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A Functional Analysis of the Tn*5* Transposase

Identification of Domains Required for DNA Binding and Multimerization

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A series of deletions were constructed in the 476 amino acid Tn*5* transposase in order to assemble an initial domain structure for this protein. The first four amino acids were found to be important for transposition activity but not for DNA binding to the Tn*5* outside end (OE). Larger amino-terminal deletions result in the complete loss of transposition *in vivo* and the concomitant loss of specific DNA binding. Four point mutants and a six base-pair deletion in the amino terminus between residues 20 and 36 were also found to impair DNA binding to the OE. Analysis of a series of carboxy-terminal deletions has revealed that the carboxy terminus may actually mask the DNA binding domain, since deletions to residues 388 and 370 result in a large increase in DNA binding activity. In addition, the carboxy-terminal deletion to residue 370 results in a significant increase in the mobility of the Tnp–OE complex indicative of a change in the oligomeric state of this complex. Further carboxy-terminal deletions beyond residue 370 also abolished DNA binding activity. These results indicate that the first four amino acids of Tnp are important for transposition but not DNA binding, a region between residues 5 and 36 is critical for DNA binding, the wild-type carboxy terminus acts to inhibit DNA binding, and that a region towards the carboxy terminus, defined by residues 370 to 387, is critical for Tnp multimeric interactions.

Keywords: transposition; Tn*5* transposase; DNA binding domain; protein oligomerization; functional analysis

1. Introduction

Tn*5* is a composite prokaryotic transposon (Figure 1). It is composed of two IS*50* elements in inverted orientation, surrounding a unique 2·7 kb region encoding resistance genes to several antibiotics (for a review, see Berg, 1989). IS*50*R encodes two proteins that are involved in the transposition reaction. These are the transposase (Tnp), which is absolutely required for the transposition reaction (Isberg *et al.*, 1982; Johnson *et al.*, 1982), and an inhibitor of transposition (Inh), which is identical to Tnp except that it lacks the amino-terminal 55 amino acid residuess of Tnp (Biek & Roth, 1980; Yin & Reznikoff, 1988).

Tn*5* transposition is likely to involve a series of specific reactions, by analogy to the other, well studied prokaryotic transposons Mu, Tn*7* and Tn*10* (Kleckner, 1990; Mizuuchi, 1992*a*). First, Tnp must recognize and bind to the ends of the transposable element. Second, these two ends must be brought together in a precise alignment to yield a higher-order nucleo-protein complex. Tnp must then catalyze the double-stranded breakage of the DNA immediately adjacent to the Tn*5* ends, and the subsequent cleavage of the target DNA site. This is followed by strand transfer of the free 3′ ends of the transposon to the 5′ ends of the target DNA (Mizuuchi, 1992*b*).

Phage Mu transposition differs from the Tn*7* and Tn*10* reactions in that only a single strand is cut adjacent to the end sequences. After strand transfer, a branched structure is formed which is processed by DNA replication to form the transposition product (Mizuuchi, 1992*b*). Since the available evidence supports a conservative mechanism for Tn*5* transposition (Berg, 1989), we assume that its transposition mechanism will be more similar to the Tn*10* and Tn*7* reactions, i.e. in which a double-stranded break is made near the ends and the element is inserted at a new site without replication of the entire element.

Tnp, therefore, must contain at least six activities which may correspond to particular domains within

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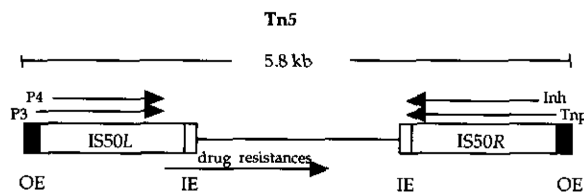


Figure 1. The structure of the 5·8 kb transposon *5* (Tn*5*). The OEs are represented by small, filled rectangles and the IEs are represented by small, open rectangles. There are 4 proteins encoded by the IS*50* elements. IS*50*R encodes the 476 amino acid residue transposase (Tnp) and the 421 amino acid residue inhibitor (Inh). The analogous proteins from IS*50*L are truncated at the C terminus by 26 residues to give P3 and P4, respectively. The unique region of Tn*5* encodes 3 antibiotic resistance genes. The transcript for these genes originates within IS*50*L.

the protein structure. It must be able to specifically recognize the transposon end sequences, interact with a second Tnp molecule bound to the opposite end, then cleave the DNA at the donor-transposon junction, bind to a non-specific DNA sequence, cleave that sequence much like a restriction endonuclease, and also facilitate the strand transfer of transposon sequences to the target DNA site. Furthermore, some transposase proteins are sensitive to regulatory interactions with host factors (Lavoie & Chaconas, 1990) or with other transposon encoded proteins (Baker *et al.*, 1991).

The regulation of Tn*5* transposition appears to be unique among prokaryotic transposons. Unlike those elements which transpose efficiently like bacteriophage Mu (Mizuuchi, 1992*a*), Tn*7* (Craig, 1989), or Tn*10* (which transposes very efficiently by increasing the level of its transposase (Roberts & Kleckner, 1988)), the Tn*5* reaction seems to be intrinsically limited. Firstly, it encodes an inhibitor of transposition, Inh, which is translated in the same reading frame as Tnp and lacks only the first 55 amino acid residues of Tnp. Inh appears to inhibit the reaction by forming mixed multimers with Tnp that are inactive for transposition (de la Cruz *et al.*, 1993). Even in the absence of Inh, however, increasing the amount of Tnp over at least a 100-fold range has a minimal effect on the transposition frequency (M. D. Weinreich, A. Gasch & W. S. Reznikoff, unpublished results). This indicates either that some host factor is limiting for the reaction (this seems very unlikely given the normal rate of transposition is 10^{-5} events per cell per generation) or that Tnp is subject to some level of post-translational inactivation. In addition, Tnp itself has been shown to inhibit the transposition of Tn*5* elements located *trans* to the site of its synthesis (DeLong & Syvanen, 1991; Wiegand & Reznikoff, 1992). Recent evidence suggests that the intrinsic limitation of Tnp activity and its ability to inhibit the transposition reaction *in trans* may be caused by Tnp–Tnp interactions which lead to an inactive multimeric species (M. D. Weinreich, A. Gasch & W. S. Reznikoff, unpublished results).

In order to understand the functional domains required for the various activities of Tnp we have begun a deletion analysis of this protein. We show that the first four amino acid residues of Tnp are very important for overall transposition activity and that residues 5 to 12 are necessary for DNA binding activity. This confirms and extends recent evidence showing that Inh, which lacks the 55 amino-terminal amino acid residues of Tnp does not bind to the Tn*5* ends (de la Cruz *et al.*, 1993). Because a collection of point mutations and a six base-pair deletion within the unique amino terminus of Tnp also greatly impair DNA binding activity but not Tnp-mediated inhibition, we suggest that the amino-terminal region of this protein encodes the DNA binding domain. Tnp does not contain a recognizable DNA-binding motif throughout its sequence and might recognize DNA through some novel structural element present in this amino-terminal region. In contrast, carboxy-terminal residues were found to actually inhibit DNA binding activity and a region between residues 370 and 387 was found to be critical for Tnp protein–protein interactions.

2. Materials and Methods

(a) *Bacterial strains and reagents*

Bacterial strains used in this study were all derivatives of *Escherichia coli* K-12, except for BL21 pLysS (Studier *et al.*, 1990) which is an *E. coli* B-strain. These were DH5α (Sambrook *et al.*, 1989), RZ201 and RZ211 (Johnson *et al.*, 1982) and MDW320 (RZ211/pOX386). Bacteria were routinely cultured in Luria broth (LB†) or on LB plates which contain 1·5% Difco Bacto-agar. Antibiotics were purchased from Sigma Chemical Co. and the concentrations used were: chloramphenicol, 20 μg/ml; ampicillin, 100 mg/μl; kanamycin, 40 μg/ml; gentamycin, 5 μg/ml; and naladixic acid, 20 μg/ml. Restriction enzymes, *Bal*31 nuclease and T4 DNA polymerase were purchased from New England Biolabs. Reverse transcriptase was from Molecular Genetic Resources, S_1 nuclease was from Boehringer Mannheim Corp. and T7 RNA polymerase was from Promega.

(b) *Plasmids*

All plasmids are listed in Figure 2 (also see Figure 7). pRZ4737 is a pACYC184 derivative of pRZ986 (Krebs, 1987) that was used to construct the N-terminal deletions in Tnp and also to overproduce Tnp. It was constructed by inserting the *Cla*I-*Sal*I fragment of pRZ986 containing the λ cI857 repressor, P_R promoter and Tnp gene into the *Cla*I-*Sal*I sites of pACYC184. The *Bsp*HI site in the vector was deleted following partial *Bsp*HI digestion by filling in with T4 DNA polymerase in the presence of the 4 dNTPs and then re-ligating. pRZ4738 was constructed by digesting pRZ4737 with *Bsp*HI, treating with S_1 nuclease to remove the overhang followed by religation of the plasmid. This resulted in a 7 bp deletion which removed the initiating methionine codon for Tnp and pRZ4738 no longer produces Tnp. pRZ4775 (and deletion derivatives) was constructed by replacing the *Hpa*I-*Not*I IS*50* fragment of pRZ4737 (and deletion derivatives) with the *Hpa*I-*Not*I fragment of pRZ7016 (Wiegand & Reznikoff,

† Abbreviations used: LB, Luria broth; OE, outside end; IE, inside end; Tnp, transposase; Inh, inhibitor.

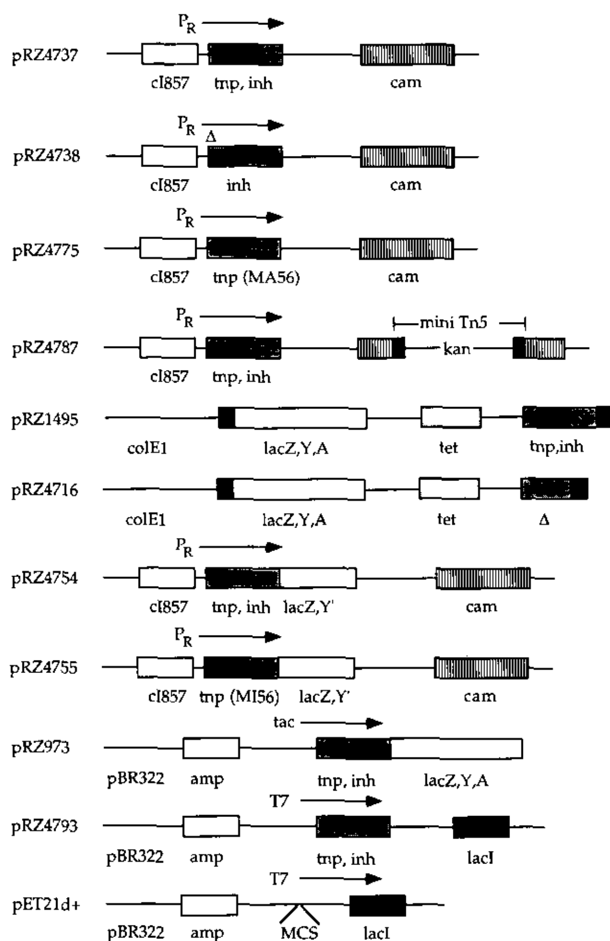


Figure 2. The structure of the plasmids used in this study. Small, filled rectangles indicate OEs and the arrows indicate the promoter for Tnp synthesis.

1992). This fragment contains the MA56 mutation that eliminates inhibitor synthesis. pRZ4787 (and deletion derivatives) contains a mini-Tn*5* element cloned downstream of the Tnp gene in pRZ4737 (and deletion derivatives) for quantifying *cis* transposition rates for the various N-terminal deletions. The 1·4 kb *Eco*RI fragment from pRZ4729 (M. D. Weinreich, unpublished results) bearing the mini-Tn*5* element was inserted into the *Eco*RI site of pRZ4737. The orientation was selected so that the direction of transcription for the kanamycin resistance gene was the same as for Tnp.

The Tnp overproducing plasmids, containing 4 point mutations and a 6 amino acid residue deletion in the N terminus, were constructed by inserting the *Bsp*HI-*Not*I IS*50*R fragment of pRZ454, pRZ455, pRZ405, pRZ457 and pRZ458 (plasmid derivatives corresponding to Tn*5*-436, -437, -405, -440 and -441 of Johnson & Reznikoff, 1984) into the *Bsp*HI-*Not*I site of pRZ4737. This resulted in plasmids pRZ4798 (AT20), pRZ4799 (AV36), pRZ4800 (Δ30-35), pRZ4801 (RH30) and pRZ4802 (DN24), respectively. The N-terminal region was sequenced to verify the presence of the mutation. It was found that two of the mutations were incorrectly assigned. pRZ454 contains an AT (GCG to ACG) mutation and not an AS mutation at position 20. pRZ455 contains an AV36 (GCC to GTC) mutation and not an AV35 change. All of these plasmids were tested for their transposition phenotype using the *trans*-papillation assay and were found to be over 100-fold defective in transposition as previously shown (Johnson & Reznikoff, 1984).

pRZ1495 contains the Tn*5lac* papillation construct (Makris *et al.*, 1988). A large deletion in the Tnp reading frame was constructed by digesting pRZ1495 with *Not*I and *Nhe*I, filling in the resulting overhangs with T4 DNA polymerase, and re-ligating to give pRZ4716. This Tnp^- derivative was complemented to transpose onto the F-derivative pOX38-Gen by supplying transposase from a second, compatible plasmid. Lac^- exconjugants containing the defective Tn*5lac* element were then screened for the ability to conjugate and one candidate (pOX386), which was conjugation proficient was saved. No papillation occurs over the course of 12 days in a strain containing this episome.

pRZ4755 was constructed to give a Tnp-β-galactosidase fusion protein in the context of the P_R Tnp overproducer, pRZ4737. The *Bsp*HI-*Afl*II(blunt) fragment of pRZ910 (Krebs & Reznikoff, 1986) containing the *tnp-lacZ* fusion was inserted into the *Bsp*HI-*Bgl*II(blunt) sites of pRZ4737. The *Afl*II and *Bgl*II sites were made blunt-ended by first digesting with these enzymes and then filling in the resulting overhangs with T4 DNA polymerase. pRZ4754, which encodes both Tnp and Inh-β-galactosidase fusion proteins, was constructed as above using plasmid pRZ901 instead of pRZ910.

pRZ4793 (de la Cruz *et al.*, 1993) was constructed to overproduce Tnp from the T7 promoter of $pET21d^+$ (Novagen) by cloning the *Bsp*HI-*Bcl*I IS*50*R fragment containing the Tnp gene into the *Nco*I-*Bam*HI sites of $pET21d^+$.

(c) *Construction of N-terminal deletions*

pRZ4737 was constructed so that it contained a single *Bsp*HI site (T'CATGA) which overlaps the start codon for Tnp. After *Bsp*HI restriction and filling in the overhang with T4 DNA polymerase, the region 5′ to the Tnp gene terminates in a blunt end with the sequence TCATG (the start codon for Tnp is underlined). This plasmid was then digested with *Eco*RI, the *Eco*RI-*Bsp*HI(blunt) fragment 5′ to the Tnp gene was isolated and then joined to a family of N-terminal deletions as described below.

To construct random deletions in the N terminus of Tnp, pRZ4737 was digested with *Bsp*HI and treated with 1 U of *Bal*31 nuclease (Sambrook *et al.*, 1989) for varying times to digest from 1 to 55 codons from the N terminus. The collection of DNAs were treated with S_1 nuclease to generate blunt-ended molecules and then digested with *Eco*RI. The large fragment containing the deleted Tnp gene was isolated and ligated with the *Eco*RI-*Bsp*HI (blunt) fragment from above, transformed into MDW 320 and plated onto lactose MacConkey agar containing chloramphenicol and tetracycline. Since the *Eco*RI site is within the *cam* gene of pRZ4737, this selection demanded that each transformant was a recombinant. Approximately 3000 colonies (<100 colonies per plate) were followed over the course of 8 days at 32°C for their papillation phenotype. Transposing and non-transposing candidates were then sequenced to determine the extent of the N-terminal deletion.

Deletions of 1, 2 and 5 amino acid residues were not recovered in the initial screen and were constructed by an analogous procedure using the Tnp-β-galactosidase fusion plasmid, pRZ4755. In this case, a more limited digestion with *Bal*31 was used and Lac^+ (in-frame) fusions were recovered and sequenced. The Δ1, 2 and 5 derivatives were cloned into the pRZ4737 background by replacing

the *Hpa*I-*Sal*I *lacZ* containing fragment of pRZ4755 with the analogous IS*50*R fragment from pRZ4737. These derivatives were then analyzed by the *trans*-papillation assay as above.

(d) *Construction of internal deletions*

Internal deletions within Tnp were constructed by digesting the Tnp-LacZ fusion plasmids pRZ973 (Krebs & Reznikoff, 1986) and pRZ4754 with a single enzyme or a pair of restriction enzymes followed by a limited S_1 nuclease digestion under conditions which allowed double-stranded digestion (Sambrook *et al.*, 1989). The plasmid DNA was then gel purified, ligated, transformed into DH5α and colonies were screened for blue color on LB plates containing 40 μg/ml XG (5-bromo 4-chloro 3-indolyl-β-D-galactoside) and either ampicillin (for pRZ973 derivatives) or chloramphenicol (for pRZ4754 derivatives). Blue candidates were screened by sequence analysis and a collection of in-frame deletions were saved (see Figure 7). After sequence analysis, these deletion derivatives were moved into pET21d$^+$ in order to place them under the control of the T7 promoter. First the *Hpa*I-*Xcm*I IS*50*R fragment containing the deletion was inserted into *Hpa*I-*Xcm*I digested pRZ4737. Then the *Bsp*HI-*Sal*I IS*50*R fragment of this plasmid (containing the deletion) was inserted into the *Nco*I-*Sal*I sites of pET21d$^+$. The resulting N-terminal junction (*Nco*I/*Bsp*HI) was sequenced to verify its wild-type structure. These pET21d$^+$ derivatives were: pRZ4842 (Δ125-455), pRZ4843 (Δ56-203), pRZ4844 (Δ126-212), pRZ4845 (Δ149-314), pRZ4846 (Δ235-371), pRZ4853 (Δ125-141), pRZ4854 (Δ308-371) and pRZ4855 (Δ279-315).

In order to complete a smaller overlapping set of deletions, several deletions were constructed using site-directed mutagenesis of single-stranded uracil containing pRZ7013 DNA (Wiegand & Reznikoff, 1992) which harbors IS*50*R. After sequence analysis, these deletions were moved into pET21d$^+$ by inserting the *Bsp*HI-*Bam*HI IS*50*R fragment into the *Nco*I-*Bam*HI sites of pET21d$^+$. The resulting N-terminal junction (*Nco*I/*Bsp*HI) was sequenced to verify its wild-type structure. The oligo-nucleotides used for site-directed mutagenesis were synthesized on an Applied Biosystems DNA Synthesizer. The sequences were: to delete codons 142 to 185, TTCCGCACCGTAGGAGTCTGTGACCGCGA; to delete codons 183 to 204, AGCATGATGACCAACGAGC-GCTTCGTGGTG; to delete codons 204 to 235, GACAAA-CTGGCGCATGGCTATCAGATCAGC; to delete codons 235 to 279, AACCAACCGGAGTTGAACGCGGTGCTG-GCC. This resulted in pRZ7013 derivatives pRZ4863, pRZ4864, pRZ4848 and pRZ4849, respectively. The pET21d$^+$ derivatives were pRZ4866 (Δ142-185), pRZ4867 (Δ183-204), pRZ4851 (Δ204-235) and pRZ4852 (Δ235-279).

(e) *Transposition assays*

The papillation assay measures the rate of Tn*5lac* transposition by following the appearance of red papillae over time, which arise following a transposition event into an active gene. The papillation assay was performed exactly as described (Weinreich & Reznikoff, 1992), only the assay was carried out at 32°C and the reporter strain (MDW 320) contained the various deletion derivatives of Tnp to be tested.

The mating-out assay (Johnson *et al.*, 1982) measures the rate of transposition into the F-derivative pOX38-Gen. It was performed by growing 4 donor cultures (RZ201, containing a Tn*5* plasmid) overnight at 33°C in LB containing gentamycin and kanamycin. In the morning, a 1 : 100 dilution of the donor was mixed with a 1 : 50 dilution of the recipient (RZ224, Nal^R) in LB and incubated with slow shaking for 6 to 8 h at 33°C. Dilutions were plated for ex-conjugants onto LB plates containing gentamycin and naladixic acid. To select for ex-conjugants containing Tn*5* insertions, LB plates containing gentamycin, naladixic acid and kanamycin were used. Colonies were counted after 36 h at 37°C.

The lambda infection assay was performed exactly as described (Weinreich & Reznikoff, 1992).

(f) *Purification of Tnp*

Purification of Tnp from pRZ4737 and various mutant derivatives was essentially as described (Wiegand & Reznikoff, 1992) with the following changes. The ammonium sulfate pellet was resuspended in 0·3M KCl-TEGX (20 mM Tris·HCl (pH 7·5), 1 mM EDTA, 20% glycerol, 0·1% TritonX-100), dialyzed overnight against this buffer, and fractionated on a Biorex-70 column. The column was washed with 2·5 vol. of 0·3 M KCl-TEGX buffer and Tnp was eluted with a 56 ml linear gradient (0·3 to 0·9 M KCl). By using the Biorex-70 column, all of the deletion derivatives eluted within several fractions of the wild-type.

(g) *In vitro transcription-translation of Tnp and deletion derivatives*

Plasmid DNAs were prepared by CsCl density centrifugation (Sambrook *et al.*, 1989). 1 μg of plasmid either uncut or linearized by various restriction enzymes was added to a cocktail (final volume 25 μl) containing T7 RNA polymerase (3U) and the translation components of the Promega TNT system, exactly as described by the manufacturer. The reaction was incubated for 2 h at 30°C and then 6 μl samples were tested immediately for DNA binding activity to the outside end (OE; described below). To visualize the Tnp proteins and deletion derivatives, [^{35}S]-methionine (Amersham) replaced the cold methionine in the translation reaction. Proteins were visualized by autoradiography after separation on 3 to 10% discontinuous SDS gels.

(h) *Gel retardation assay*

For the gel retardation assay, various amounts of wild-type Tnp or Tnp derivatives were incubated with approximately 10 fmol of ^{32}P-labeled OE DNA in binding buffer (20 μl final vol.) for 30 min at 30°C. The DNA containing the OE was prepared by isolating the 60 bp *Eco*RI-*Hin*dIII fragment from pRZ7067-WT or pRZ7067-7G (Makris *et al.*, 1988). The 7G mutation in the OE abolishes Tnp binding and is used as a measure of specificity (Jilk, unpublished results). Binding buffer contained 20 mM KPO_4 (pH 7·6), 0·1 mM DTT, 150 mM Kglutamate, 10 mM $MgCl_2$, 0·5 mM EDTA, 200 μg/ml BSA, 0·1% TritonX-100 and a 240-fold excess (by weight) of calf thymus DNA. Sucrose and xylene cyanol were added and the reaction was loaded onto an 8% polyacrylamide gel in 0·5 × TBE and electrophoresed at 4°C for 2 h at 300 V. Gels were dried and autoradiographed.

(i) *Western blotting*

Immunoblotting was performed exactly as described (Wiegand & Reznikoff, 1992).

3. Results

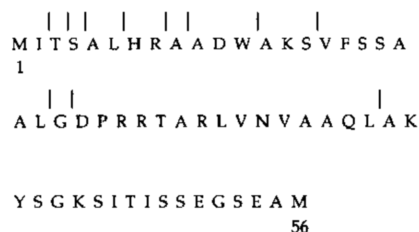
Tnp is identical to Inh except that it has an additional 55 N-terminal amino acid residues. Therefore, this N-terminal region must be critical for transposition activity since Inh does not promote Tn*5* transposition. In order to test which regions of the N terminus are most important for Tnp activity, a series of unidirectional N-terminal deletions in the Tnp gene were constructed using *Bal*31 nuclease. These derivatives were then screened for their transposition activity *in vivo* and DNA binding activity *in vitro* (see Materials and Methods). The *trans*-papillation assay was initially used to measure the transposition rates of the deletion derivatives. In this assay, the transposition activity of a Tnp derivative is measured *in trans* by its ability to promote the movement of a defective Tn*5lac* transposon. A fraction of the transposition events generate hybrid β-galactosidase protein fusions which are scored as Lac^+ papillae within a Lac^- colony on indicator plates. The accumulation of papillae over time is a measure of the transposition rate.

(a) *The N terminus of Tnp is necessary for DNA binding*

We found that small deletions in the N terminus of Tnp result in decreased transposition and that larger deletions abolish transposition activity *in vivo*. The collection of in-frame deletions that were recovered is shown in Figure 3A along with their transposition rates relative to the wild-type (Figure 3B). No candidate was recovered with a transposition frequency greater than the wild-type. The only mutants which retained activity were those deleting one, two, or three amino acids. (Since a methionine codon at position one is necessary for translation in *E. coli*, a deletion of two amino acids indicates that amino acid residues 2 and 3 were deleted.) These deletion mutants resulted in a progressive three- to fivefold reduction in the transposition frequency although they had very similar Tnp expression levels compared to the wild-type protein (below). Further deletions of 5, 7, 8, 11, 14, 21, 22 and 37 amino acid residues resulted in the complete loss of transposition activity (Figure 3B).

A Western blot showing the relative expression levels of the entire collection of N-terminal deletions is given in Figure 4. Since Tnp steady state levels were not readily detected at 32°C, the relative expression levels were visualized after a brief (1 h) induction at 42°C. This experiment indicates that the wild-type, Δ1, Δ2, Δ3, Δ11 and Δ37 derivatives are approximately equally abundant (±50%). The remaining derivatives show a reproducible decrease in abundance relative to the wild-type. This may reflect that these derivatives are not as stable as the wild-type protein or might simply reflect decreased translational efficiency due to altered sequences surrounding the initiating methionine codon. Tnpα, which is also indicated in this Figure, is an

A



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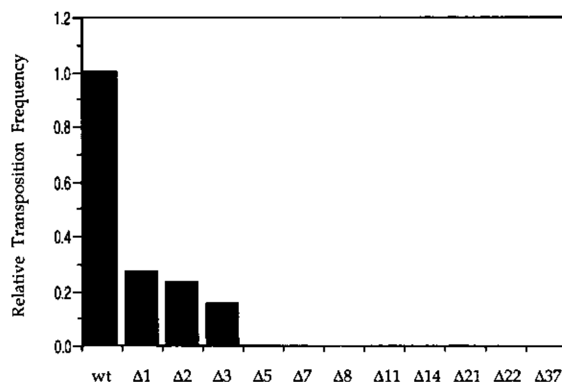

Figure 3. N-terminal deletions in Tnp. A, The amino acid sequence of the 55 amino acid residue N terminus of Tnp is given. A vertical slash indicates the position of each deletion that was recovered. B, The relative activity of the deletion derivatives is given with respect to the wild-type. The *trans*-papillation assay was used to quantify the transposition levels. The assay was performed at 32°C using pRZ4737 and deletion derivatives as the source of Tnp.

N-terminal degradation product of Tnp (see Discussion).

These N-terminal deletion mutants of Tnp are also defective in promoting transposition *in cis*.

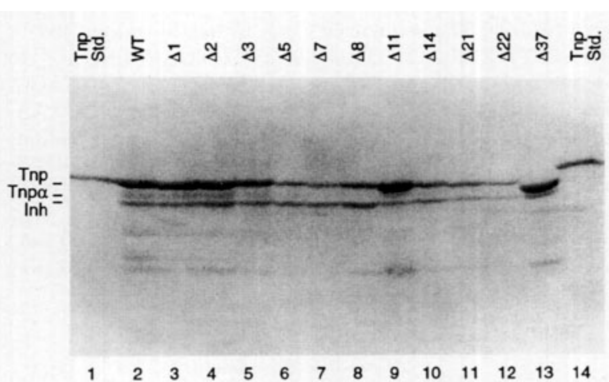

Figure 4. Expression levels of deletion derivatives. Each plasmid in DH5α was grown to an A_{550} of approximately 0·6 at 32°C in LB containing chloramphenicol. The cultures were shifted to 42°C for 60 min and then collected and resuspended in 1× protein sample buffer. Approximately equivalent amounts of cells were loaded onto a 3 to 10% SDS gel, the samples were separated for 3 h at 220 V and then transferred to nitrocellulose and probed with a polyclonal antibody against Tnp as described (Wiegand & Reznikoff, 1992). Tnpα is an N-terminal degradation product of Tnp.

Table 1
Transposition frequencies in cis

Tn5 plasmid	Transposition frequency†	Relative frequency
pRZ4737	$(2\cdot8 \pm 0\cdot34) \times 10^{-5}$	1·0
pRZ4737 Δ1	$(7\cdot8 \pm 1\cdot5) \times 10^{-6}$	0·28
pRZ4737 Δ3	$(6\cdot0 \pm 1\cdot6) \times 10^{-6}$	0·21
pRZ4737 Δ5	$< 1\cdot2 \times 10^{-7}$	<0·004
pRZ4737 Δ7	$< 1\cdot5 \times 10^{-7}$	<0·005
pRZ4737 Δ11	$< 1\cdot4 \times 10^{-7}$	<0·005

† Transposition frequencies were determined by the mating-out assay as described in Materials and Methods.

Since the Tn5 transposase is approximately 100-fold more active *in cis* than *in trans* (Johnson *et al.*, 1982) and it was possible that only the *trans* activity of the Tnp was decreased, the transposition activity of a subset of these N-terminal deletions was also tested using the *cis* mating-out assay. The *cis* transposition frequencies (Table 1) exactly parallel the *trans* activities and indicate that removal of residues 2 to 4 is tolerated but subsequent deletions of five or more residues also abolish transposition *in cis*.

To determine if the loss of transposition activity was due to a defect in DNA binding, the wild-type, Δ3, Δ11 and Δ37 Tnp derivatives were purified and tested for their binding activity to the OE. The mobility shift assays indicate that the Δ3 derivative has a binding activity indistinguishable from the wild-type (Figure 5, lanes 1 and 2) and a titration of the wild-type and Δ3 proteins revealed no differences in their DNA binding activity (not shown). The Δ11 and Δ37 derivatives, however, showed no DNA binding activity (Figure 5, lanes 3 and 4) and increasing the amount of the Δ11 and Δ37 proteins by 2·5-fold did not result in any detectable binding (data not shown). These results suggest that the Δ3 derivative has a fivefold lower transposition activity for reasons other than altered DNA binding, since it has the same DNA binding activity as the wild-type protein. In addition, the loss of DNA binding to the OE by Δ11 and Δ37 is sufficient to explain the loss of transposition activity *in vivo*.

To determine whether the loss of DNA binding activity by Δ11 was due to a specific defect in DNA binding rather than a gross conformational change, a second activity of Tnp was assayed *in vivo*. Tnp has been shown to actually inhibit Tn5 transposition *in trans*, in a dose dependent manner, as well as activate transposition in *cis* (DeLong & Syvanen, 1991; Wiegand & Reznikoff, 1992). Inh synthesis was eliminated from the wild-type, Δ3 and Δ11 variants by substituting the methionine start codon of Inh with one for alanine. This MA56 mutation does not perturb the transposition activity of Tnp or its DNA binding activity (Wiegand & Reznikoff, 1994). These derivatives were then tested for inhibition activity at 32°C or after a brief induction at 42°C. The results indicate that Δ3 and Δ11 have inhibitory properties nearly identical to the wild-type protein *in vivo* (Table 2). The twofold decrease in inhibition by Δ3 is in agreement with its approximately twofold lower expression level compared to the wild-type (Figure 4 and data not shown). Therefore, these mutant proteins are taking on whatever folded conformation is required for this inhibitory activity in the cell.

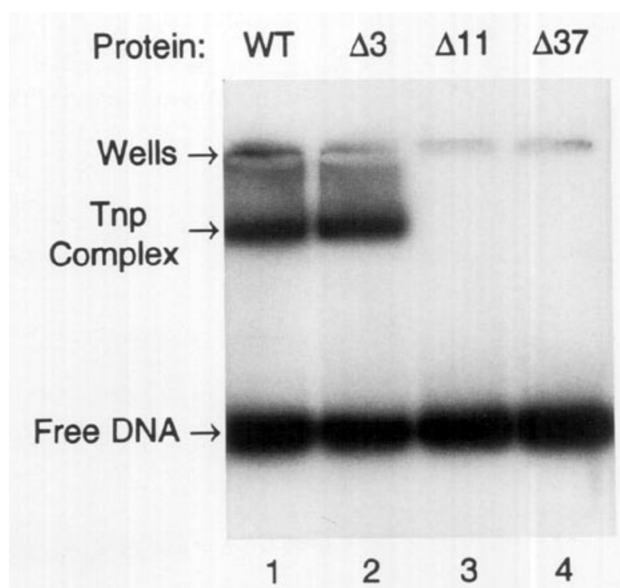

Figure 5. DNA binding activity of wild-type Tnp and deletion derivatives. Equivalent amounts (0·4 μg) of purified proteins were added to a 60 bp DNA fragment containing the OE and analyzed by gel retardation as described in Materials and Methods.

(b) *Point mutations in the N terminus also impair DNA binding*

To test whether other amino acid residues in the unique N-terminal region of Tnp were also important for DNA binding we cloned a series of previously isolated point mutations and a six base-pair deletion located in the N terminus (Johnson &

Table 2
Trans-inhibition of deletion derivatives

Plasmid	Average transposition frequency†	Relative frequency
A. *32°C cultures*		
Tnp⁻Inh⁻	$4\cdot5 \times 10^{-5} (\pm 0\cdot72)$	1·0
W.T.	$1\cdot2 \times 10^{-5} (\pm 0\cdot24)$	0·27
Δ3	$2\cdot7 \times 10^{-5} (\pm 0\cdot63)$	0·60
Δ11	$1\cdot0 \times 10^{-5} (\pm 0\cdot19)$	0·22
B. *With 42°C induction*		
Tnp⁻Inh⁻	$2\cdot6 \times 10^{-5} (\pm 0\cdot38)$	1·0
W.T.	$2\cdot7 \times 10^{-7} (\pm 0\cdot36)$	0·010
Δ3	$6\cdot3 \times 10^{-7} (\pm 1\cdot2)$	0·024
Δ11	$3\cdot6 \times 10^{-7} (\pm 0\cdot54)$	0·014

† Inhibition by Tnp and deletion derivatives was measured using the λ infection assay described in Materials and Methods. The plasmids in these experiments are thermo-inducible for Tnp (MA56) synthesis. Cells were assayed both at 32°C when Tnp synthesis is repressed and after a 30 min induction to 42°C to increase the synthesis of Tnp derivatives in the cell.

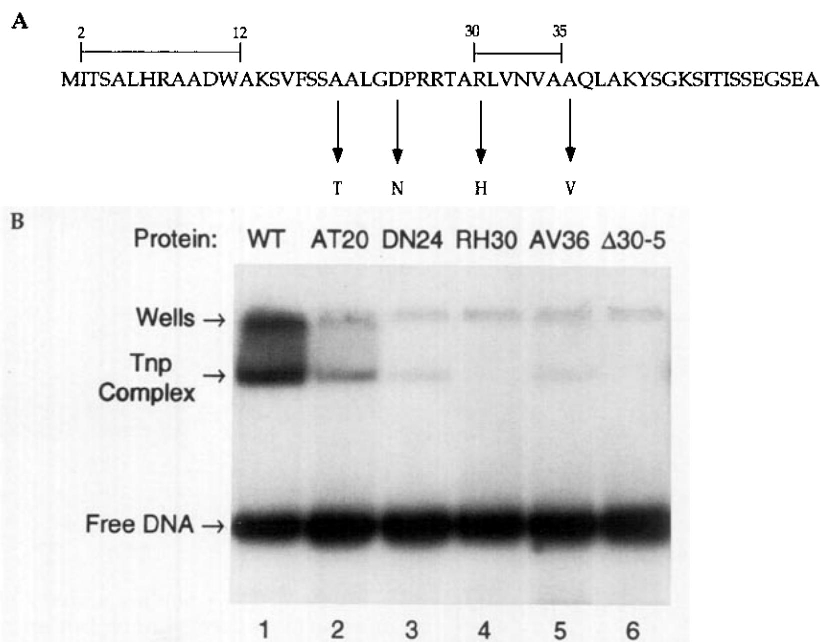

Figure 6. A, Mutations in the N terminus of Tnp. The position of 4 point mutations and a 6 bp deletion is shown within the unique N terminus of Tnp. The position of the Δ11 mutation which removes residues 2 to 12 is also indicated. Δ11 has no DNA binding activity to the OE. The relative *trans* activities were $<1{\cdot}0\%$ of the wild-type and are in complete agreement with those *cis* frequencies previously reported (Johnson & Reznikoff, 1984). B, DNA binding of purified Tnp mutants to the OE. Each mutant protein was purified (AT20, DN24, RH30, AV36 and Δ30-35) exactly as the wild-type protein and equivalent amounts of protein (0·3 μg) were assayed for binding to the OE as described in Materials and Methods.

Reznikoff, 1984) into a vector suitable for Tnp overproduction. These substitutions (AT20, DN24, RH30, AV36 and Δ30-35; Figure 6A) all resulted in less than 1% of the wild-type transposition frequency (Johnson & Reznikoff, 1984; data not shown). The Tnp derivatives were induced and purified as described in Materials and Methods. All mutant proteins showed similar levels of induction compared to the wild-type and behaved exactly like the wild-type protein during purification. When assayed for DNA binding to the OE using gel retardation, each substitution results in a protein with a significant decrease in DNA binding activity. The RH30 and Δ30-35 mutations yield proteins with no detectable DNA binding activity and the AT20, DN24 and AV36 substitutions result in significantly reduced DNA binding (Figure 6B). This demonstrates that N-terminal amino acid residues between 20 and 36 are also important for DNA binding. Furthermore, each of these substitutions with the exception of AT20, results in a protein which has wild-type or nearly wild-type levels of trans-inhibition (data not shown). This indicates that these derivatives are specifically defective in DNA binding and that their tertiary structures are not extensively altered *in vivo*.

(c) *The C terminus of Tnp inhibits DNA binding and contains a region essential for protein oligomerization*

In order to define further the region of Tnp which is required for DNA binding, C-terminal and internal in-frame deletions were constructed in Tnp (shown in Figure 7) and then tested for their DNA binding characteristics (see Materials and Methods for details of construction). Because many C-terminal and internal deletions of Tnp resulted in insoluble Tnp proteins from bacterial extracts, each of these mutant derivatives was expressed *in vitro* using a coupled transcription-translation rabbit reticulocyte lysate and then tested directly for DNA binding activity to the OE.

The full length Tnp protein translated *in vitro* gave a retarded complex with the same mobility as that formed with purified Tnp from *E. coli* (Figure 8, lanes 1, 2; and data not shown). This indicates that no *E. coli* host proteins are necessary for formation of this Tnp complex. The mutant 7C-OE DNA was used in alternating lanes to demonstrate the specificity of the retarded complexes. The two bands near the top of the gel represent non-specific complexes with the OE DNA, since they are present when the reticulocyte lysate alone is used in the binding reaction (i.e. in the absence of added template DNA to program Tnp synthesis). Furthermore, all of the deletions resulted in approximately the same amount of protein as the wild-type in the translation reaction (data not shown).

Deletion of 89 residues from the C terminus (Δ388–476) results in a significant increase in the DNA binding activity and also in the formation of an additional complex with a faster mobility (Figure 8 lanes 3, 4). Deletion of 89 residues also does not appreciably alter the mobility of the upper

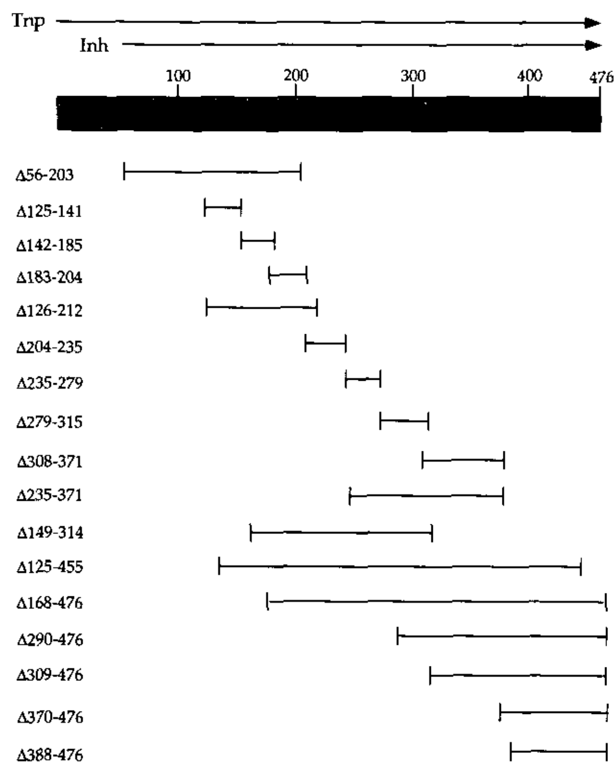

Figure 7. The collection of C-terminal and internal deletions tested for DNA binding to the OE. Deletions were isolated as described in Materials and Methods and are depicted here with their endpoints relative to IS*50*R.

complex with respect to the wild-type complex. A further deletion of only 18 amino acids (Δ370-476), however, results in a large increase in DNA binding but also shifts the complex exclusively to a faster moving one (Figure 8 lanes 5, 6). This large

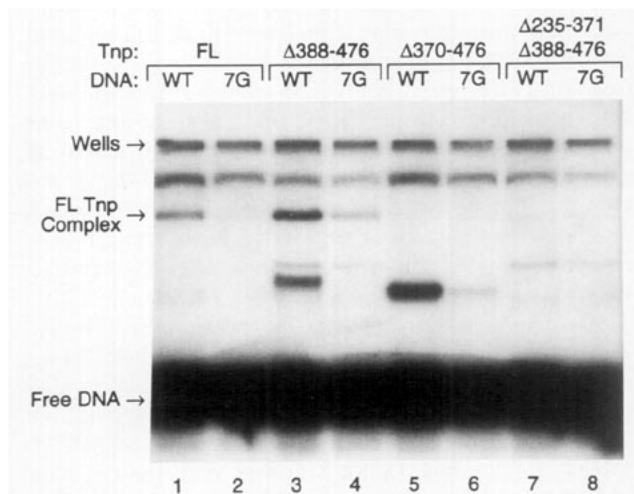

Figure 8. Gel retardation assays of *in vitro* translated Tnp derivatives. Proteins were translated in a rabbit reticulocyte lysate as described in Materials and Methods and 6 μl portions were analyzed for DNA binding activity to the wild-type OE and the mutant (7G) OE containing DNA. The carboxy-terminal deletions in Tnp were made by restricting pRZ4793, encoding full length Tnp, to completion with *Bsa*AI (Δ388–476) or *Hin*dIII (Δ370–476).

increase in mobility relative to the wild-type complex suggests that a change in the oligomeric state of the protein–DNA complex has occurred (see Discussion). Further deletions from the C terminus to positions 309, 290 and 168 result in proteins with no observable DNA binding activity (not shown).

A set of 12 internal deletions was constructed between residues 56 to 371 to further define the sequence requirements for DNA binding (Figure 7). These were expressed *in vitro*, both in the context of the wild-type C terminus and with a C-terminal truncation to residue 388 (since this increased the binding activity of the wild-type protein) and tested for binding to the OE. With the exception of Δ235-371, which exhibits very weak activity and decreased specificity compared to the wild-type (Figure 8, lanes 7, 8), none of these derivatives exhibited detectable DNA binding to the OE (data not shown). These results indicate that the DNA binding domain is located between residues 5 and 369.

(d) *Identification of a potential leucine zipper domain in Tnp and Inh*

The deletion extending from the C terminus through residue 370 removes one amino acid residue in a region that has similarity to a leucine zipper, residues 349 to 370 (Figure 9). Since the leucine zipper is a domain which mediates protein–protein interactions (Landschulz *et al.*, 1988) and Δ370-476

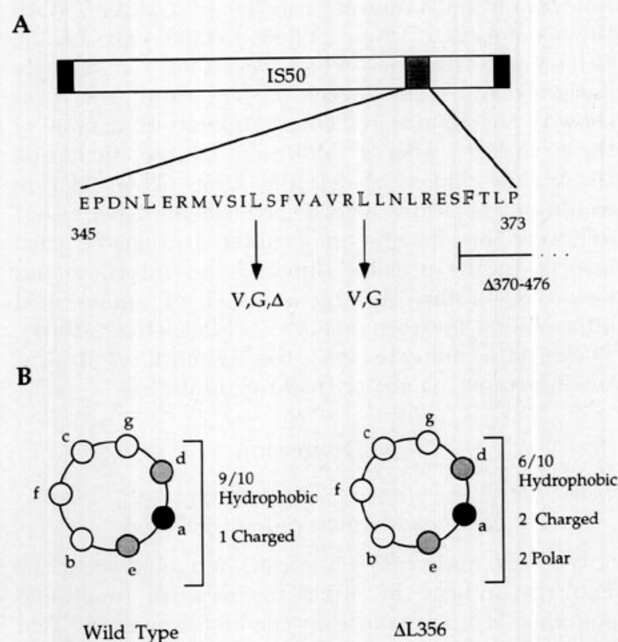

Figure 9. A, The position of a potential leucine zipper in IS*50*. The conservative changes to the leucines that were made are indicated. Also indicated is the end point of the Δ370-476 deletion that impinges on this region. B, A helical wheel projection of the putative leucine zipper α-helix. A filled circle represents the leucines in the heptad repeat and the bracket indicates the hydrophobic character of the amino acid residues surrounding the leucine on 1 face of the helix. The ΔL356 mutation is also indicated in this fashion.

Table 3
Inhibition by Leucine Zipper Mutants

Plasmid	Transposition frequency	Fold inhibition
pRZ7038 Tnp^- Inh^-	$1·6\times10^{-3}$	1·0
pRZ7013 Tnp^+Inh^+	$3·1\times10^{-6}$	516
pRZ7013 LG356	$2·4\times10^{-6}$	666
pRZ7013 LV356	$3·0\times10^{-6}$	533
pRZ7013 LG363	$4·8\times10^{-6}$	333
pRZ7013 LV363	$3·3\times10^{-6}$	484
pRZ7013 LG356 LG363	$1·5\times10^{-4}$	(11)†
pRZ7013 LG356 LV363	$2·8\times10^{-6}$	571
pRZ7013 LV356 LG363	$3·2\times10^{-5}$	(50)†
pRZ7013 LV356 LV363	$1·8\times10^{-6}$	889
pRZ7013 ΔL356	$1·4\times10^{-6}$	1142

Inhibition was determined by the λ infection assay described in Materials and Methods. This assay measures the inhibition activity of the resident pRZ7013 derivatives on the transposition rate of a wild-type Tn*5* carried on a defective λ phage. The standard errors for these measurements were less than 30%.

† Western blot analysis has demonstrated that these mutants have significantly lower steady-state levels of Tnp and Inh.

has presumably lost the ability to interact with Inh (see Discussion), we made conservative changes to the leucines in this putative domain and tested whether they affected inhibition *in vivo*. The mutations were introduced into IS*50*R on the high copy plasmid pRZ7013 and the inhibition activity of Tnp and Inh was measured using the λ infection assay. In this assay, Tnp and Inh inhibit the transposition of a wild-type Tn*5* element carried on a replication and integration deficient λ phage. With the exception of the LV356 LG363 and LG356 LG363 substitutions, which result in significantly lower steady-state levels of Tnp and Inh (not shown), the results in Table 3 indicate that none of these changes affects inhibition *in vivo*, including the severe change of deleting L356. This deletion would put all amino acid residues in the α-helix that followed this leucine in a different register with respect to the possible dimer–dimer interface and would destabilize a large number of amino acid interactions between helices (O'Shea *et al.*, 1991). These data indicate that this domain, if it is a leucine zipper, is not critical for inhibition.

4. Discussion

(a) *The N terminus of Tnp is important for two functional aspects of transposition*

We have undertaken a mutational analysis of the Tn*5* transposase in order to map its functional domains. It was found that the first five amino acid residues of Tnp are absolutely required for its function. A deletion of a single amino acid residue results in a threefold reduction in activity. A deletion of residues 2 to 4 results in a fivefold reduction in the transposition frequency, but with no detectable loss in DNA binding activity *in vitro*. This suggests either that the first few amino acid residues of Tnp (1 to 4) are important for some transposition activity other than DNA binding, or that our *in vitro* binding experiments do not accurately reflect the *in vivo* binding behavior of these mutants. Deletions of five or more amino acid residues from the N terminus result in a total loss of transposition *in vivo* and, for the Δ11 and Δ37 derivatives which were tested, a loss of binding to the Tn*5* OE.

We propose that the N terminus encodes two functions, one unknown and one critical for DNA binding, for the following reason. Recently it has been found that high level Tnp production is both cytotoxic to *E. coli* and results in defects in cell division (M. D. Weinreich, H. Yigit & W. S. Reznikoff, unpublished results). This cytotoxic effect does not depend on the ability of Tnp derivatives to bind to DNA, since the series of N-terminal point mutants and Δ30-35 which are defective in DNA binding, also kill cells when overproduced. Instead, this phenomenon is strictly correlated with the presence of the first two to three amino acid residues of Tnp (M. D. Weinreich, H. Yigit & W. S. Reznikoff, unpublished results). Suppose the only transposition defect of the Δ3 derivative was reduced affinity for the OE *in vivo*. Then, its high level expression should also be lethal since the killing caused by Tnp derivatives does not depend on their ability to bind to the OE. Since overproduction of the Δ3 derivative is not toxic to *E. coli*, however, this suggests that the first several amino acid residues of Tnp are important for some aspect of transposition other than DNA binding.

It has been shown previously that a fraction of Tnp is associated with the inner cell membrane in *E. coli*, although the significance of this association has not been elucidated (DeLong & Syvanen, 1990; Isberg & Syvanen, 1985). Interestingly, Inh does not show such an association by itself. This indicates that there is some region in the unique N terminus of Tnp which is critical for this localization. The finding that the first three amino acid residues of Tnp are important for transposition and absolutely required for cytotoxicity raises at least the possibility that these first few amino acid residues might be required for this localization. If true, this membrane localization would be functionally significant for transposition. A first test of this model would be to follow the membrane localization of wild-type Tnp and the N-terminal deletions.

A second region of the N terminus is clearly required for DNA binding since Δ11 and Δ37 Tnp derivatives no longer bind to the OE. We also purified and tested the DNA binding activity of four Tnp derivatives containing point mutations (AT20, DN24, RH30 and AV36) and one in-frame deletion (Δ30-35) which were previously shown to be defective in transposition (Johnson & Reznikoff, 1984). All of these were defective in DNA binding and the RH30 and Δ30-35 derivatives had no detectable DNA binding activity. It seems unlikely that each of these point mutations is grossly altering the conformation of Tnp which would indirectly affect DNA binding (especially the conservative AV36 mutation) since they show similar levels of protein

expression to the wild-type, behave identically to the wild-type during purification and also exhibit wild-type levels of Tnp-dependent inhibition. This data together with the N-terminal deletion analysis shows that residues from 5 to 36 are critical for DNA binding, either by encoding part of the DNA binding domain or by allowing some protein–protein interaction which is a prerequisite for DNA binding.

This N-terminal deletion analysis also indicates that Tnpα is a degradation product of Tnp missing approximately 30 to 35 N-terminal amino acid residues and possesses no DNA binding activity. It was noted previously, that fractions of Tnp which contained a proteolytic fragment of Tnp, Tnpα, exhibited greater DNA binding than fractions which contain mostly Tnp alone (de la Cruz *et al.*, 1993). Tnpα was in fact shown to be present with Tnp in bound OE complexes (de la Cruz *et al.*, 1993; Wiegand & Reznikoff, 1994). It remained a formal possibility therefore, that Tnpα is responsible for the binding activity in our Tnp preparations. Firstly, it is not produced from an independent translational start site in Tnp because pRZ4738 (MA56) which has a seven base-pair deletion removing the start codon for Tnp does not encode this product (data not shown). We can also conclude that Tnpα is an N-terminal degradation product of Tnp because its mobility on SDS gels does not change in deletions removing up to 22 amino acid residues from the N terminus. If this were a C-terminal proteolytic product this would not be the case. It is absent in the Δ37 deletion indicating that the proteolytic site has been removed. Furthermore, it does not bind to the OE on its own because Tnp Δ11 preparations, which have this Tnpα contamination (e.g. see Figure 4 lane 9), exhibit no DNA binding activity to the OE.

(b) *The DNA binding domain of Tnp is located between residues 5 and 369*

The DNA binding characteristics of Tnp variants having C-terminal or internal deletions were tested to localize further the DNA binding domain. A deletion of 89 amino acid residues from the C terminus (Δ388-476) leads to a significant (5 to 10-fold) increase in DNA binding activity. This suggests that the wild-type C terminus normally interferes with DNA binding, perhaps by partially masking the binding domain. A further deletion of 18 residues (Δ370-476) also results in greater overall binding activity but additionally results in the loss of the wild-type complex and a shift to a complex with a much faster mobility. C-terminal truncations to residues 309, 290 and 168 resulted in proteins with undetectable DNA binding activity.

Tnp derivatives containing internal deletions between residues 56 and 371 were also tested for DNA binding activity. With the exception of Tnp-Δ235–371, none of these deletion derivatives exhibited any DNA binding activity. The weak DNA binding by Tnp-Δ235–371, however, does not show the same specificity to the OE as the wild-type. We cannot draw a firm conclusion from this negative evidence, however, since each of the C-terminal and internal deletion derivatives which lack DNA binding activity may be improperly folded. So, the OE DNA binding domain of Tnp lies between residues 5 and 369 (defined by the N and C-terminal deletions) and may be contained between residues 5 and 235, but additionally requires other structural features of the protein for stable DNA binding. Either two regions of Tnp are required to comprise the DNA binding domain or secondary structural elements of one part of the protein are critical for maintaining an active DNA binding domain. This protein appears not to have a modular structure containing an independently folded DNA binding domain, since we have been unable to localize this domain further with a deletion analysis.

The simplest interpretation is that the OE DNA binding domain of Tnp is located in the N terminus. We do not think that the loss of DNA binding by the N-terminal mutants is due to the loss of a protein-protein contact between Tnp monomers which is required for DNA recognition. The OE does not contain any obvious dyad symmetry element typically present at recognition sites for dimeric DNA binding proteins. Also, it has previously been shown that the wild-type Tnp–OE complex contains a heteromultimer of Tnp with either Inh or Tnpα (de la Cruz *et al.*, 1993; Wiegand & Reznikoff, 1994) and this is the only complex that we have detected with purified Tnp. Since Inh does not bind to the OE on its own and it is a dimer in solution, it must form mixed multimers (presumably dimers) with Tnp which then bind to the OE resulting in a complex inactive for transposition (de la Cruz *et al.*, 1993). Because Inh lacks the first 55 amino acid residues of Tnp and cannot form these presumptive N-terminal dimer contacts required for DNA binding, we suspect that Tnp–Tnp dimerization at the N terminus is not necessary for DNA binding.

(c) *The C terminus of Tnp inhibits DNA binding and is also essential for Tnp–Inh oligomerization*

Deletions into the C terminus of Tnp increase its apparent DNA binding activity. The Δ388-476 and Δ370-476 derivatives also still retain specificity for the OE sequence since they bind poorly to the mutant 7G-OE DNA. The five- to ten fold increase in DNA binding activity that occurs with these deletion derivatives indicates that C-terminal residues of Tnp normally interfere with efficient OE recognition. This could result if these residues mask the DNA binding domain of Tnp perhaps by directing a functionally inactive conformation for this protein.

The Tnp derivative with the C-terminal deletion (Δ370-476) results not only in an apparent increased affinity for the OE but also shifts the Tnp-OE complex exclusively to a faster mobility one on polyacrylamide gels. This large shift in mobility strongly suggests a difference in the multimeric state of Tnp from that of the wild-type complex. It

has already been shown that proteolytic fragments of Tnp which have lost the C-terminal approximately 100 to 150 amino acid residues generate an OE complex that has an increased mobility similar to our C-terminal deletion of 107 amino acid residues (Δ370-476). Importantly, these complexes were shown not to contain Inh or Tnpα and were deduced to be monomeric (Wiegand & Reznikoff, 1994). Taken together, this strongly suggests that residues 370 to 387 are critical for dimerization with Inh and, therefore, the inhibitory activity of Inh. If this region is also critical for Tnp–Tnp interactions, then multimerization may be the mechanism which inhibits binding to the OE by occluding the DNA binding domain within an inactive Tnp dimer. This is consistent with the fact that only 3 to 5% of purified Tnp exhibits specific binding to the OE. This small percentage of molecules active for DNA binding, however, consist of Tnp–Inh multimers which are presumably inactive for transposition. Since we have yet to observe a Tnp–OE complex containing full length Tnp alone, this may indicate that the majority of Tnp in our preparations is present as an oligomeric species, incompetent for DNA binding.

Deletion of 26 C-terminal amino acid residues of Tnp results in the complete loss of transposition activity *in vivo* (Johnson *et al.*, 1982; Rothstein & Reznikoff, 1981). So, the C terminus is essential for transposition but at the same time inhibits the DNA binding activity of Tnp. This may be part of a mechanism which intrinsically limits the activity of Tnp so that transposition does not exceed levels acceptable to the cell.

We discovered a region which has similarity to a leucine zipper motif (residues 349 to 370) directly adjacent to the C-terminal deletion which results in an increased mobility Tnp–OE complex. The leucine zipper is an α-helical structural motif involved in protein dimerization (Landschulz *et al.*, 1988) and residues 349 to 370 are predicted to be α-helical. We tested the importance of this putative dimerization motif for inhibition by making conservative changes in leucines which are critical for protein–protein contacts in these motifs (Landschulz *et al.*, 1988). Also, a single amino acid residue deletion in this domain was constructed which would disturb the register of amino acid residues in the putative helix after the deletion. The inhibitory activity of Inh is almost certainly due to mixed-dimer formation with Tnp, resulting in subunit poisoning. None of these substitutions had any effect on inhibition *in vivo*. Importantly, the ΔL356 change had no effect on inhibition. If this putative leucine zipper domain is critical for Tnp–Inh interactions, it would have produced a severe defect in inhibition since the precise positioning of amino acid residues in the α-helices is critical for dimer formation (O'Shea *et al.*, 1991). These results indicate that this putative leucine zipper domain is not essential for Tnp–Inh formation. However, preliminary data are consistent with this general region being necessary for inhibition *in vivo*.

Two other approaches can be taken to more precisely define the amino acid residues required for DNA binding. The first is a genetic screen that will identify second site revertants in Tnp which recognize altered OE sequences. The second is a high resolution cross-linking study of Tnp to the OE to identify amino acid residues in close proximity to the OE sequence. We have also tried using limited proteolysis with trypsin to identify a peptide that will bind to the OE. However, there is a hyperaccessible site about 30 to 35 amino acid residues from one terminus (presumably the N terminus) and this cleavage rapidly results in the loss of all DNA binding activity for Tnp (data not shown).

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