Identification of amino acid residues essential for the catalytic activity of *Drosophila* P element transposase

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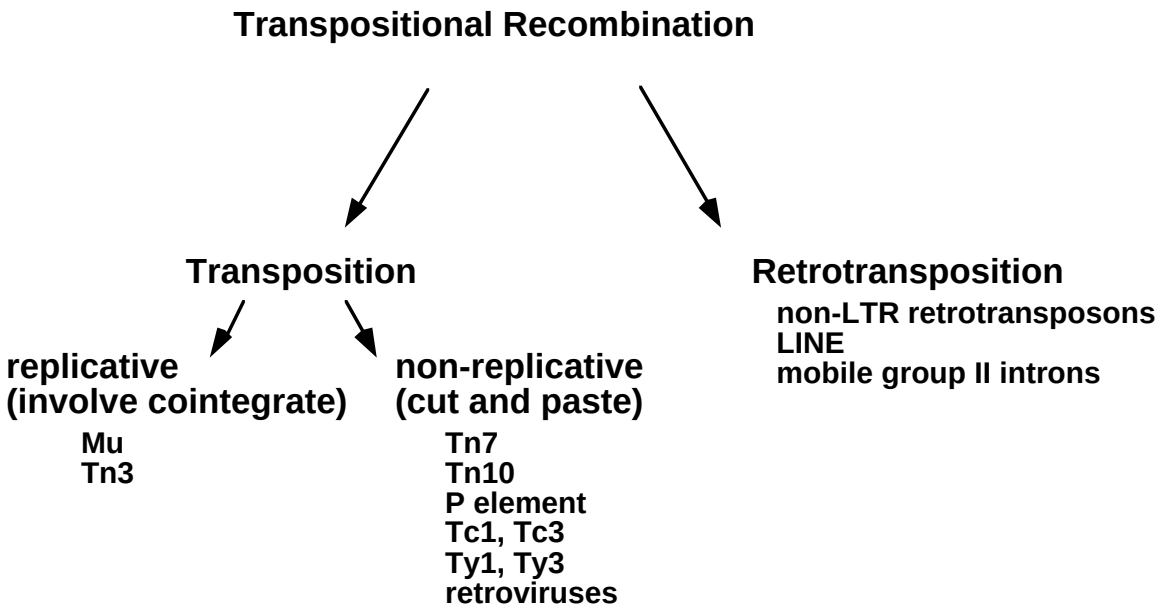
1. Introduction

1.1 Transposition

Transposable elements are discrete DNA segments that can translocate between non-homologous insertion sites. The transposon family of mobile genetic elements is widespread among organisms, with transposons having been identified in virtually all organisms examined (Berg and Howe, 1989).

Transposable elements are involved in a wide variety of biological transactions including genome alterations by element insertion and deletion, homologous recombination between element copies, viral integration and replication, and the dispersal of a variety of determinants, most notably antibiotic resistance genes (Craig, 1997).

There are two major pathways for transpositional recombination, usually referred to as transposition and retrotransposition.



In transposition, element translocation results from DNA breakage and joining reactions. The ends of the mobile element are disconnected from a donor site by DNA cleavage reactions, and these exposed ends are then joined to a

target site by DNA strand transfer reactions (Craig, 1996; Mizuuchi, 1992a; Plasterk, 1995). This pathway of translocation of a DNA substrate is used by elements that exist only as DNA. Their category is further subdivided into a) non-replicative transposition, as found in transposition of bacterial Tn7 and Tn 10 elements, the *Drosophila* P element, *C.elegans* Tc1 and Tc3 elements, yeast Ty1 and Ty3 and in retroviral integrases, and b) replicative transposition, as found during the replicative lifecycle of bacteriophage Mu and with bacterial transposon Tn3 (see previous page). The mechanism(s) of replicative and non-replicative transposition will be discussed in the next section.

In the other pathway for transposition, often called retrotransposition, DNA, RNA, and reverse transcriptase all participate directly in recombination (Craig, 1997; Eickbush, 1992). Recombination initiates by target DNA breakage at the site of element insertion, a target 3'-OH exposed by this break then provides a primer for reverse transcription which uses an element RNA as its template. The ultimate result of this retrotransposition reaction is the insertion of a DNA-form of the element into the target site through this copying mechanism. Elements using this pathway are termed non-LTR (Long Terminal Repeat) retrotransposons, and include the human LINE element (Long Interspersed Nuclear Element), and mobile group II introns (Craig, 1997). Fundamental to both transposition and retrotransposition is the assembly of a functional protein-DNA complex, which is likely to be the key regulating step.

1.2. Mechanism of replicative versus non-replicative transposition

In the transposition reaction, transposase performs two distinct and sequential reactions on its DNA substrate. In both replicative and non-replicative transposition pathways, the first step is a pair of site-specific, endonucleolytic cleavages that separate the 3'-OH of the transposon DNA ends from the 5' phosphoryl ends of the adjoining host (Fig. 1A) (Grindley and Leschziner, 1995). In other words, a nuclear substitution reaction (Sn2) using water as a nucleophile creates the first DNA break.

In the second step, the mechanism of transposition is determined by whether cleavage occurs at the 5' ends (Fig. 1A). If the 5' end of the element is cleaved to generate an excised transposon intermediate, the element transposes by a cut-and-paste mechanism. Tn7 (Bainton *et al*., 1991), Tn10 (Benjamin and Kleckner, 1992), the Tc elements (Luenen *et al*., 1994; Vos *et al*., 1996), and the P element (Kaufman and Rio, 1992) all transpose by such a mechanism. Bacteriophage Mu also transposes by the cut-and paste mechanism during the lysogenic pathway of its lifecycle (Pato, 1989). The nature of the nontransferred strand cleavage, however, is variable and occurs either 3 bp outside for the Tn7 element (Bainton *et al*., 1991; Gary *et al*., 1996), 2 bp inside for the Tc element (Luenen *et al*., 1994; Vos *et al*., 1996), at the transposon termini for the Tn10 element (Benjamin and Kleckner, 1992), or 17 bp within the transposon termini for the P element (Beall and Rio, 1997).

If the 5' end of the element is not cleaved, the element transposes by a replicative transposition mechanism in which the transposon remains attached to both the donor site and the target site. The intermediate is replicated by host replication proteins to produce two copies of the element in a structure called a cointegrate (Fig. 1A, right panel). Bacteriophage Mu and the Tn3 element can transpose by this type of mechanism (Craigie and Mizuuchi, 1985; Lavoie and Chaconas, 1996; Mizuuchi, 1992b).

Analogous to Mu, retroviral integration, like HIV integration, does not require processing at the 5' end of the reverse-transcribed genome by the integrase protein prior to insertion into the host genome (Fig.1A, left panel) (Engelman *et al*., 1991). A staggered target-site cleavage is made by most transposases and integrases, and DNA repair of the gaps that flank the newly inserted element generated the characteristic target-site duplications present after insertion.

Thus, all these DNA transposition reactions are related by 3'-end breakage, whereas the 5'-end processing may vary.

1A.

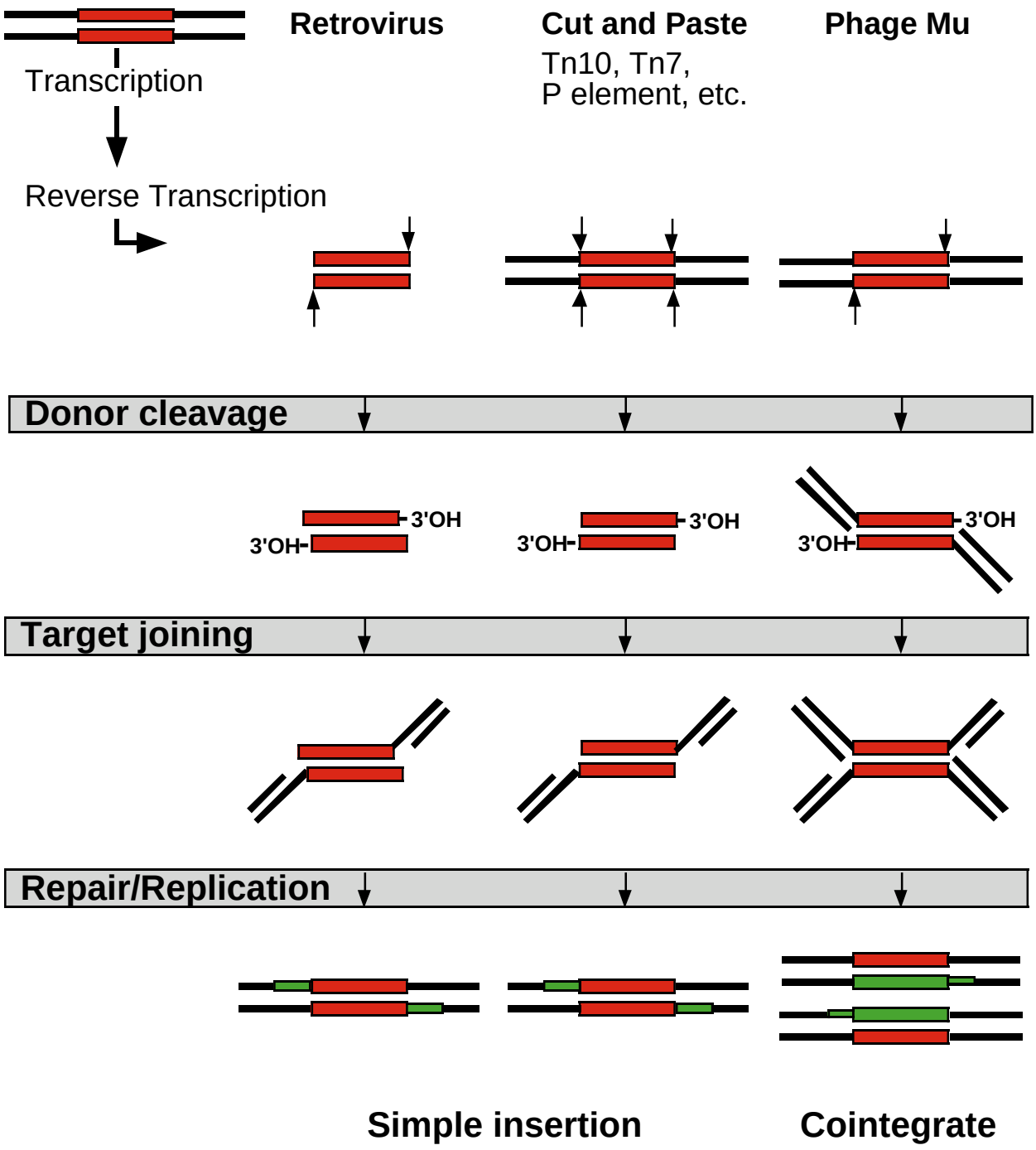

Retrovirus
Cut and Paste
Tn10, Tn7,
P element, etc.
Phage Mu
Transcription
Reverse Transcription
Donor cleavage
3'OH
3'OH
3'OH
3'OH
3'OH
3'OH
Target joining
Repair/Replication
Simple insertion
Cointegrate

Figure 1A. Schematic diagram of the transposition reactions that underlie the translocation of three mobile elements (redrawn from Craig, 1995). For P element transposition, the first step of the reaction, DNA cleavage, results in excision of the P element by the transposase protein through double-stranded DNA breaks at each end of the P element. The element is then inserted into a new target site by strand transfer of the 3'-OH groups at the P element termini to the 5'-phosphate groups at the target site. The resulting intermediate consisting of a double-stranded DNA break at the donor site and a gapped DNA at the target site. The DNA gaps must be repaired by the *Drosophila* repair enzymes in order to complete the transposition reaction and to prevent chromosome loss. [Red boxes, mobile elements; black lines, flanking donor and target DNA; small black arrows, cleavage at 3'-OH and 5'-OH ends; green boxes, DNA replication.]

1.3. Energetics of strand transfer: one-step transesterificaton

Neither of the two transfer reactions in the transpositional recombination of Tn10 (Morisato and Kleckner, 1987), phage Mu (Maxwell *et al*., 1987), and retroviral DNA integration (Bushman and Craigie, 1991) depends on hydrolysis of any high energy cofactor such as ATP. ATP is required for Tn7 transpositional recombination (Bainton *et al*., 1991), but its role, as in the Mu reaction, appears to be in the target DNA selection process rather than in the chemistry of the transfer steps (Mizuuchi, 1992a). In contrast, the *Drosophila* P element transposase requires GTP as a cofactor for transposition. However, GTP hydrolysis is not required for the steps of transposition *in vitro*, because nonhydrolyzable GTP analogs can completely substitute for GTP (Kaufman and Rio, 1992). GTP hydrolysis may be required for subsequent steps of the transposition reaction that occur after strand transfer. Thus, strand transfer must proceed via a transesterification step(s) rather than by sequential steps of phosphodiester bond hydrolysis and ligation (which would require exogeneous energy).

Strong evidence that the phosphoryl transfers involved in 3'-end processing and strand transfer both occur by a single-step mechanism has been obtained for HIV integrase (Engelman *et al*., 1991) and Mu transposase (Mizuuchi and Adzuma, 1991)

using chiral thiophosphates at the cleaved position; each single reaction analyzed results in an inversion of the stereochemical configuration of the phosphorothioate. These findings render strong support to a one-step mechanism and thus argue against the involvement of a covalent intermediate.

1.4. Active complex composition

The number of subunits and the size of the transposase/integrase protein varies widely between different elements. The Tn7 transposon for instance, encodes five proteins, two of which, TnsA and TnsB, in conjunction with combinations of the three additional Tn7-encoded proteins, carry out the cleavage and strand transfer steps of the transposition reaction (Bainton *et al*., 1991). In contrast, Tn10 transposon encodes a single polypeptide that performs the catalytic steps of transposition (Benjamin and Kleckner, 1992). In phage Mu, the active form of the transposase protein is a tetramer (Mizuuchi, 1992b) in which the same two monomers within a MuA tetramer provide the catalytic domains for the strand cleavage and strand transfer reactions (Namgoong and Harshey, 1998). However, a functional Mu tetramer complex can only assemble at the Mu termini after interaction with the *E.coli* HU protein bound to the left end of the Mu genome and *E.coli* IHF bound to the Mu enhancer element (Lavoie and Chaconas, 1993).

For the retroviruses such as MLV and HIV, large nucleoprotein complexes containing the integrase protein and host factors assemble onto the ends of the reverse-transcribed proviral genome prior to processing and insertion into the host genome (Farnet and Bushman, 1997).

1.5. Target site selection

There is a wide variety of patterns of target site selection used by the different transposable elements, which suggests that many strategies successfully promote element propagation and optimization of the element–host

relationship. To avoid insertion into essential genes, transposon Tn7, for example, inserts into a specific site in bacterial chromosomes that provides a "safe" place where insertion will not adversely affect the host bacterium (Craig, 1997). Some other elements, including the yeast Ty1 and Ty3 elements and the *Drosophila* P element, usually insert upstream of promoters, thereby decreasing the probability of element insertion into essential protein coding sequences. However, this type of insertion often inactivates the promoter resulting in a mutant phenotype which is very deleterious (Engels, 1989). Some very "resourceful" mobile elements are also introns, so if they insert into an essential gene, they can be removed from the mRNA by RNA splicing (Craig, 1997). The DNA sequences required for integration of retroviruses are short, imperfect inverted repeats at the outer ends of the retroviral long terminal repeats (LTR) (Vink and Plasterk, 1993). There is no systematic evidence for preferential integration into nonessential sequences by any retrovirus, however, anecdotal evidence suggests that some important genes are used more frequently (Hubbard *et al*., 1994) while other genes are used less frequently than expected as integration targets (Frankel *et al*., 1985; King *et al*., 1985). There does seem to exist a preference for insertion of retroviruses into bent DNA, as found for example in nucleosomes, however, not all bends in DNA result in preferred targets (Craig, 1997; Muller and Varmus, 1994).

1.6. Catalytic Motif

Despite the many obvious differences in the life-style and "features" of the various mobile DNA elements, they appear to be fundamentally related by the structure of the catalytic domain of the encoded transposase protein, the DD(35)E motif. This motif, so-called because of the usually 35 amino acid spacing between the last two residues, is thought to coordinate divalent metal-ion binding during catalysis (Engelman *et al*., 1993; Kulkosky *et al*., 1992). Even conservative substitutions at these positions have a drastic effect on cleavage and strand transfer (Baker and Luo, 1994; Engelman *et al*., 1993; Kim *et al*.,

1995; Kulkosky *et al*., 1992). The presence of these conserved, essential carboxylates and the requirement for divalent metal ion(s) during catalysis led to the suggestion that these enzymes may promote phosphoryl transfers by a process similar to the two-metal ion mechanism proposed for the 3'-5' exonuclease of DNA polymerase (Joyce and Steitz, 1994) and RNAse H (Davies *et al*., 1991). In fact, structural analysis has revealed that the integrases (HIV,RSV) are members of a superfamily of nucleic acid-processing enzymes that include RNAse H (Yang *et al*., 1990b), the Holliday junction-resolving enzyme RuvC (Ariyoshi *et al.,* 1994) and the Mu transposase (Bujacz *et al*., 1996; Bujacz *et al*., 1995; Dyda *et al*., 1994; Rice and Mizuuchi, 1995; Rice *et al*., 1996).

1.7. V(D)J Recombination

V(D)J recombination is the process by which functional immunoglobulin and T cell receptor genes are assembled from multiple gene coding segments in developing lymphocytes. The segments are composed of variable (V), diversity(D), and joining (J) gene segments that are distributed throughout a wide portion of the genome. One of each type of gene segment is joined together in a site-specific recombination reaction that is tightly regulated and involves the RAG1 and RAG2 gene products (Oettinger *et al*., 1990; Schatz *et al*., 1989). Each coding segment is flanked by a conserved recombination signal sequence (RSS) that consists of a heptamer and a nonamer sequence separated by either a 12 or 23 bp spacer sequence. Efficient recombination requires one RSS of each type, a restriction known as the 12/23 rule (Tonegawa, 1983).

Together, the RAG1 and RAG2 proteins bind two recombination signals, bring them into close juxtaposition (this process is termed synapsis), and cleave the DNA, thereby separating the signals from the flanking coding segments (Eastman *et al*., 1996; Van Gent *et al*., 1996b).

The DNA-bending high-mobility group proteins HMG1 and HMG2 substantially enhance the efficiency of coordinate cleavage (Sawchuk *et al*.,

1997; Van Gent *et al*., 1997), in part by improving binding to the 23-signal, and by their general ability to bind and to modulate DNA structures. DNA bending induced by the HMG proteins can facilitate the formation of higher-order nucleoprotein complexes, suggesting that these proteins may have an architectural role in assembling such complexes (Grosschedl *et al*., 1994).

After HMG-protein assisted DNA binding of RAG1 and RAG2, cleavage occurs in two steps, with a nick first introduced adjacent to the heptamer to expose a 3'-OH group on the coding flank, followed by a direct nucleophilic attack of the 3'-OH on the opposite DNA strand (McBlane *et al*., 1995). The products are blunt, 5'-phosphorylated signal ends and covalently sealed hairpin coding ends.

The chemical steps of V(D)J recombination are similar to the steps of retroviral integration and transposition in that they proceed through a common pathway that involves exposure of a 3'OH group and is attack on the target phosphodiester bond in a magnesium-dependant reaction (Craig, 1995; Van Gent *et al*., 1996a). Also, the RAG proteins remain stably associated with a synapsed pair of recognition elements after DNA cleavage, as is common in transposition (Agrawal and Schatz, 1997; Mizuuchi, 1992b).

These findings together with the fact that the RAG1 and RAG2 genes have a compact genomic organizaton, as would be expected for components of a transposable element, support the idea that the antigen receptor gene segments and the RAG1 and RAG2 proteins may have evolved from an ancestral transposon (Agrawal *et al*., 1998; Hiom *et al*., 1998; Lewis and Wu, 1997; Litman *et al*., 1993; Thompson, 1995). Further evidence in support of this idea comes from data demonstrating that the RAG1 and RAG2 proteins can perform strand transfer *in vitro* (Agrawal *et al*., 1998; Hiom *et al*., 1998). It was postulated that the split nature of immunoglobulin and T-cell-receptor genes derives from germline insertion of this element into an ancestral receptor gene soon after the evolutionary dievergence of jawed and jawless vertebrates (Agrawal *et al*., 1998; Litman *et al*., 1993; Rast *et al*., 1997).

1.8. The *Drosophila* P element

P elements were discovered as the agents that cause a syndrome of genetic traits in *Drosophila* known as hybrid dysgenesis (Engels, 1989; Rio, 1990). This collection of abnormalities, including temperature-dependent sterility, elevated rates of mutation, chromosome rearrangement, and recombination is usually seen only in the progeny of a cross of males with autonomous P elements and females that lack P elements. These two kind of strains are called "P" and "M" because they contribute paternally and maternally, respectively, to hybrid dysgenesis. No dysgenic traits are observed in the progeny of the reciprocal M male by P female cross or in progeny from P x P or M x M crosses. Furthermore, the symptoms of hybrid dysgenesis are restricted entirely to the germ lines of the progeny from a dysgenic cross. These phenotypes are caused by the high rates of P element transposition that occur in this tissue. Thus, P element transposition is regulated in two ways: genetically (it occurs only in P male x M female progeny) and tissue specifically (it occurs only in the germ lines of dysgenic progeny) (Rio, 1991).

The full-length P element is 2.9 kb in length (Fig 1B). A heterogeneous class of internally deleted elements also exists, some of which appear to encode truncated proteins with distinct biological activities, such as the KP element, which contains an internal deletion from amino acid 807 to 2561 (Fig. 1B) (Rio, 1991). In a typical P strain, there are approximately 10-15 complete elements and 30-40 smaller, deleted elements. Mutational analysis has shown that all four open reading frames are required for production of a functional 87 kD transposase protein (Karess and Rubin, 1984; Rio *et al*., 1986). In the soma, removal of the third intron, or IVS3 sequence, from the P element pre-mRNA is inhibited, resulting in the production of a 66 kD protein (Fig. 1B) (Laski *et al*., 1986; Rio *et al*., 1986). Both the 66 KD protein and the KP protein are

1B.

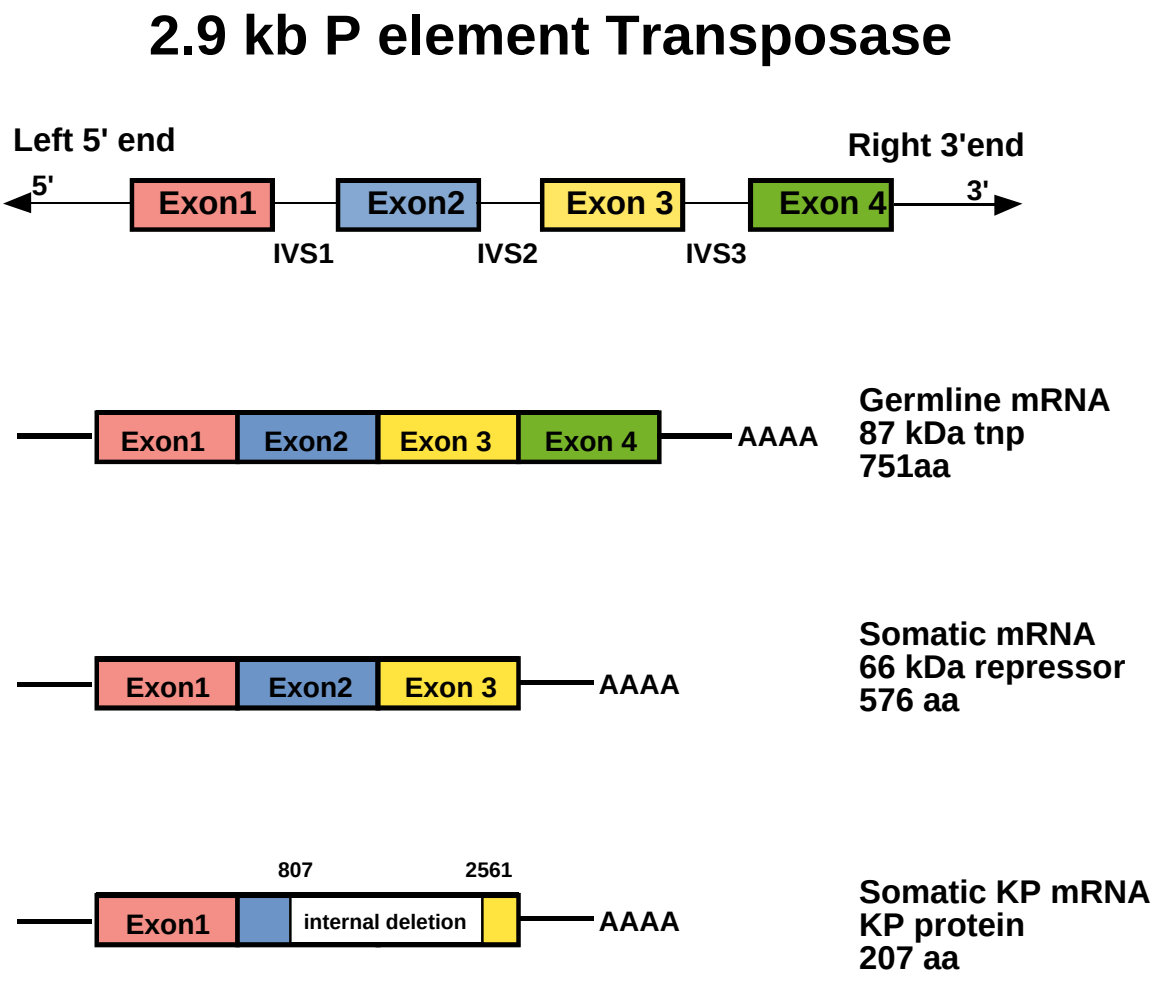

2.9 kb P element Transposase
Left 5' end
Right 3'end
5'
Exon1
Exon2
Exon 3
Exon 4
3'
IVS1
IVS2
IVS3
Exon1
Exon2
Exon 3
Exon 4
AAAA
Germline mRNA
87 kDa tnp
751aa
Exon1
Exon2
Exon 3
AAAA
Somatic mRNA
66 kDa repressor
576 aa
807
2561
Exon1
internal deletion
AAAA
Somatic KP mRNA
KP protein
207 aa

Figure 1B. Schematic structure of the 2.9 kb P element, including the derived mRNAs and proteins. The full-length P element is 2.9 kb and contains four open reading frames encoding exons 1 to 4. Removal of all three intervening sequences (IVS1-3) allows for production of an 87 kD transposase protein that catalyzes transposition. Transposase synthesis is restricted to the germ line because splicing of the third intron (IVS3) is inhibited in somatic tissues. IVS3-containing transcripts produce a 66 kD transpositional repressor protein in both somatic and germ line tissues by using a stop codon within the IVS3 sequence. In addition to full-length P elements, many *Drosophila* strains contain internally deleted P elements such as the KP element shown at the bottom of the figure. The KP element contains an internal deletion from nucleotides 807-2561 of the P element sequence. As a result, a 24 kD protein is produced that also represses transposition and contains the amino-terminal 199 amino acids of the transposase protein in addition to 8 amino acids of unique sequence at the C-terminus.

repressors of transposition *in vivo* (Andrews and Gloor, 1995; Misra and Rio, 1990; Rasmusson *et al*., 1993; Robertson and Engels, 1989). The KP protein is also a repressor *in vitro* (Lee et al., 1998).

1.9. Structure of the P element

A key to understanding the mechanism of P element transposition lies in the structure, function, and biochemical activities of the P element transposase. Parts of the P element primary structure/function relationship have been determined (Fig. 1C). All P elements contain approximately 150 bp of sequence at their termini that are required for transposition. Within these *cis*-acting sequences are the 10 bp consensus transposase binding sites located 52 bp or 40 bp away from the left and right ends of the P element termini, respectively (Fig. 1C) (Kaufman *et al*., 1989), which are required for transposition *in vivo* (Mullins *et al*., 1989) and *in vitro* (Kaufman and Rio, 1992). In addition, 11bp inverted repeats approximately 140 bp within the P element end sequences are enhancers of transposition *in vivo* (Mullins *et al*., 1989). The 31 bp inverted repeats (IR) sequences on both sides of the element are thought to interact with the *Drosophila*-encoded protein IRBP (inverted Repeat Binding Protein) which

could be required for or simply enhance transposition (Rio *et al*., 1986). P element transposase requires both 5' and 3' P-element termini for efficient DNA cleavage to occur, suggesting that a synaptic complex forms prior to cleavage (Beall and Rio, 1997). Transposase makes a staggered cleavage at the P element termini in which the 3' cleavage site is at the end of the P element, whereas the 5' cleavage site is 17 bp within the P element 31 bp inverted repeats, directly adjacent to the IRBP-binding site (Beall and Rio, 1997). P element termini were shown by LMPCR (Ligation Mediated Polymerase Chain Reaction) to be protected from exonucleolytic degradation following the cleavage reaction, suggesting that a stable protein complex remains bound to the element termini after cleavage.

In addition to the *cis*-acting structures at the DNA ends of the transposable element, essential for functional transposition, several domains within the P element transposase protein's amino acid sequence have been identified (Fig.1C, lower part of diagram): The site-specific DNA-binding domain has been mapped to the amino terminal 88 amino acids of the transposase protein, to a region that contains a potential zinc-binding motif (Lee *et al*., 1998). Studies by Mul and Rio showed that P element transposase is a GTP-binding protein whose nucleotide-binding region has several conserved sequence motifs know to also be specifically required for GTP binding in other proteins (Mul and Rio, 1997). Amino acids 260-415 were found to be required for GTP binding. In addition, there are three different potential leucine zipper motifs in the transposase coding sequence (amino acids 101-122, 283-311, and 497- 525) which could mediate multimerization of the transposase protein (Rio *et. al*., 1986; O'Hare and Rubin, 1983). The amino-terminal leucine zipper motif spanning amino acids 101-122 mediates dimerization of the KP repressor protein *in vitro* (Lee *et al*., 1996). Both dimers and tetramers of the transposase protein have been detected *in vitro* (Mul and Rio, unpublished). However, the active oligomeric form of the transposase protein has yet to be determined. Within the N-terminus of the transposase protein are several potential sites of phosphorylation by the DNA–dependent

1C.

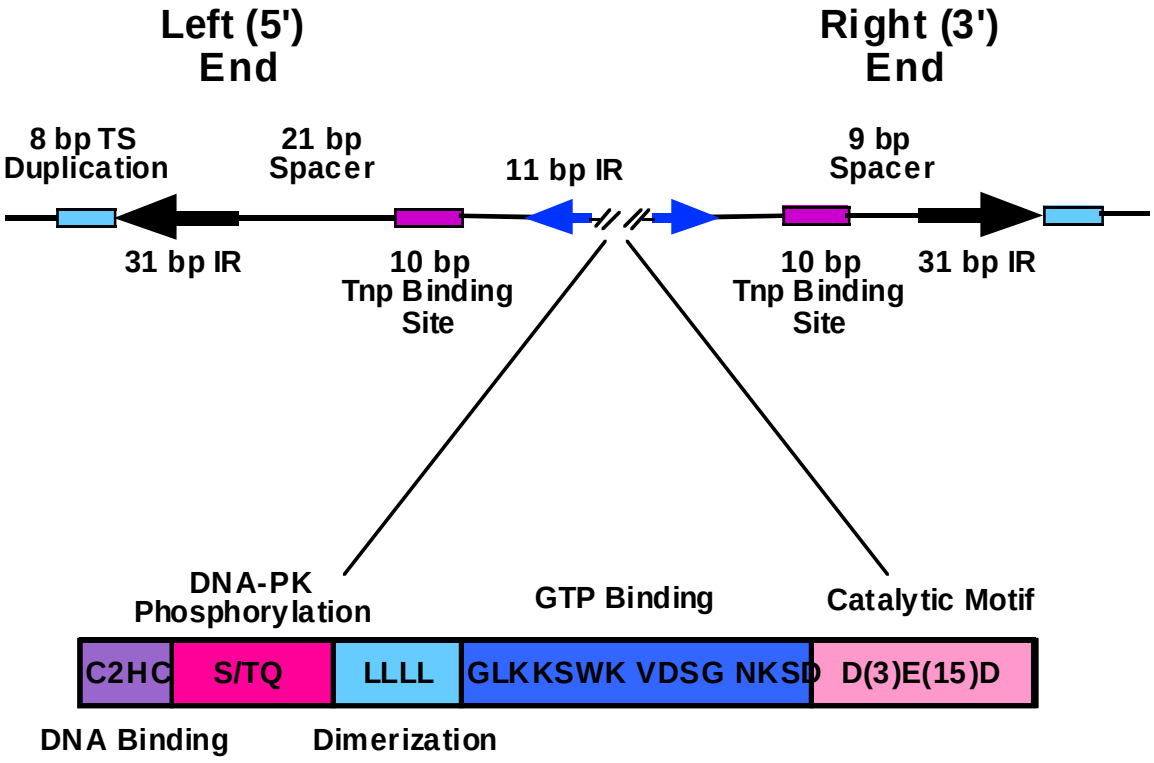

Left (5')
End
Right (3')
End
8 bp TS
Duplication
21 bp
Spacer
11 bp IR
9 bp
Spacer
31 bp IR
10 bp
Tnp Binding
Site
10 bp
Tnp Binding
Site
31 bp IR
DNA-PK
Phosphorylation
GTP Binding
Catalytic Motif
C2HC
S/TQ
LLLL
GLKKSWK VDSG NKSD
D(3)E(15)D
DNA Binding
Dimerization

Figure 1C. Schematic diagram of left (5') and right (3') end of the *Drosophila* P-element and its catalytic domains. The P-element transposon contains several sequence elements at its termini that are essential in *cis* for transposition: The transposase-binding site contains a 10-bp consensus sequence located internally at each end of the P-element. Interaction between this site and the transposase protein is essential for transposition. A terminal 31-bp inverted repeat located at each end of the transposon is also necessary for mobilization and is recognized by the *Drosophila* inverted repeat binding protein (IRBP). Finally, there is an internal 11-bp inverted repeat that has been shown to act as a transpositional enhancer *in vivo*. The lower part of the diagram depicts the functional domains of the transposase protein: The DNA binding domain (purple box) and the DNA-PK phosphorylation domain (red box) are located in the N-terminal region. The leucine zipper motif for dimerization (light blue box) and the GTP binding domain (dark blue box) are positioned in the central region of the protein. The catalytic D(3)E(15)D motif (pink box) of transposase is located in the C-terminal half of the protein.

protein kinase (DNA-PK) directly adjacent to the site-specific DNA binding domain (Fig. 1C). The catalytic component, DNA-PKcs is thought to be targeted to DNA in mammalian cells by its cofactor, the Ku heterodimer. P element transposition is thought to be regulated throughout the cell cycle, occurring predominantly in G2 (Engels *et al*., 1990). DNA-PK activity has been detected in *Drosophila* extracts (Finnie *et al*., 1995). Moreover, in mammalian cells, there is 5-to 10-fold more DNA-PK activity in the G2 phase of the cell cycle (Jin *et al*., 1997). Therefore, phosphorylation by DNA-PK may be one way that the P element transposase activity is regulated throughout the cell cycle.

1.10. P element transposition mechanism

P elements move via a non-replicative cut-and past mechanism that is catalyzed by an 87 kD P element encoded transposase protein (Engels, 1989). Transposase binds specifically to internal sequences at both ends of the transposon (Kaufman *et al*., 1989) and is believed to excise the donor P element as a double-strand DNA intermediate. Following excision, the free 3’ OH groups of the released P element DNA are thought to make a nucleophilic attack on transposase-activated 5’ phosphoryl groups at the target sequence (Kaufman

and Rio, 1992). After insertion of the P element, host proteins presumably repair the single-stranded gaps flanking the element (Engels, 1989) and the double-strand breaks at the donor sites (Beall and Rio, 1996) (see Fig. 1A). *In vitro* studies using circular plasmids as donor and target DNAs indicated that optimal transposition efficiency requires GTP as well as Mg^{++} (Kaufman and Rio, 1992); (Mul and Rio, 1997). When the GTP requirement was switched to XTP *in vivo* by changing one amino acid within the transposase protein that recognizes the guanine ring to an amino acid that recognizes the xanthine ring, the mutant transposase protein was only active *in vivo* when xanthosine or xanthine were added exogenously to the cell culture media. This validated that GTP is a required cofactor for the transposase protein (Mul and Rio, 1997).

1.11. Catalytic DDE Motif

That retroviral integrases/transposases from different organisms are indeed fundamentally related is especially apparent considering the signature array of conserved acidic amino acids, the DD(35)E motif. These conserved amino acids are critical for the 3' end processing reactions, suggesting that they are part of (or at least closely related to) the active sites of the enzymes (Craig, 1995).

Alignments of the DDE motifs of various retroviruses, retrotransposons and IS (insertion) elements and transposable elements have been performed by several groups (Baker and Luo, 1994; Capy *et al*., 1996; Kulkosky *et al*., 1992), which clearly demonstrated a relationship between most of these transposable elements by their DD(35)E signature amino acid array. In a study by Capy *et al.,* 1996, retroviral integrases and four main groups of transposases, namely the mariner-Tc1 superfamily (Emmons *et al*., 1983; Jacobson *et al*., 1986), the IS family (Galas and Chandler, 1989; Rezsohazy *et al*., 1993), the *hAT* superfamily (including the elements hobo of *Drosophila melanogaster, Ac* of *Zea mays*, and Tam3 of *Antirrhinum majus*) (Calvi *et al*., 1991), and P elements, were compared. Resulting from this alignment and comparison of catalytic motifs by

Capy *et al.*, some similarities between ISs (insertion elements) and members of the mariner-Tc1 superfamily were found, but the two remaining groups of elements, the *hAT* and P superfamilies, showed no similarities neither between themselves nor with the other groups (Capy *et al*., 1996). It appears therefore, that P element transposase protein is different from other recombinase proteins in that no obvious candidate(s) for a catalytic DDE motif can be found in a primary amino acid sequence alignment neither with other members of its family nor with other groups of recombinases. Therefore, the putative DD(35)E motif of the P element transposase protein has to be determined experimentally.

1.12. Aim of this work

In the work presented here, the identification of the catalytic motif of the *Drosphila* P element transposase protein was attempted. Since the approach of aligning P element transposase with other known recombinase proteins did not result in any significant homologies, an attempt to further narrow down possible candidates for this motif by comparing conserved, hydrophobic amino acids adjacent to the DD(35)E motif from retroviral integrases with the P element transposase was made. A selection of glutamate and aspartate residues in the protein's C-terminus were mutated to alanine, and the mutant proteins' activity tested in an *in vivo* excision assay. Three residues (D528, E531, and D545) were identified in this manner, all of which almost completely abolished *in vivo* excision activity. The involvement of these residues in P element catalytic transposase activity was further confirmed employing three *in vitro* assays: *in vitro* strand-transfer, *in vitro* cleavage, and *in vitro* LMPCR. In the *in vitro* strand transfer assay, mutations of the D528 and E531 to both alanine or cysteine respectively, greatly reduced activity and significantly altered metal-binding specificity. However, activity of D545Atnp and D545Ctnp was only slightly altered in *in vitro* strand transfer assays. To elucidate which step of the P element transposition reaction D545Atnp inhibited, the single mutant protein was tested in *in vitro* cleavage and LMPCR assays, along with the wild-type and the

triple mutant protein. Both the single and the triple mutant completely abolished *in vitro* cleavage and LMPCR activity.

It was concluded that the residues D528, E531, and D545 are, or are a part of, the *Drosophila* P element catalytic DDE, in this case DED, motif.

2. Materials

2.1. Chemicals and Other Materials

Chemicals were obtained from the following companies: Sigma (St. Louis, MO, USA),	Fisher Scientific (Fair Lawn, NJ, USA), Difco Laboratories (Detroit, MI, USA),
Bio-Rad Laboratories (Hercules, CA, USA)	
Streptavidin Agarose	Pierce (Rockford, IL, USA)
Heparin Agarose	Sigma
Acrylamide powder, Dowex affinity resin	Bio-Rad
Heparin-Agarose	Sigma
Ni^{++}-NTA superflow affinity resin, Qiaex Gel Extraction Kit and Qiagen Maxi Prep Kit	Qiagen (Chatsworth, CA USA)
Sequenase Version 2.0 Kit	USB (Cleveland, OH, USA)
Phenol	Gibco, BRL (Gaithersburg, MD, USA)
Chemiluminescence Western Blotting Reagents	Amersham (Arlington Heights,IL, USA)
Hybond N^{+} Nitrocellulose Pure Nitrocellulose Transfer and Immobilization Membrane,	Schleicher + Schuell (Keene, NH, USA)

Gel Blot Paper	
3 MM Chromatography Paper	Whatman (Maidstone, U.K.)
(α^{35}S) ATP (3000 Ci/mM),	Amersham
(α^{32}P) dCTP(3000Ci/mM)	(Arlington Heights, IL, USA)
(γ^{32}P)ATP (7000Ci/mM)	ICN (Costa Mesa CA, USA)
Kodak XAR Films	Eastman Kodak Company (New Haven, CT, USA)

A Sorvall RC 5B Superspeed Refrigerated Centrifuge with either a GS3 (for 100-500 ml samples) or an SS34 (for 10-40 ml samples) rotor was used for all large scale centrifugations up to 12 000 rpm. A Beckman L8-80 Ultracentrifuge with either a Vti65.2 rotor, Vti 45 or Vti 80 rotor was used for centrifugations between 30 000 and 60 000 rpm.

For 1500-4000rpm tissue-culture spins (L2 Schneider, and Sf9 insect cell lines), a Beckman GS-6R centrifuge, an ICE Clinical Centrifuge, and a Sorvall RC-3B were used.

2.2. Enzymes

T4-Ligase, T7-polymerase,T4-DNA polymerase, alkaline phosphatase, proteinase K, *Taq* -polymerase, *Pfu*-polymerase, lysozyme, bovine serum albumin (BSA), RNAse (DNAse free), DNAse (RNAse free), molecular weight standards for protein gels, molecular size standards for DNA, nucleotides and desoxynucleotides, and restriction endonucleases were purchased from Boehringer Mannheim (Indianapolis, IN, USA), New England Biolabs (Beverly, MA, USA), Gibco BRL (Life Technologies, Inc.; Gaithersburg, MD, USA), or

Amersham Pharmacia Biotech (Arlington Heights, IL, USA).

2.3. Antibodies

α KH (anti-rabbit)	generous gifts from the
αRD6 (anti-mouse)	members of the Rio lab
αHR2 (anti-rabbit)	
αPG4 (anti-mouse)	
α20G2 (anti-mouse)	
αRC8 (anti-mouse)	
Blotting Grade Goat-Anti-Mouse IgG (H+L) or Goat-Anti-Rabbit IgG Horseradish Peroxidase Conjugate	Bio-Rad

2.4. Plasmids and *E.coli* strains

pBluescript (=pBSK(+))	Stratagene
pBSK(+)pAC-TnpD486A/E531A,	generous gifts of Y.Mul
pBSK(+)pAC-TnpD478A/E444A,	(for description, see 3.1.6.)
pBSK(+)pAC-TnpD528A/E628A,	
pBSK(+)pAC-TnpD545A/E580A,	
pBSK(+)pAC-TnpD586A/E621A,	
pBSK(+)pAC-TnpD620A/E621A,	
pBSK(+)pAC-TnpD620A/E655A,	
pBSK(+)pAC-TnpE600A,	
pBSK(+)pAC-TnpE605A,	
pBSK(+)pAC-TnpD615A,	
pBSK(+)pAC-TnpE689A,	
pBSK(+)pAC-TnpD642A	

pISP-2/Km	derived from pISP-2 (Rio *et al*., 1986)
pFastBac	Gibco, BRL
DH5α	Gibco BRL
MC1061 recA$^-$	Invitrogen (Carlsbad, CA, USA).
single-stranded DNA template for M13 mutagenesis	generous gift of E.Beall

2.5. Tissue culture

2.5.1. Cell lines

Sf9 (*Spodoptera frugiperda*)	ATCC,USA
Schneider L2 (*Drosophila*)	(Summers & Smith)

2.5.2. Chemicals for tissue culture medium

TMN-FH, amino acids for M3 medium	Sigma
Bactopeptone, Yeast extract	Difco
$NaH_2P0_4{\cdot}H_20$	Malinckrodt
Antibiotics:	
Penicillin, Streptomycin, Hygromycin	Gibco, BRL
Fetal Calf Serum	Gibco, BRL

3. Methods

3.1. Molecular Techniques with Recombinant DNA

3.1.1. Synthesis and preparation of oligonucleotides

All oligonucleotides were synthesized on an ABI model 392 DNA synthesizer. After deprotection, acetyl groups were removed by a one hour incubation in ammonium hydroxide at 65^0 C. To remove the ammonium hydroxide, the samples were centrifuged under vacuum until dry, and either resuspended in 200 µl dH_20 for immediate use, or further purified by denaturing urea polyacrylamide gel electrophoresis.

3.1.2. Gel purification of oligonucleotides

Lyophilized oligonucleotides were resuspended in 50 µl of 0.1 M NaOH/ 1 mM EDTA, after which 100 µl formamide dye (1 ml deionized formamide containing 250 mM EDTA, pH 8.0, and 1% each of xylene cyanol and bromophenol blue) were added, the samples heated to 90^0 C for 5 min, and subsequently loaded onto a denaturing polyacrylamide gel. The percentage of acrylamide used depended upon the length of the oligonucleotide. For the length of 25-40 nucleotides, a 15% gel was made as follows: 60g Urea, 45 ml 40% acrylamide stock (19% to 1%), 24.6 ml dH_20, 6 ml 20XTBE, 1.2 ml 10% APS, and 75 µl TEMED. Gels were run at 30 V for approx. 2 hours in 1X TBE buffer (0.09 M Tris-borate, 0.002 M EDTA at pH 8.0). The oligonucleotide bands were then visualized by shadowing with a short wave UV lamp, and the full-length oligonucleotide band (usually the slowest migration and most intense band of the ladder) cut out with a scalpel. DNA was extracted from the gel pieces by three incubations with 3 ml TE each at 37^0 C, 2 x 3-4 h, and 1 x overnight, squeezing the eluate through a 10 ml syringe for each extraction. Flow-throughs

were combined and extracted with n-butanol to concentrate. Each butanol-extraction was done with an equal volume to that of the aqueous layer, and solutions were spun in a tabletop centrifuge for 1 minute at 1600 xg to separate the two layers. When the volume was about 400 μl, the purified oligonucleotide suspension was phenol/chloroform extracted and ethanol precipitated by adding $MgCl_2$ to 10 mM final concentration, $Na(OAc)_2$ to 0.3 M final concentration, and 2.5 volumes 100% ethanol. The DNA pellet was washed with 95% ethanol, dried in a speed vacuum centrifuge for 15 min, and resuspended in 200 μl dH_20. OD_{260} readings were taken to determine DNA concentration.

3.1.3. DNA Preparation

3.1.3.1. Preparation and Transformation of *E.coli* electrocompetent cells

Two liters of LB (1% tryptone (w/vol), 0.5% yeast extract (w/vol), 150 mM NaCl, pH 7.4) containing 50 μg/ml streptomycin were inoculated with 1/100 volume of a fresh overnight culture of *E.coli* mc1061. Cells were grown with vigorous shaking at 37^0 C to an OD_{600} between 0.5 and 1.0. Flasks were chilled on ice for 30 min and then centrifuged at 8000 rpm in a Sorvall GSA rotor for 10 min at 4^0 C. All subsequent steps were carried out on ice. Cell pellets were resuspended in a total of 1 liter cold wash media (1 mM HEPES-NaOH, pH 7.0), and re-centrifuged as above. This washing process was repeated twice more, after which cell pellets were resuspended in 40 ml cold, sterile 10% glycerol, and centrifuged in a Sorvall SS34 rotor at 8000 rpm for 15 min at 4^0 C. Ensuing, the cell pellet was resuspended in 2.5 ml 10% glycerol total volume so that the final volume of this 50% slurry was about 5.0 ml. Cells were frozen in 40 μl aliquots in a dry ice/ethanol bath, and aliquots stored at -80^0 C.

For electroporation, DNA in a volume less than 2 μl was added to a cell aliquot, electropulsed at 2.5 V, 250Ω, and 25 μF and immediately resuspended in 1 ml

LB + 10 mM $MgSO_4$ + 0.4% glucose. Culture tubes were incubated for 1 h shaking at 37^0 C, and plated on selective media.

3.1.3.2. Preparation and Transformation of *E. coli* $CaCl_2$ competent cells

400 ml of LB containing the appropriate antibiotics were inoculated with 10 ml of an overnight culture of the desired bacterial strain (DH5α), and incubated shaking vigorously at 37^0 C. When the OD_{600} was 0.4-0.5, flasks were chilled on ice for 30 min, and then spun at 4000 rpm for 10 min at 4^0 C. The supernatant was discarded, and the pellet resuspended in 20 ml of ice-cold 0.1 M $CaCl_2$. After 30 min incubation on ice, cells were again centrifuged as above, the pellet resuspended in 16 ml of ice-cold $CaCl_2$ and incubated on ice for 2 hours. To store competent cells, glycerol was added to a final concentration of 20%, and aliquots frozen in liquid nitrogen and stored at -80^0C.

For transformation, plasmid DNA was incubated with 50-100 μl competent cells for 20 min on ice, followed by a 90 sec. heat-shock at 42^0 C, 2 min on ice, and incubation with 1 ml of LB for 1 h at 37^0 C. Transformed cells were plated on selective media.

3.1.3.3. Plasmid DNA minipreparations

A 5 ml overnight culture from a single colony was grown in LB containing 50 μg/ml ampicillin and/or the appropriate antibiotic. 1.5 ml of the culture was pelleted by a 2 min centrifugation in a tabletop microcentrifuge. The medium was withdrawn by aspiration and the remaining pellet resuspended in 300 μ l P1 Buffer (Qiagen) containing 100 μg/ml RNAse A. Bacterial cells were lysed by the addition of 300 μl P2 (Qiagen), incubated 5 min at room temperature, and neutralized by adding 300 μl of chilled P3 (Qiagen), after which the bacterial lysates were incubated on ice for 5 min. They were then spun for 10 min in a

tabletop centrifuge, and the supernatant precipitated with 750 μl isopropanol. After another centrifugation for 10 min in a tabletop centrifuge, the supernatant was aspirated, the pellet resuspended in 200 μl TE, and phenol/chloroform extracted. The plasmid DNA was further concentrated by ethanol precipitation, and redissolved in 20 μl of TE (10 mM Tris, HCl, pH 8.0, 1 mM EDTA). DNA prepared in this fashion was pure enough to be used for DNA sequencing.

3.1.3.4. Plasmid preparation by CsCl gradients or Qiagen Maxi Prep Kit

Overnight cultures of transformed *E.coli* DH5α cells were grown on a shaker at 37^0 C in 5 ml LB containing the appropriate antibiotic(s). Each overnight culture was then diluted 1:100 into 500 ml of medium, and again grown overnight at 37^0 C on a shaker. The cultures were spun down at 6000 rpm for 10 min in a Sorvall GSA rotor, and the cell pellet resuspended in 8 ml Solution I (50 mM glucose, 25 mM Tris-HCl, pH 8.0, 10 mM EDTA) containing 1 ml of fresh lysozyme (40 mg/ml). After 30 min incubation at room temperature, cells were lysed by treatment with 18 ml of freshly made solution II (1% SDS, 0.2 M NaOH), and incubated on ice for 5 min. The lysed bacterial solution was neutralized with 9 ml of solution III (2M $H(OAc)_2$, 3M $K(OAc)_2$, pH 5.6), and incubated on ice again for 10 min, after which it was centrifuged at 8000 rpm in a Sorvall GSA rotor. The supernatant was filtered through a cheesecloth into a 50 ml tube, and the tube filled with isopropanol, inverted to mix, and incubated on ice for 10 min. After a 10 min centrifugation in a Sorvall GSA rotor, the supernatant was discarded, the pellet air dried, and resuspended in 3 ml TE. The resuspended DNA pellet was transferred to a 15 ml Corex tube containing 3 ml 5M LiCl (chilled at -20^0 C) and spun for 5 min in a Sorvall SS34 rotor at 8000 rpm, after which the resulting supernatant was transferred to a 30 ml Corex tube containing 6 ml isopropanol and spun again at 8000 rpm for 10 min. The supernatant was discarded, and the pellets air dried. After resuspending the DNA pellets in 4 ml TE, 4 g of CsCl and 320 μg/μl ethidium bromide were added to the DNA mix, the

solution transferred into heat seal tubes and spun in a Beckman Ultracentrifuge Vti65.2 rotor at 55 000rpm for 12-24 hours at 18^0 C. The DNA plasmid bands were dripped using a 16 Gauge needle syringe under longwave UV light for detection. To remove the ethidium bromide, dripped DNA bands were put over 1.5 ml Dowex columns, washed in 1/2 volumes TE/1M NaCl, and the resulting clear flow-through dialyzed against 1 l TE at 4^0 C in the coldroom. Samples were stored at -20^0 C.

Concentration of the DNA was measured by taking OD readings at 260 nm. One A_{260} unit equals 50 μg/ml DNA dsDNA.

Plasmid preparation using the Qiagen Maxi Prep Kit

Overnight cultures of transformed *E.coli* DH5α cells were grown on a shaker at 37^0 C in 5 ml LB containing the appropriate antibiotic(s). Each overnight culture was then diluted 1:100 into 500 ml of medium, and again grown overnight at 37^0 C on a shaker. The cultures were spun down at 6000 rpm for 10 min in a Sorvall GSA rotor, and the purification of the plasmids done according to the Qiagen plasmid maxi protocol distributed by the manufacturer with the Purification Kit.

3.1.3.5. Amplification of DNA fragments by the polymerase chain reaction (PCR) and *DpnI* mutagenesis

The PCR amplification reactions were performed employing both the Perkin Elmer Cetus DNA Thermal Cycler and the PTC-100 Programmable Thermal Controller by MJ Research, Inc. Standard reactions were prepared in sterile 0.5 ml tubes as follows: 5 μl of 10X reaction buffer, 75-100 ng of double-stranded DNA template, 40 pmol of oligonucleotide primer #1, 40 pmol of oligonucleotide primer #2, 5 μl of dNTP mix (1.25 mM), 1 μl Taq /Pfu polymerase (2.5 U/μl) and dH_20 to a final volume of 50 μl. Cycling parameters depended on

the size of the template DNA and on the stringency of the oligonucleotide-primer/DNA match. For *DpnI* mutagenesis employing a 7.1 kb DNA template, parameters were as follows: One cycle (95^0 C for 3 min, 45^0 C for 2 min, 72^0 C for 16 min), and 17 cycles (94^0 C for 1 min, 50^0 C for 2 min, 70^0 C for 16 min). Finally, reactions were incubated at 72^0 C for 4 min to allow for complete extension. After cooling down to room temperature, the PCR-products were purified by phenol/chloroform extraction and ethanol precipitation, and a small aliquot was run on an agarose gel as a control for amplification efficiency. For *DpnI* mutagenesis, the PCR amplified DNA was digested with *DpnI* restriction enzyme, and directly transformed into $CaCl_2$-competent DH5α cells and plated on selective media together with a control of undigested DNA. All point-mutated plasmids were confirmed by DNA sequencing.

3.1.4. Modification and purification of DNA fragments

3.1.4.1. Restriction Endonuclease digestion and restriction analysis

Restriction endonuclease cleavage was accomplished by incubating the enzyme(s) with the DNA under the conditions described by the supplier. Usually, the enzyme and buffer were diluted tenfold into the reaction, and the DNA was digested for 2 h at 37^0C. For restriction analysis, the DNA of interest was cleaved with a variety of restriction endonucleases, either individually or in combination using the conditions as stated above.

To check proper digestion, an aliquot was run on a 1% agarose gel containing ethidium bromide (0.5 μg/ml) in 1XTAE (0.04 M Tris-acetate, 0.001 M EDTA pH 8.0) or 1XTBE (0.09 M Tris-borate, 0.002 M EDTA pH 8.0) buffer, and visualized by UV-transillumination.

To ensure proper activity of other enzymes after the treatment with restriction endonucleases, the latter had to be inactivated. This was done either by heat-inactivation for 20 min in a 65^0 C waterbath, or by phenol/chloroform extraction and

ethanol precipitation.

3.1.4.2. Dephosphorylation

In order for the digested vector not to religate with itself in a subsequent ligation reaction, it had to be dephosphorylated prior to ligation. This was done by adding 2-3 volumes of TE-buffer and 2 μl alkaline phosphatase and incubating for 30 min at 37^0 C. After dephosphorylation, the phosphatase was removed by phenol/chloroform extraction and the vector was precipitated with ethanol.

3.2.4.3. Ligation of DNA

To ligate insert- and vector DNA, they were mixed in 1:5 ratio (between 20 and 30 ng vector, and 100-150 ng insert), adding 1 μl 10X ligation buffer (0.66 M Tris-HCl, pH 7.6, 100 mM $MgCl_2$, 150 mM DTT, 10 mM ATP, 10 mM spermidine), 1 μl BSA (2 mg/ml), 1 μl T4-Ligase (1 U), and dH_2O to a final reaction volume of 10 μl and incubated overnight at 16^0C.

3.1.4.4. Agarose gel electrophoresis

Analytical agarose gels were run in horizontal gels (13 x 14 cm or 5 x 7.5 cm) using 1X TBE as a running buffer. Preparative gels were run only on 13 x 14 cm gels using 1X TAE as a running buffer. Depending on the size of the DNA fragments to be separated, the agarose-concentration was varied between 0.8 and 1.8%. Ethidium bromide (0.5 μg/ml) was added to the gels before pouring them. The samples were mixed with 1/5 volumes of 6x gel loading buffer (0.25% bromophenol blue, 0.25% xylene cyanol FF, 30% glycerol), before loading the gel. The gels were run at voltages between 50V and 200V, and then photographed by UV illumination (302 nm).

3.1.4.5. Purification of DNA

Phenol/chloroform extraction (Sambrook *et al*., 1989)

For phenol extraction of proteins, DNA-solutions were mixed with 0.5 vol phenol (Tris-buffered, pH 8) and 0.5 vol. chloroform/ isoamylalcohol (24:1), vortexed and incubated 5 min at room temperature to form an emulsion. The mixture was then centrifuged at 12000 xg for 5 min in a microcentrifuge and the upper, aqueous phase re-extracted with chloroform/ isoamylalcohol (24:1).

DNA precipitation with ethanol

To precipitate DNA, 2.5 vol of ice-cold 100% Ethanol and 1/10 vol. of 3 M sodium acetate were added to the DNA-solution, mixed and incubated on ice for 5 min. The solution was then centrifuged at 4^0 C at 12000 xg for 20 min, washed twice with 70% Ethanol, dried in a vacuum centrifuge, and resuspended in dH_20 or TE-buffer (10 mM Tris HCl pH 8.0, 1 mM EDTA pH 8.0).

Elution of DNA from agarosegels using the Qiaex Gel Extraction Kit

The DNA band of interest was excised in a minimum gel slice, solubilized, washed, and eluted from the Qiaex beads according to the manufacturer's protocol.

3.1.5. DNA sequencing

To verify accurately synthesized PCR products and the correct orientation of inserts in the ligation, the plasmids were sequenced using the Sequenase Version 2.0 Kit (USB). Template DNA (3-5 µg) was denatured by incubation at 37^0C for 30 min with 0.2 M NaOH, and 0.2 mM EDTA. After ethanol

precipitation, the DNA was resuspended in dH_20 and 1.5 pmol oligonucleotide primer and 2 μl 5X sequencing buffer (USB) per reaction were added to a total reaction volume of 10 μl. The primer was annealed to the DNA by incubation at 65^0 C for 2 min and then allowed to cool at room temperature for approx. 30 min. DNA sequencing was then carried out in two steps: In the first (labeling) step, 1 μl DTT (0.1 M), 2 μl labeling mix (diluted 1:5 in dH_20), 1 μl α-^{35}S-ATP (3000 Ci/mmol), and 2 μl Sequenase (diluted 1:8 in enzyme dilution buffer) were added to the reaction and incubated 2-5 min at 20^0 C. This step incorporated labeled nucleotides into DNA chains of variable length. In the second (termination) step, 3.5 μl aliquots of the sequencing reaction were pipetted into 2.5 μl aliquots of ddNTPs, and the reaction incubated at 40^0 C for 5 min. Processive DNA synthesis eventually stopped after growing chains had been terminated by a dideoxynucleotide. Finally, all reactions were stopped by the addition of 4 μl EDTA/formamide stop solution. The DNA was denatured by heating for 2 min at 95^0 C and run on a high-resolution denaturing polyacrylamide gel (6% (vol/vol) acrylamide (20:1 acrylamide:bis), 6% 10X TBE (vol/vol), 8 M urea) designed to resolve radiolabeled extension products on the basis of size. Gels were run at approx. 1500 Volts to insure denaturing conditions. The gel was transferred onto 3 MM Whatman Chromatography Paper and dried on a BIO RAD vacuum gel dryer. DNA bands were visualized by autoradiography at RT on a Kodak X-AR film.

3.1.6. Cloning of pBSK and pUChyg vectors for tissue culture transfections

P Bluescript plasmid pBSK(+)PAC-Tnp was derived from pBSK(+) (Stratagene), the 2.6 kb actin 5C fragment from pAC (Ashburner, 1989), and the transposase cDNA with 25% of the N-terminus chemically resynthesized to alter the codon usage to the most frequently occurring codons in *Drosophila* (Lee et al., 1996). pBSK(+)pAC-TnpD486A/E531A, pBSK(+)pAC-TnpD478A/E444A,

pBSK(+)pAC-TnpD528A/E628A, pBSK(+)pAC-TnpD545A/E580A, pBSK(+) pAC-TnpD586A/E621A, pBSK(+)pAC-TnpD620A/E621A, pBSK(+)pAC-TnpD620A/ E655A, pBSK(+)pAC-TnpE600A, pBSK(+)pAC-TnpE605A, pBSK(+)pAC-TnpD615A, pBSK(+)pAC-TnpE689A, pBSK(+)pAC-Tnp D642A (gifts of Yvonne Mul), and pBSK(+)pAC-TnpD486A, pBSK(+)pAC-TnpD528A, pBSK(+)pAC-TnpE531A and the pBSK(+)pAC-Tnp double-and triple-mutants were generated by M13 single strand mutagenesis as described in section 2.1.8. pBSK(+)pAC-TnpD545A, pBSK(+)pAC-TnpE580A, and pBSK(+)pAC-TnpE628A were made by *DpnI* mutagenesis as described in the *QuikChange Site-Directed Mutagenesis Kit* (Stratagene) and in section 2.1.3.5. Mutations were introduced by annealing oligonucleotides containing the desired nucleotide changes to the single-or double stranded DNA in a PCR amplification reaction. Incorporation of the desired mutation was confirmed by DNA sequence analysis using Sequenase 2.0 as described by the manufacturer (US Biochemical) and the following primers: 5'-TATTT ATACAAGCCATCAAGCG-3' (1528-1508) for D486A, 5'-TGATGG CTTGTATAAATATTTGCAAGA-3' (1513-1527) for D528A, E531A, and D545A, 5'-CTCATCATCGACAGGCTCATCATC-3' (1815-1792) for E580A, and 5' –GATGATGAGCCTGTCGATGAGATG-3' (1807-1830) for E628A. Mutant DNA was subcloned using *NheI* and *NotI* restriction sites. pUChygMT-tnp was derived from the same transposase cDNA as pUChygMT (generous gift from C. Thummel, University of Utah). pUChygMT-tnp tDED and pUChygMT-tnp D545A were generated by subcloning fragments from respective pBSK(+)pAC-Tnp clones using *NheI* and *BamHI* restriction sites, and confirmed by DNA sequencing. pISP-2/Km contains a 0.6 kb non-autonomous P-element derived from pISP-2 (Rio et al., 1986) and the kanamycin resistance fragment form plasmid pKm109-9 (Reiss et al,. 1984).

3.1.7. Site-directed mutagenesis using a single-stranded template
(Kunkel *et al*., 1987), modified

For phosphorylation of the mutagenic oligonucleotide, 200 pmol DNA oligo, 2 µl 10X Kinase Buffer (660 mM Tris-HCl, pH 7.6), 100 mM $MgCl_2$, 10 mM Spermidine, 150 mM DTT), 1 µl 10 mM ATP, and 1 µl T4 Polynucleotide Kinase were mixed and dH_20 added to a final volume of 20 µl. The reaction was incubated at 37^0 C for 1 hour, and subsequently at 65^0 C for 10 min. To anneal the phosphorylated, mutagenic oligonucleotide to the single-stranded template, 0.5 pmol of single-stranded DNA template was mixed with 10 pmol of phosphorylated oligo and 1 µl 10X annealing buffer (200 mM Tris-HCl, pH 7.5, 100 mM $MgCl_2$, 500 mM NaCl, 10 mM DTT) and H_20 to a final volume of 10 µl. The reaction mixture was heated for 5 min at 75^0 C and cooled over 30 min to room temperature. For the extension reaction, 10 µl of annealed ssDNA/oligo mixture was incubated with 1 µl 10 X extension buffer (200 mM Tris-HCl, pH 7.5, 100 mM $MgCl_2$, 100 mM DTT), 4 µl dNTPs (500 µM final), 1 µl ATP (500 µM final), 1 U T4 DNA ligase , 0.5 U T4 DNA polymerase and 2.5 µl dH_2O on ice for 5 min. The extension reaction was then placed at room temperature for 5 min, and subsequently incubated at 30^0 C for 2 h, and for 10 min on ice. The reaction was phenol/chloroform extracted in a final volume of 100 µl, ethanol precipitated, washed with 70% ethanol, dried and resuspended in 20 µl TE. 5 µl were transformed into a dut^+, ung^+ strain (like DH5α), and plated on media containing selective antibiotics.

3.1.8. Cloning of Baculovirus constructs

Transposase cDNA containing a C-terminal His-tag was cloned into pFastBac (Gibco) using *BamHI/NotI* restriction sites. Point mutations of the tnp DED motif were generated by *DpnI* mutagenesis with oligonucleotides containing the desired nucleotide changes as described above, and clones verified by

sequencing. pFastBac clones containing the point mutations D528Atnp, E531Atnp, D545Atnp, D528A/E531A/D545 tnp, D528Ctnp, E531Ctnp, D545Ctnp and wt tnp were used for generating high titer Baculovirus stocks using *the Bac-to-Bac Baculovirus Expression System* (Gibco BRL) as described by the manufacturer. In brief, the recombinant plasmids were transformed into *DH10Bac* competent cells. Colonies containing recombinant bacmids were identified by disruption of the *lacZ*α gene, i.e. by blue and white screening. High molecular weight mini-prep DNA was prepared from selected *E. coli* colonies containing the recombinant bacmid, and this DNA was used to transfect Sf9 insect cells using CellFectin as described by the manufacturer.

3.2. Molecular Techniques with Recombinant Proteins

3.2.1. SDS polyacrylamide gel electrophoresis (SDS-PAGE)

(Laemmli, 1970), modified

One-dimensional gel electrophoresis under denaturing conditions (in the presence of 0.1% SDS) separates proteins according to their molecular weight as they move through the polyacrylamide gel matrix towards the anode.

Glass plates (10 x 8 cm) were used and assembled with spacers as described by the manufacturer. The gels were prepared as follows: For a 7.5% gel, 5 ml dH_2O was mixed with 2.5 ml 30% acrylamide (30% acrylamide, 0.8% bis) and 2.5 ml lower Tris-buffer (to make 500 ml 4X buffer, 90.85 g Tris base, and 20 ml 10% SDS were mixed, the pH adjusted to 8.8 with HCl, and filled to 500 ml with dH_2O). To polymerize the gel, 50 µl of 10% APS and 15 µl of TEMED were added, and the resolving gel was poured to approx. 4/5 of the total gel size and covered with 1 ml of isoamylalcohol. After polymerization the isoamylalcohol was removed by aspiration and the stacking gel was poured: 1.5 ml dH_2O was mixed with 0.63 ml of 4X upper Tris-buffer (for 100 ml 4X, 6.06 g Tris base and 4 ml 10% SDS were mixed, the pH adjusted to 6.8 with HCl, and

filled up to 100 ml with dH_2O) and 0.38 ml 30% acrylamide, and polymerized by addition of 15 µl of 10% APS and 7 µl TEMED. A teflon comb was inserted into the stacking gel. After polymerization, the comb was removed and the gel assembled in the gel apparatus, adding approx. 200 ml of 1X SDS running buffer (250 mM Tris, 2.5 M glycine, 1% SDS) to each of the gel chambers.

An aliquot of the protein to be analyzed was diluted 1:1 (vol/vol) with 2X SDS-sample buffer (100 mM Tris HCl (pH 6.8), 200 mM DTT, 4% SDS, 0.2% bromophenol blue, 20% glycerol), boiled 5 min at 100 ^{0}C, spun down briefly in a microcentrifuge, and loaded onto the gel. The gel was run at 15 A until the protein(s) had migrated through the stacking gel, and the voltage was then increased to 20 A until the bromophenol blue had reached the end of the resolving gel.

3.2.2. Staining with Coomassie Brilliant Blue

(Sambrook *et al*., 1989)

The detection of protein bands in the gel with Coomassie Brilliant Blue staining depends on nonspecific binding of a dye, Coomassie Brilliant Blue R, to proteins. The detection limit is 0.3-1 µg/protein band.

To stain a gel, it was placed in 250 ml of staining solution (0.25% Brilliant Blue, 25% isopropanol, 10% acetic acid) for 1 h at room temperature with shaking. The gel was then washed with destaining solution (25% isopropanol, 10% acetic acid) until the background stain was negligible, rinsed in dH_2O twice for 10 min each, and dried between cellophane paper.

3.2.3. Silver staining

(Heukeshoven and Dernick, 1988)

After electrophoresis, the gel was fixed twice for 10 min in 50% methanol, and twice for 10 min in 5% methanol on an orbital shaker. After fixing, the gel was incubated 20 min in 0.03 M DTT to reduce the proteins and then incubated

with agitation for 30 min in staining solution (0.1% $AgNo_3$, 0.02% formaldehyde), after which the gel was developed with 2.6% $NaCO_3$, and 0.01% formaldehyde until the protein bands of interest appeared as intense as desired. The reaction was stopped by the addition 5 g of solid citric acid. After 15 min of incubation, the gel was washed three times in dH_20 and dried on cellophane paper.

3.2.4. Western blotting

For Western Blotting, the proteins were separated by standard techniques on SDS PAGE. After electrophoresis, the gel was placed on two sheets of Gel Blot filter paper, previously soaked in transfer buffer (48 mM Tris Base, 39 mM glycine, 20% methanol, 0.037% SDS). The uncovered side of the gel was overlaid with prewet nitrocellulose, precut to match the gel size, and the nitrocellulose was overlaid with additional two sheets of pre-soaked filter papers. The filter paper containing the gel and nitrocellulose was sandwiched between sponge pads and placed in a plastic support, and the entire assembly was placed in a tank containing transfer buffer. The proteins were transferred electrophoretically from the gel onto the nitrocellulose membrane at 500 mA.

After protein transfer, the filter was blocked with 3% non-fat dry milk in TBS (20 mM Tris pH 7.4, 150 mM NaCl) for 30 min on an orbital shaker. The blocking solution was decanted and the first antibody was added in a solution containing 3% non-fat dry milk in TBS. The filter was incubated at room temperature for 1 h with shaking, and then washed twice in TBS containing 0.05% Tween 80 and once in TBS for 15 min each. A 1/3000 dilution of the BIO-RAD Goat anti-Mouse or anti-Rabbit IgG (H+L) Horseradish Peroxidase conjugate in TBS with 3% non-fat dry milk was then added, and the filter was incubated at room temperature for 1 h on an orbital shaker, and subsequently washed twice in TBS containing 0.05% Tween 80, and once in TBS for 10 min each. The peroxidase detection was done using the Boehringer Mannheim Chemiluminescence Western Blotting Reagents or the Renaissance Western

Blotting Kit (NEN DuPont) and Kodak XAR films according to the manufacturer's protocol.

3.2.5. Preparation of biotinylated DNA-Streptavidin agarose DNA affinity columns

For 1 ml settled resin, 0.54 mg biotinylated oligo (Bio TdT3: 5'**b**GATCCAGGTGGTGTCGGATCCAGGTGGTGTCGGATCCAGGTGGTGTCG, **b**=biotinylation site) and 0.57 mg of non-biotinylated oligo (TdT 3 bot.: 5' GATCCGACACCACCTGGATCCGACACCACCTGGATCCGAC ACCACCTG) were combined in a final volume of 100 µl (or larger if necessary) containing 10 mM $MgCl_2$ and 100 mM NaCl. The mixture was placed in a beaker with boiling water and allowed to slow cool for 4-5 hours. The oligo mix was then filled up to a total volume of 1 ml with TE/100 mM NaCl and a small aliquot taken to measure the OD_{260} pre-binding. 1 ml of streptavidin resin was washed thoroughly with excess TE/100 mM NaCl and added to the rest of the oligo mixture, rotating overnight at 4^0 C. A sample of the supernatant post-binding was taken to measure OD_{260} to estimate capacity. The resin was then washed extensively with TE/100mM NaCl and stored at 4^0 C. For longer periods of storage time, 0.02% sodium azide was added.

3.2.6. Purification of transposase from Schneider L2 cells

Wildtype and mutant P-element transposase (tDED and D545A) were purified from the *Drosophila* Schneider L2 stable cell line pUChygMT-Tnp, pUChygMT-Tnp tDED, and pUChygMT-Tnp D545A as described (Mul and Rio, 1997). pUChygMT-Tnp and the respective pUChygMT-Tnp tDED, and pUChygMT-Tnp D545A mutant Schneider L2 cell lines were generated by

transfection with calcium phosphate co-precipitation using pUChyg plasmid, followed by selection with 200 μg/ml hygromycin (Rio and Rubin, 1985). At 20-22h after induction of the metallothionein promoter with 0.7 mM $CuS0_4$, the cells were harvested by centrifugation, washed with phosphate-buffered saline (PBS) + 1g/l $MgCl_2$, spun at 2500 rpm for 5 min and the resuspended in Buffer A (15 mM KCl, 10 mM Hepes-KOH, pH 7.6, 2 mM $MgCl_2$, 1 mM EDTA, 1 mM EGTA, 50 MM NaF, 0.2 mM PMSF, and 0.5 mM DTT) on ice. All the following steps were carried out on ice, and centrifugations performed at 4 ^{O}C. Cells were then dounce-homogenized, and 1/10 volume of Buffer B (1M KCl, 50 mM Hepes-KOH, pH 7.6, 30 mM $MgCl_2$, 1 mM EDTA, 1 mM EGTA, 0.2 mM PMSF, and 0.5 mM DTT) added before centrifugation for 10 min at 8000 rpm. The nuclear pellet was resuspended in isotonic buffer (9A:1B) and 1/10 volume of saturated $(NH_4)_2SO_4$, pH 7.6 added, and subsequently incubated for 30 min at 4 ^{O}C. The nuclear lysate was then spun at 35 000 rpm for 1h in a Beckman 45 Ti rotor, after which the supernatant was saturated with finely ground $(NH_4)_2SO_4$ to 70% over the course of one hour. The ammonium sulfate precipitated nuclear extract was then spun at 12 000 rpm for 10 min, after which the resulting pellet was resuspended in HGKED buffer (25 mM HEPES-KOH, pH 7.6, 10% glycerol, 0.04 m KCl, 1 mM EDTA, 1 mM EGTA, 50 mM NaF) containing 0.04 M KCl, and dialyzed twice against 2 liters of HGKED (containing 0.04 M KCl) for 6-7 hours total. After dialysis, the extract was spun briefly at 12000 rpm for 10 min, and the supernatant extract frozen in liquid nitrogen and stored at –80 ^{O}C. The nuclear extract was chromatographed on heparin-agarose (Kaufman *et al.,* 1989). The flow-through (H0.1FT) contained highly active transposase, as determined by a genetic base plasmid assay (Kaufman and Rio, 1992). The H0.1FT transposase containing fractions were chromatographed on a nonspecific DNA affinity resin (TdT) as described (Kaufman *et al.,* 1989; Beall and Rio, 1997). The transposase protein was eluted with increasing KCl, and the 0.3 M KCl peak fraction (T0.6) was used in the cleavage and subsequent ligation-mediated PCR (LMPCR) assays. TdT-transposase-fractions contained

approx. 5 ng/µl transposase protein as judged by silver-stained-SDS-polyacrylamid gels containing known amounts of bovine serum albumin.

3.2.7. Bradford Protein Assay

To measure total protein concentration, the BIORAD Bradford colorimetric assay was used. A standard curve was made with bovine serum albumin (1-10 µg/ml BSA) according to the manufacturer's protocol, and concentration of the assayed protein was made based on this curve.

3.2.8. Purification of Baculovirus protein from Sf9 cells

Sf9 insect cells were grown in well-aerated suspension flasks using TNM-FH medium with 10 % fetal calf serum. $2x10^8$ cells were infected at an M.O.I. (multiplicity of infection) of 10 with Baculovirus containing the different transposase point mutations described above. Cells were grown stirring at 27°C for 60 hours, harvested by centrifugation, washed once with PBS and the pellets frozen in liquid nitrogen. For extract preparation, pellets were thawed on ice and resuspended in Lysis Buffer (50 mM NaH_2PO_4, pH 8.0, 300 mM NaCl, 10 mM imidazole, 0.2 mM PMSF and 7 mM ß-mercaptoethanol). The suspensions were sonicated four times for 20 seconds each at highest setting, and then spun in a Beckman Ti70 rotor at 35000 rpm for 30 min at 2^0 C. The resulting supernatant was incubated with 1ml of Ni^{++}-NTA-superflow beads for 2 h rotating at 4^0 C. Beads were washed extensively in wash buffer (50 mM NaH_2PO_4, pH 8.0 ; 300 mM NaCl; 20 mM imidazole, 0.2 mM PMSF and 7 mM ß-mercaptoethanol) after which proteins were eluted with elution buffer (50 mM NaH_2PO_4, pH 8.0 ; 300 mM NaCl; 250 mM imidazole, 0.2 mM PMSF and 7 mM ß-mercaptoethanol) in four elution steps of 15 min each, rotating at 4^0 C. Elutions were pooled and dialyzed against HGKED buffer (25 mM HEPES-KOH (pH 7.6), 10% glycerol, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 0.2 mM PMSF) containing 0.1 M KCl, for six

hours, changing buffer once. Extracts were either used for *in vitro* strand transfer at this point, or the supernatant was further incubated with TdT-streptavidin beads for 2 hours rotating at 4°C. After incubation, beads were washed extensively in HGKED (containing 0.1 M KCl), and subsequently the protein was eluted stepwise with HGKED buffer containing increasing KCl concentrations (0.3 M KCl, 0.6 M KCl,1 M KCl). The 0.3 M KCl eluate (T0.3-2) was used for the *in vitro* strand transfer assay.

3.3. Tissue culture

3.3.1. Maintenance of Schneider L2 cells

3.3.1.1. Thawing cells

Vials containing ca $6x10^6$ cells were quick-thawed in a 37^0 C waterbath, and taken up in 7 ml of M3 medium containing 5% fetal calf serum (for a 20 liter prep of M3, the following components were dissolved in 15 liter of dH_20: 30 g a-alanine, 5 g b-alanine, 12.08 g arginine, 6 g anhydrous asparagine, 6 g aspartic acid, 4 g cysteine-HCL, 12 g glutamine, 143 g K-glutamate (L-glutamic acid monopotassium salt), 130.6 g Sodium glutamate (L-glutamic acid monosodim salt), 10 g glycine, 11 g histidine, 5 g isoleucin, 8 g leucine, 17 g lysine-HCl, 5 g methionine, 5 g oxaloacetic acid, 5 g phenylalanine, 8 g proline, 7 g serine, 10 g threonine, 5 g tyrosine, (free base), 2 g tryptophan, 8 g valine, 200 g glucose, 50 g bactopeptone, 40 g Yeast Extract, 15.2 g $CaCl_2$ (anhydrous), 43 g $MgSO_4$ (anhydrous), 15.6 g $NaH_2PO_4 \cdot H_20$, 21 g BIS-Tris, 1 g choline, 10 g $KHCO_3$, and 1 g streptomycin sulfate. The solution was brought to near volume and adjusted to pH to 6.8 with 50% NaOH, requiring ca 15-20 ml of 50% NaOH. The medium was brought to its final volume of 20 l, filter-sterilized, and stored at 4^0 C.)
Cells were spun down for 5 min at 1500 rpm, the supernatant aspirated, and cells

taken up in 10 ml M3+FCS into a 25 cm^2 Tissue-culture flask (T-flask). Cells were grown at 25^0 C. After reaching confluency, cells were split and grown in 75 cm^2 T-flasks.

3.3.1.2. Splitting L2 cells

Cells were split 1:5 every 2-3 days, depending on when they had reached confluency, meaning that cells had grown to a density of approx. 4-6x10^6 cells/ml. To split cells, they were pipetted up and down in the medium contained in the flask with a 5 ml pipette until more than 80% had become detached from the flask bottom. Cells were then pipetted into a fresh 75 cm^2 or 150 cm^2 T-flask containing enough M3 medium to insure 1:5 dilution and placed at 25^0 C.

3.3.1.3. Growing L2 Cells in spinner flasks

Since large amounts of cells (15-30 l of 6x10^6 cells/ml) were needed for protein preparations, L2 cells were expanded into 2-4 150 cm^2 T-flasks until they reached confluency 4-6 x10^6 cells/ml. They were then split into sterile 1 l bottles and media added to a density of 3.5 x10^6 cells/ml, not to exceed 300 ml. After reaching confluency, they were split into 6 l spinner flasks, and split 1:1 when cell density was between 6 -8 x10^6 cells/ml.

3.3.1.4. Freezing cells

A large culture of the cells to be frozen down was grown in a 150 cm^2 T-flask. After reaching confluency, cells were resuspended and spun down at 1500 rpm for 5 min, after which they were taken up in 1.5 ml freezing medium, which consists of the medium the cells are normally grown in supplemented with 20% FCS and 10% DMSO. For L2 cells this medium thus consisted of M3+20%

FCS+10% DMSO. 0.5 ml cell aliquots were made and stored in a foam box at –80^0 C overnight, after which they were transferred to liquid nitrogen.

3.3.1.5. Stable transfection of Schneider L2 cells

For stable transfection of Schneider L2 cells, cells were transfected with pUChygMT-Tnp or a derivative thereof by $CaCl_2$/HEBS precipitation as described in section 2.3.3. After DNA had been successfully transfected, cells were incubated overnight at 25^0 C, and split 1:4 into fresh medium in a 25 cm^2 T-flask the next day. Cells were incubated overnight at 25^0 C again, and then hygromycin added to the medium (M3 +5% FCS) to a final concentration of 200 μg/ml. Cells were split 1:4 into medium containing 200 μg/ml hygromycin every 3-4 days for 1-2 weeks. The hygromycin concentration was then lowered to 50-100 μg/ml, and hygromycin was omitted when cultures were expanded beyond 1 l for large scale protein preparations.

3.3.2. Maintenance of Sf9 insect cells

Sf9 insects cells were grown in 200 ml spinner flasks at 27^0 C in TNM-FH medium + 10% FCS (to make 10 l of TNM-FH medium, a packet of TNM-FH powder (Sigma) was dissolved into 9 l of dH_20, after which 3.5 g of sodium bicarbonate, 0.5 g streptomycin sulfate, and 0.32 g penicillin were added. The pH was adjusted to 6.2 with 1M HCl or 1M NaOH, the volume brought up to 10 l and the medium filtered immediately. TNM-FH was stored at 4^0 C.) When cells reached confluency, i.e. a density of 10^6 cells/ml they were split 1:4 by removing cells from the spinner flask and adding new medium to a density of 25x10^5. Cells were split every two days.

3.3.2.1. Baculovirus transfection-lipofection of Sf9 cells

Transfection

To transfect Sf9 cells for baculovirus production, $1x10^6$ Sf9 cells were seeded in a 30 mm well of a 6-well dish in TNM-FH + 10% FCS. Cells were allowed to attach for 1 hour at 27^0 C, after which the medium was aspirated off and 4 ml of serum-free TMN-FH medium was added. Plates were rocked for 5 min at room temperature and transfection mixtures (per well) prepared as follows: Mix A (1.7 µl Baculo-Gold DNA (0.1 µg/µl Pharmagen), 1.3 µg recombinant plasmid carrying foreign gene, *e.g*. a pVL1392/1393 derivative), Mix B (12 µl Lipofectin (Gibco), 28 µl serum free medium). Mix A was added to mix B and incubated at room temperature for 15 min, after which 430 µl of serum–free medium was added. Medium was aspirated off the cells, and transfection mixture spread over the well dropwise. Transfections were rocked at room temperature for 12 h, and subsequently the supernatant was aspirated off and 3 ml of complete TMN-FH medium containing 10% FCS added. The 6-well dish was sealed with Parafilm-foil, and the transfections incubated for 4 days at 27 inside a large zip-lock bag. The supernatant was then pipetted off, and spun at 1500 rpm for 5 min to be used for further amplification.

Primary amplification

In this procedure virus titer was increased prior to plaque purification. $7x10^6$ cells were plated per 10 cm dish and allowed to attach for 1 h at 27^0 C. 1-10 10 cm dishes were plated per recombinant virus. After cells had attached, the medium was aspirated off, and 1.5 ml transfection supernatant (from step I) and 1.5 ml complete TMN-FH medium added to the cells, which were thereafter

rocked for 1h at room temperature. 8 ml of complete TMN-FH medium were added, and the dishes placed at 27^0 C in a zip-lock bag for 4 days. Ensuing, the supernatant was pipetted off again, spun for 5 min at 1500 rpm and then used for plaque purification. The stock was stored wrapped in aluminum foil (protected from light) at 4^0 C.

3.3.2.2. Baculovirus plaque assay with neutral red staining

(O'Reilly *et al*., 1997)

To determine the titer of a baculovirus stock, plaque assays were performed. Four 60 mm dishes with $2x10^6$ cells/dish were seeded per virus stock to be titered. Cells were incubated at 27^0 C for 1 h to attach to the dish. In that time, virus stocks were diluted 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6}, 10^{-7} into 1 ml serum-free TMN-FH each. After cells had attached, the medium was aspirated off the cells and 1 ml of dilutions put onto each labeled plate. Plaque assays were rocked at room temperature for 1 hr, after which the virus inoculum was removed completely and 5 ml TMN-FH + 0.5% agarose overlay added onto each plate. After the overlay had hardened, plates were incubated at 27^0 C in a zip-lock bag for 4 days. To prepare the neutral red overlay, a 0.5% melting agarose/200 μg/ml neutral red mix was prepared, and 2 ml overlay added to each plate. After overlay had hardened, plates were placed at 27^0 C in a zip-lock bag overnight. The next morning plaques were counted, which appeared as light, whitish areas surrounded by the live neutral red staining cells, and the virus stock titer calculated taking the mean of the individual dilutions.

3.3.2.3. Amplification of Baculovirus plaques and preparation of high titer virus stocks

Primary amplification

After performing a neutral red plaque assay on the primary amplification after transfection, two plaques were picked to amplify for each virus. Several well-separated plaques were picket using a short, cotton-plugged pasteur pipette by pipetting the agarose plug into 1 ml complete TMN-FH medium. The plaque(s) were allowed to diffuse into the medium overnight at 4 ^{0} C, and 0.5 ml used to infect 1x10^{6} attached cells in one well of a 6-well tissue culture plate each. Infected cells were rocked for 1 h at room temperature, after which the individual 6-well plates were sealed with Parafilm and placed at 27 ^{0} C in a zip-lock bag for 4 days. The supernatant was then removed and freed of remaining cells by centrifugation at 1500 rpm for 5 min, and the virus stock stored at 4^{0} C protected from light. The cell pellets were saved for western blotting in order to confirm proper, full-length expression of the desired protein by the newly created baculovirus.

Secondary amplification

Four 10 cm dishes were seeded at 5x10^{6} cells per plate, cells were left to attach for 1 h at room temperature, and then infected with 1 ml of the primary plaque amplification with 3 ml complete TMN-FH as described above. After incubation with virus inoculum, 9 ml of complete TMN-FH medium were added and plates incubated for 4-5 days at 27^{0} C in a zip-lock bag. Virus supernatant was harvested by centrifugation for 5 min at 1500 rpm, and virus stock titered and stored as described above.

3.4. Activity assays for *Drosophila* P element transposase

3.4.1. *InVivo* P-element excision assay

Drosophila Schneider L2 cells were transfected by calcium phosphate co-precipitation with pBSK(+)pAC-Tnp, or the respective pBSK(+)pAC-Tnp-mutant for expression of transposase under control of the *Drosophila* actin 5C promoter and with pISP-2/Km as a reporter plasmid. For transfection, $1x10^6$ cells per well were plated out in a 6-well dish and allowed to attach overnight at 25^0 C. 10 μg of transposase-expressing plasmid and 10 μg reporter plasmid were added to 0.35 ml 0.25 M $CaCl_2$, and this mixture was added dropwise into 0.35 ml 2X HEBS. After precipitating for 20 min at RT the mixture was then added to the cells and incubated 24 h at 25^0 C. To recover plasmid DNA from the Schneider L2 cells 24h after transfection, the cells were harvested, washed four times with PBS and incubated in lysis buffer (10 mM Tris-HCl, pH 8.0, 10 mM EDTA, pH 8.0, 0.6% SDS) for 5 min at room temperature. Chromosomal DNA was precipitated with one-fourth volume 5 M NaCl overnight at 4^0 C. The plasmid DNA in the supernatant was extracted with phenol and chloroform, ethanol-precipitated, resuspended in water, electroporated into *E.coli* strain MC1061 and selected for kanamycin resistance by plating on selective media. The excision frequency was calculated as $[N_{(Amp+Kan)}/N_{(Amp)}$ in which $N_{(Amp+Kan)}$ and N_{amp} represent the number of bacterial colonies resistant to both kanamycin (Kan) and ampicillin (Amp) or to ampicillin only, respectively. Thus, the results are corrected for the recovery of the plasmid DNA from the L2 cells. The wild-type transposase activity for each individual assay is set at 100%, which equals a frequency of 38 ± 6.5 x 10^6. For the various transposase mutants, activity is given as the percentage of wild-type activity. Typically, the number of Amp^R colonies was 83 ± 31 x 10^6 per 10^6 L2 cells, and the amount of DNA recovered form 2-4 x 10^6 cells was analyzed.

10XHEBS: 8 g NaCl, 1.08 g dextrose, 0.373 g KCl, 0.1 g Na_2HPO_4, 4.766g HEPES, pH to 7.1 with 4M NaOH, add dH20 to 100 ml. Store at –20 ^oC.

10XPBS: 40 g NaCl, 1 g KCl, 4.6 g Na_2HPO_4, 1 g KH_2PO_4, add dH20 to 500 ml, autoclave, store at room temperature.

3.4.2. *In vitro* strand transfer assay:

The DNA oligonucleotides used for the strand transfer assays are as follows:

P1: 5'-CGTTAAGTGGATGTCTCTTGCCGACGGGACCACCTTATGTTATTTCATCATG-3'

P2-17: 5'- AGGTGGTCCCGTCGGCAAGAGACATCCACTTAACG-3'

The oligonucleotides were gel purified and 11.6 pmol of P1 radiolabeled at the 5' end using 1 μl T4 polynucloetide kinase (USB), 3 μl [γ ^{32}-P] ATP (7000Ci/mmol, ICN), 2 μl 10X Kinase buffer (0.66 M Tris-HCl, pH 7.6, 100 mM $MgCl_2$, 10 mM Spermidine, 0.15 M DTT) and 2 μl BSA (2 mg/ml) in a total volume of 20 μl made up with dH_2O . The kinasing reaction was incubated at 37^0 C for 1 h, heated at 100^0 C for 5 min, and then chilled on ice. Subsequently, 11.6 pmol of P2 was annealed to the radiolabeled P1 oligonucleotide in a reaction buffer containing 0.1 M NaCl . The reaction was again heated to 90^0 C for 5 min, and then slow cooled to room temperature, after which the duplex DNA strand transfer substrate were removed from the unincorporated [γ ^{32}-P] using MicroBiospin 30 Chromatography columns (Bio Rad).

Reaction conditions for the standard strand transfer assay were as follows: 0.5 pmol of radiolabeled strand transfer substrate was incubated with 50-100 ng TO.3-2 transposase-containing Baculovirus protein fractions (wildtype, D528A, E531A, D545A, tDED, and D528C, E531C, and D545C) or Ni^{++}-NTA-superflow fractions (wt, D528C, E531C, D545C, Sf9-mock) in a volume of 6 μl in chromatography buffer (HGKED: 20 mM Hepes-KOH, pH 7.6, 20% glycerol,

100mM KCl, 0.5 mM EGTA, 0.5 mM EDTA, 0.2 mM PMSF, 0.5 mM DTT), with the addition of 100 μg/ml bovine serum albumin. If reactions were preincubated with $Mg(OAc)_2$ and GTP, they contained 5 mM $Mg(OAc)_2$ and 2 mM GTP. Binding was carried out on ice for 15 min. The reaction was initiated by addition of: 0.35X HGKED (0M KCl), 200ng Bluescript tetramer target DNA, and, if not already present in the preincubation mix, 5 mM $Mg(OAc)_2$, 2 mM GTP to a total volume of 20 μl, and [KCl ≤ 35 mM]. $MgCl_2$, $MnCl_2$, and $CaCl_2$ were added to a final concentration of 20 mM. Reactions were performed at 30^0 C for 2 hours, terminated by the additions of 125 μl of stop solution (50 mM Tris-HCl, pH 7.5, 10 mM EDTA, 0.3 M NaCl, 1% SDS, 250 μg/ml yeast RNA), and incubated at 37^0 C for 30 min with 0.1 mg/ml proteinase K. The reactions were extracted with 25:24:1 phenol:chloroform:isoamylalcohol and ethanol precipitated. The pellets were resuspended in 15 μl TE containing 100 μg/ml RNAse A and analyzed by agarose gel electrophoresis. Strand transfer of the free substrate oligonucleotide to the plasmid DNA target results in both relaxed circular single-ended (SET) or linearized double-ended transfer (DET) products.

3.4.3. *In vitro* cleavage and LMPCR assays

For DNA cleavage, transposase-containing fractions (~2 μg) were incubated with 100 ng circular plasmid pISP2/Km as the substrate DNA in HGKED reaction buffer containing 0.1 M KCl for 15 min on ice. The cleavage reaction was initiated by adding 2 mM GTP, 10mM $MgCl_2$, and HGED buffer (containing no KCl) to a total volume of 20 μl and incubated at 27^0 C for 1 hour, after which reactions were stopped by addition of 125 μl footprinting stop solution (1% SDS, 0.2M NaCl, 20 mM EDTA, 250 μg/ml yeast RNA) and incubated at 37^0 C for 30 min with 0.1 mg/ml proteinase K. The reactions were then phenol/chloroform extracted, precipitated with 100%ethanol, washed with 70% ethanol, and dried in a speed vacuum centrifuge. The pellets were resuspended in 10 μl TE containing 100 μg/ml RNAse A. Half of the reaction products were analyzed by agarose gel

electrophoresis and standard DNA southern blot hybridization (described in section 2.4.3.) following capillary transfer to Hybond N^+ membrane (Amersham). Products were detected with a α-^{32}P random-hexamer labeled *EcoRI/EagI* pISP2/Km restriction fragment (described in section 2.4.4.).

For LMPCR analysis, large scale cleavage reactions were performed (fivefold increase), and 1/6 of the total cleaved substrate ligated to either FM25-2+4E or FM25-2+3F oligonucleotides, which anneal specifically to either four basepairs of the 3' P-element overhang or to three basepairs of the 5' substrate overhang generated by DNA cleavage, respectively. Reaction mixtures were as follows: 1.5µl 5X cleavage reaction DNA, 1 µl 10X ligase buffer (0.66M Tris-HCl, pH 7.6, 0.1 M $MgCl_2$, 0.1 M spermidine, 0.15 M DTT, 0.01 M ATP), 1 µl 50 pmol/µl oligo (Fm25-2+4E or FM25-2+3F), 1 µl BSA (2 mg/ml), 1 µl T4 DNA ligase (1 U/ml) and filled up with dH_2O to 10 µl . Reactions were incubated at 15^0 C overnight, and then heat-treated at 65^0 C for 5 min. 90 µl of dH_20), 10 µg glycogen and 100 µl phenol/chloroform were added subsequently, and after phenol extraction the reaction were precipitated with ethanol, washed, dried and resuspended in 30 µl dH_20. 1/6 of the reaction mixture (5µl) was used for LMPCR, adding the following: 2.5 µl 10X PCR buffer as supplied by the manufacturer (Gibco BRL), 2 µl 2.5 mM dNTPs, 1 µl 25 pmol/µl ligated oligo, 1 µl 25 pmol/µl specific oligo (2778-2804 or 2946-2972), 0.5 µl Taq polymerase (5 U/µl) and the reaction filled to a total volume of 25 µl with dH_20. PCR cycling parameters were as follows: 1 cycle (95^0 C for 3 min), 25 cycles (95^0 C for 30 sec, 50^0 C for 30 sec., 72^0 C for 1 min), and 5 min at 72^0 C, after which the PCR reaction was slow cooled to room temperature, and 10 µl of each reaction were run on an 8% native acrylamide gel using pBR322-MspI as used as a size marker. LMPCR products were visualized by ethidium bromide staining.

PCR primer pairs and product sizes:

FM25-2+4E: GCGGTGACTCGGGAGATCTGAGATGCATA and 2778-2804 : ATCGCTGTCTCACTCAGACTCAATACG generate a 135bp product.

FM25-2+3F: GCGGTGACTCGGGAGATCTGAGATGATG and

2946-2972: AACCTGCGTGCAATCCATCTTGTTCAA generate a 99 bp product.

3.4.4. Southern transfer and hybridization

Agarose gels with samples and size- markers were run at 120 V for 2 hours, bands visualized by ethidium bromide staining, and the gel prepared for Southern transfer by incubating 45 min each in 500 ml denaturing solution (0.5 M NaOH, 1.5 M NaCl) followed by incubation in 500 ml neutralizing solution (1 M Tris-HCl, pH 7.4, 3 M NaCl). The gel was placed into a dish filled with 20 x SSC with a sponge topped by three pieces of soaked Whatman 3 MM onto the Whatman filter paper. It was covered with one piece of Hybond-N^+ paper the size of the gel and 3 additional pieces of Whatman paper. The DNA bands were transferred to the Hybond-N^+ paper overnight by capillary action and then UV crosslinked onto the filter paper ($1200x10^2$ μJ). The filter was then prehybridized in a bottle with 30 ml aqueous hybridization buffer (15ml 20 X SSCP, 2.4 ml (5 mg/ml) herring sperm DNA, 6 ml 100 X Denhardt's, 3ml 10% SDS, 36 ml dH_20) for 4 hours rotating at 65^0 C , and the hybridized in 15 ml of preheated buffer with ca $1x10^6$ cpm/ml denatured probe overnight at 65^0 C . Subsequently, the filter was washed 2x20 min in preheated 0.1 X SSC/0.5% SDS, air dried and exposed to film.

20X SSCP:

For 2 l: 800 ml 1 M sodium phosphate, pH 6.8, 176.4 g sodium citrate, 280.5 g NaCl, to 2 l with dH_2O

20X SSC:

For 5L: 876.5 g NaCl, 441.25 g Na citrate, to 5 l with dH2O

100X Denhardt's Solution:

For 1L: 20 g polyvinyl pyrrolidone, 20 g BSA, 20 g ficoll 400, 10 ml 0.25 M EDTA, add dH_2O to 1l.

3.4.5. α-^{32}P random-hexamer labeling of small DNA fragments

To make radiolabeled probe for use in Southern Blot assays, 1 µl of 1:100 diluted random hexamers (Boehringer) and 100 ng of the *EcoRI/EagI* pISP2/Km restriction fragment were filled up to a total reaction volume of 3 µl with dH_20. The reaction was covered with 50 µl mineral oil and heated at 100^0 C for 3 min, after which it was immediately placed on ice. The following was added to the reaction: 2 µl 10 X random hexamer buffer (900 mM Hepes-NaOH, pH 6.6, 100 mM $MgCl_2$), 2 µl (2 mM dNTPs –dCTP), 3 µl α^{32}P-dCTP (3000 Ci/mMol), 2 µl Klenow (2 U/µl labeling grade), 0.4 µl DTT (0.5 M), and 7.6 µl dH_20. The labeling reaction was allowed to proceed for 3-12 h at room temperature, after which the reaction was stopped by addition of 90 µl stop buffer (0.1% SDS, 10 mM EDTA), vortexed, spun down, and the aqueous phase pipetted into a new tube for immediate use, or frozen at –20 ^{0}C.

4. Results

4.1. Identification of amino acid residues involved in the catalytic activity of P element transposase using point-mutational analysis

P-element transposition occurs by a cut-and-paste mechanism: once transposase is positioned at the transposon termini and assembled into an active complex, it executes the DNA breakage reactions that cut the transposon away from flanking DNA at the donor site, and then joins the exposed ends to the target DNA (see Introduction Fig. 1A) (Kaufman and Rio, 1992). This pathway of transposition is used by other elements such as bacterial Tn10, Mu, Tn7, and the eukaryotic Tc1/mariner elements (Craig, 1997). The catalytic residues for many recombinases have been identified by point-mutational analysis and sequence alignments. The catalytic core consists of a triad of acidic amino acids, the DD(35)E motif, which is thought to coordinate metal ion-binding required for catalysis (Baker and Luo, 1994; Bolland and Kleckner, 1996; Katz *et al*., 1992; Kulkosky *et al*., 1992; Sarnovsky *et al*., 1996). Even conservative substitutions at these positions have a drastic effect on cleavage and strand transfer activity (Baker *et al*., 1994; Kim *et al*., 1995; Kulkosky *et al*., 1992).

Drosophila P element transposase shows no similarities with the DDE signature of other transposable elements. However, aligments of the catalytic core structures of transposition proteins do not always show strong homologies, yet they can be fundamentally related to one another as has been spectaculary demonstrated by the structures of the catalytic domain of bacteriophage Mu and HIV at the atomic level (Craig, 1995).

The aim of this work was to identify and characterize the catalytic residues constituting the DD(35)E motif of the *Drosophila* P element transposase. In order to analyze the amino acid sequence of P element transposase for potential, catalytic aspartic acid (D) and glutamic acid (E) residues, searching was narrowed to the C-terminal half of the protein. It appeared likely that the residues

important for catalytic activity should be present in this region, since the C-terminally truncated repressor proteins 66K and KP were shown to be defective in P element transposition (Lee *et al*., 1998) (see Introduction Fig. 1B). Interestingly, this region of primary sequence of transposase was found to contain a relatively high density of D and E residues (Fig. 1A). As a first round of selection, sequence alignments and comparisons with the catalytic domains of retroviral integrases were performed. This approach did not yield any distinctive catalytic core structure, due to the lack of primary sequence homologies in general (data not shown). Subsequently, a series of transposase double mutations were generated, changing both a D and an E residue located in close proximity to each other, not necessarily being part of the same, putative D-D-E combination, into alanine (Fig. 1A, aspartates and glutamates boxed in blue, red and green) (Y. Mul, umpublished). Particular residues were picked on the basis of homologies to hydrophobic residues surrounding the HIV integrase DDE motif, and by partial alignment to a recombinase DDE alignment scheme made by Baker and Luo, 1994.

```
  1    MKYKFCCKAVTGVKLIHVPKCAIKRKLWEQSLGCS    36
 37    LGENSQICDTHFNDSQWKAAPAKGQTFKRRRLNADA    72
 73    VPSKVIEPEPEKIKEGYTSGSTQTESCSLFNENKSL   108
109    REKIRTLEYEMRRLEQQLRESQQLEESLRKIFTDTQ   144
145    IRILKNGGQRATFNSDDISTAICLHTAGPRAYNHLY   180
181    KKGFPLPSRTTLYRWLSDVDIKRGCLDVVIDLMDSD    216
217    GVDDADKLCVLAFDEMKVAAAFEYDSSADIVYEPSD   252
253    YVQLAIVRGLKKSWKQPVFFDFNTRMDPDTLNNILR   288
289    KLHRKGYLVVAIVSDLGTGNQKLWTELGISESKTWF    324
325    SHPADDHLKIFVFSDTPHLIKLVRNHYVDSGLTING    360
361    KKLTKKTIQEALHLCNKSDLSILFKINENHINVRSL   396
397    AKQKVKLATQLFSNTTASSIRRCYSLGYDIENATET   432
433    ADFFKLMNdWFdIFNSKLSTSNCIECSQPYGKQLDI   468
469    QPDILNRMSeIMRTGILdKPKRLPFQKGIIVNNASL   504
505    DGLYKYLQENFSMQYILTSRLNQdIVeHFFGSMRSR   540
541    GGQFdHPTPLQFKYRLRKYIIARNTEMLRNSGNIEE   576
577    DNSeSWLNLdFSSKENENKSKDDePVDDePVDEMLS    612
613    NIdFTEMdeLTEDAMeYIAGYVIKKLRISdKVKENL    648
649    TFTYVDeVSHGGLIKPSEKFQEKLKELECIFLHYTN   684
685    NNNFeITNNVKEKLILAARNVDVDKQVKSFYFKIRI   720
721     YFRIKYFNKKIEIKNQKQKLIGNSKLLKIKL        751
```

Figure 1A. Primary amino acid sequence of the P element transposase protein. Potential catalytic D and E residues which were mutated to alanine are marked with blue, red and green boxes. Amino acid residues boxed in green or blue had little or no effect on in *vivo* excision activity. Amino acids boxed in red almost completely abolished *in vivo* excision activity as described in the text.

4.2. Mutations in potential catalytic amino acid residues affect *in vivo* activity of transposase

A genetic assay was used as previously described by Mul and Rio (1997) to test the mutant proteins for *in vivo* activity relative to wild-type activity (Fig. 1B). The *in vivo* assay is based on transient transfection of *Drosophila* Schneider L2 cells with a plasmid that expresses either wild-type or mutant transposase protein. In addition, a reporter plasmid is introduced in order to monitor the transposase-induced P element excision frequency (Fig. 1B). The reporter DNA contains a non-autonomous P element inserted directly downstream of the start codon for the bacterial kanamycin (Kan) resistance gene. Excision of the P element and processing of the DNA ends places the translation start codon in frame with the rest of the kanamycin resistance coding sequence. Following recovery of plasmid DNA from the L2 cells, the DNA is introduced into *Escherichia coli* and excision events are measured by genetic selection for kanamycin resistance. This assay was used for quantitative measurement of transposase activity *in vivo* in order to compare the catalytic activities of the wild-type and mutant transposase proteins. However, only a fraction of the plasmids that have undergone excision are detected due to the requirement for the restoration of the kanamycin open reading frame in this assay. As a result, the determined excision frequency is an underestimate of the actual activity of the transposase proteins being examined. As expected from previous data (Mul and Rio, 1997), it was found that in *Drosphila* cells expressing wild-type transposase resulted in excision of the P element at a frequency of 4×10^{-5}, while in the absence of transposase, no excision products were recovered.

1B.

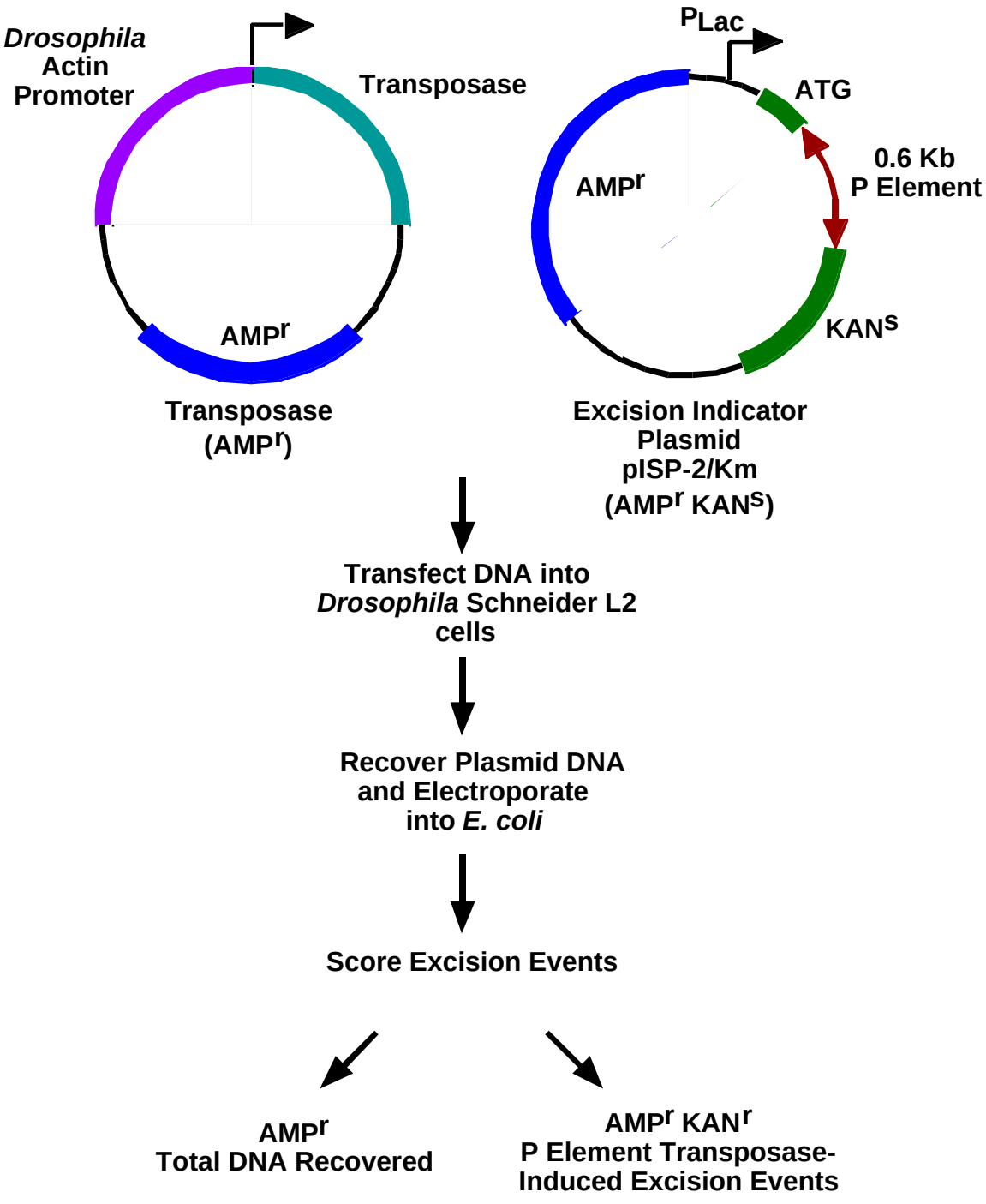
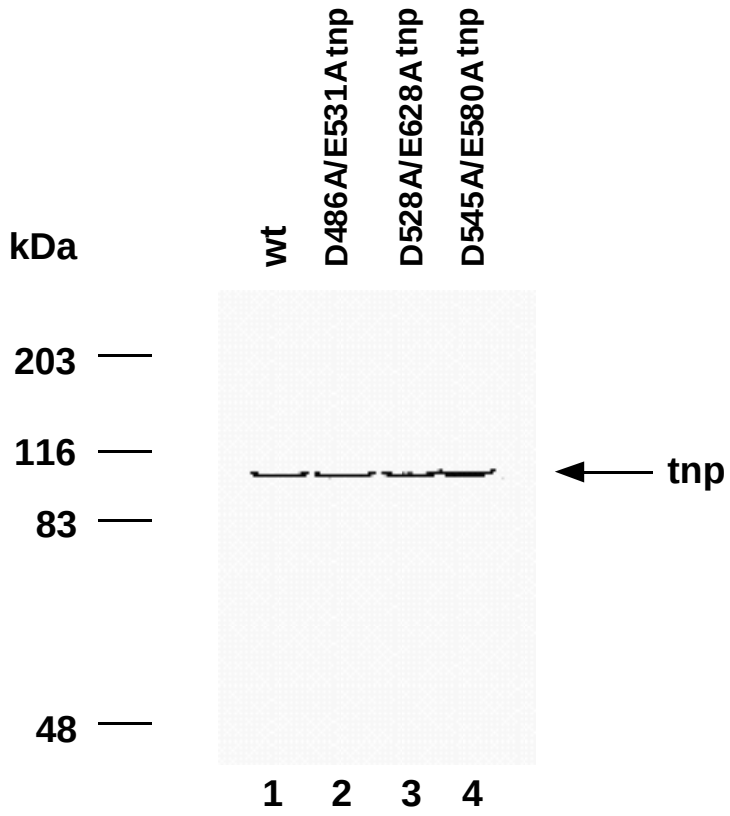

Drosophila Actin Promoter
Transposase
AMP^r
Transposase (AMP^r)
P$_{Lac}$
ATG
0.6 Kb P Element
AMP^r
KAN^s
Excision Indicator Plasmid pISP-2/Km (AMP^r KAN^s)
Transfect DNA into *Drosophila* Schneider L2 cells
Recover Plasmid DNA and Electroporate into *E. coli*
Score Excision Events
AMP^r Total DNA Recovered
AMP^r KAN^r P Element Transposase-Induced Excision Events

Figure 1B. Schematic diagram of the *Drosophila* cell culture transposase assay. *Drosophila* Schneider L2 cells were transfected with a plasmid encoding either wild type or mutant transposase proteins under the control of the constitutive *Drosophila* actin promoter (left) and a reporter plasmid, pISP-2Km (right), which contains the kanamycin resistance gene open reading frame interrupted by a 0.6 kb nonautonomous P element. Transposase activity was detected following excision of the P element by the gain in kanamycin resistance following plasmid recovery from the transfected cells and electroporation into *E. coli* cells. The excision frequency was determined by comparing the total amount of DNA recovered (ampicillin resistant colonies) to the number of plasmids that had undergone excision (ampicillin and kanamycin resistant colonies).

All alanine substitution mutants shown in Fig. 1A, boxed in blue, red, and green were tested in the assay described in Fig. 1B. Figure 2B shows a selection of the mutants tested. (Single or double mutants which had no significant effect on the *in vivo* activity were omitted in Fig. 2 for clarity.) From these double-D/E-A substitution mutants, three (D486A/E531A, D528A/D628A, and D545A/E580A0 completely or almost completely abolished transposase activity *in vivo* (Fig. 2B). In contrast, the transposase mutants D487A/E444A, D586A/E621A, and D620A/E621A were still partially active in the *in vivo* excision assay. Interestingly, transposase D620A/E655A was more active than the wild-type protein (Fig. 2B.). More active forms of transposase may have been eliminated during evolution due to excessive damage to the *Drosophila* host genome. It was concluded from these results that a subset of the amino acids D486, D528, E531, D545, E580, and D628 could be the essential residues for catalytic activity of *Drosophila* P element transposase protein.

In order to demonstrate that the differences in transposase activity were not due to lower expression and/or stability of the mutant proteins, western blot analysis of the L2 cell extracts after transfection (Fig. 2A) was performed and equal levels of protein expression were confirmed. To locate the specific single substitution mutants responsible for the loss of catalytic activity *in vivo*, single

2A.


kDa
wt
D486A/E531A tnp
D528A/E628A tnp
D545A/E580A tnp
203
116
83
48
tnp
1
2
3
4

2B.

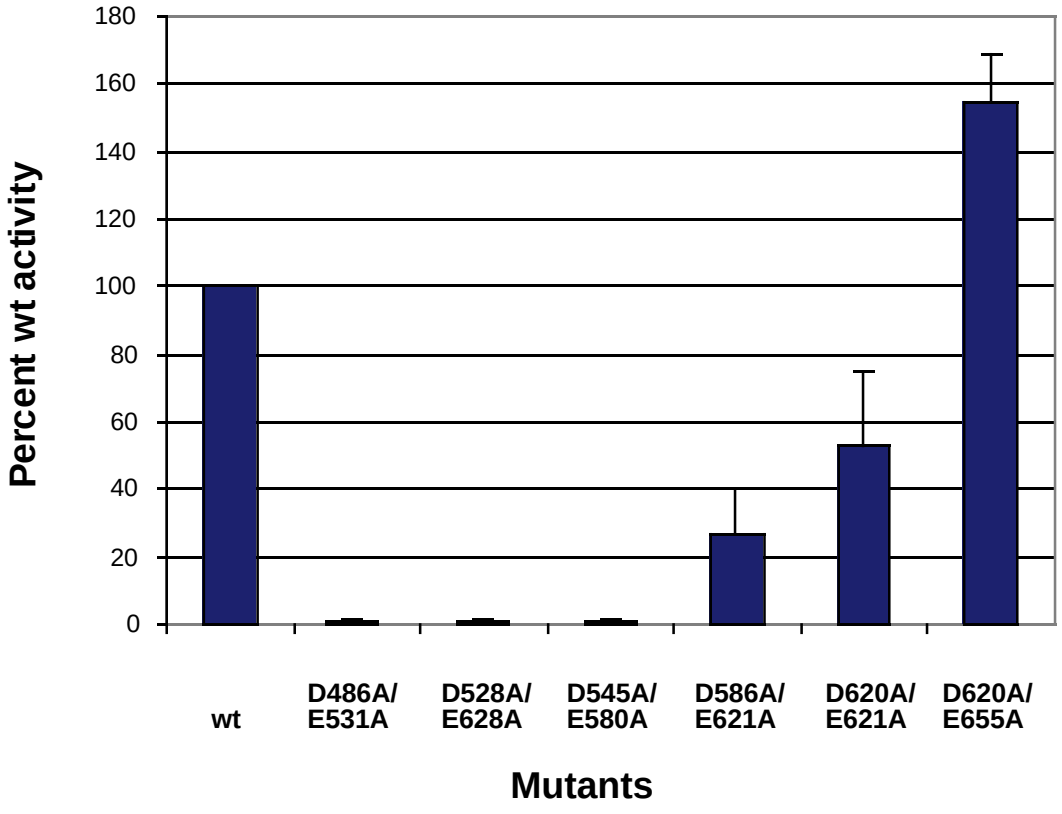
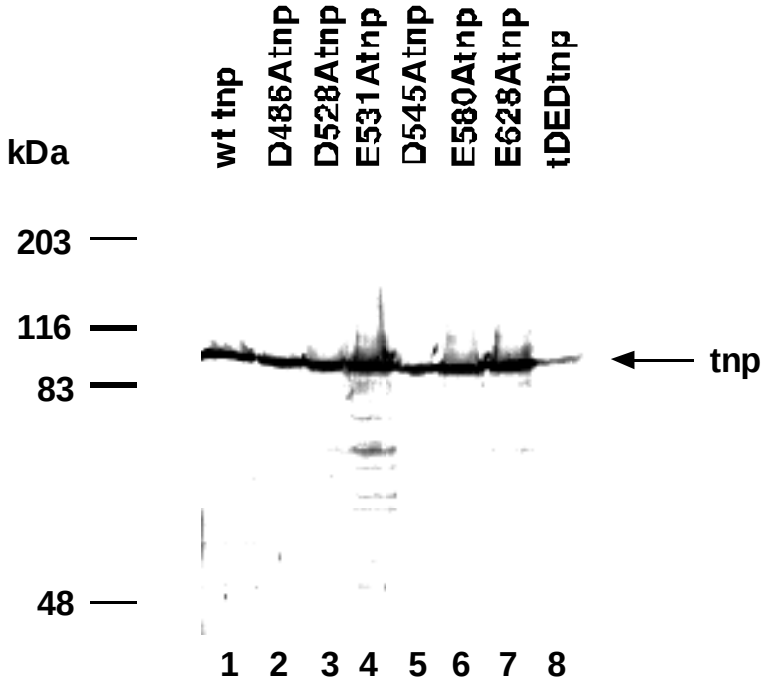

Percent wt activity
180
160
140
120
100
80
60
40
20
0
wt
D486A/ E531A
D528A/ E628A
D545A/ E580A
D586A/ E621A
D620A/ E621A
D620A/ E655A
Mutants

Figure 2. Comparison of wild-type and double-mutant transposase activity *in vivo*. A. Western Blot using anti-KP antibody of wildtype (lane 1), and double mutants D486A/E531A (lane 2), D528A/E628A (lane 3), and D545A/E580A (lane 4) of transposase protein crude cell extracts after L2 cell transfection. Equal amounts of protein were detected for wildtype and mutant transposase. Transposase is indicated by an arrow. Approximately 400 ng of protein were loaded in each lane. **B.** Bar graph depicting *in vivo* excision activity. Each of the indicated mutant transposase proteins was tested for activity four individual times in the assay described in Fig. 1B. Shown are the mean activity values and standard deviations. The wild-type transposase activity for each individual assay is set at 100%, which equals an excision frequency of 38 ± 6.5 x 10^{-6}. For the various transposase mutants, activity is given as the percentage of wild-type activity: D486A/E531A [0.0 ± 0.0], D528A/E628A [0.3±0.4], D545A/E580A [0.7±0.9], D586A/E621A [27±13]. D620A/E62A [53±22], D620A/E655A [155 ±14] (numbers following ± are indicative of the standard deviation).

mutants of the double mutant pairs D486A/E531A, D528A/D628A, and D545A/E580A were generated. The six single mutants were then tested in the *in vivo* assay as described in Fig. 1B, and the excision events scored for by genetic selection as described. Substitution mutants D528Atnp, E531Atnp, D545Atnp and the combined triple mutant tDED tnp were all found to be inactive *in vivo* (Fig. 3B). Western Blot analysis of L2 cell extracts showed that expression levels of all of the single mutants were equal to wild-type levels (Fig. 3A, lanes 1-7), however, the levels for the triple DED (D528A/E531A/D545A) mutant were consistently lower than those of the wild type and of the single mutants (Fig.3A, lane 8).

These results led to the conjecture that the above three residues, D528, E531, and D545, although in slightly different order (D-E-D), possibly constitute, or form part of the *Drosophila* P element catalytic DDE triad found in most recombinase proteins. To further confirm this hypothesis, wild-type and mutant transposase proteins were purified using the Baculovirus system and Sf9 cells, and stable transfections of L2 insect cells, and the purified protein(s) tested in three different *in vitro* activity assays.

3A.


wt tnp
D486Atnp
D528Atnp
E531Atnp
D545Atnp
E580Atnp
E628Atnp
tDEDtnp
kDa
203
116
83
48
tnp
1 2 3 4 5 6 7 8

3B.

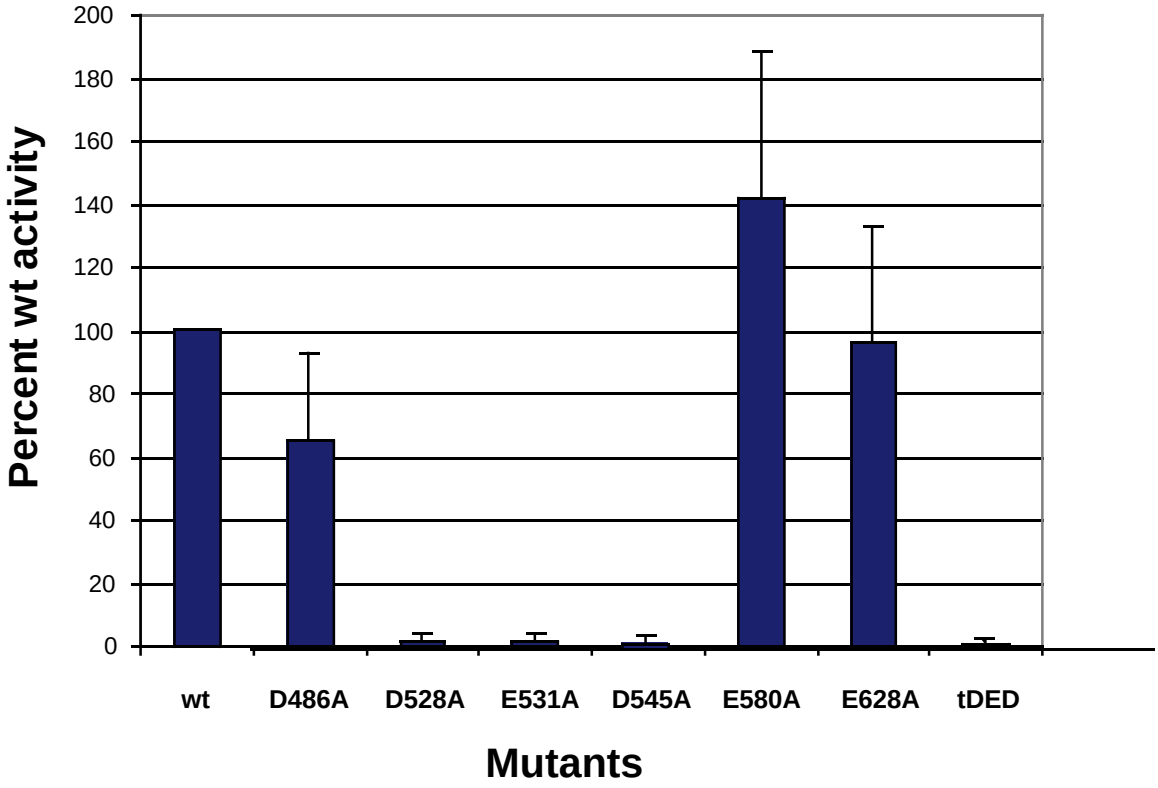

Percent wt activity
200
180
160
140
120
100
80
60
40
20
0
wt
D486A
D528A
E531A
D545A
E580A
E628A
tDED
Mutants

Figure 3. Comparison of wild-type and single-mutant transposase activity *in vivo*. A. Western Blot using anti-KP antibody of wild-type (lane 1), and single mutants D486A (lane 2), D528A (lane3), E531A (lane 4), D545A (lane 5), E580A (lane 6), E628A (lane 7), and tDED (lane 8) of the transposase proteins crude cell extracts after L2 cell transfection. Equal amounts of protein were detected for wild-type and single-mutant transposases. The triple mutant (tDED) showed a slightly lower level of protein expression. Transposase is indicated by an arrow. Approx. 400 ng of protein extracts were loaded in each lane. **B.** Bar graph representing the *in vivo* activity of transposase alanine substitution mutants. Each of the indicated mutant transposase proteins was tested for activity four times in the assay described in Fig. 1B. Wild-type (wt) transposase activity is set at 100%, and activity of the mutants is shown as percent of wt. Alanine substitution mutants tnpD528A [1.6±2.7], tnpE531A [1.8±2.4], and tnpD545A [0.03±0.06], as well as the combined triple mutant tDED [0.7±0.3] severely reduced or abolished activity *in vivo*. Alanine substitution mutations D486A [65±28], E580A [142.4±46], and E628A [96.2±37] had little or no effect on *in vivo* activity. Standard deviations are indicated as thin vertical lines.

4.3. Mutations in the potential catalytic motif of P element transposase affect *in vitro* activity

4.3.1. *In vitro* strand transfer assay

The P element transposition reaction can be divided into two steps: donor DNA cleavage and strand transfer into a target DNA. To determine which step of the transposition reaction the D528A/E531A/D545A mutants were affecting, the mutant transposase proteins were first tested for *in vitro* strand transfer activity. Following excision of a P element from the donor DNA, the transposase protein catalyzes the integration of the P element into a new target site by a reaction termed strand transfer (Fig. 4A). Strand transfer can be performed *in vitro* using short, radiolabeled, double-stranded DNA oligonucleotide substrates carrying a 31 bp terminal inverted repeat and a transposase-binding site along with the authentic 17 bp staggered cleavage site (Beall and Rio, 1998). Transposase activity is measured by the transfer of either one or two oligonucleotide substrates to a circular plasmid target as determined by a shift in the radioactivity from the

4A.

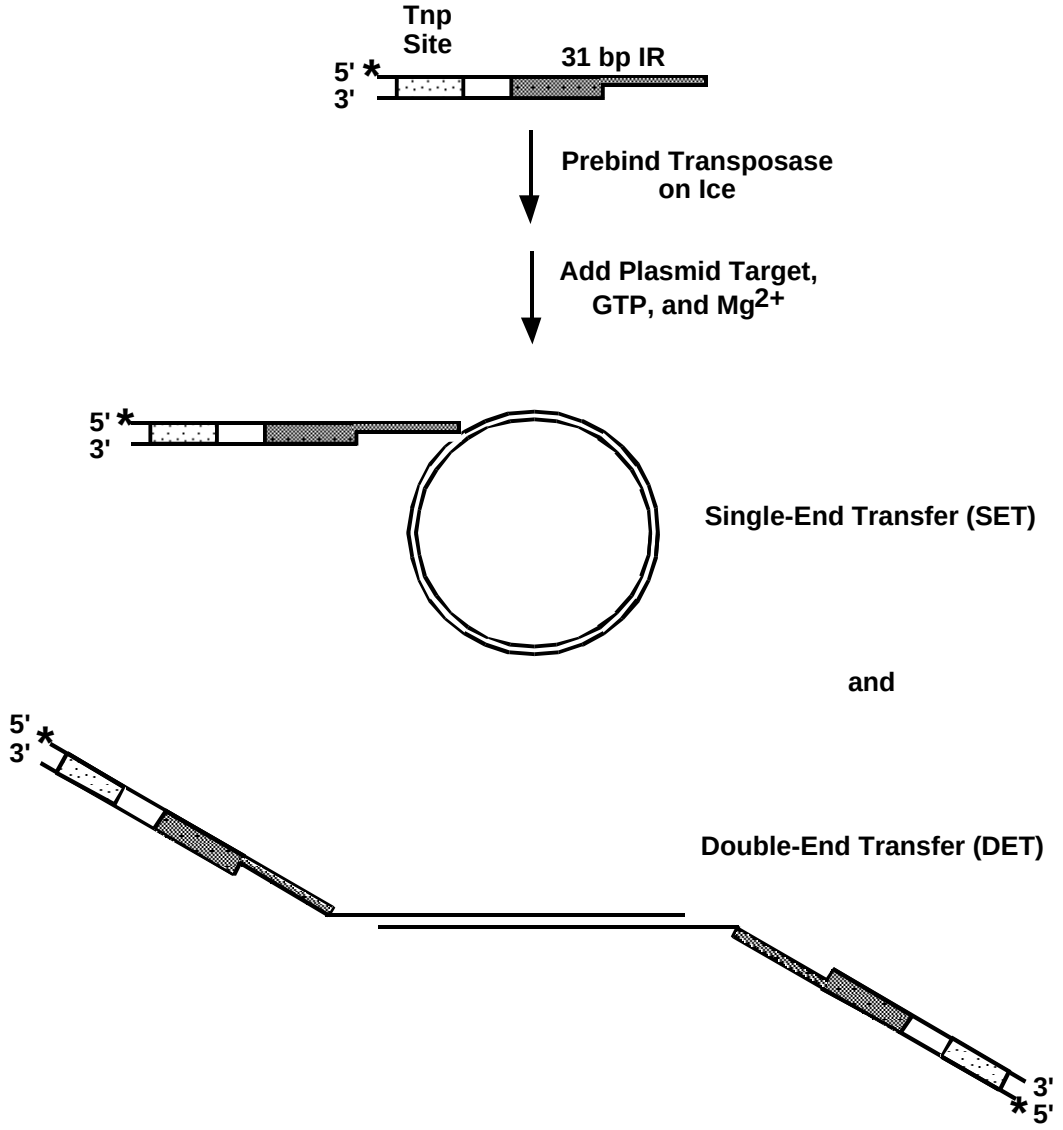

Tnp
Site
31 bp IR
5'
3'
Prebind Transposase
on Ice
Add Plasmid Target,
GTP, and Mg2+
5'
3'
Single-End Transfer (SET)
and
5'
3'
Double-End Transfer (DET)
3'
5'

Figure 4A. Schematic diagram of *in vitro* strand transfer assay.
Oligonucleotides derived from the right (3') P element end that contain the transposase binding site (light gray box) and the 31 bp inverted repeat (dark gray box) were annealed to produce substrates that mimic a cleaved P element terminus. Transposase-containing fractions were incubated with radiolabeled (asterisk) substrate DNA on ice, and the strand transfer reaction initiated by adding GTP, Mg^{++} and the plasmid DNA target. Reaction products were run on a 0.7% TAE agarosegel, and visualized by autoradiography. Strand transfer of the free substrate oligonucleotide to the plasmid DNA target results in both relaxed circular, single-ended (SET) or linearized, double-ended (DET) transfer products as shown in the diagram.

free substrate to the plasmid target DNA following agarose gel electrophoresis. The reaction products are classified as either single-end transfer product (SET) or double-end transfer product (DET) (see Fig. 4A).

The DDE motif is thought to provide a binding site for a divalent metal at or near an active site(s) for DNA hydrolysis and strand transfer (Craig, 1997). Mg^{++} is a critical cofactor in P element transposition *in vitro*; no transposition is observed in the absence of divalent metal (Kaufman and Rio, 1992). Since the homology between the P element transposase DED motif and the usual DD(35)E triad found in other recombinases is only limited, establishing that the DED is indeed the metal-dependent active site required additional evidence.

Before testing the mutant transposase proteins in *in vitro* strand transfer, reaction conditions for this assay were optimized by a series of control experiments with the wild-type protein only (Fig. 4B). The following variables were examined: The preincubation mix (see Materials and Methods) either contained 5 mM $Mg(OAc)_2$ and 2 mM GTP (Fig. 4B, lanes 1-4 and 6-8), only 1 mM GTP and 5 mM $Mg(OAc)_2$ (lanes 9-12), or did not contain $Mg(OAc)_2$ and GTP at all (Fig. 4B, lanes 12-18). The latter conditions will be referred to as 'standard'. Also, the reactions were allowed to proceed either with additional metal ions in the form of $MgCl_2$, $MnCl_2$, and $CaCl_2$ added into the preincubation mix (Fig. 4B, lanes 6-8 and 16-18), or added after preincubation (Fig. 4B, lanes 1-3, 9-11, and 12-14). The most prominent effects on *in vitro* strand transfer activity were seen when

4B.

	Me^{++} postinc.				Me^{++} preinc.				Me^{++} postinc.						Me^{++} preinc.			
GTP postinc.												+	+	+	+	+	+	+
PI+1mM GTP									+	+	+							
PI+2mM GTP	+	+	+	+		+	+	+										
Mg^{++}	+					+			+			+				+		
Mn^{++}		+					+			+			+				+	
Ca^{++}			+					+			+			+				+
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18

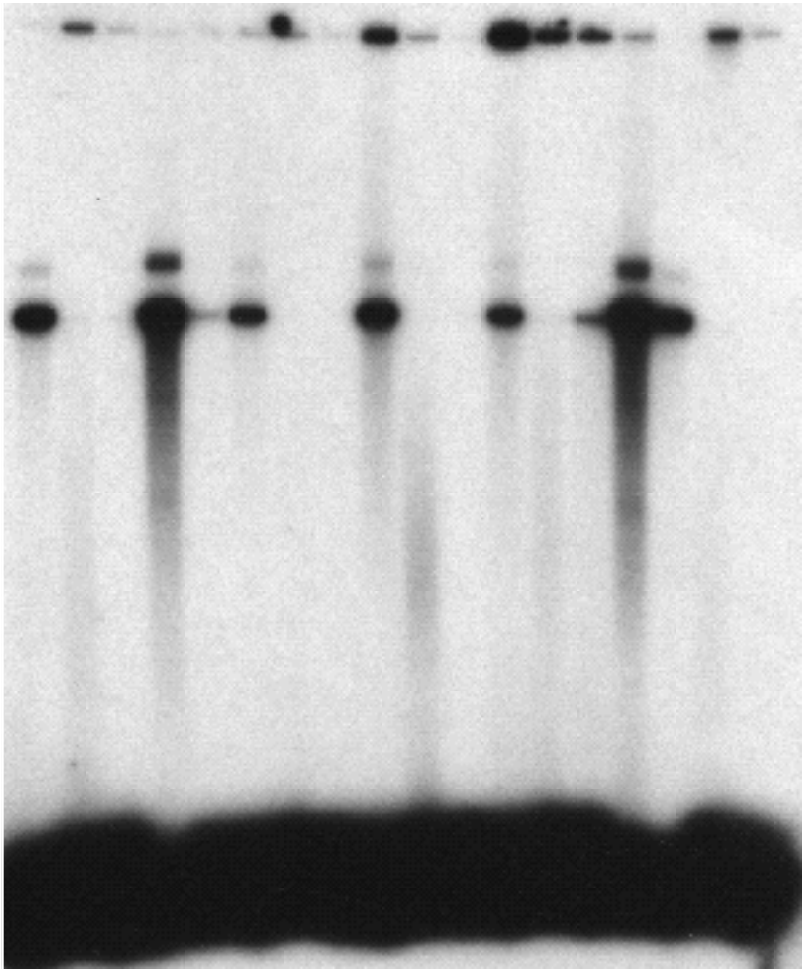


Figure 4B. Autoradiogram of strand transfer reactions with wildtype transposase protein. Baculovirus-expressed wildtype transposase protein with a C-terminal His-tag was tested in the strand transfer assay as described in Fig. 4A. Shown is an autoradiogram from an experiment in which different reaction conditions were tested. All reactions contained 2 mM GTP and 5 mM $Mg(OAc)_2$ in the preincubation mix (PI) (lanes 1-4, and 6-8), or incubation mix (GTP postinc.) (lanes 12-18), unless preincubation was performed with only 1 mM GTP (lanes 9-11). Preincubation with additional metal ions (lanes 6-8 and 16-18) and postincubation with additional metal ions (lanes 1-3 and 9-14) were all done at a final Me^{++} concentration of 20 mM each. For Me^{++} preincubation, Mg^{++} (lane 6 and 16), Mn^{++} (lane 7 and 17), and Ca^{++} (lane 8 and 18) were added to the preincubation reaction on ice. For metal postincubation, Mg^{++} (lanes1, 9 and 12), Mn^{++} (lanes 2,10 and 13), and Ca^{++} (lanes 3,11 and 14) were added to the reaction after preincubation on ice, together with the plasmid DNA target. Lanes 4, 5, and 15 do not contain any extra metal in the standard reaction mix, lane 5 is the –GTP strand transfer control reaction. After completion of the strand transfer reaction, 1/2 of the total reaction was loaded on a 0.7% TAE gel. Single-end transfer (SET) and double-end transfer (DET) products are indicated by arrows. For each reaction, 0.5 pmol of radiolabeled substrate DNA and approx. 100 ng of wildtype transposase protein (as estimated by silver staining) were used.

a) the protein was or was not preincubated with $Mg(OAc)_2$ and GTP, and b) when additional metal ions were added after preincubation. Lowering of the GTP concentration and preincubation with metal ions did not show any significant effects, so these reaction conditions were omitted in future experiments.

4.4. Alanine substitution mutations D528Atnp, E531Atnp, and D545Atnp change the metal-ion specificity of the *in vitro* strand transfer reaction

To determine whether the mutant proteins would inhibit transposase-mediated strand transfer, and whether specific metal ions were capable of rescuing strand transfer activity *in vitro*, strand transfer reactions were performed using Mg^{++}, Mn^{++}, and Ca^{++} -ions (20 mM final concentration). C-terminally His-

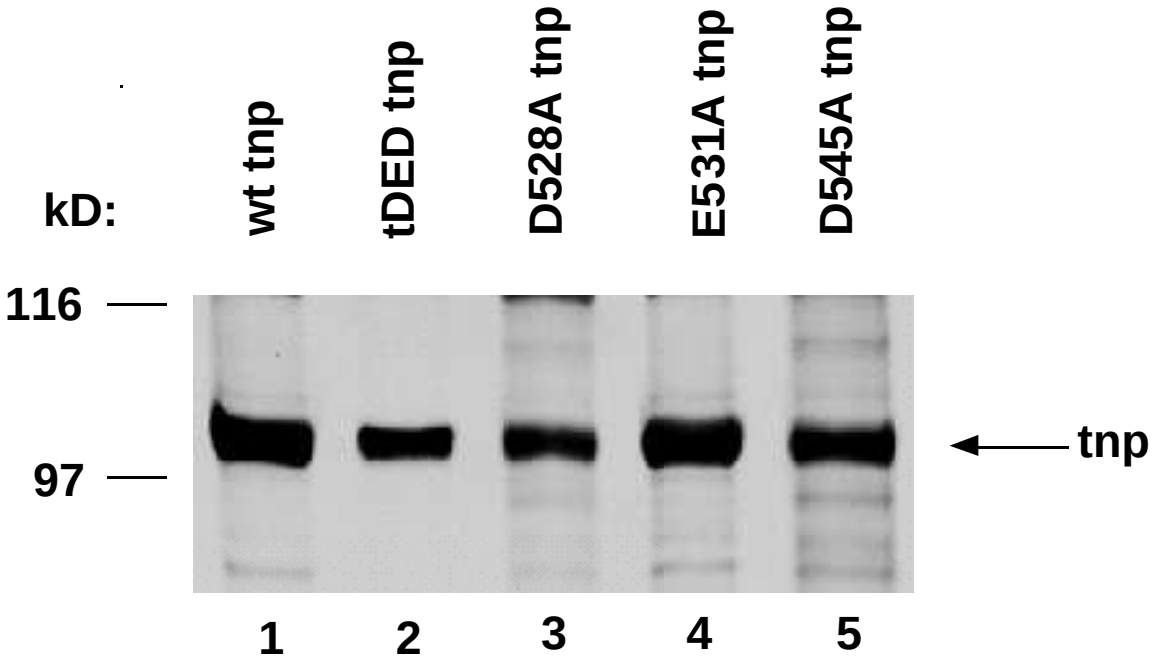


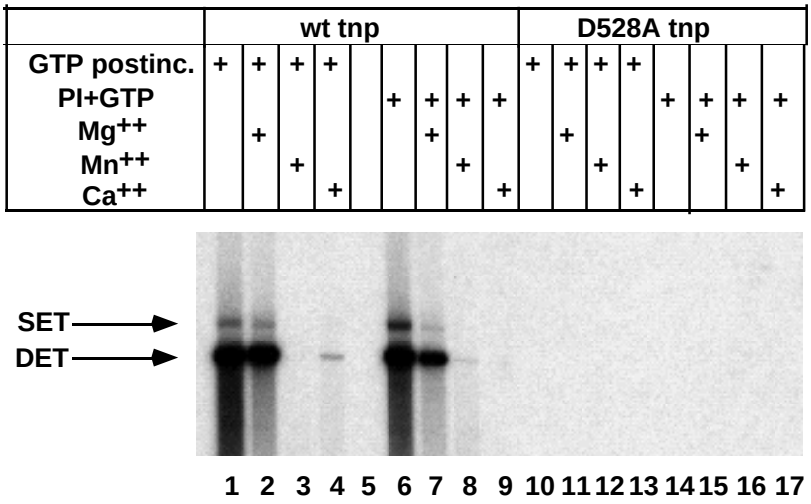
Figure 4C. Transposase-containing protein fractions used to study *in vitro* activities. Analysis of the purified P element transposase-containing fractions by SDS-PAGE on a 7.5% acrylamide gel stained with silver. Positions of the molecular weight markers in kD are indicated on the left of the panel. 5 µl of highly purified protein after Ni^{++}-NTA super-flow and TdT-Streptavidin DNA affinity chromatography were loaded in each lane: lane 1, wt tnp; lane 2, triple DED tnp; lane 3, tnpD528A; lane 4, tnpE531A; lane 5, tnpD545A. Transposase is indicated by an arrow.

tagged transposase protein expressed using the Baculovirus system, and the respective mutant proteins D528Atnp, E531Atnp, and D545Atnp were purified via Ni^{++}-NTA-Superflow and DNA-Streptavidin affinity chromatography, and the 0.3 M KCl fractions used in the activity assay. Equal protein concentration and purity was demonstrated by SDS-PAGE followed by silver staining (Fig 4C, lanes 1-5).

As expected, the level of wild-type *in vitro* strand transfer activity was consistently higher than that of any of the mutants, as seen by the strong strand transfer activity of the wild-type protein in Fig. 5A, lanes 1-9 after only 4 hours of exposure, as compared to the absence of any visible band for D528Atnp in the same figure. Even after a 7 day exposure of the respective strand transfer gels, activities of D528A tnp (Fig. 5B, lanes 1-8), E531A tnp (Fig. 5B, lanes 9-16), D545A tnp (Fig. 5B, lanes 17-24), and tDED (Fig. 5B, lanes 25-27) were notably lower than that of the wild-type protein, as seen by the weaker band intensity of the reactions containing mutant proteins versus wild-type.

The addition of metal ions to the strand transfer reactions had different effects on wildype versus mutant protein activity: Additional $MgCl_2$ had a slightly reducing effect on the levels of wild-type strand-transfer activity compared to standard conditions (Fig. 4B, lanes 1 and 4; Fig. 5A, lanes 1-2 and 6-7), however it failed to stimulate D528Atnp at all (Fig. 5B, lanes 2 and 6), and only slightly stimulated E531 tnp activity (Fig. 5B, lanes 10 and 14). Addition of any of the three metal ions to reactions containing D545 tnp appeared to have little overall effect, since this mutant already showed strand transfer activity, albeit reduced compared that of the wild-type, under standard reaction conditions (Fig. 5B, lanes 17-24). Interestingly, $MnCl_2$ inhibited wild-type strand transfer activity (Fig. 5A, lanes 3 and 8), yet was able to stimulate activity of D528Atnp (Fig. 5B, lanes 3 and 7), E531 tnp (Fig. 5B. lanes 11 and 15), and even tDED (Fig. 5B, lane 26), although stimulation of triple mutant activity was very weak. Conversely, where $CaCl_2$ seemed to reduce wild-type activity compared to levels under standard conditions (Fig. 5A, lane 4) it greatly stimulated E531Atnp activity in conditions

5A.


wt tnp
D528A tnp
GTP postinc. + + + + + + + + + + + + + + + +
PI+GTP + + + + + + + +
Mg++ + + + + + +
Mn++ + + + + + +
Ca++ + + + + + +
SET
DET
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17

5B.

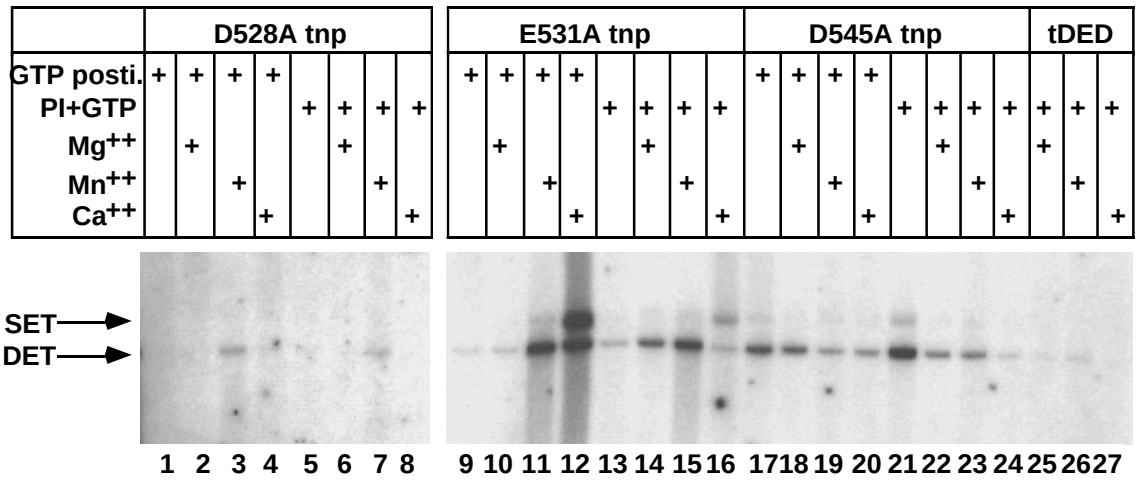

D528A tnp
E531A tnp
D545A tnp
tDED
GTP posti. +
PI+GTP +
Mg++ +
Mn++ +
Ca++ +
SET
DET
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27

Figure 5. Autoradiogram of strand transfer assays performed with wild-type and D528A, E531A, D545A and tDED transposase mutant proteins. C-terminally His-tagged, Baculovirus-expressed transposase proteins D528A, E531A, D545A, tDED and wild-type were tested in the strand transfer assay as described in Fig. 4A. Shown are autoradiograms from an experiment in which transposase-containing fraction were either preincubated (PI) with reaction buffer containing 2 mM GTP and 5 mM $Mg(OAc)_2$, (Fig. 5A, lanes 6-9, and 14-17, Fig. 5B, lanes 5-8, 13-16, 21-27) or where 2 mM GTP and 5 mM $Mg(OAc)_2$ were added after preincubation (Fig.5A, lanes 1-4, 10-13, Fig. 5B, lanes 1-4, 9-12, and 17-20). Reactions containing 20 mM final concentration of $MgCl_2$, $MnCl_2$, and $CaCl_2$ are indicated in the panel above the autoradiograms with a + sign. Lane 5 in Fig. 5A. is the –GTP control reaction. **A.** Autoradiogram of wt and D528A reactions electrophoresed on a 0.7% TAE agarose gel, after 4 h exposure of the gel to X-ray film. **B.** Autoradiogram of D528A, E531A, D545A and tDED reactions after 7 days exposure to X-ray film. Single-end transfer (SET) and double-end transfer (DET) products are indicated by arrows. For each reaction, 0.5 pmol of radiolabeled substrate DNA and ~ 100 ng of respective wt or mutant transposase proteins were used.

without $Mg(OAc)_2$ and GTP preincubation (Fig. 5B, lane 12). This metal did not, however, stimulate D528A tnp (Fig. 5B, lanes 4 and 8) nor E531Atnp activity when preincubated with $Mg(OAc)_2$ and GTP.

Taken together, these results demonstrate that the alanine substitution mutations D528Atnp, E531Atnp, D545Atnp and tDEDtnp have a marked effect on protein's loss of catalytic activity under standard conditions, and by the metal-ion induced rescue of their *in vitro* strand transfer activity. Thus, it is very likely that the amino acids D528, E531, and D545 in fact represent the metal binding, i.e. catalytic motif, or parts of the latter, in the *Drosophila* P-element transposase protein.

4.5. Cysteine substitution mutations D528Ctnp, E531Ctnp, and D545Ctnp alter metal ion specificity in *in vitro* strand transfer

To establish that the D528/E531/D545 amino acid motif is indeed the metal-dependent active site of the P element transposase protein, the differential

chemistry of metal-sulfur and metal-oxygen interactions was used in an attempt to provide further evidence that an essential metal acts in close proximity to the tDED motif. Similar metal ion specificity experiments have been done with ribozymes in which the metal requirements of thiol-containing substrates were explored (Piccirilli *et al*., 1993; Sontheimer *et al*., 1997; Weinstein *et al*., 1997). The finding that Mn^{2+} was able to support the cleavage of the thiol-substituted RNA substrate far better than could Mg^{2+}, i.e. that a change in metal ion specificity was observed suggested an interaction between the metal and the substrate in the active sites of these enzymes.

Inference that the aspartates (D) and glutamates (E) of the DDE triad function in catalysis by binding a divalent metal ion(s) comes from metal ion substitution experiments with mutant Mu, Tn7 and Tn10 transposase proteins; when the D or E residues are mutated to cysteine, the thiophilic divalent metal ion Mn^{++} rescues the strand transfer (and cleavage) defects (Junop and Haniford, 1997; Sarnovsky *et al*., 1996). To test whether a similar shift in metal-ion specificity also occurs with P element transposase, cysteine-substitution mutations of the D528/E531/D545 motif were used in our *in vitro* strand transfer assay, supplemented with different metal ions. C-terminally His-tagged proteins, expressed in the Baculovirus system, of mutants D528Ctnp, E53Ctnp, and D545Ctnp were purified via Ni^{++}-NTA-Superflow and DNA-Streptavidin affinity chromatography, and the 0.3 M KCl fractions used for *in vitro* strand transfer. The D528Ctnp, E53Ctnp, and D545Ctnp-fractions had very low *in vitro* strand transfer activity ensuing this mode of purification however (data not shown), therefore the protein used for the assays presented in this work were solely purified using Ni-NTA-Superflow chromatography, without the succeeding DNA-Streptavidin affinity chromatography step. To ascertain that indeed transposase and/or the respective mutants were responsible for strand transfer activity, and activity was not due to other contaminating proteins still left in the relatively crude fractions, uninfected Sf9 cell extracts were also purified via Ni^{++}-NTA-Superflow, and used as a negative control. Figure 6A, lanes 1-5 shows a western blot of the

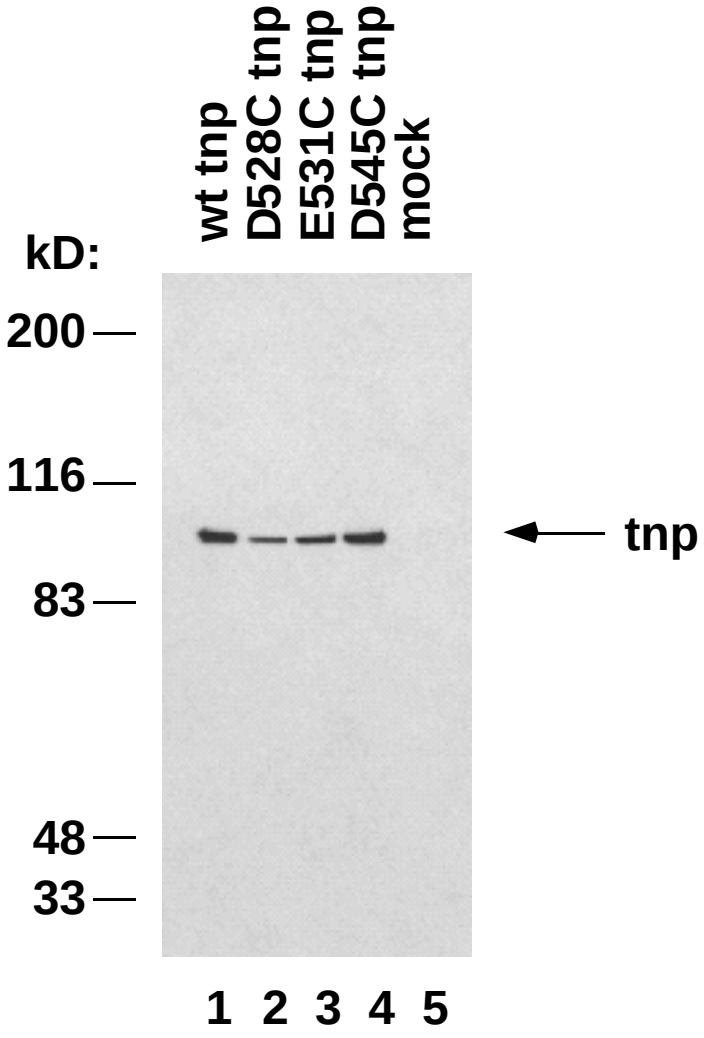


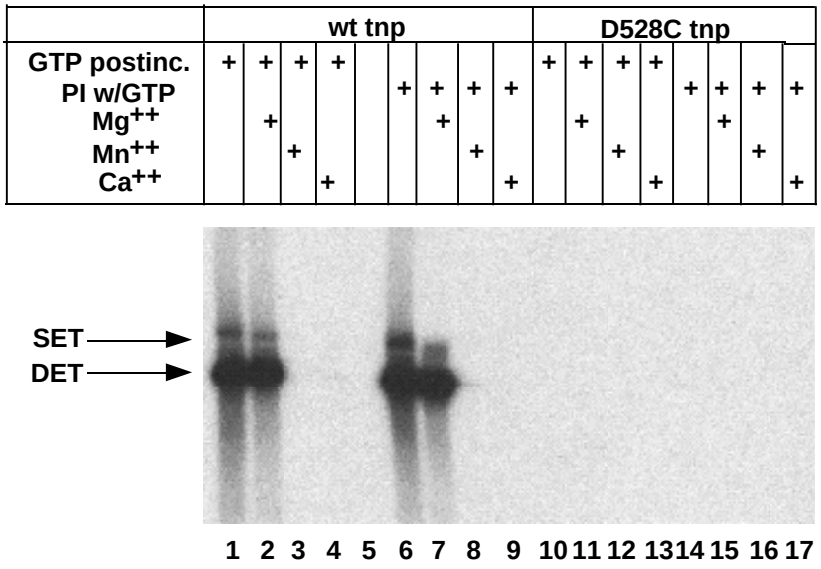
Figure 6A. Transposase-containing protein fractions used to study *in vitro* strand transfer activity. Western Blot with anti-Kp antibody of Baculovirus extract after Ni^{++}-NTA Superflow chromatography of wt tnp (lane 1), D528Ctnp (lane 2), E531Ctnp (lane3), D545Ctnp (lane 4) and of uninfected Sf9 cell extract (lane 5) used for *in vitro* strand transfer. Positions of the molecular weight markers in kD are indicated on the left of the panel. Transposase protein is indicated by an arrow. Approx. 300 ng of protein were loaded in each lane.

proteins after Ni^{++}-NTA-Superflow chromatography used for the *in vitro* strand transfer assay, demonstrating comparable expression levels.

Conditions for *in vitro* strand transfer of the cysteine substitution mutants were kept exactly as for the alanine substitution mutants, and uninfected Sf9 cell extracts were used as a mock control extract.

Similar to the observations in the experiments using the alanine substitution mutations, levels of wild-type strand transfer activity (Fig. 6B, lanes 1-9) were higher than those of D528Ctnp (Fig. 6B, lanes 10-18 after overnight exposure, and Fig. 6C, lanes 1-8 after 3 day exposure) and E531Ctnp (Fig. 6C, lanes 9-15). In fact, E531Ctnp showed no activity at all under any of the conditions tested. However, levels of strand transfer activity in reactions containing D545Ctnp were almost as high as wild-type levels under standard conditions (Fig. 6C, 17), and in reactions preincubated with $Mg(OAc)_2$ and GTP (Fig. 6B, lane 21). Additional $MgCl_2$ in reactions containing D545Ctnp seemed to slightly reduce strand transfer activity with and/or without $Mg(OAc)_2$ and GTP pre-incubation (Fig. 6C, lanes 18 and 22). The same effect of $MgCl_2$ was also observed for the wild-type protein (Fig. 6B, lanes 2 and 7). $MnCl_2$ reduced strand transfer activity for both D545Ctnp (Fig. 6C, lanes 19 and 23) and the wild-type protein (Fig. 6B, lanes 3 and 8). (Compare also to Fig. 5A, lanes 3 and 8). Activity for D528Ctnp was seen most prominently without the addition of extra metal ions, but with or without $Mg(OAc)_2$ and GTP pre-incubation (Fig. 6C, lanes 1 and 5). $MgCl_2$ strongly reduced strand transfer activity for the D528C transposase mutant, and both $MnCl_2$ and $CaCl_2$ abolished its activity completely (Fig. 6C, lanes 3-4 and 7-8). No activity was seen under any conditions for the uninfected Sf9 cell extract, making it very likely that indeed the transposase protein was responsible for the strand transfer activity observed in this assay and not a residual contaminant within the Sf9 cell extracts.

6B.


wt tnp
D528C tnp
GTP postinc.
PI w/GTP
Mg++
Mn++
Ca++
SET
DET
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17

6C.

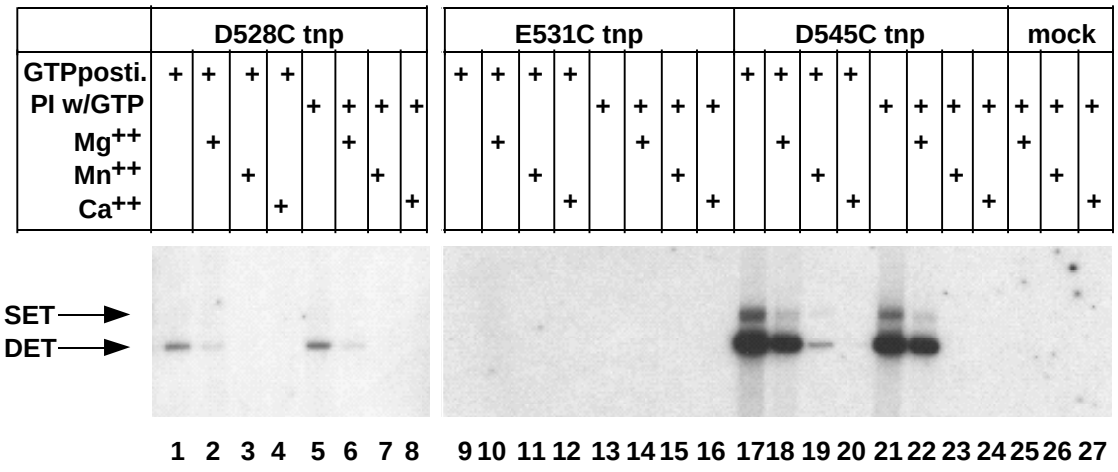

D528C tnp
E531C tnp
D545C tnp
mock
GTPposti.
PI w/GTP
Mg++
Mn++
Ca++
SET
DET
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27

Figure 6. Autoradiogram of strand transfer assays performed with wildtype and D528C, E531C, and D545C transposase extracts.
C-terminally His-tagged Baculovirus-expressed proteins D528C, E531C, D545C transposase and uninfected Sf9 cell extract were tested in the strand transfer assay under the same conditions as described in Figure 5 (A and B). **B.** Autoradiogram of wild-type and D528C reactions after 4 h exposure to X-ray film. Lane 5 is the –GTP control reaction. **C.** Autoradiogram of D528C, E531C, D545C and mock infected Sf9 cell extract reactions after 3 days exposure to X-ray film. Single-end transfer (SET) and double-end transfer (DET) products are indicated by arrows. For each reaction, 0.5 pmol of radiolabeled substrate DNA and approx. 100 ng of respective wt or mutant transposase protein extract were used for each reaction.

4.6. *In vitro* DNA cleavage activity is abolished by mutations in P element transposase DED-motif

Since it was intriguing that D545Atnp, although inactive *in vivo*, could still perform strand transfer *in vitro*, identification of the step at which the mutant protein inhibited the P element transposition reaction was attempted. D545Atnp, tDEDtnp, and wt tnp purified from *Drosophila* Schneider L2 cells (Fig. 7B). The detection level for wildtype transposase was consistently 3-5 fold higher than that of the transposase mutant proteins in the western blots (Fig. 7B). This could be due to subpotimal expression of the mutant proteins in Schneider L2 cells, or a less stringent interaction of the transposase mutant proteins with the antibodies used for western blotting (anti-KP, anti-RD6, anti-HR2) due to folding defects. HO.1 fractions of wt, D545A, and tDED were tested in an *in vitro* cleavage assay (Fig. 7A). In the DNA cleavage assay, a plasmid containing a 628 bp P element is used as the cleavage substrate by the transposase protein in a reaction containing GTP and Mg^{++} (Fig. 7A). Cleavage products, including the excised P element, were then either run on agarose gels and detected by southern blotting (Fig. 7C), or used in an LMPCR reaction with primers specific for the excised P element end (Fig. 8B). Wild-type transposase yielded a 628 bp fragment in the

7A.

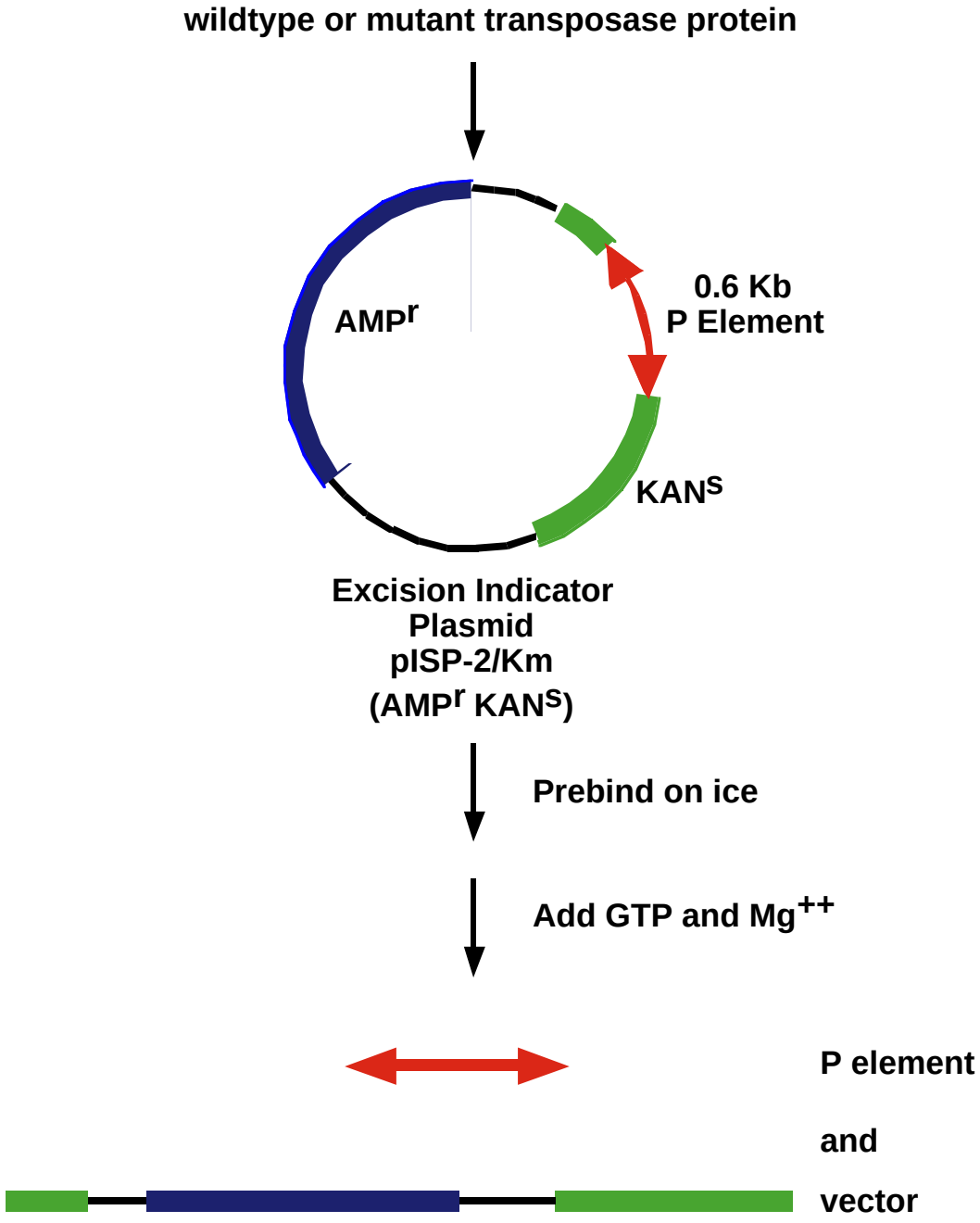
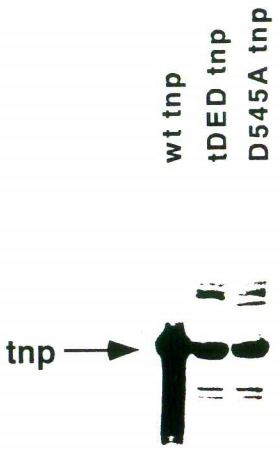

wildtype or mutant transposase protein
AMP^r
0.6 Kb
P Element
KAN^S
Excision Indicator
Plasmid
pISP-2/Km
(AMP^r KAN^S)
Prebind on ice
Add GTP and Mg^{++}
P element
and
vector
Southern Blotting or LMPCR

Figure 7A. Schematic diagram of *in vitro* cleavage assay. Wildtype or mutant transposase-fractions were incubated for 15 min on ice with pISP-2/Km indicator plasmid, which contains a non-autonomous 628 bp P element. The cleavage reaction was started by the addition of 2mM GTP and 10mM $MgCl_2$ in HGED reaction buffer, and allowed to proceed for 1 h at 27 ^{0}C. After the reaction was stopped, the DNA transposition reaction products were extracted and isolated as described in methods section 3.4.3. The DNA was electrophoresed on a 1%TBE agarose gel and transferred to a nitrocellulose membrane. The excised P element DNA was detected by probing the membrane with a radioactive probe corresponding to a portion of the transposon DNA.

cleavage assay with and without the addition of DNA bending HMG1 and 2 proteins (Fig. 7C, lanes 1-5). Neither tDEDtnp nor D545Atnp yielded any cleavage products (Fig. 7C, lanes 6-9 and 10-13), not even in the presence of HMG1 and 2, which have been shown in other systems to facilitate the formation of higher-order nucleoprotein complexes (Grosschedl *et al*., 1994).

To confirm these observations, the products of the cleavage reaction were used in an LMPCR assay, which has previously been shown to be a more sensitive method for the detection of excision products than the Southern Blot technique. In the LMPCR reactions, two primer pairs specific for the 3' P element ends termed A (specific for the free DNA plasmid-ends generated by P element excision), and B (specific for the excised P element ends) were used (Fig. 8A). Wild-type transposase yielded LMPCR products for both primer pairs (Fig. 8B, lane 1 and 6) in the presence of GTP. It also showed some residual activity in the –GTP control reaction for primer pair B (lane 7). This finding could be explained by the fact that some GTP remains in the transposase fractions after purification, which can then give a low amount of cleavage activity. Also, P element ends yielding LMPCR products detected with primer pairs B might be more stable because they are bound by transposase and thus protected from nuclease degradation (lane 2), which is not so in the case of the free DNA backbone ends, i.e. with primer pair A. Products in lane 7 appear to run slightly slower than those in lane 6. This is a technical imperfection within the native

7B.


wt tnp
tDED tnp
D545A tnp
tnp
1 2 3

7C.

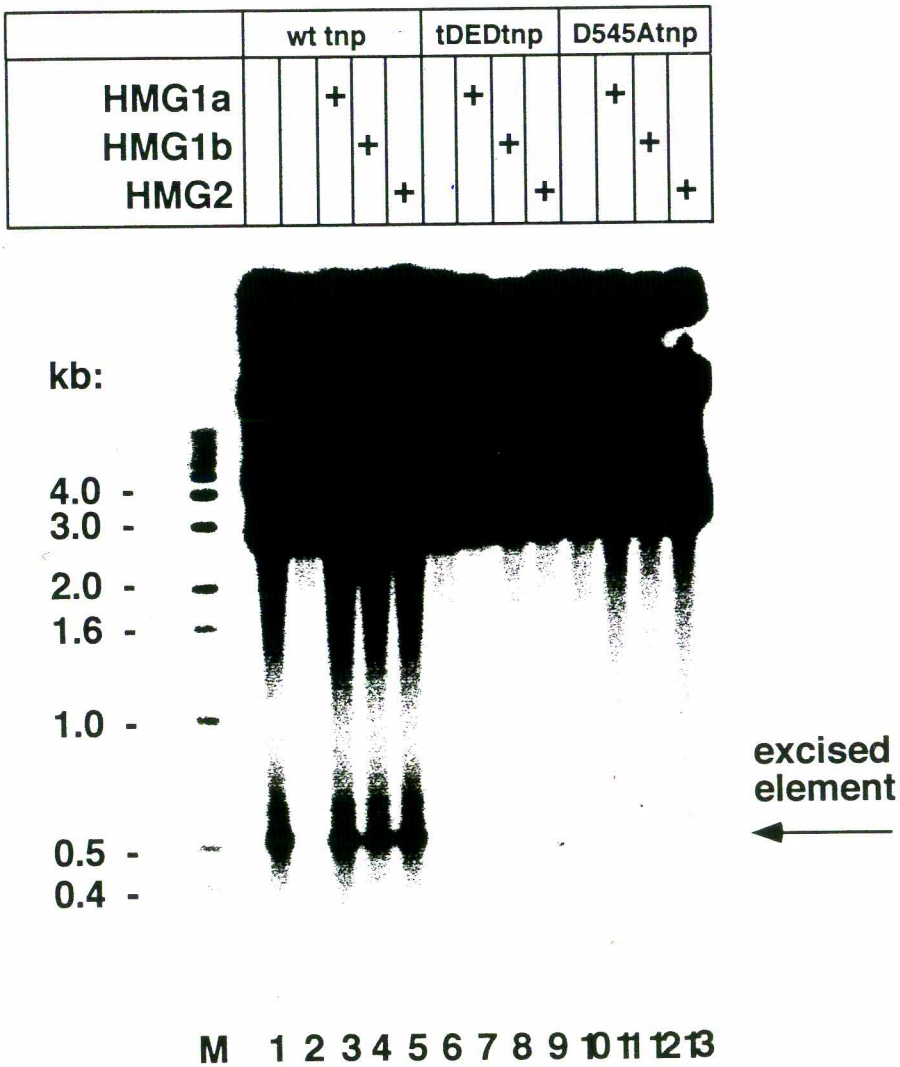

wt tnp
tDEDtnp
D545Atnp
HMG1a
HMG1b
HMG2
kb:
4.0 -
3.0 -
2.0 -
1.6 -
1.0 -
0.5 -
0.4 -
excised element
M 1 2 3 4 5 6 7 8 9 10 11 12 13

Figure 7B. Western Blot of Schneider L2 cell extract of wildtype tnp, tDED tnp, and D545Atnp used for the *in vitro* cleavage assay. Western Blot of transposase protein purified from *Drosophila* cell culture nuclear extract using heparin agarose chromatography. Approximately 10 μl of the 0.1 M KCL fraction (HO.1) is loaded in each lane. Lane 1, wt tnp; lane 2, tDED (D528A/ E531A/D545A) tnp; lane 3, D545A tnp. Transposase protein is indicated by an arrow. **C. Autoradiogram of *in vitro* cleavage assay.** Heparin-agarose 0.1 M KCl fractions of wildtype, tDED, and D545A transposase were tested in the cleavage assay as described above. Highly purified HMG1(a and b) and HMG2 were added to the reactions as indicated by the panel above the figure. DNA from the cleavage assay was electrophoresed, blotted onto nitrocellulose membrane and probed with a α-^{32}P random-hexamer labeled *EcoRI/EagI* pISP2/Km restriction fragment. Bands were visualized by autoradiography. The molecular weight markers are indicated in kilobases on the left side of the panel. Lane1-5, wildtype tnp; lanes 6-9, tDED (528A/E531A/D545A) tnp; lanes 10-13, D545A tnp. Lane 2 is the –GTP control reaction. The 628 bp excised P element is indicated by an arrow.

acrylamide gel and has no significance. Cleavage activity and subsequent ability to yield LMPCR products was completely abolished for the triple alanine mutant (tDEDtnp) (lanes 3 and 8), and for D545Atnp (lanes 4 and 9). This implies that in the case of the single and triple mutant no overhangs by element excision were created to which the site-specific PCR primers could have ligated, in other words, no DNA cleavage has taken place.

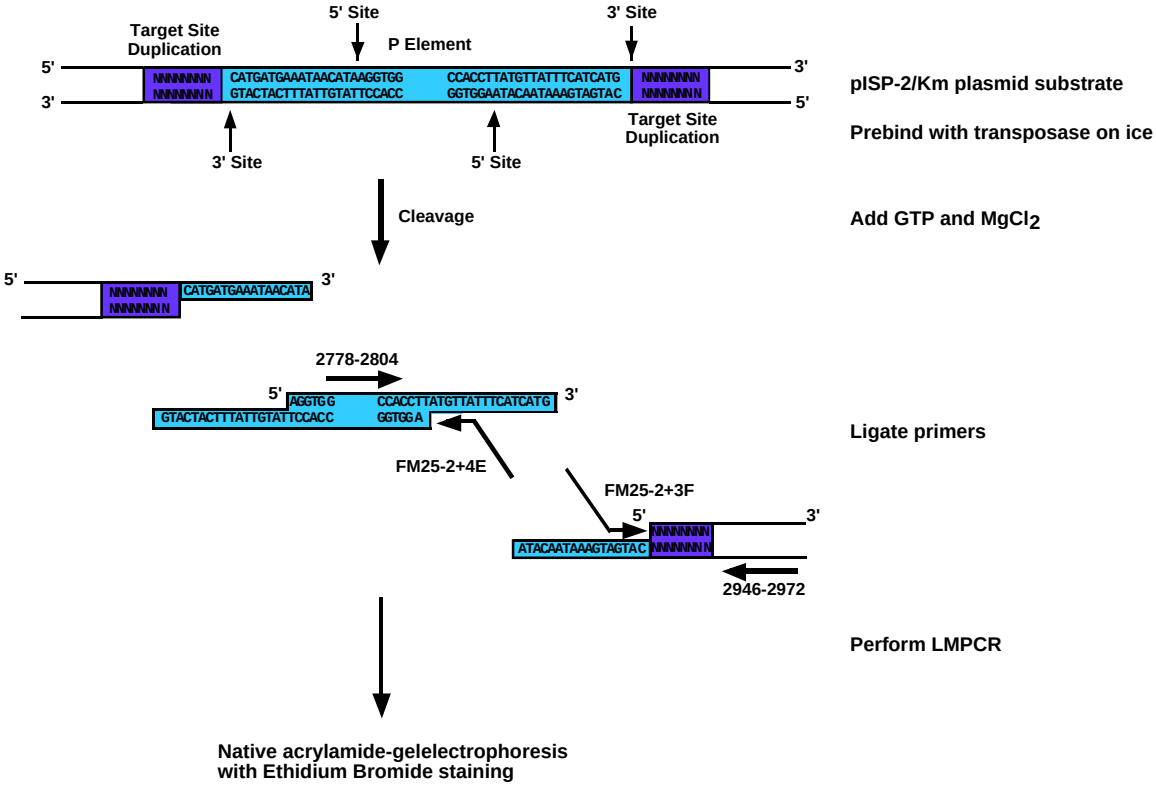


Figure 8A. Schematic diagram of LMPCR assay. For the LMPCR (ligation-mediated polymerase chain reaction) assay, large scale cleavage reactions were performed (fivefold increase) as described in Fig. 7A., and 1/6 of the total cleaved substrate ligated to either FM25-2+4E or FM25-2+3F oligonucleotides, which anneal specifically to either four basepairs of the 3' P-element overhang or to three basepairs of the 5' substrate overhang generated by DNA cleavage, respectively (Beall and Rio, 1997). Ligation was allowed to proceed overnight at 16^0 C, and 1/6 of the reactions then used for PCR with the following primer pairs: FM25-2+3F and 2946-2972 (A) to generate a 99 bp product, or (B) FM25-2+4E and 2778-2804 to generate a 135 bp product. LMPCR products were run on an 8% native acrylamide gel and visualized by ethidium bromide staining.

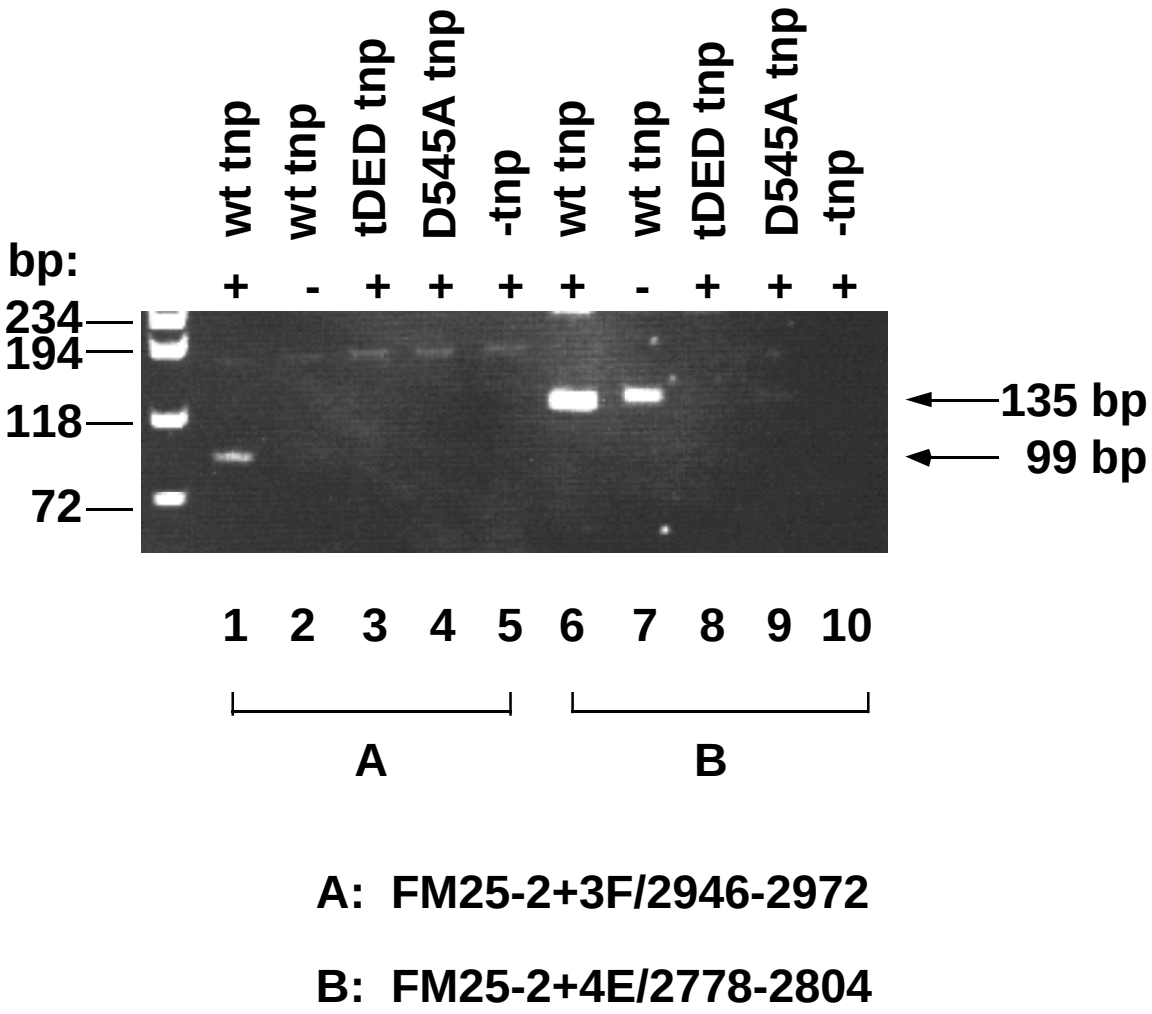


Figure 8B. 8% native acrylamide gel of LMPCR products stained with ethidium bromide. Only wild-type transposase yields LMPCR products with both primer pairs A and B as described in Fig. 8A, indicative of functional DNA cleavage. Lanes 1, 2, 6, and 7 are the LMPCR reactions performed with wt tnp, lanes 2 and 7 are (–GTP) control reactions, as indicated by the minus sign above the lanes. Lanes 5 and 10 are (– transposase) control reactions of the LMPCR reaction mix. Lanes 3 and 8 are reactions performed with the tDED mutant protein, lanes 4 and 9 those of the D545A single mutant. Both single and double mutant protein show no DNA-cleavage-dependent LMPCR activity.

5. Discussion

P elements move via a non-replicative cut-and-paste mechanism that is catalyzed by an 87 kD P element-encoded transposase protein (Berg and Howe, 1989). Transposase binds specifically to sequences internal to both ends of the transposon (Kaufman *et al*., 1989), and is believed to excise the donor P element as a double-strand DNA intermediate, and to create an 8-bp target site duplication upon insertion into its target DNA (Engels *et al*., 1990; Kaufman and Rio, 1992; O'Hare and Rubin, 1983). The P element is unique in that GTP is required as a cofactor, in addition to magnesium, for activity (Kaufman and Rio, 1992; Mul and Rio, 1997).

The aim of this work was to identify the residues involved in the catalytic activity of the P element transposase protein. This so-called DDE motif is thought to coordinate divalent metal ion binding for catalysis of DNA transposition. P element transposase shows no similarities with the DDE signature of other transposable elements.

5.1. Identification and characterization of catalytic amino acid residues in the *Drosophila* P element transposase protein

Since no region in the *Drosophila* P element transposase primary amino acid sequence shows any similarity to other recombinase proteins' catalytic motifs (Capy *et al*., 1996), identification of putative members of the P element DD(35)E motif was difficult. In addition, the protein's C-terminus contains an especially high density of aspartate and glutamate residues, so no "obvious" candidates for the motif could be assigned. Alignments were proposed on the basis of hydrophobic residues found around the DD(35)E motif in retroviral integrases, and these residues among with others identified by partial sequence homology with a DDE motif alignment by Baker and Luo, 1994, employed for

point mutational analysis.

A series of seven transposase double mutants and five transposase single mutants was generated, changing either an aspartate (D) or a glutamate (E) residue, or both into alanine (A), and these mutants were tested for *in vivo* excision activity (Fig. 1A). All alanine substitution mutants shown in, Fig. 1A boxed in blue, red and green were tested for *in vivo* excision activity as described in Fig.1B. (All figures referred to in the discussion section are pertaining to section III. unless otherwise noted). The *in vivo* excision assay was performed four independent times with each mutant or mutant pair.

There were three double mutants among the mutants tested that severely reduced *in vivo* activity (Fig. 2B). When singled out, residues D528, E531, and D545, when mutated to alanine, almost completely abolished *in vivo* excision activity (activity less than 1% as compared to wild-type activity set at 100%). In addition, the double mutant D444A/E478A also severely reduced activity to 5.8± 2.0 % of wild-type activity (data not shown). These two residues were not considered for the P element catalytic motif however, since all catalytic aspartates and glutamates previously identified (Baker and Luo, 1994; Bolland and Kleckner, 1996; Engelman and Craigie, 1992) resulted in complete or almost complete abrogation of *in vivo* activity (less than 1% of wild-type activity).

5.2. *In vitro* strand transfer activity is greatly reduced by mutations D528A, E531A, and D545A in the potential catalytic motif of P element transposase

In order to test whether the loss of *in vivo* activity by the substitution mutations D528Atnp, E531Atnp, and D545Atnp could be assigned to a specific step in the *in vitro* activity assays, *in vitro* strand transfer and DNA cleavage assays were performed with wild-type and mutant transposase proteins. Equal amounts of Baculovirus-expressed proteins were used for *in vitro* strand transfer, and protein purified from stable L2 Schneider cell lines for *in vitro* cleavage.

Unexpectedly, wild-type transposase showed slightly less *in vitro* strand transfer activity when standard reactions were supplemented with extra $MgCl_2$ (Fig. 4B, compare lanes 1 and 4). A possible explanation for this finding could be that excess salt from the $MgCl_2$ confines normal (unsupplemented) levels of strand transfer activity. Transposase mutants D528A and E531A abolished *in vitro* strand transfer activity under standard conditions, i.e. when no additional metals were added to the reactions (Fig. 5B, lanes 1 and 9). Surprisingly, D545Atnp still showed *in vitro* strand transfer activity under standard conditions, albeit at reduced levels when compared to wild-type (Fig. 5B, lane 17, compare to Fig. 5A, lane 1). When Mg^{++}, Mn^{++}, and/or Ca^{++} (20 mM final concentration) were added to the reaction mixtures containing wild-type or mutant transposase protein, the most prominent effect was seen in the case of Mn^{++} addition: when $MnCl_2$ was present in the strand transfer reaction, wild-type activity was completely abolished (Fig, 5A, lane 3). However, D528Atnp and E531Atnp activity were partially restored (Fig. 5B, lanes 3,7,11, and 15) under these conditions. Similar observations have been made by Baker and Luo, 1994, where elevated levels of Mn^{++} (5-40 mM) were capable of restoring activity to asparagine substitution mutants of the MuA catalytic DDE motif. A plausible explanation for the strand transfer defect of the alanine substitution mutants of P element transposase could be that the loss of a negative charge at each position compromises the interaction of the protein with the divalent cation essential for catalysis, and could alter the shape of the metal binding-pocket or cleft. The ability of Mn^{++} to activate mutant protein activity suggests that the absence of activity seen under standard conditions (i.e. 5 mM Mg^{++}) may be due to poor association between P element transposase and this divalent metal ion, and that this situation can be partially reversed by substituting for a different metal with a better “fit” into the catalytic pocket, i.e. a different, in this case bigger hydrated ionic radius (hydrated ionic radius of Mn^{++} is 0.80; that of Mg^{++} is 0.66) (Hecht, 1996).

Manganese-ion addition seemed to slightly reduce D545A strand transfer

activity when compared to activity of the standard reaction conditions without metal addition (Fig. 5B, compare lanes 19 and 17, 23 and 21). The “behavior” of the D545A mutants with or without metal addition more closely resembled that of the wild-type than that of the other two mutant proteins discussed above.

A very prominent effect was also seen with the E531A mutant when reactions were supplemented with $CaCl_2$ (Fig. 5B, lane 12), which increased overall levels of strand transfer, especially those of single-ended strand transfer. Analogous to the reasoning adapted for Mn^{++} activation of the mutant transposase proteins above, the change from an acidic to a neutral amino acid (E→A) at position E531 might induce a change in the protein’s conformation, so as to enable $CaCl_2$ to bind more efficiently. Contrarily, $CaCl_2$ is unable to bind to the protein in its wild-type configuration (Fig. 5A, lane 4). Again, the difference in metal-binding could be caused by access limitations posed by the transposase protein’s catalytic pocket on the metal-ion size: Ca^{++} has a bigger hydrated metal ion radius (0.99) than Mg^{++} (0.66) (Hecht, 1996). Conceivably, $CaCl_2$ can only access the catalytic pocket of the mutant E531A, but not that of the wild-type transposase protein. Likewise, Mg^{++} can no longer activate strand transfer of E531A (Fig.5B, lane 10), because of its suboptimal fit into the mutant protein’s metal binding cleft. Moreover, the observation that $CaCl_2$ activates strand transfer of the E531A mutant to a greater extent without GTP preincubation (Fig. 5B, lane 12) than with GTP preincubation (Fig. 5B, lane 16), further argues for a conformational change within the mutant transposase protein.

These results strongly suggest that the amino acids D528, E531, and D545 in fact are, or are part of, the catalytic metal binding domain of the P element transposase protein, since the alanine substitution mutants of this motif show a profound effect on metal-binding and subsequent ability to activate *in vitro* strand transfer.

5.3. Mutations D528Ctnp, E531Ctnp, and D545Ctnp alter metal ion specificity in *in vitro* strand transfer

In an attempt to provide further evidence that an essential metal acts in close proximity to the tDED motif, the differential chemistry of metal-sulfur and metal-oxygen interaction was used. Baculovirus-expressed transposase substitution mutants D528C, E531C, and D545C were tested in *in vitro* strand transfer assays for possible metal-induced restoration of activity. It has been demonstrated previously in studies on metal ion catalysis during splicing of pre-messenger RNA that substitution of an oxygen leaving group by sulfur provides a means to test metal ion-leaving group interactions because various metals differ in their ability to coordinate sulfur (Piccirilli *et al*., 1993; Sontheimer *et al*., 1997; Weinstein *et al*., 1997). Mn^{++} readily accepts sulfur as a ligand, whereas Mg^{++} does not. Thus, a switch in metal specificity from Mg^{++} to Mn^{++} following sulfur substitution was seen as evidence of a direct metal ion-leaving group interaction.

Transposase substitution mutants D528C, E531C, and D545C showed no restoration of strand transfer activity when standard reaction mixtures were supplemented with Mn^{++} (Fig. 6B). In fact, addition of any extra metal inhibited catalytic activity of D528Ctnp (Fig. 6B, lanes 2-4 and 6-8), and no activity at all was seen for E531Ctnp under any conditions. Cysteine substitutions at these two amino acid residues could have caused a conformational change in the protein's catalytic metal binding pocket as discussed above for the alanine substitution mutants, resulting in a configurational size restriction of the pocket. Conceivably, the smaller size of the metal binding pocket could then enable some Mg^{++} binding with D528Ctnp (Fig. 6B, lanes 1 and 5), but no metal-ion access at all with E531Ctnp (Fig. 6b, lanes 9-16). A further explanation for the substantial difference between the ability to rescue strand transfer of the E531A mutant (Fig.5B, lanes 11-16) and the inability to do so of the E531C mutant under any conditions (Fig. 6B, lanes 9-16) could be that this residue, in addition to its function in catalytic metal-ion binding, could play a substantial role in the

overall formation and stabilization of the protein's tertiary structure. In the wild-type protein, the negatively charged E531 residue could thus constitute a pivotal amino acid for appropriate folding of the transposase protein into its active conformation. It could also be important for the stabilization of the adjacent leucine-zipper motif (amino acids 497-525), which is potentially involved in mediating multimerization of the transposase protein. Thus, if E531 is mutated to cysteine, it is no longer able to stabilize the protein's active configuration or, even more erroneously, possibly contacts random amino acids within the protein and stabilizes an inactive configuration and/or multimer.

D545Ctnp showed highest levels of activity under standard conditions, without extra metal (Fig. 6B lanes 17 and 21). Addition of Mg^{++} slightly reduced strand transfer activity, however, addition of Mn^{++} completely abolished strand transfer when reactions were preincubated with $Mg(OAc)_2$ and GTP, and severely reduced activity when $Mg(OAc)_2$ and GTP were added after preincubation (Fig. 6B, lanes 23 and 19). Mg^{++} in form of $Mg(OAc)_2$ had to be added to all reaction mixtures to ensure accurate GTP activity, which relies on the presence of Mg^{++}.

A possible explanation for the inability of Mn^{++} to rescue strand transfer activity as anticipated could be, that the thio-substituted catalytic site still binds Mg^{++}, but the bound metal ion is no longer effective in catalysis. Similar observations have been made by Weinstein *et al.,*1997, in experiments where 3'-(thioinosylyl) -(3'→5')-uridine substrates were used in a reaction that emulates exon ligation: Under conditions were $MgCl_2$ was used as a competitor in reactions with the thio-substituted catalytic site, the Mn^{++}-enhanced transesterification reaction was inhibited by Mg^{++}.

To sample a range of thiophilic metals (and Lewis acids), strand transfer reactions with the transposase cysteine substitution mutants were also conducted preincubating with Co^{++}, Cd^{++}, and Zn^{++} in combination with 5 mM $Mg(OAc)_2$. *In vitro* strand transfer reactions were performed in buffer containing 20 mM HEPES-KOH at pH 7.6 with 20 mM and 10 mM final metal concentration.

No restoration of strand transfer activity was seen with Co^{++}, Cd^{++}, and Zn^{++} under these conditions (data not shown).

In concordance with experiments by Weinstein *et al.,*1997, who had tested different pH conditions in order to optimize their assays, *in vitro* strand transfer with the transposase cysteine substitution mutants was carried out at different pH ranges: reaction buffer containing 20 mM MOPS at pH 6.5, and buffer containing 20 mM Tris at pH 8.5, respectively, were used, and reaction mixtures of both buffer conditions supplemented with 10 mM final concentration of Mg^{++}, Mn^{++}, Ca^{++}, Co^{++}, Cd^{++}, and Zn^{++} in the preincubation mixture. No rescue of strand transfer was observed under these conditions. Strand transfer reactions were also carried out at different DTT concentrations (5 – 20mM final concentration) to rule out the possibility that proteins were not in a reduced state, which is very likely to be necessary for their respective activities. Different DTT concentrations had no effect on overall activity levels, however (data not shown).

Since the strand transfer reaction is performed at ≤ 35 mM KCl, and all supplementary metals had been used in form of chloride salts, i.e. $MgCl_2$, $MnCl_2$, $CaCl_2$, $CoCl_2$, $CdCl_2$, and $ZnCl_2$, inhibition of strand transfer activity by excess salt was considered. *In vitro* strand transfer reactions were therefore carried out supplemented with 20 mM $Mg(OAc)_2$, $Mn(OAc)_2$, and $Ca(OAc)_2$, however, no rescue of activity was observed other than that previously seen with D545Ctnp and Mg^{++} (data not show).

Thus, activity of substitution mutants D528C, E531C, and D545C could not be rescued by addition of thiophilic metal ions under any of the conditions tested. However, successful rescue by Mn^{++} of a cysteine substitution mutation in recombinase catalytic motifs has only been reported once in the literature by Sarnovsky *et al.,* 1996: Cysteine-substitution at the D114 position of TnsA of the Tn7 transposon showed Mn^{++}-specific rescue of 5' cleavage activity. A weak case is also made by Junop and Haniford, 1997, who mention Mn^{++}-specific rescue of cysteine substitution mutations of the putative DD(35)E motif of Tn10, however, these observations are quoted as unpublished. Therefore Mn^{++}-rescue

of point-mutated cysteine residues in a putative DDE motif can not be considered the unconditional test for the identification of a recombinase catalytic triad.

A possible explanation for the inability of the cysteine substitution mutants to rescue strand transfer activity could be that there might not be an essential interaction between a metal ion and the catalytic domain in the strand transfer step of transposition: metal binding might only be crucial for the target capture and cleavage steps of the transposition reaction, but not substantial for the ensuing strand transfer step. To elucidate this hypothesis, cysteine substitution mutants could be tested for restoration of *in vivo* excision, or *in vitro* cleavage activity in reactions supplemented with Mn^{++} or other thiophilic metals.

A further method to identify and/or confirm metal binding sites within the transposase protein would be the utilization of Fenton-chemistry. This technique makes use of the ability of Fe^{++} to functionally or structurally replace Mg^{++} at ion-binding sites and to generate short-lived and highly reactive hydroxyl radicals that can cleave nucleic acid and protein backbones in spatial proximity of these ion-binding sites (Berens *et al*., 1998; Tullius *et al*., 1987). Such cleavage was demonstrated previously for malic enzyme from pigeon liver (Chou *et al*., 1995; Wei *et al*., 1995; Wei *et al*., 1994), glutamine synthetase from *E. coli* (Farber and Levine, 1986), and the Tet repressor (Ettner *et al*., 1995). This method was also successfully employed to map the catalytic residues in the active center of the RNA polymerase β'- subunit (Zaychikov *et al*., 1996). The β'- subunit was found to be cleaved at the conserved motif (NADFDGD), and substitution of the three aspartate residues in this motif created a catalytically inactive mutant that failed to support Fe^{++}-induced cleavage of DNA or proteins, although it could still bind promoters and form open complexes (Zaychikov *et al*., 1996).

5.4. Transposase mutants tDED (D528A/E531A/D545A) and D545A abolish *in vitro* DNA cleavage activity

Since it was intriguing that D545Atnp, although inactive *in vivo*, could still

perform strand transfer *in vitro*, identification of the step at which the mutant protein inhibited the P element transposition reaction was attempted. A likely hypothesis was that D545A blocked the DNA cleavage step, but given an artificial oligonucleotide mimicking an excised P element end, could still perform strand transfer with reduced levels *in vitro*. 0.1 M KCl heparin-agarose fractions of wild-type and mutant (D545Atnp, tDEDtnp) transposase protein purified from *Drosophila* cells L2 was tested in *in vitro* cleavage. As expected, only the wild-type protein showed cleavage activity (Fig. 7C), and not even the addition of DNA-bending HMG1 and 2 protein to the reactions containing mutant (D545Atnp, tDEDtnp) transposase protein enabled cleavage activity to the single or the triple mutant. HMG 1 and 2 (high mobility group proteins 1 and 2) have been shown to enhance cleavage activity in other recombination systems by their ability to bend and to modulate DNA (Sawchuk *et al*., 1997; Van Gent *et al*., 1997).

To further confirm these results, LMPCR reactions were performed. Cleavage activity and subsequent ability to yield LMPCR products was abolished for the triple alanine mutant (tDEDtnp) (Fig. 8B, lanes 3 and 8) and for D545Atnp (Fig. 8B, lanes 4 and 9). Thus, it seems probable that the alanine substitution mutation D545A abolishes or severely reduces cleavage activity, which leads to a complete loss of activity *in vivo*. Given an artificial P element end in an *in vitro* reaction, however, it can still perform some, albeit reduced, strand transfer.

5.5. Conclusion

Unlike most of the transposase and integrase proteins in which the catalytic triad conforms to the DD(35)E consensus, the P element catalytic motif as determined thus far appears to be DE(15)D. Hence, the spacing between D528 and E531 is very narrow, which is not observed in any other recombinases analyzed so far. However, in the caboxylate-chelated two-metal-ion catalytic mechnism originally proposed for the 3’ to 5’ exonuclease of Klenow fragment (Beese and Steitz, 1991; Beese and Steitz, 1993) and later extended to the

ribozymes (Steitz and Steitz, 1993), and RNAse H (Davies *et al*., 1991; Yang *et al*., 1990a), the metal-ion coordinating motif (D355, E357, D424, D501) of the 3' to 5' exonuclease activity of *E.coli* DNA polymerase (Beese and Steitz,1991) also contains an aspartate and a glutamate in very close proximity to each other (D355 and 357). Similar mechanisms of metal-ion coordination would be feasible for the hydrolysis as well as the polynucleotidyl transfer on both RNAs and DNAs.

A further discrepancy between the putative P element catalytic DE(15)D and the "normal" DD(35)E motif is the amino acid spacing between the middle and last catalytic residue of the triad. However, the perfect DD(35)E spacing originally identified in retroviral integrases is, in fact, not always entirely conserved in other recombinational systems: where TnsA (D114, E149) and TnsB (D273, D361, E396) transposase proteins of the Tn7 system *do* comply to the DD(35)E spacing (Sarnovsky *et al*., 1996), transposases of Tn10 (D97, D161, E292) (Kennedy and Haniford, 1996), and Mu (D269, D336, E396) (Baker and Luo, 1994; Krementsova *et al*., 1998) *do not*. As discussed previously, P element transposase also fails to show any significant homologies in putative alignments with other recombinase-family members. Therefore a divergence from the common, catalytic motif spacing would almost be anticipated for this protein.

It was also surprising that the three catalytic residues for the transposase protein are all present in exon 3, which is present in the catalytically inactive 66 kD transpositional repressor protein (see Introduction, Fig. 1C). The C-terminus (exon 4) must contribute to the catalytic function of transposase by a yet unidentified mechanism. Exon 4 could be necessary for complex assembly of transposase, conceivably providing bridging interactions between the different monomers required to assemble the active multimeric form of the protein. Moreover, the protein's C-terminus could be needed for appropriate protein folding into its active configuration, or could be involved in positioning of the active protein multimer on the DNA substrate, providing essential binding sites

for accurate DNA-protein interactions. Similar observations have been made with bacteriophage Mu transposase, where the core domain alone is catalytically inactive. This was shown to be due at least in part to the fact that MuA is only active when it is assembled into a higher order protein-DNA complex (Lavoie and Chaconas, 1996; Mizuuchi, 1992b). The C-terminal domain of MuA appears to contain an additional component of the active site: a short peptide corresponding to amino acids 575-600 has been shown to possess nonspecific endonuclease activity in the presence of Mg^{++} (Wu and Chaconas, 1995). This part of the protein was shown to be critical for the chemical steps of the normal reaction, and it apparently functions in concert with the acidic residues in the core domain of another MuA monomer in the active higher order protein-DNA complex (Aldaz *et al*., 1996; Yang *et al*., 1996).

5.6. Outlook

After completion of the *in vivo* and *in vitro* experiments with the D528/E531/D545 residues described in this thesis, additional *in vivo* experiments were performed using the D444A and the E478A mutants previously disregarded as being putative members of the catalytic motif of P element transposase. Very surprisingly, it was demonstrated that the D444A mutant almost completely abolished *in vivo* excision activity [3.1±2.1], whereas excision levels of the E478A mutant were equal to, or higher than those of the wild-type protein. This led to the hypothesis that D444 could also be a part the metal-coordinating, catalytic motif of the P element transposase. Together with the previously identified D528/E531/D545 triad, this acidic residue would constitute an uncommon, four-member type catalytic transposase motif **D**(84)**D**(3)**E**(15)**D**. Four member catalytic metal binding motifs have been identified in *E.coli* DNA polymerase (Beese *et al*., 1993), and in the *E.coli* RuvC resolvase (Ariyoshi *et al*., 1994) of the polynucleotidyl superfamily, but not any transposase protein investigated to

date.

Whether D444 is a member of the metal-coordinating motif or not, the catalytic residues of the P element transposase determined in this thesis seem to diverge from the usual spacing and/or number of catalytic residues constituting the metal-binding motifs of other known transposases.

Final evidence for the composition and organization of the P element catalytic core will require determination of the protein's 3-D structure. This poses a complex task, however, since sufficient amounts of the 87 kD protein are difficult to purify, especially because P element transposase is believed to exist in diverse phoshorylation forms, each of which would have to be separated from one another prior to any crystallization attempts.

Once crystallization of P element transposase is successful, it will be very interesting to determine the proteins precise metal-coordination mechanism, as well as its evolutionary relationship to other recombinases.

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7. Summary

Drosophila 2.9 kb P element contains four exons that encode several products: the full length 87 kD transposase, the 66 kD repressor protein, and a heterogeneous class of internally deleted elements, some of which appear to encode truncated proteins with distinct biological activities, such as the KP element. P elements move via a non-replicative cut-and-paste mechanism that is catalyzed by the 87 kD P element-encoded transposase, which is expressed solely in the germ line.

The goal of this thesis was to identify the amino acid residues that make up the catalytic motif of the *Drosophila* transposase protein. This work describes the isolation of P element transposase mutants and the characterization of single-amino acid substitution mutations that render P element transposase specifically defective in donor cleavage and strand transfer.

Three residues (D528, E531, and D545) were identified in an *in vivo* excision assay, all of which almost completely abolished transposase activity. The involvement of these residues in transposase catalytic activity was further confirmed in three independent activity assays: *in vitro* strand-transfer, *in vitro* cleavage, and *in vitro* LMPCR. In the *in vitro* strand transfer assay, mutations of the D528 and E531 to either alanine or cysteine respectively, greatly reduced activity and significantly altered metal-binding specificity. Mutations D545Atnp and D545Ctnp only slightly altered in *in vitro* strand transfer activity, however. To elucidate which step of the P element transposition reaction D545Atnp inhibited, the single mutant protein was tested in *in vitro* cleavage and LMPCR assays, together with the wild-type and the triple mutant protein (tDED). Both the single and the triple mutant completely abolished *in vitro* cleavage and LMPCR activity. It was concluded that the residues D528, E531, and D545 are, or are a part of, the *Drosophila* P element catalytic motif and presumably participate in coordinating the divalent metal ion required for catalysis.

7.1. Zusammenfassung

Das mobile P Element der Fruchtfliege *Drosophila* enthält vier Exons, die zur Kodierung dessen verschiedener Genprodukte notwendig sind: dem vollständigen 87 kD Transposase Protein, dem 66 kD Repressor Protein, sowie einer heterogene Subklasse von Proteinen mit internen Deletionen. P Elemente verändern ihre Position im Genom durch einen “cut-and-paste’” Mechanismus, indem das DNA Element an einer Stelle des Genoms ausgeschnitten (Exzision), und and einer anderen Stelle wieder integriert (Integration) wird. Sowohl Exzion als auch Integration werden durch das nur in Keimzellen exprimierte Transposase Protein katalysiert.

Ziel der vorliegenden Arbeit war die Isolierung und Charakterisierung von Transposase-Punktmutanten, die einen spezifischen Defekt in der Exzisions-und Integrations-Aktivität des Transposase Proteins aufweisen. Mit Hilfe eines *in vivo* Aktivitäts-Tests wurden drei Aminosäurereste (D528, E531 und D545) identifiziert, deren Mutation jeweils eine Inaktivierung des Proteins bewirkten. Die Wichtigkeit dieser Mutanten für die katalytische Aktivität des Transposase Proteins wurde anhand von drei weiteren *in vitro* Aktivitäts-Tests, dem “strand transfer-”, dem “cleavage-” und dem “LMPCR-assay” bestätigt. Dabei konnte gezeigt werden, daß Mutationen der Aminosäurereste D528 und E531 zu Alanin oder Cystein die *in vitro* Strand Transfer-Aktivität (Integrationsaktivität) im Vergleich zum Wildtyp deutlich verringerten, und die Metallbindungsspezifität stark veränderten. Mutationen des Aminosäurerrestes D545 zu Alanin oder Cystein hatten nur einen schwachen Einfluß auf die Aktivität und die Metallbindungsspezifität im “strand transfer” Aktivitäts-Test. Um festzustellen, welchen Schritt im Exzision/ Integrationsmechanismus des P Elements die D545A Mutante beeinflusste, wurde das gereinigte Protein dieser Einfachmutante neben den Proteinen des Wildtyps und der Dreifach-Mutante tDED (D528A/E531A/D535A) in zwei *in vitro* DNA-Exzisions Aktivitäts-Test getestet. Sowohl die Einfach- als auch die Dreifach-Mutante zeigten eine völlige

Inaktivierung der *in vitro* DNA Exzisions-Aktivität.

Aus diesen Ergebnissen wurde gefolgert, daß die Aminosäuerereste D528, E531 und D545 entweder das vollständige katalytische Motiv des P Element Transposase Proteins, oder zumindest Teile davon ausmachen, und folglich an der Koordinierung der zur Katalyse notwendigen, divalenten Metallionen beteiligt sind.

8. Addendum

Abbreviations

A	Ampère
APS	Ammoniumpersulfate
ATP	Adenosine-Triphosphate
BSA	Bovine Serum Albumin
bp	Basepairs
cpm	Counts per minute
dH_20	Double distilled H_20
ds DNA	Double-stranded DNA
ss DNA	Single-stranded DNA
dNTP	Desoxynucleotidetriphosphate
DTT	Dithiothreitol
h	Hours
HIV	Human Immunodeficiency Virus
HEPES	N-(2-Hydroxyethyl)-Perazine-N'-2-ethane-sulfonic acid
IRBP	Inverted repeat binding protein
kbp	Kilobasepairs
kDa	Kilodaltons
lac operon	Lactose operon
LTR	Long terminal repeat
mcs	Multiple cloning site
min	Minutes
µl	Microliter
MLV	Murine Leukemia Virus
MOPS	Morpholinopropanesulfonic acid
PMSF	Phenylmethylsulfonylfluoride
M_r	Relative Mass
PBS	Phosphate-buffered Saline
RNA	Ribonucleic Acid
RSS	Recombination signal sequence
RSV	Rous Sarcoma Virus
rpm	Rotations per Minute
SDS	Sodium Dodecyl Sulfate
sec	Seconds
TBS	Tris-buffered Saline
TdT	Terminal desoxytransferase
TEMED	N-N-N'-N'-Tetramethylethylene-diamine
tnp	Transposase

tDED	Triple transposase mutant (D528A/E531A/D545)
U	Unit (of enzyme activity)
V	Volt
vol	Volume
w	Weight
wt	Wild-type
xg	Relative Centrifugal Force
3-D	Three dimensional

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Erklärung

Ich versichere, daß ich meine Dissertation

Identification of amino acid residues essential for the catalytic activity of *Drosophila* P element transposase

selbständig und ohne unerlaubte Hilfe angefertigt und mich dabei keiner anderen als der von mir ausdrücklich bezeichneten Quellen und Hilfen bedient habe.

Die Dissertation wurde in der jetzigen oder einer ähnlichen Form noch bei keiner anderen Hochschule eingereicht und hat noch keinen sonstigen Prüfungszwecken gedient.

Berkeley, den 5.11.1999

Katharina Ahrens