of inhibitor for wild-type and EK mutant transposase proteins, we constructed two sets of plasmids. The plasmids contain wild-type Tn*5* or the EK derivatives cloned onto a high-copy-number vector. One set of plasmids carries the wild-type inhibitor start codon (pRZ7017), whereas the other set contains the MA56 mutation (pRZ7018).

The MA56 mutation by itself causes a 10-fold increase in Tn*5* transposition (Fig. 4, columns 1 and 4). This result is consistent with the loss of the inhibitor protein and suggests that the alanine at position 56 does not greatly alter the transposase function. The EK110 and EK345 mutations increase transposition 10- and 16-fold, respectively, when



	WT	EK 110	EK 345	MA56	MA56/ EK 110	MA56/ EK 345
Trp. Freq.	2.96±2.16 x10^{-6}	3.01±1.89 x10^{-5}	4.74±2.23 x10^{-5}	3.14±1.88 x10^{-5}	1.85±0.69 x10^{-4}	2.29±1.53 x10^{-4}
Relative Trp. Freq.	1	10.2	16.0	10.6	62.5	77.4

FIG. 4. Tn*5* transposition frequencies in the presence and absence of inhibitor. The mating-out assay was used to determine whether the increase of transposition caused by the EK mutations is dependent on the presence of inhibitor protein. From left to right, the plasmids used were pRZ7017, pRZ7017 EK110, pRZ7017 EK345 (Tn*5* on pTZ19U expressing both transposase and inhibitor), pRZ7018, pRZ7018 EK110, and pRZ7018 EK345 (Tn*5* MA56 on pTZ19U expressing only transposase). Relative transposition frequencies are normalized to transposition of wild-type Tn*5*.



	MA56	MA56/ EK 110	MA56/ EK 345
Trp. Freq.	1.45±0.88 x10^{-6}	1.63±0.53 x10^{-5}	2.18±1.07 x10^{-5}
Relative Trp. Freq.	1	11.2	15.0
Trans-Activity (%)	4.6	8.8	9.5

FIG. 5. Wild-type and EK transposase activities in *trans*. This assay is a modification of the mating-out transposition assay. Transposase is expressed from pRZ7016 (IS*50*R MA56 on pBKS-) and *trans*-activates transposition of a reporter element from a second compatible plasmid, pRZ4711 (outside ends flanking a kanamycin resistance gene on pACYC184), to the F factor. The transposase-expressing plasmids contain the MA56 mutation to prevent inhibitor expression from interfering with the assay and were (from left to right) pRZ7016, pRZ7016 EK110, and pRZ7016 EK345. At the bottom of the figure is a list of the relative increases in transposition for the EK mutations and the *trans* activities of the transposase proteins compared with their corresponding *cis* activities (Fig. 4, columns 4 to 6). *Trans* activity is calculated as the ratio of transposition frequency in *trans* to the frequency in *cis* times 100 and is expressed in percent.

measured in the presence of inhibitor (Fig. 4, columns 2 and 3). These transposition increases are greater than those observed for Fig. 2, presumably due to the higher copy number of the transposon carrying plasmids in this experiment (see Discussion). The corresponding MA56/EK double mutants show increases of six- and sevenfold over the MA56 construct (Fig. 4, column 4 versus columns 5 and 6), so most of the effect of the EK mutations is not dependent on the presence of the inhibitor protein. We conclude that the presence of the inhibitor protein is not necessary for the hypertransposing phenotype. It must, therefore, be due to the alteration in the transposase protein.

Activities of transposase EK mutations are increased in *trans*. Transposase activates transposition to a greater extent when the protein is expressed on the same replicon that contains the transposon ends (*cis* complementation) compared with activating transposition of an element on a separate replicon (*trans* complementation). After the observation that the EK mutations increase the transposition frequency in *cis*, we wanted to test whether this increase is also manifested in a *trans*-complementing situation.

To test this *trans* activity, transposase was expressed from IS*50*R on a high-copy plasmid, pRZ7016. All of the IS*50*R constructs in this experiment carry the MA56 mutation, allowing only transposase and not inhibitor expression, which simplifies the interpretation of the results. Transposition was scored as the movement of a kanamycin resistance gene flanked by two Tn*5* outside ends from a second, compatible plasmid (pRZ4711) to the F factor of the host cell. This reporter Tn*5* element does not encode transposase or inhibitor.

Figure 5 shows the results of this *trans*-activity assay. As expected, the level of transposition promoted in *trans* is about 20-fold lower than that in *cis* (compare Fig. 4, column 4, with Fig. 5, column 1). The comparison of *cis*-activated transposition to frequencies determined in *trans* is listed as the *trans* activity for each transposase protein. Note that the *trans* activities for the EK110 and EK345 mutants are 8.8 and 9.5% of their respective *cis* activities compared with 4.6% for the control transposase. Therefore, the mutations increase transposition in *trans*, and the relative increase is even larger than that in *cis*. These increased *trans* activities of the EK transposase mutants, although consistently observed in additional assays, vary between 8 and 30% of the respective *cis* activities. The reason for this variability is not known.

Binding of EK mutant transposase proteins to outside end DNA reveals a novel retarded complex. The fact that we observed a greater transposition increase in *trans* than in *cis* led us to believe that the EK mutations affect an early step in the transposition process, perhaps enhancing the affinity of transposase for the outside ends. Once the predicted complex of transposase and the transposon end DNA is formed, *cis* and *trans* complementation of transposition should become indistinguishable. Therefore, we wanted to compare the specific binding of wild-type and EK mutant transposase proteins with the outside end DNA of Tn*5*.

The EK mutations were cloned onto a transposase-overproducing plasmid, pRZ7015. In this construct, transposase is under the control of the bacteriophage λ P_r promoter, which can be activated by denaturation of the temperature-sensitive λ CI 857 protein at 42°C. After overexpression,

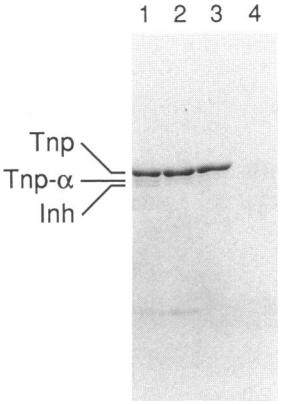


FIG. 6. Polyacrylamide gel electrophoresis of purified transposase proteins. Wild-type transposase and the two EK mutant proteins were overproduced in DH5α cells from pRZ7015 plasmids and purified by medium-pressure heparin-agarose column chromatography. The protein fractions (2.5 μg) were heated in sample buffer, electrophoresed on a polyacrylamide gel (0.1% SDS, 3% stacker, 10% running gel), and stained with Coomassie blue. Lanes: 1, wild-type transposase; 2, EK110 transposase; 3, EK345 transposase; 4, proteins prepared identically from DH5α pBR322 cells as control for host protein contaminations. This lane was loaded with equal volume rather than equal protein concentration. The transposase (Tnp) and two minor contaminating species, inhibitor (Inh) and the transposase degradation product (Tnp-α), bands are indicated.

wild-type and mutant transposase proteins were purified by heparin-agarose column chromatography (4). A Coomassie-stained polyacrylamide gel of the purified proteins shows that transposase represents approximately 80% of the total protein (Fig. 6). The remaining species are contaminating inhibitor and Tnp-α, a proteolytic cleavage product of transposase (3).

Increasing amounts of purified transposase were incubated with end-labeled DNA containing the outside end of Tn*5*, and the reactions were then separated on a nondenaturing polyacrylamide gel. Figure 7A shows that transposase forms specific complexes with the outside end DNA. The migration of the DNA fragment in the absence of proteins (lane 1) and in the presence of mock extracts, purified from cells that did not express transposase (lane 11), are shown for comparison. Incubation of outside end DNA with transposase resulted in the formation of a unique retarded complex (complex I, lanes 2 to 4). When EK mutant transposase proteins were tested for DNA binding, an additional, slightly faster migrating complex appeared (complex II, lanes 5 to 10).

The gel was scanned with a Betascope blot analyzer, and the distribution of the ^{32}P label is summarized in Fig. 7B. This quantitation and overexposure of the gel (data not shown) indicate that both complexes are present in both wild-type and mutant transposase retardation reactions. Measuring the total retardation of free DNA into complexes I and II, we found that the DNA-binding affinities of the EK mutant transposase proteins are slightly lower than those of wild-type. The ratio of complex II to complex I, however, is much greater for the EK mutants: 0.33 for EK110 and 0.63 for EK345 compared with 0.06 for wild-type transposase. Notice that EK345, the mutant with the stronger genetic phenotype, results reproducibly in a greater complex II-to-complex I ratio than did the weaker EK110 mutant. This indicates a correlation of complex II formation rather than total DNA-binding activity with the genetically determined transposition frequency. We believe that this retardation pattern might lead to an explanation for the action of the mutations, and we are currently investigating the nature of complex II.

Transposase as inhibitor of Tn*5* transposition. After constructing the MA56 mutation, we showed that the replacement of the inhibitor start codon resulted in the physical absence of the protein on a Western blot (see above). A second assay was then performed to confirm the functional absence of an inhibiting factor. We chose the bacteriophage λ infection assay, which was originally used to identify the function of the inhibitor protein (12).

Cells were infected with Tn*5* inserted into a genetically defective bacteriophage λ vector (λ NK467) which can neither replicate nor integrate into the host chromosome. The infecting λ Tn*5* DNA is undermethylated, a condition which allows very little inhibitor protein expression and results in an initial burst of transposase production (18). Transposition events driven by this transposase were scored as the recovery of kanamycin-resistant host cells after infection. However, when a plasmid expressing inhibitor protein at a steady-state level is present in the cell, Tn*5* transposition from the λ phage is greatly reduced (12). To test the effect of different levels of Tn*5* proteins, we used both medium- and high-copy-number plasmids (see Fig. 3).

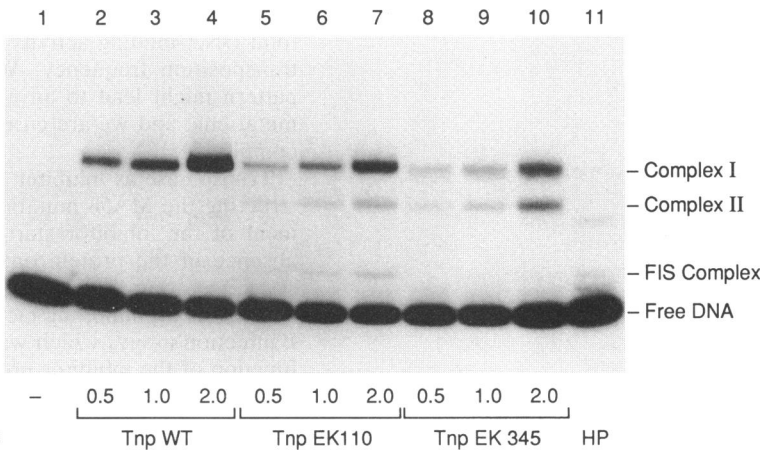
Transposition frequencies in cells carrying plasmids with the following features were compared: vectors containing IS*50* end sequences but expressing no Tn*5* proteins as a negative control (pRZ7039 and pRZ7038), vectors containing IS*50*R expressing both transposase and inhibitor (pRZ7024 and pRZ7013), and vectors containing IS*50*R MA56 expressing only transposase (pRZ7029 and pRZ7016).

As expected, transposition decreases about 50-fold when both transposase and inhibitor are expressed at steady-state levels from a medium-copy-number plasmid (Fig. 8A, columns 1 and 2). Since transposase is mainly *cis* acting, its expression from the plasmid should not significantly activate transposition of the λ Tn*5* element and, therefore, not interfere with the assay.

To our surprise, we found that transposition is decreased, even in the absence of the inhibitor protein. When transposase, but not inhibitor, is expressed from the plasmid, transposition is threefold lower than that in control cells not containing Tn*5* proteins (compare Fig. 8A column 5 and column 1). This result is more pronounced when the Tn*5* proteins are present at higher concentrations by expression from high-copy-number plasmids. Transposition in the presence of a higher steady-state level of transposase reaches only 3% of the frequency measured for the control cells (Fig. 8B, columns 5 and 1). We interpret this phenomenon as evidence that Tn*5* transposase has two functions: activation of transposition in *cis* and inhibition of transposition in *trans*.

We then tested the EK transposase mutants for this inhibitory effect and observed approximately twofold higher transposition frequencies, and, therefore, lower inhibition, than measured with the corresponding wild-type proteins. The transposition increase was observed both with the absence of inhibitor protein (Fig. 8, columns 6 and 7) and when inhibitor and transposase were expressed in steady-state levels (Fig. 8, columns 3 and 4). This indicates that the EK mutations reduce the inhibitory effect of transposase, although we have not ruled out that this result might be

A



B

Transposase Gel Retardation

Protein		WT			EK110			EK345		
(μg)		0.5	1	2	0.5	1	2	0.5	1	2
Free DNA	(%)	88.5	82.4	64.8	93.0	88.4	77.1	93.2	89.5	81.3
Retarded DNA	(%)	11.5	17.6	35.2	7.0	11.6	22.9	6.8	10.5	18.7
Complex I	(%)	11.0	16.5	33.3	5.3	8.7	17.0	4.4	6.5	10.8
Complex II	(%)	0.5	1.1	1.9	1.7	2.9	5.9	2.4	4.0	7.9
Average Ratio of Com.II/Com.I		0.06±0.01			0.33±0.01			0.63±0.09		

FIG. 7. Gel retardation of Tn*5* outside end DNA by wild-type and EK transposase proteins. (A) A representative autoradiograph of a 5% polyacrylamide gel run in 0.5% TBE at 4°C. Lane 1 shows the ^{32}P-labelled 266-bp DNA fragment containing the Tn*5* outside end in the absence of added proteins. The remaining lanes show the retardation of this fragment after a 30-min incubation with increasing amounts of protein. Lane 11 represents a negative control reaction of DH5α pBR322 host proteins, equivalent to the amount of contamination in the lanes with the highest transposase concentration (lanes 4, 7, and 10). One of the retarded bands was identified as a complex formed by the host Fis protein (25). The origin of the other two complexes is unknown. A total of 0.5, 1, and 2 μg of wild-type transposase (lanes 2 to 4), EK110 transposase (lanes 5 to 7), and EK345 transposase (lanes 8 to 10) were incubated with the DNA. Two specifically retarded bands appear and are marked as complex I and complex II. (B) The autoradiograph was quantitated by using a Betascope blot analyzer, and the relative distributions of the ^{32}P-labelled DNA fragment are listed. The number of total counts per lane ranged from 5.7×10^5 to 8.3×10^5. The ratio of band intensity of complex II to that of complex I was calculated for each reaction, and the averages are listed for wild-type and EK mutant transposase proteins.

partially due to the increased *trans* activity of the mutant transposase proteins.

DISCUSSION

This study independently confirms the recent finding that Tn*5* transposase is not only a *cis*-acting activator of the transposition process but also a *trans*-active inhibitor (6). This observation uncovers yet another layer of regulation to modulate the frequency of Tn*5* transposition. Transposable elements need to prevent uncontrolled transposition, which would be detrimental for the host cell and, in turn, for the transposon itself.

One regulatory mechanism to control Tn*5* transposition is the limitation of transposase expression (13). An additional level of control is the regulation of transposase activity by the inhibitor protein. This protein is missing critical amino acids required for transposition activation but retains enough similarity to transposase to be involved in, and thereby interfere with, the transposition reaction.

This paper presents a novel aspect of the regulation of transposase activity. The transposase protein itself was found to inhibit transposition and can thereby act as the functional equivalent of the inhibitor protein. Western blots ruled out the possibility that transposase is simply proteolytically cleaved after translation to yield the 55-amino-acid-

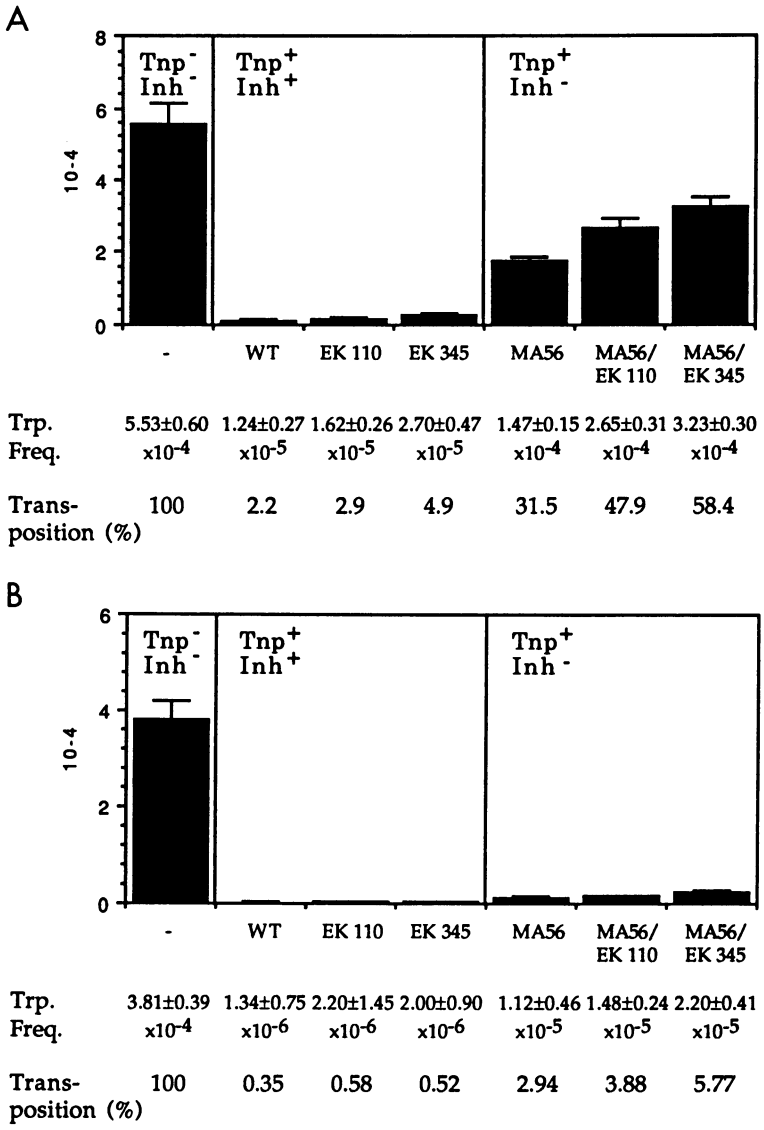


FIG. 8. Transposase inhibits transposition in *trans*. Transposition frequencies were determined by the bacteriophage λ infection assay which measures transposition of a wild-type Tn*5* from an infecting λ phage into the host cell chromosome. Plasmids in the cell were expressing either no IS*50*R proteins (column 1), transposase and inhibitor (columns 2 to 4), or transposase alone (columns 5 to 7) at steady-state levels. The assay measures the ability of these proteins and their EK mutant derivatives to interfere with the transposition reaction. The inhibition of transposition is expressed as percent transposition remaining relative to the control cells (column 1). (A) IS*50*R proteins are expressed from medium-copy-number pBR322-based plasmids. These plasmids are (from left to right) pRZ7039, pRZ7024, pRZ7024 EK110, pRZ7024 EK345, pRZ7029, pRZ7029 EK110, and pRZ7029 EK345. (B) IS*50*R proteins are expressed from high-copy-number pBKS-based plasmids. These plasmids are (from left to right) pRZ7038, pRZ7013, pRZ7013 EK110, pRZ7013 EK345, pRZ7016, pRZ7016 EK110, and pRZ7016 EK345.

shorter inhibitor protein. Since there seems to be no feedback regulation for either transposase or inhibitor protein expression, the inhibitory function of transposase would prevent uncontrolled transposition in the case of accidental, or deliberate, transposase overproduction. The transposase mutations characterized in this paper partially overcome this regulation, resulting in a hypertransposing phenotype.

To explain these observations, we based a model on the rationale that the mechanism of transposase-mediated inhibition is likely to be similar to the mechanism of the inhibitor protein. The inhibitor could act in two ways: by binding to the transposon ends and occluding these sites from interaction with transposase or by contacting the transposase protein directly to form inactive hetero-oligomers. Preliminary DNA-binding experiments show that the inhibitor protein does not bind specifically to the transposase-binding site (3), which argues against the first mechanism. Favoring the second possibility are the following considerations. We expect that transposase contains a multimerization site. Oligomerization during the transposition process would allow the interaction of the transposon ends (9). Indeed, transposase elutes as an apparent tetramer when it is passed through a gel exclusion column (4). Since the inhibitor protein consists of the carboxy-terminal 9/10th of the transposase, it seems reasonable that the oligomerization site is shared by these two proteins. Furthermore, indirect evidence that inhibitor contacts transposase in vivo was presented (5).

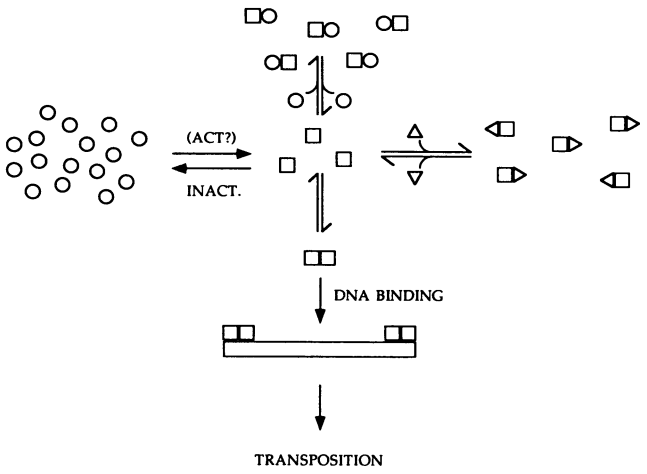


FIG. 9. Model of transposition regulation. Transposase is present in the cell in two forms: a *cis*-activating protein and a *trans*-inhibiting species. Transposition is inhibited by the formation of hetero-oligomers of active transposase (□) with inhibitor (△) and with the inactive transposase (○) protein (for details, see Discussion).

In Fig. 9, we present a model of transposition regulation which is consistent with the observed data. We postulate the existence of two different transposase species: active transposase capable of promoting transposition and inactive transposase which has inhibitory function. The active transposase oligomerizes, interacts with the transposon ends, and carries out the reactions necessary for transposition. The inhibitor binds to the active transposase monomers and forms inactive hetero-oligomers. This diminishes the concentration of the active species and thereby reduces the frequency of transposition. The inactive transposase species exerts its inhibitory effect in the same fashion.

The existence of two transposition-inhibiting protein species may explain why we observe only a limited increase of the transposition frequency in the absence of the inhibitor protein (Fig. 4). The model predicts that both inhibitor and inhibiting transposase need to be absent to allow a further increase. However, we have not ruled out the possibility that the alanine missense mutation in transposase results in decreased activity or that host factors become rate limiting for the transposition reaction.

The model leaves open whether initially inactive transposase is made, which needs to be activated, or whether active transposase is the first product, which subsequently is inactivated. We favor the second possibility since it would explain why transposase is *cis* activating but *trans* inhibiting, i.e., by the time newly translated transposase reaches Tn*5* end sequences on a second replicon, most of the transposase would be inactivated.

This inactivation step could be due to a wide array of transposase alterations, and it may or may not be dependent on host factors. Transposase could, for example, be phosphorylated or dephosphorylated, undergo a conformational change, or be proteolytically cleaved. We routinely observe a transposase degradation product, Tnp-α, in purified transposase preparations (Fig. 6). Preliminary experiments indicate that Tnp-α is caused by cleavage in the N-terminal 55 residues specific to transposase (3). We are testing the possibility that Tnp-α is the inhibiting transposase species.

The presented model also provides an explanation for the phenomenon that the frequency of Tn*5* transposition in the cell is fairly independent of the copy number of the plasmid on which the transposon is located (10). Since the plasmid copy number is directly related to the number of donor sites for the transposition process, a constant overall transposition frequency must result from a decrease of transposition per transposable element. According to our model, a high-copy-number plasmid carrying Tn*5* leads to the buildup of a high concentration of the *trans*-active inhibiting transposase and inhibitor protein species in the cell. The local concentration of the *cis*-acting active transposase around each transposon, however, remains similar to the low-copy-plasmid situation. The shift in ratio of activating versus inhibiting molecules leads thereby to a decrease in transposition frequency for each individual transposable element.

This buildup of *trans*-active inhibiting transposase is also the reason why the effect of inhibiting Tn*5* transposition from an infecting bacteriophage λ, demonstrated in Fig. 8 and 9, is more pronounced when the IS*50*R proteins are expressed from a high-copy-number plasmid.

How does the proposed model of transposition regulation explain the phenotype of the EK mutations? One possibility that fits the observations is that the EK mutations shift the ratio of activating versus inhibiting transposase, for example, by delaying the inactivation process. This would result not only in the observed increase in transposition frequency but also in a greater increase in *trans*, since the active transposase has more time to reach transposon DNA end sequences located on a different replicon.

We, furthermore, observed that the EK mutations show a greater transposition increase when they are present on a high-copy-number plasmid (compare Fig. 2 and 4). This is the expected result since the EK mutations would partially overcome the above-mentioned copy number effect.

The fact that the inhibition assay (Fig. 8) shows only a modest restoration of transposition frequencies for the EK mutations might be due to the specific design of the assay, in which we are providing wild-type transposase in *cis* on the λ phage. Therefore, we are observing only the effect of the decrease in inhibiting EK mutant transposase rather than the coupled increase in active and decrease in inhibiting transposase species. The higher transposition frequencies of the EK mutants in this assay, however, could also reflect the increased ability of the plasmid-encoded mutant transposase proteins to activate transposition of the λ Tn*5* element in *trans*.

Assuming a changed ratio of active to inactive EK mutant transposase, the gel retardation patterns can be interpreted in the following way. The upper retarded band (Fig. 7A, complex I), which is slightly weaker for the EK mutants, results from a nonproductive interaction of active and inactive transposase hetero-oligomers with the Tn*5* end sequences. The lower retarded band (complex II), appearing only faintly for the wild type and much stronger for the EK mutants, represents the DNA interaction with an active transposase complex. This interpretation is consistent with the observed correlation of the intensity of complex II with the genetically determined transposition frequency.

The altered migration of complex II could be due directly to the difference between the active and inactive transposase molecules: a change in protein conformation or molecular weight, for example. However, several other molecular explanations are possible. If DNA is bent after binding to transposase, then the two complexes could differ in the angle of this bend. Or complex II could contain a transposase-induced nick in the DNA. Finally, complex I could contain

more transposase molecules than complex II. We are currently testing these possibilities. Investigating the DNA-binding characteristics of the EK mutant transposase proteins could help to elucidate the early steps of the transposition process.

Finally, we are using the purified hyper-*trans*-active EK mutant transposase proteins to develop an in vitro assay for Tn*5* transposition. This assay is critical for a biochemical analysis of the molecular mechanism of the transposition reaction. To observe transposition in vitro, transposase protein will be added to outside end DNA sequences and the reaction will be tested for the formation of transposition products. We expect to see initially a very low signal, since *trans*-activated Tn*5* transposition occurs extremely infrequently in vivo. The hyper-*trans*-active character of the EK mutant transposase proteins, however, will increase our chances for the successful development of the Tn*5* in vitro transposition assay.

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