

The C-terminal α Helix of Tn5 Transposase Is Required for Synaptic Complex Formation*

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An important step in Tn5 transposition requires transposase-transposase homodimerization to form a synaptic complex competent for cleavage of transposon DNA free from the flanking sequence. We demonstrate that the C-terminal helix of Tn5 transposase (residues 458–468 of 476 total amino acids) is required for synaptic complex formation during Tn5 transposition. Specifically, deletion of eight amino acids or more from the C terminus greatly reduces or abolishes synaptic complex formation *in vitro*. Due to this impaired synaptic complex formation, transposases lacking eight amino acids are also defective in the cleavage step of transposition. Interactions within the synaptic complex dimer interface were investigated by site-directed mutagenesis, and residues required for synaptic complex formation include amino acids comprising the dimer interface in the Tn5 inhibitor x-ray crystal structure dimer. Because the crystal structure dimer was hypothesized to be the inhibitory complex and not a synaptic complex, this result was surprising. Based on these data, models for both *in vivo* and *in vitro* synaptic complex formation are presented.

Tn5 is a composite, prokaryotic transposon consisting of two IS50 elements (termed IS50R and IS50L) flanking a region of DNA encoding three antibiotic resistance genes. Tn5 is bracketed by 19-bp¹ inverted repeat transposase recognition sequences termed outside ends (OEs). Two OEs (or similar end sequences) are absolutely required for movement of the Tn5 transposon (1). IS50R encodes two proteins, a 476-amino acid (aa) *cis*-acting transposase (Tnp) protein, which is essential for transposition, and a 421-aa inhibitor protein (Inh), which inhibits transposition both in *cis* and in *trans* (2) by forming nonproductive multimers with Tnp (3). Because translation of Inh begins from a downstream, in-frame, independent start codon, Inh is identical to Tnp except that 55 aa on the N terminus are missing. IS50L encodes proteins P3 and P4 corresponding to Tnp and Inh of IS50R. These proteins are non-functional because of an ochre codon at residue Glu-451 (4, see Ref. 5 for review).

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¹ The abbreviations used are: bp, base pairs(s); Tnp, transposase; Tnp-FL, hyperactive, full-length Tn5 transposase; Inh, Tn5 transposase inhibitor protein; aa, amino acid(s); OE, outside end; ME, mosaic end; dbb, donor backbone; PEC, paired ends complex; PCR, polymerase chain reaction.

Tn5 transposes by a cut and paste mechanism (Fig. 1) (6). First, a monomer of Tn5 Tnp binds each OE sequence (7). Interaction of these bound Tnp monomers through Tnp-Tnp dimerization forms a complex nucleoprotein structure termed a synapse (3, 8). Nicking of one DNA strand then occurs via nucleophilic attack of a water molecule (activated by a Tnp-coordinated Mg²⁺) on the phosphodiester backbone between the +1 position of the OE and the –1 position of the flanking DNA (donor backbone). This released 3'-OH then attacks the opposite DNA strand creating a hairpin intermediate (8) and releasing the blunt-ended transposon from the donor backbone (dbb) DNA (6). A second activated water molecule then resolves the hairpin intermediate. Strand transfer then occurs via a transesterification reaction in which the 3'-OH groups of the transposon attack the phosphodiester backbone of the captured target DNA in a staggered fashion. Formation of a covalent bond between the oxygen of the 3'-OH groups of the transposon ends and the 5'-phosphate groups of the target integrates the transposon (10). Integration results in a 9-bp duplication of target DNA (4).

Application of this mechanism *in vitro* requires only four components; a transposon consisting of DNA flanked by Tn5 specific end sequences such as the OEs or hyperactive mosaic ends (MEs), a functional Tnp having hyperactive E54K and L372P mutations (referred to as Tnp-FL throughout this manuscript), transposition buffer containing Mg²⁺, and target DNA (6).

Understanding the correlation between the structure of Tn5 Tnp and protein function during transposition is important for elucidating the complete mechanism of transposition and understanding its regulation. Previous studies have implied a role for the N-terminal 113 amino acids of Tnp in OE DNA binding (7, 11), structurally determined the location of the DDE catalytic triad (12), and hypothesized aa 369–387 to be important for conformational stability (11), yet a role for the C-terminal region (aa 441–476) in transposition has not been elucidated nor has the region of Tnp required for synaptic complex formation (dimerization) been established.

Previous experiments hypothesized two regions of Tnp to be important for dimerization (11). The first, aa 114–314, encompasses the catalytic core of Tnp. The second, aa 441–476, includes the C-terminal α helix. These two dimerization domains were thought to have distinct functions; the catalytic dimerization domain was hypothesized to be required for synapsis whereas the C-terminal dimerization domain was necessary for inhibition. This theory was based, in part, on a functional domain comparison of Tn5 Tnp with Tn10 Tnp, a catalytically identical prokaryotic transposase. A homologous catalytic dimerization domain was detected in Tn10 Tnp, but the C-terminal dimerization domain was not found. Two different amino acid sequence alignments of Tn10 Tnp and Tn5 Tnp reveal no similarity between the two proteins in this C-termi-

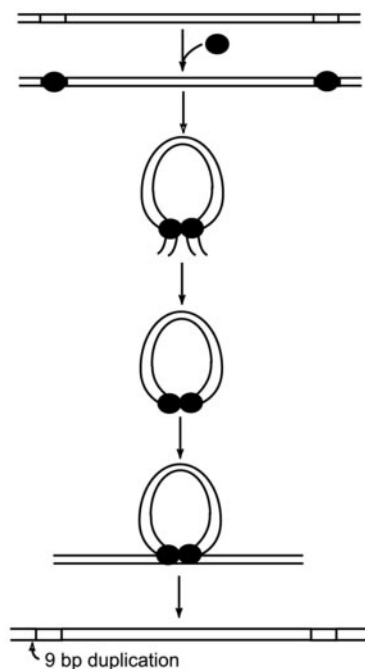


FIG. 1. **Mechanism of transposition.** Tn5 is transposed by a cut and paste mechanism. First a monomer of Tn5 transposase (Tnp) binds each OE. Interaction of these bound Tnp monomers through Tnp-Tnp dimerization forms a synapse. Cleavage of the transposon via a hairpin intermediate can then occur. This releases the transposon from donor DNA. Strand transfer via a transesterification reaction in which the 3'-OH groups of the transposon attack the phosphodiester backbone of the target DNA in a staggered fashion then occurs. This is followed by formation of a covalent bond between the oxygen of the 3'-OH groups of the transposon ends and the 5'-phosphate groups of the target. Formation of this bond integrates the transposon. Integration results in a 9-bp duplication of target DNA.

nal region (11).² Because Tn5 also encodes Inh, which inhibits transposition through production of nonfunctional Inh-Tnp multimers (3), and this type of regulation by dimerization to an inhibitor protein is unique to Tn5, it was concluded that the C-terminal dimerization domain was primarily important for inhibition.

This hypothesis was supported upon elucidation of the x-ray crystal structure of the Tn5 Inh dimer (12). In the dimer structure, residues in the C terminus of each monomer form a scissor-like dimerization interface (Fig. 2). The authors concluded that the structure could not represent the synaptic complex because the distance between monomer active sites in the dimer structure is 65 Å. If no conformational change occurs following cleavage and the transposon is inserted into target DNA with a 9-bp stagger, the active sites in the synaptic complex would be approximately 35 Å apart. Because a dramatic conformational change along multiple axes would be required for the C-terminal dimerization helix to be involved in synaptic complex formation, the authors hypothesized the dimer structure to be the inhibitory complex. Investigation of this hypothesis by analysis of a site-directed mutant (A466D) confirmed the requirement of dimerization of the C-terminal α helices for inhibition (13).

To determine if the C-terminal dimerization region was only required for inhibition, a complete set of C-terminal Tnp deletion mutants was constructed by *in vitro* transposition (14), and their phenotypes were determined *in vivo* (data not shown). After screening 1000 colonies, only deletions C-terminally truncated by seven amino acids or less were found to be

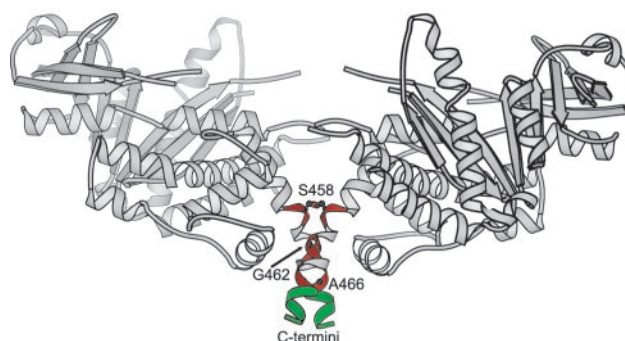


FIG. 2. **Location of C-terminal Tnp mutants.** The x-ray crystal structure of the Tn5 inhibitor (Inh) dimer is shown (12). C-terminal residues Ser-458, Gly-462, and Ala-466 were mutated to disrupt the C-terminal dimerization interface. These residues are colored red, and their side chains are represented as "ball and stick" in each Inh monomer. The dimer interface interactions are primarily hydrophobic, and the crossover point of the two C-terminal helices within the interface is at residue Gly-462. Dimerization of the C-terminal α helices brings Gly-462 and Ala-466 residues from each monomer into close proximity. Another important interaction includes hydrogen bonding of each Ser-458 residue with the Lys-459 residue (not shown) of the opposite monomer. Residues shown in green were deleted progressively (from the C terminus) to determine the number of C-terminal residues required for a functional Tnp. All remaining residues shown in gray were not mutated.

functional (data not shown). These experiments established a requirement for the C terminus in transposition.

In this manuscript we present evidence that the C-terminal α helix of Tnp is required for both *in vivo* and *in vitro* transposition; deletion of eight or more amino acids from this helix resulted in loss of function. We determined that the C-terminal α helix is necessary for synapsis, but does not directly affect the cleavage step of transposition. Investigation of the synapsis dimer interface revealed interactions identical with those seen in Inh dimer crystal structure. This inferred that the interface observed in the Inh x-ray structure is required for synaptic complex formation.

EXPERIMENTAL PROCEDURES

Plasmids and Protein Purification—All DNA manipulations were performed in *Escherichia coli* strain DH5 α (15). All DNA polymerase chain reaction (PCR) primers were obtained from Integrated DNA Technologies. All mutant Tnp constructs contain hyperactive E54K and L372P mutations (7, 16) and the M56A mutation (17), which prevents translation of the Inh protein. C-terminal point mutations and truncations were first cloned into pET-21d(+) (Novagen) for *in vivo* analysis. C-terminal truncations were constructed by amplifying the bases corresponding to aa 1 to the truncation point from pRZ10300 by PCR using Pfu polymerase (Stratagene), a primer which amplified the N-terminal *Xba*I site of pRZ10300 and an *Eco*RI-tailed primer. The PCR products were digested with *Xba*I (Promega) and *Eco*RI (Promega) and cloned into the large *Xba*I-*Eco*RI fragment of pET-21d(+). pRZ10300 was originally constructed by L. Mahnke as follows. The *Xba*I-*Sal*I fragment from pRZ7075 (18) was cloned into the large *Xba*I-*Sal*I fragment from pBAD18-Cm (19) to create pRZ10250. The hyperactive E54K and L372P mutations were then introduced by substituting the *Xba*I-*Bgl*II Tnp fragment from pRZPET2 (6) into the large *Xba*I-*Bgl*II fragment of pRZ10250 to construct pRZ10300.

Point mutants G462D and S458A were constructed by overlap PCR (20). Bases corresponding to the C-terminal region of Tnp (aa 301–476) were amplified from pRZPET2 by PCR using Pfu polymerase and internal mismatched primers containing the point mutation. The external primers included the *Nhe*I site in Tnp and the *Dra*III site C-terminal of the Tnp gene. PCR products were digested with *Nhe*I (New England Biolabs) and *Dra*III (New England Biolabs) and ligated to the large *Nhe*I-*Dra*III fragment of pRZPET2.

The point mutation A466D was originally cloned into pRZ7075 by L. Mahnke as follows. Overlap PCR using mismatched internal primers was used to amplify bases corresponding to the C-terminal region of Tnp. External primers included the N-terminal *Nhe*I site of Tnp and the C-terminal *Bcl*I site of Tnp. The PCR product was digested with *Nhe*I

² M. Steiniger-White, unpublished data.

and *BclI* (New England Biolabs) and cloned into the large *NheI*-*BclI* fragment of pRZ7075 to create pRZ10350. For this study, E54K and L372P mutations were incorporated by digesting both pGRPET2 and pRZ10350 with *PvuI* (Amersham Pharmacia Biotech) and *AluNI* (New England Biolabs). The *PvuI*-*AluNI* fragment from pRZ10350 containing the A466D mutation was then ligated to an 847-bp *PvuI*-*AluNI* fragment from pRZPET2 and a 456-bp *AluNI*-*AluNI* fragment from pRZPET2 containing the E54K and L372P mutations.

All C-terminal point mutations and truncations were also substituted into pGRTYB35 (9) for protein purification. The C-terminal region of each mutant was amplified by PCR using a primer that included the *NheI* site of Tnp and a *KpnI*-tailed primer. Each PCR product was digested with *NheI* and *KpnI* (New England Biolabs) and ligated to the large *NheI*-*KpnI* fragment of pGRTYB35. All mutants were purified as described previously (9). It should be noted that proteins expressed from this vector each contain one extra C-terminal glycine residue.

For purposes of this manuscript, C-terminal truncations will be denoted by Tnp Δ followed by the number of amino acids remaining in the transposase, the C-terminal point mutations will be denoted as Tnp followed by the amino acid change and the amino acid number, and the full-length Tnp containing just E54K, L372P, and M56A mutations will be referred to as Tnp-FL (see Table I).

In Vivo Transposition Assay—*In vivo* transposition frequencies were determined by a mating out assay similar to that described previously (17). R212Z donor cells containing an F-factor, pOX38-Gen (17), a transposon (encoding chloramphenicol resistance) containing plasmid, pFMA50–187 (8), and a pET-21d(+) plasmid expressing a C-terminal mutant Tnp (see above) were grown overnight at 37 °C and mixed in a 1:1 ratio with an overnight culture of recipient cells, 14R525 (21). Because T7 polymerase was not present in R212Z cells, expression of mutant Tnps is due to read-through transcription from neighboring genes. The level of protein produced under these conditions keeps the concentration of Tnp below toxic levels while also allowing investigation by *in vivo* assays. After 5 h of gentle shaking at 37 °C, aliquots of the cells were plated on Luria-Bertani (LB) agar containing gentamycin (5 mg/ml) and nalidixic acid (20 mg/ml) and also on LB agar containing gentamycin (5 mg/ml), nalidixic acid (20 mg/ml), and chloramphenicol (20 mg/ml). The ratio of colonies growing on gentamycin, nalidixic acid, and chloramphenicol (exconjugants that have a transposon on the conjugated pOX38-Gen F-factor) to colonies growing on gentamycin and nalidixic acid (total exconjugants that may or may not contain a transposon on the conjugated pOX38-Gen F-factor) is interpreted as the transposition frequency. The mating out assay was repeated four times for each mutant Tnp. It should be noted that the transposon used in this assay had OEs instead of the hyperactive MEs used in the *in vitro* assays.

In Vitro Transposition Assay—*In vitro* transposition efficiency for each mutant was determined as described previously (6). 0.49 pmol of pGRST2 (13) and 7.50 pmol of each C-terminal mutant (or control Tnp-FL) were added to transposition buffer (0.1 M potassium glutamate, 25 mM Tris acetate, pH 7.5, 10 mM magnesium acetate, 50 μ g/ml bovine serum albumin, 0.5 mM β -mercaptoethanol, 2 mM spermidine, 100 μ g/ml tRNA; final concentrations) (6) and diluted to a final reaction volume of 40 μ l. Following a 30-min incubation at 37 °C, 5 μ l of each reaction were added to 2.5 μ l of 1% SDS and heated at 68 °C for 10 min to remove the Tnp from the DNA. Reaction products were then separated by agarose gel electrophoresis and visualized by ethidium bromide staining. Percent of donor backbone release was calculated as follows with the aid of Molecular Dynamics Image Quant software: %ddb = donor backbone/total substrate \times 1.4 \times 100. Because the released dbb and original substrate are different lengths, equal ethidium bromide uptake by both DNAs will correspond to different numbers of molecules. To compensate for this, the donor backbone/total substrate ratio has to be multiplied by 1.4.

Synapsis Assay—Synaptic complex formation was assayed *in vitro* for each mutant as follows. The DNA substrate was prepared by digestion of the pGRST2 plasmid with *SmaI* and *PvuII* to give a 566-bp fragment containing the 456 bp of transposon DNA (including the 19-bp ME (22)) and 110 bp of dbb. 0.54 pmol of this DNA substrate and 5.4 pmol of each C-terminal mutant (or control Tnp-FL) were added to transposition buffer without Mg²⁺ and diluted to a final reaction volume of 25 μ l. Following incubation for 75 min at 37 °C, 10 μ l of each reaction were run on a 1.2% agarose gel, and paired end complex (PEC) formation was visualized by ethidium bromide staining. The percentage of shifted DNA was quantitated using Molecular Dynamics Image Quant software and calculated by dividing shifted DNA by the total amount of DNA.

Cleavage Assay—To confirm that the C-terminal mutations affect

TABLE I
Tn5 transposase (Tnp) C-terminal truncations and point mutations analyzed in this study

Mutant name	Mutation(s)
Tnp-FL	E54K, L372P, ^a M56A ^b
Tnp Δ 466	C-terminal 10 aa deleted ^c
Tnp Δ 467	C-terminal 9 aa deleted
Tnp Δ 468	C-terminal 8 aa deleted
Tnp Δ 469	C-terminal 7 aa deleted
Tnp Δ 470	C-terminal 6 aa deleted
TnpA466D	A466D
TnpG462D	G462D
TnpS458A	S458A

^a E54K and L372P are hyperactive mutations found in all mutants.

^b M56A is a mutation that prevents translation of the Inh and is also found in all mutants.

^c Tnp has a total of 476 aa.

only synaptic complex formation, cleavage for some of the mutants was assayed. Equal amounts of PECs for Tnp-FL, Tnp Δ 469, Tnp Δ 470, and TnpS458A (see above) were diluted in transposition buffer. The concentration of Mg²⁺ was then adjusted to 10 mM, and reactions were diluted to a final volume 10 μ l. Following incubation for 15 min at 37 °C, 1.0 μ l of 100 μ g/ml RNase was added followed by a 5-min incubation at 25 °C. 1.0 μ l of 5% SDS was then added and the reactions were heated at 68 °C for 5 min. RNase was added to the reaction to digest tRNA in the transposition buffer. Products were separated on a 1.7% agarose gel and visualized by ethidium bromide staining. The 456-bp cleavage product was quantitated using Image Quant software. The 110-bp cleavage product was not quantitated because of incomplete tRNA digestion and low abundance.

RESULTS

In Vivo Analysis of Tn5 Tnp C-terminal Mutants—Previous studies of the C-terminal region of the Tn5 inhibitor protein (Inh), a protein identical with Tn5 Tnp except lacking the N-terminal 55 amino acids, have revealed its importance in the mechanism of Tn5 inhibition (13). To determine the role of C-terminal dimerization in the mechanism of transposition, a set of nested C-terminal truncations was made by *in vitro* intramolecular transposition (data not shown) (14). An *in vivo* screen was used to test these deletions for transposition activity, and no truncation having more than seven amino acids deleted was found to be functional (data not shown). These preliminary experiments established a role for the C terminus in transposition. To precisely determine the number of C-terminal residues required for activity, progressive single amino acid deletions were constructed. C-terminal point mutations were made to specifically disrupt the C-terminal dimerization domain seen in the Inh x-ray crystal structure (see Table I and Fig. 2) (12). Each C-terminal mutation was amplified by PCR and cloned into a pET-21d(+) vector. Expression levels of each mutant protein were determined to be approximately equal in *E. coli* strain BL21 (DE3) pLysS (data not shown).

The transposition frequency effected by each Tnp mutant was quantitated using an *in vivo* mating out assay (Fig. 3). This assay measures the frequency of transposition onto an F-factor and is quantitated by comparing the number of exconjugants having a transposon on their F-factor to the total number of exconjugants. In this system, Tnp-FL effects a transposition frequency of 1.6×10^{-5} .

Removing six amino acids from the C terminus (Tnp Δ 470) of Tnp results in a transposition frequency of 4.5×10^{-7} , whereas removing seven amino acids (Tnp Δ 469) gives a transposition frequency of 8.2×10^{-9} . These mutants have a 45-fold and

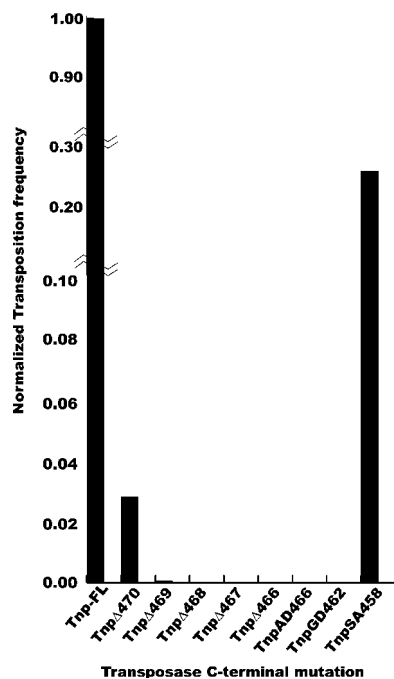


FIG. 3. *In vivo* transposition analysis of C-terminal Tnp mutants. Shown is a graphical representation of *in vivo* transposition frequencies of C-terminal deletion and point mutants as measured by a mating out assay. The transposition frequency for Tnp-FL was set equal to 1, and the transposition frequencies for each mutant were normalized to this value. The double broken lines on the y axis indicate non-linearity of this axis. Mutants having transposition frequencies less than 5.0×10^{-9} (transposition frequencies below this value are undetectable using this assay) are represented without bars.

2500-fold lower *in vivo* transposition frequency, respectively, when compared with Tnp-FL. No *in vivo* transposition activity could be measured (indicating transposition frequency of less than 5.0×10^{-9}) for Tnps lacking eight, nine, or ten amino acids at the C terminus (TnpΔ468, TnpΔ467, TnpΔ466, respectively), although it should be noted that an extremely low level activity was seen for both TnpΔ468 and TnpΔ467 using the alternate, qualitative *in vivo* papillation assay (data not shown) (23). No transposition activity was detected for C-terminal point mutants TnpG462D or TnpA466D using either the mating out assay or the papillation assay for transposition (data not shown) while point mutant TnpS458A effects a transposition frequency of 4.1×10^{-6} . This mutation only reduces transposition frequency 5-fold.

Determination of *in Vitro* Transposition Activity of Tnp C-terminal Mutants—*In vitro* transposition reactions (6) were performed to determine the ability of C-terminal mutants to cleave substrate DNA. Briefly, the transposition substrate pGRST2 was incubated with each purified C-terminal mutant Tnp (or control Tnp-FL) in a buffer containing Mg^{2+} . Following removal of the Tnp from the reaction products, they were visualized on an agarose gel. Cleavage efficiency was determined by quantitation of percent donor backbone release (see “Experimental Procedures”).

Incubation of Tnp-FL with pGRST2 in buffer containing Mg^{2+} causes single end cleavage of the substrate DNA which linearizes the supercoiled substrate giving a 4000-bp band. Subsequent cleavage at the second transposon end causes release of the transposon (1300 bp) from the dbb (2700 bp). The transposon can then integrate into itself (intramolecular transposition) and unreacted supercoiled substrate (intermolecular transposition). Intermolecular transposition products are detected as high molecular weight bands, whereas intramolecular transposition products cannot be seen in these reactions be-

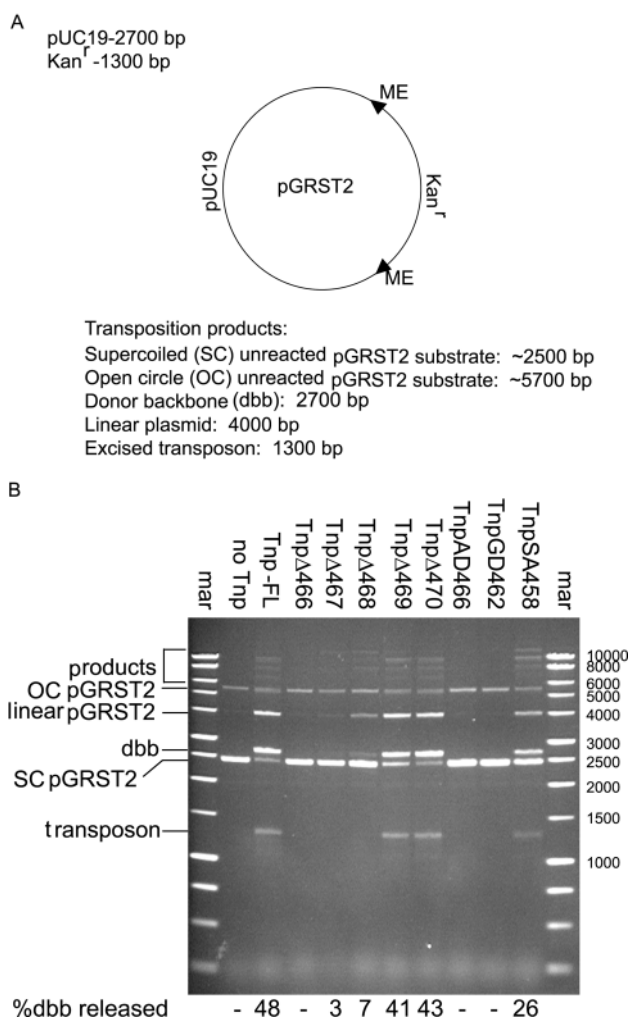


FIG. 4. *In vitro* transposition analysis of C-terminal Tnp mutants. A, the plasmid substrate, pGRST2, used for *in vitro* transposition assays is shown together with expected transposition products. B, pGRST2 was incubated with each C-terminal Tnp mutant (or control Tnp-FL) in a buffer containing Mg^{2+} . After removal of Tnp from the reaction products, they were separated on an agarose gel and stained with ethidium bromide. Transposition products and unreacted substrate are labeled. Products were identified by comparison to DNA markers (*mar*) of known size. The percentage of dbb released from substrate is indicated for each mutant.

cause of their low abundance.

No cleavage or transposition products are detected when pGRST2 is incubated with TnpΔ466, TnpA466D, or TnpG462D (Fig. 4, lanes 4, 9, and 10). A small amount of linear substrate (indicating single end cleavage) and intermolecular transposition products can be seen when the substrate is incubated with TnpΔ467 (Fig. 4, lane 5). Incubation of pGRST2 with TnpΔ468 results in 7.4% dbb release (Fig. 4, lane 6), while TnpΔ469 and TnpΔ470 release 41% and 43% of dbb from substrate, respectively (Fig. 4, lanes 7 and 8). TnpΔ469 and TnpΔ470 have approximately 85% of the cleavage activity seen for Tnp-FL, which releases 48% of the dbb (Fig. 4, compare lanes 7 and 8 with lane 3). Point mutant TnpS458A releases 26% of dbb from pGRST2 substrate (Fig. 4, lane 11). This mutation reduces cleavage efficiency approximately 50% when compared with Tnp-FL (Fig. 4, compare lane 11 with lane 3).

It should be noted that differences in mutant effects between *in vivo* and *in vitro* transposition frequencies may be due, in part, to the use of an OE-flanked transposon for *in vivo* transposition experiments whereas a transposon flanked by hyperactive MEs was used for *in vitro* experiments.

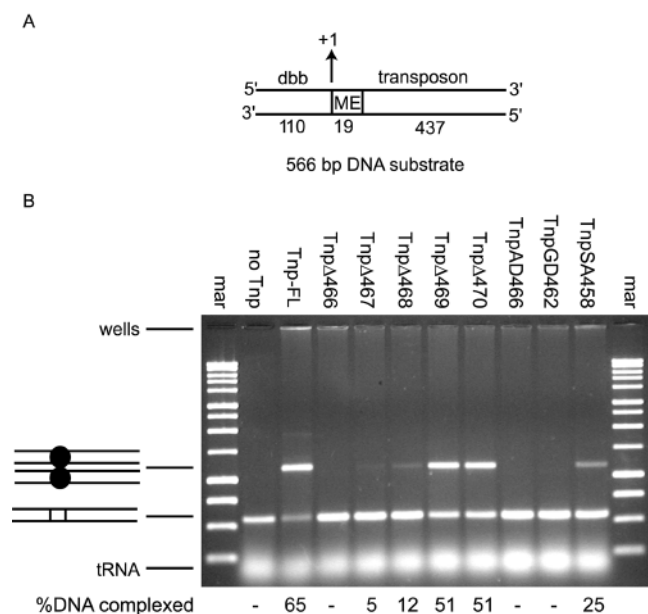


FIG. 5. Analysis of synaptic complex formation. *A*, the 566-bp DNA substrate used for analysis of synaptic complex formation is shown. This substrate has 456 bp of transposon DNA (which includes the 19-bp ME) and 110 bp of dbb DNA. The location of cleavage of the transposon from dbb DNA during transposition is labeled +1. *B*, the synopsis substrate was incubated with each C-terminal Tnp mutant (or control Tnp-FL) in a buffer without Mg^{2+} . The synopsis reactions were then analyzed by electrophoresis on an agarose gel. Synaptic complex formation is represented by PECs, which consist of both synopsis substrate DNAs and Tnp. These PECs have a slower mobility than uncomplexed DNA and are represented schematically by two substrate DNAs and two Tnp molecules (black spheres). Uncomplexed DNA is represented schematically as one synopsis substrate DNA. tRNA is a component of the reaction buffer, and DNA molecular weight markers (*mar*) were used to identify the uncomplexed DNA. The percent of substrate DNA complexed by each mutant is also indicated.

Synaptic Complex Formation for C-terminal Tnp Mutants—The C-terminal deletions and point mutants assayed for *in vitro* transposition activity were found to be defective in cleavage (see above). We then hypothesized that the C-terminal dimer interface observed in the Inh crystal structure was required for synopsis and that these mutants may be impaired in synaptic complex formation rather than directly defective in cleavage. To test this theory, pGRST2 plasmid was digested to give a 566-bp linear DNA substrate having 110 bp of dbb and 456 bp of transposon DNA (including the 19-bp ME). This substrate was incubated with all mutant proteins (or control Tnp-FL) in buffer without Mg^{2+} . The resulting PECs, which represent synaptic complex formation, and uncomplexed substrate DNA were visualized on an agarose gel. Percent PEC formation was determined by dividing the amount of shifted DNA by the total DNA.

Incubation of small (40–85 bp) radiolabeled substrates containing the ME with highly purified Tnp-FL results in formation of PECs that can be detected in polyacrylamide gels (24). PECs have also been formed using larger DNA substrates and visualized in agarose gels.³ Finally, precleaved transposons can be incubated with Tnp-FL to form synaptic complexes (25). Based on these data, we conclude that Tnp-FL forms PECs when incubated with the 566-bp substrate and that these PECs have a slower mobility than uncomplexed substrate DNA (Fig. 5, lane 3).

When TnpΔ466, TnpA466D, and TnpG462D were incubated with the 566-bp substrate, no PECs were detected (Fig. 5, lanes

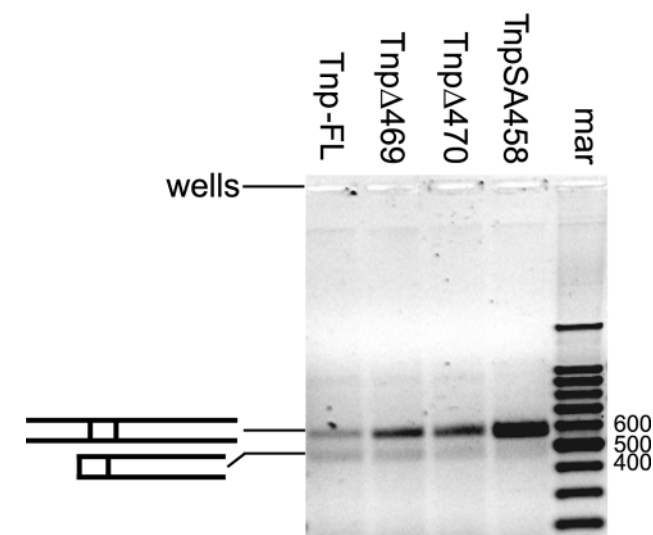


FIG. 6. Cleavage of synopsis substrate by C-terminal Tnp mutants. To rigorously show that C-terminal deletion mutants were only defective in synaptic complex formation and not defective in the cleavage step of transposition, PECs formed for Tnp-FL, TnpΔ469, and TnpΔ470 (Fig. 5) were diluted to equal the number of PECs formed for S458A (Fig. 5). The concentration of Mg^{2+} was then adjusted. Uncomplexed DNA carried over from the synopsis experiments is approximately 550 bp as compared with DNA markers (*mar*) of known size and is schematically shown as one uncomplexed synopsis substrate. The 456-bp cleavage product is represented schematically as transposon plus ME.

4, 9, and 10). TnpΔ467 and TnpΔ468 shift 5% and 12% of the DNA substrate to the PEC species, respectively (Fig. 5, lanes 5 and 6). These truncations decrease PEC formation 92% and 85% when compared with Tnp-FL, which complexes 65% of the substrate (Fig. 5, compare lanes 5 and 6 with lane 3). Both TnpΔ469 and TnpΔ470 shift 51% of the DNA substrate (Fig. 5, lanes 7 and 8). PEC formation is only decreased by 21.5% when compared with Tnp-FL for these C-terminal truncations (Fig. 5, compare lanes 7 and 8 with lane 3). Point mutant TnpS458A shifts 25% of the 566-bp DNA substrate (Fig. 5, lane 11). This represents a 61.5% decrease in PEC formation when compared with Tnp-FL (Fig. 5, compare lane 11 with lane 3).

It should be noted that percent DNA substrate shifted to PECs closely parallels the percent dbb release for each C-terminal mutant as measured by the *in vitro* transposition assay (see above).

Cleavage Assay—To rigorously show that these C-terminal mutants are only defective in synaptic complex formation and not in cleavage, mutants having lowered activity (TnpΔ470, TnpΔ469, and TnpS458A) and Tnp-FL were shown to produce equal amounts of cleavage products when equal amounts of synaptic complexes were incubated with Mg^{2+} . After quantitation of PEC formation, PECs formed with Tnp-FL, TnpΔ470, and TnpΔ469 were diluted to equal the number of PECs formed with TnpS458A. Mg^{2+} was then adjusted to 10 mM. Incubation of preformed PECs with Mg^{2+} induces cleavage of the substrate into the 110-bp dbb fragment and the 456-bp fragment transposon DNA. Following incubation, the Tnp-FL and mutant Tnps were removed from the DNA and the reaction products were separated on an agarose gel (Fig. 6).

Because the PECs were not purified from uncomplexed substrate DNA before cleavage, the original substrate (566 bp) is seen in each lane. The amount of original substrate is representative of each mutant's ability to form PECs. For example, because Tnp complexes approximately 65% of the DNA substrate (see above), little uncomplexed substrate can be seen in this experiment (Fig. 6, lane 1). The 456-bp cleavage product is

³ I. Y. Goryshin, unpublished data.

detected as a faint band just below uncomplexed substrate. Quantitation of this fragment for each mutant reveals an equal amount of cleavage product. Accurate quantitation of the 110-bp cleavage product was not possible because incomplete digestion of the tRNA interfered with the signal. Regardless of this, visualization of the 110-bp cleavage product would have been very difficult due to its small size and low abundance. This experiment confirms that the complexes formed with the 566 bp substrate (see above) were indeed PECs because the complexes were competent for cleavage.

DISCUSSION

The C Terminus of Tn5 Tnp Is Required for Transposition Both in Vivo and in Vitro—Previous experiments hypothesized two regions of Tn5 Tnp to be important for dimerization (11). These dimerization regions included the catalytic core and the C-terminal α helix. The dimerization region encompassing the catalytic core was thought to be required for synaptic complex formation whereas the C-terminal dimerization domain was primarily responsible for inhibition. This hypothesis was supported by the x-ray crystal structure of the Tn5 Inh dimer (12). In this structure, the C-terminal helices of each Inh monomer interact to form the dimer interface. Because a dramatic conformational change would be required for this dimer to represent a synaptic complex, the authors concluded that the structure was the inhibitory complex. Analysis of a C-terminal helix site-directed mutant of Inh confirmed that this interface was required for inhibition (13).

To determine if the C-terminal α helix was also required for transposition, a complete set of nested C-terminal deletions was created using the *in vitro* transposition system and a vector containing the Tnp gene (data not shown) (14). These C-terminal deletions were screened for functionality using a papillation assay (data not shown), but only Tnps with seven or less amino acids deleted from the C terminus were functional. These data led to the conclusion that the C terminus was required for transposition.

Transposition frequencies of C-terminal deletion mutants Tnp Δ 466, Tnp Δ 467, Tnp Δ 468, Tnp Δ 469, and Tnp Δ 470 were quantitated both *in vivo* and *in vitro* to determine the number of residues in the C-terminal α helix required for function. *In vitro* data show that deletion of six or seven amino acids from the C-terminal helix of Tnp-FL only slightly impairs transposition activity, whereas deletion of eight, nine, or ten amino acids greatly reduces or abolishes activity. These data suggest that the final seven residues of Tnp-FL are not required for activity, but the remaining C-terminal residues are very important for functionality. While progressive deletion of C-terminal residues also causes reduction and loss of transposition activity *in vivo*, the precise deletion point at which activity is abolished is not defined.

Synaptic Complex Formation Requires the Tn5 Tnp C-terminal Helix—After establishing a role for the C terminus in Tn5 transposition, the C-terminal deletion mutants were used to determine which step of transposition requires the C-terminal helix. PEC formation is the first step of transposition that can be analyzed *in vitro* (full-length Tnp monomer binding to end sequence DNA cannot be assessed independently). The number of PECs formed by each C-terminal deletion closely parallels the level of *in vitro* transposition activity for each mutant. Complete loss of both PEC formation and *in vitro* transposition activity results when 10 C-terminal residues are deleted. Deletion of eight or nine residues causes a dramatic decrease in *in vitro* transposition activity, and these mutants form few PECs. Only minor decreases in *in vitro* transposition activity and PEC formation are detected upon deletion of six or seven C-terminal amino acids.

Although residues 458 to 469 of the C-terminal helix are sufficient for almost complete Tnp-FL activity and PEC formation, deletion mutants lacking only six and seven C-terminal residues do have a 15% decreased *in vitro* activity and PEC formation relative to Tnp-FL. These final seven residues (470–476) may provide stabilization of the synaptic complex by extending the dimer interface required for PEC formation or through interaction of these residues with the opposite monomer in the synaptic complex. The residues could also stabilize Tnp-FL prior to synaptic complex formation.

The close parallel between the ability to form PECs and *in vitro* transposition activity for each mutant suggests that the defect in PEC formation accounts for all loss of activity. To verify that catalysis is not affected by the C-terminal mutations, cleavage was induced in equal amounts of Tnp Δ 469, Tnp Δ 470, and Tnp-FL PECs. Following induction, equal amounts of cleavage products were observed for each mutant. Although Tnp Δ 469 and Tnp Δ 470 don't form PECs as efficiently as Tnp-FL, their ability to cleave transposon from dbb DNA is not affected by the mutations. This implies that the C-terminal helix is required only for synaptic complex formation and not catalysis.

The ability of each deletion to integrate a precleaved transposon flanked by MEs into a supercoiled pUC19 plasmid was also investigated (data not shown). This assay shows reduction of strand transfer activity as C-terminal residues are deleted and therefore mirrors the result of both the *in vitro* transposition assay and PEC formation assay. The loss of strand transfer activity probably reflects each deletion's loss of ability to form PECs and is not directly related to cleavage efficiency (see above).

The role of the C-terminal helix in each major step of transposition (PEC formation, cleavage, and strand transfer) was investigated. The C-terminal α helix is imperative for synaptic complex formation but does not directly affect cleavage or strand transfer.

These data explain why protein P3, a naturally occurring Tnp truncation translated from IS50L, is nonfunctional. Because P3 is truncated at residue Glu-451, it is missing the C-terminal α helix and cannot form synaptic complexes.

The Synaptic Complex Dimerization Interface—Analysis of Tnp C-terminal Tnp deletion mutants determined that the C-terminal helix was required for dimerization to form synaptic complexes during Tn5 transposition. We next wanted to determine if the specific dimerization interface used for synaptic complex formation was the same as the interface formed by the C-terminal helices in the Tn5 Inh dimer x-ray crystal structure.

The dimerization interface observed in the Inh structure involves residues 458 to 468 of each Inh monomer. These amino acids interact to form a "scissor-like" interface with a crossover point at glycine 462. Although hydrophobic interactions are the major stabilizing force, hydrogen bonding of each Ser-458 to the Lys-459 of the opposite monomer may also contribute to the stability of the interface.

To probe the dimerization interface required for synaptic complex formation, three point mutations were investigated. The mutation of alanine 466 to aspartic acid was studied previously in an Inh background, and this residue was found to be essential for inhibition and Inh dimerization. Mutations of serine 458 to alanine and glycine 462 to aspartic acid were created specifically for this study. If the dimer interface necessary for synaptic complex formation was the same as the interface observed in the crystal structure, mutations A466D and G462D should prevent dimerization due to repulsion of like charged residues at the dimerization interface. The effect of the

S458A mutation was expected to be less dramatic because this mutation only negates two potential hydrogen bonds.

Mutations A466D and G462D abolish *in vivo* transposition activity, *in vitro* transposition activity, and PEC formation. Therefore, the dimerization interface required for synapsis must include the same residues as the interface seen in the Inh dimer. The S458A mutation only reduces transposition activity and PEC formation 2-fold implying that the hydrophobic interactions between residues 460 and 468 of each monomer must be sufficient for synaptic complex formation and hydrogen bonding of Ser-458 to Lys-459 of the opposite monomer contributes only slightly to the stability of the interface.

Model for Synaptic Complex Formation—This manuscript discusses the requirement of the Tn5 Tnp C-terminal α helix for synaptic complex formation. Because Tn5 is a *cis*-acting protein, a convenient model can be formulated for *in vivo* synaptic complex formation of Tnp. As the Tnp is translated, a DNA binding domain binds end sequence DNA (7). Following complete translation of Tnp, the N terminus bound to end sequence DNA cannot interact with the C terminus. Indirect evidence of an N-terminal, C-terminal Tnp interaction has been well documented in the literature (13, 26). Because the C terminus isn't neutralized by interaction with the N terminus, two C termini can dimerize to bury nonpolar α helical residues. This initial dimerization complex may resemble the Inh crystal structure dimer. Conformational changes may then occur to bring the active sites closer together resulting in a synaptic complex that is competent for cleavage.

In vitro, transposition cannot be coupled to translation of Tnp. This explains why wild type Tnp is not active *in vitro*. If the C terminus of Tnp interacts with the N terminus, the C-terminal α helix cannot be utilized for synaptic complex formation nor can the N terminus bind end sequence DNA. The hyperactive L372P mutation required for *in vitro* activity is hypothesized to disrupt these N-terminal, C-terminal Tnp interactions. This increases Tnp flexibility and allows the N terminus to bind end sequence DNA. Following binding, the two monomer C termini are free to dimerize. Conformational changes may then occur to form a synaptic complex competent for cleavage.

A second dimerization domain encompassing the Tnp catalytic core was predicted from previous studies (11). Evidence presented in this manuscript supporting the requirement of C-terminal α helices for synaptic complex formation does not preclude the use of this second predicted dimerization domain in later steps of transposition (*i.e.* cleavage, target capture or strand transfer). Although, if a second dimerization domain can be utilized following cleavage, synaptic complex formation with C-terminal dimerization must first occur before a conformational change allows utilization of a second dimerization region (see above).

Tnp Dimerization Domain Comparisons—This work defines the C-terminal α helix of Tn5 Tnp as being essential for synaptic complex formation. An investigation of IS911 dimerization domains has revealed that a leucine zipper between amino acids 63 and 95 of the OrfAB transposase is required for dimerization and that this dimerization is a prerequisite for end binding (27, 28). A more closely related, mechanistically iden-

tical transposase, Tn10 Tnp, has no region of amino acid homology to the C-terminal helix of Tn5 Tnp. Therefore Tn10 synaptic complex formation must utilize a different Tnp dimerization domain and occur by a mechanism distinct from Tn5. Dimerization interactions for other Tnps such as Tn7 and Mu have not been directly investigated. From these comparisons we conclude that Tn5 Tnp utilizes a novel kind of C-terminal dimerization for synaptic complex formation.

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Note Added in Proof—Following completion of this work, the x-ray crystal structure of Tn5 transposase complexed with OE DNA (preintegration complex) was solved (Davies, D. R., Goryshin, I. Y., Reznikoff, W. S., and I. Rayment (2000) *Science*, in press). The C-terminal α helix, discussed in this manuscript, was the primary protein-protein dimerization interface seen in the co-crystal structure. Therefore, the biochemical data presented here correlate well with structural data.

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