doi:10.1016/S0022-2836(02)00877-X available online at http://www.idealibrary.com on IDEAL® J. Mol. Biol. (2002) 322, 971–982

JMB

Evidence for "Unseen" Transposase–DNA Contacts

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In this study, evidence of novel, important interactions between a hyperactive Tn*5* transposon recognition end sequence and hyperactive Tn*5* transposase (Tnp) are presented. A hyperactive Tn*5* end sequence, the mosaic end (ME), was isolated previously. The ME and a wild-type end sequence, the outside end (OE), differ at only three positions, yet transposition on the ME is tenfold higher than on the OE *in vivo*. Also, transposition on the ME is much more efficient than transposition on the OE *in vitro*. Here, we show that the decreased activity observed for the OE is caused by a defect in paired ends complex (PEC) formation resulting from the orientation of the A–T base-pair at position 4 of this end. Efficient PEC formation requires an interaction between the C5-methyl group (C5-Me) on the non-transferred strand thymine base at position 4 (T4) and Tnp. PEC formation on nicked substrates is much less affected by the orientation of the A–T base-pair at position 4, indicating that the C5-Me group is important only for steps preceding nicking. A recently determined co-crystal structure of Tn*5* Tnp with the ME is discussed and a model explaining possible roles for the base-pair at position 4 is explored.

Keywords: Tn*5*; transposition; protein–DNA interactions and crystallography

Introduction

Tn*5* is a prokaryotic, composite transposon consisting of two insertion sequences, IS*50R* and IS*50L*, flanking a region of DNA containing three antibiotic-resistance genes. Tn*5* is delineated by 19 bp inverted repeat transposase (Tnp) recognition sequences termed outside ends (OEs); each IS element is flanked by an OE and an inside end (IE). IS*50R* encodes the 476 amino acid residue Tnp protein, which is essential for transposition.[1]

Tn*5* transposes by a cut and paste mechanism.[2] First, monomers of Tnp are postulated to bind each OE sequence.[3] Homodimerization of these bound Tnp monomers *via* their C termini forms a synaptic or paired ends complex (PEC).[4–7] All chemical steps of Tn*5* transposition occur within this complex.[8] Following PEC formation, nicking of the transferred strand DNA occurs *via* nucleophilic attack of a water molecule (activated by a Tnp-coordinated Mg^{2+}) on the phosphodiester backbone between the +1 position of the OE and the −1 position of the flanking DNA (donor backbone or dbb) resulting in the generation of a 3′-OH group. This 3′-OH group then attacks the non-transferred DNA strand, creating a hairpin intermediate[9] and releasing the dbb from the blunt-ended transposon.[10] A second activated water molecule then resolves the hairpin intermediate. The resulting post-cleavage PEC then captures a target DNA molecule and strand transfer occurs *via* a transesterification reaction in which the 3′-OH groups of the transposon attack phosphodiester bonds of the target in a staggered fashion. Formation of a covalent bond between the 3′-OH groups of the transposon ends and the 5′-phosphate groups of the target integrates the transposon.[11] Due to the staggered strand transfer attack, integration results in a 9 bp duplication of target DNA flanking the transposon ends.[12]

The role of each base of the 19 bp recognition end sequence (ES) in the mechanism of transposition

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Abbreviations used: dbb, donor backbone; ES, end sequence; Tn, transposon; Tnp, transposase; PEC, paired ends complex; ME, mosaic end; IE, inside end; OE, outside end; IS, insertion sequence; ND, non-detectable.

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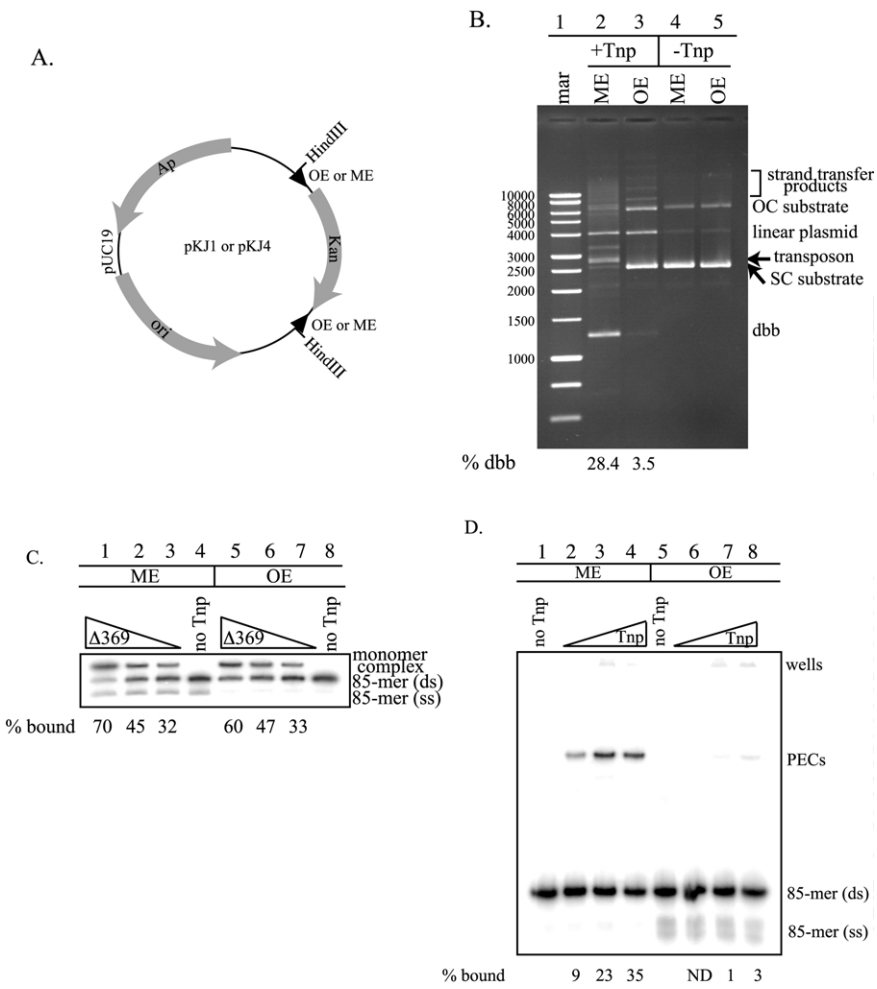

Figure 1. An increase in PEC formation results in eightfold higher *in vitro* transposition activity on the ME. (a) The plasmid substrates, pKJ1 and pKJ4, used for *in vitro* transposition assays are shown. (b) pKJ1 (containing a transposon with OEs) and pKJ4 (containing a transposon with MEs) plasmid substrates were incubated with Tnp for 30 minutes in a buffer containing Mg^{2+}. After removal of Tnp from the reaction products, they were separated on an agarose gel. Incubation of Tnp with pKJ4 and pKJ1 in buffer containing Mg^{2+} first causes single end cleavage of the substrate DNA giving a 4000 bp band. Subsequent cleavage at the second ES causes release of the transposon (2700 bp) from the dbb (1300 bp). The free transposon can then integrate, or strand transfer, into unreacted supercoiled substrate (intermolecular transposition). Intermolecular transposition products are detected as high molecular mass bands. These transposition products and unreacted substrate are labeled. Products were identified by comparison to DNA markers of known size. The percentage of dbb released from substrate is indicated for each non-control reaction. This assay shows that the OE is eightfold less active *in vitro* than the ME following a 30 minute reaction. (c) Non-nicked ME and OE substrate DNAs were incubated with Tnp EKΔ369 in a buffer without Mg^{2+} and then electrophoresed on a native polyacrylamide gel to visualize monomer complexes. Monomer complexes consist of one Tnp EKΔ369 molecule bound to one substrate DNA and are shifted relative to unbound non-nicked substrate. A small amount of single-stranded, non-nicked substrate is seen. The percentage of substrate bound by Tnp is indicated. This assay shows that a defect in monomer binding is not responsible for the tenfold less *in vitro* activity seen with the OE. (d) The non-nicked OE and ME substrate DNAs were incubated with Tnp in a buffer without Mg^{2+} and then electrophoresed on a native polyacrylamide gel to visualize PECs. The PECs are shifted relative to the non-nicked substrate DNAs. A small amount of single-stranded, non-nicked susbstrate is seen. The percentage of substrate complexed by Tnp is indicated. This experiment indicates that PEC formation on the OE is impaired, and that this defect decreases *in vitro* transposition activity on the OE.

has been investigated by genetic analyses.[12–14] These analyses showed that the majority of OE and IE single-point mutations result in a decreased *in vivo* transposition frequency and that no single-point mutation increased the *in vivo* transposition frequency significantly. To study the relationship between the OE and the IE, degenerate oligonucleotides containing either an A–T or a G–C base-pair (in both orientations) at the seven non-identical positions between the OE and the IE were cloned into plasmids and assayed for *in vivo* transposition activity.[13] Surprisingly, reversion of the bases at positions 10, 11, 12, and 15 of the IE to their corresponding bases in the OE resulted in a hyperactive ES. This combination of mutations acts synergistically to increase transposition frequency relative to the IE *in vivo*.[13]

This hyperactive ES, which is a combination of the OE and the IE, is termed the mosaic end (ME). The ME differs from the OE at only three positions; 4, 17, and 18; yet transposition on a supercoiled plasmid containing MEs is much more efficient than transposition on a supercoiled plasmid containing OEs (this work). Also, activity on the ME is tenfold higher than on the OE *in vivo* (this work). The basis for this difference in activity between the OE and the ME has never been investigated specifically. Previously published mutational analyses have failed to consistently define a role for the base-pairs at positions 4, 17 and 18 of the ES. One set of experiments compared *in vivo* transposition frequencies of mutant OEs (paired with either an OE or an IE) to the transposition frequency of two wild-type OEs in both *dam*$^+$ and

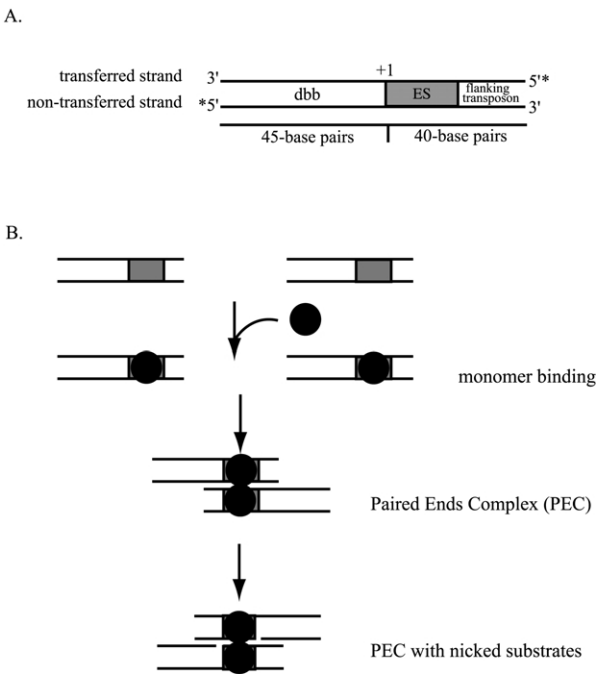

Figure 2. First steps of transposition on small oligonucleotides. (a) The substrate for *in vitro* reactions is shown. The 85 bp substrate has 45 bp of dbb and 40 bp of transposon DNA including the 19 bp recognition end sequence (ES), shown in gray. The asterisk (*) indicates the locations of the ^{32}P label on each substrate. Transferred and non-transferred strands are defined. The location of the nick in the nicked substrates is defined by +1. (b) The first three steps of Tn*5* transposition are explained with small oligonucleotide substrates. Tn*5* transposase (Tnp) first binds the recognition ES (gray box) as a monomer, creating a monomer complex containing one Tnp molecule bound to one substrate DNA molecule. Dimerization of the bound Tnp molecules then forms a PEC containing two Tnp molecules and two substrate DNAs. Following formation of a PEC, transferred strand nicking can occur in the presence of Mg^{2+} between +1 of the ES and −1 of the dbb resulting in a PEC having two Tnp molecules and two nicked DNA substrates.

dam$^-$ *Escherichia coli* strains. All mutations at position 4 in both strains reduced *in vivo* transposition frequency. Mutations at positions 17 and 18 increased transposition in the *dam*$^-$ strain, but decreased transposition in the *dam*$^+$ strain.[12] Similar experiments performed in the Reznikoff laboratory showed no decrease in *in vivo* transposition activity when the OE non-transferred strand A at position 4 was changed to either a G or a C in a *dam*$^+$ strain, while mutations at every other position studied reduced *in vivo* transposition activity. Unfortunately the bases at positions 17 and 18 were not mutated.[14] These inconsistencies have made it difficult to understand the exact role of the bases at positions 4, 17 and 18 in Tn*5* transposition.

No clue as to the role of these bases is found by examining the Tn*5* Tnp-OE post-cleavage PEC co-crystal structure.[15] Because no base-specific contacts between Tnp and the base-pairs at positions 4, 17, or 18 are observed in this structure, we assumed that the ME base-pair differences would cause either conformational changes in the end sequence DNA or specific contacts between Tnp and base-pairs 4, 17, and 18. But, when the Tnp-ME post-cleavage PEC co-crystal structure was determined (this work), no statistically significant conformational difference is observed nor is a base-specific contact seen between Tnp and base-pairs 4, 17 and 18.

Many questions arise from these inconsistencies. For instance, which step of transposition confers the hyperactive phenotype to the ME? Does the affected step of transposition occur before the post-cleavage step represented by the co-crystal structures? Which of the three differing bases (or combination of bases) is/are responsible for affecting this step of transposition? What does answering these questions tell us about the overall Tn*5* transposition mechanism?

Here, we present evidence that PEC formation with the OE is impaired relative to the ME, while the ability of Tnp to bind the OE as a monomer is unaffected. Also, the identity of the base-pair at position 4 is critical for efficient PEC formation, while mutation of the base-pairs at positions 17 and 18 in the ME to their corresponding OE bases has no effect on PEC formation. By examining alternate bases at position 4, we conclude that efficient PEC formation on the ME requires the C5-methyl group (C5-Me) on the non-transferred strand thymine base at position 4 (T4). Also, PEC formation is inhibited by either a repulsive interaction between O4 of the transferred strand T at position 4 of the OE and the backbone O of Gly245 or the orientation of N6 and O4 of the A–T base-pair of the OE. Tnp binding of a nicked substrate is much less affected by the identity of the non-transferred strand base at position 4, indicating that the C5-Me group is critical only for steps preceding nicking. Finally, a recently determined post-cleavage PEC co-crystal structure of Tn*5* Tnp with the ME is presented. A model explaining the potential roles of the base-pair at position 4 is explored and the overall significance of the interactions of this base-pair with Tnp is addressed.

Results

Determination of *in vitro* transposition activity with the ME and OE

An *in vitro* transposition assay was used to analyze cleavage of OE or ME delineated transposons from flanking donor DNA by Tnp. Briefly, plasmid substrates pKJ1 (having a transposon with OEs) and pKJ4 (having a transposon with MEs) were incubated with a hyperactive Tnp in a buffer containing Mg^{2+}. Following removal of

Table 1. Mutant Tn5 transposase (Tnp) recognition end sequences analyzed in this study

End sequence[a]	Substrate name[b]
CTG**T**CTCTTGATCAGA**TC**T[c]	IE
CTG**A**CTCTTATACAC**AG**T[c]	OE
CTG**T**CTCTTATACACA**TC**T	ME
CTG**A**CTCTTATACACA**TC**T	ME-4A
CTG**T**CTCTTATACACA**AG**T	ME-17A/18G
CTG**C**CTCTTATACACA**TC**T	ME-4C
CTG**G**CTCTTATACACA**TC**T	ME-4G
CTG**U**CTCTTATACACA**TC**T	ME-4U

Recognition end sequences analyzed in this study are listed.
[a] Changes made to positions 4, 17, and 18 of the end sequence are shown in bold. All sequences are written 5′ to 3′ and are of the non-transferred strand of the end sequence.
[b] The substrate names refer to the position and base change of the mutation.
[c] The seven differing positions between the IE and the OE are underlined.

the Tnp from the reaction products, they were visualized on an agarose gel. Transposition activity was determined by quantification of percentage donor backbone release (see Materials and Methods).

Incubation of Tnp with pKJ4 in buffer containing Mg^{2+} first causes single end cleavage of the substrate DNA giving a 4000 bp band (see Figure 1(a)). Subsequent cleavage at the second ME causes release of the transposon (2700 bp) from the dbb (1300 bp). The free transposon can then integrate, or strand transfer, into unreacted supercoiled substrate (intermolecular transposition). Intermolecular transposition products are detected as high molecular mass bands.

Following a 30-minute incubation of pKJ4 with Tnp, the released dbb reaction product constitutes 28.4% of the total reaction products (see Figure 1(b), lane 2). Also, the free transposon and strand transfer reaction products are plainly visible when compared to the no Tnp control (see Figure 1(b), lane 4). Following a 30-minute incubation of pKJ1 with Tnp, the released dbb reaction product constitutes only 3.5% of the total reaction products (see Figure 1(b), lane 3). This represents an eightfold increase in transposition activity on the ME when compared to the OE. Although transposition activity on the OE is low, both linear reaction products and strand transfer products are detected when compared to the no Tnp control (see Figure 1(b), lane 5).

Tnp monomer binding to the ME and OE

The ME was found to be eightfold more active than the OE *in vitro* (see above). To begin investigating the cause of this discrepancy, the ability of monomeric Tnp to bind the OE was assayed. This experiment determines whether the OE can complete the first step of transposition (see Figure 2(b)). We are able to independently test monomer Tnp binding using a C-terminal deletion mutant of Tnp, Tnp EKΔ369, which is not able to form PECs because it lacks the C-terminal dimerization domain.[4,16] Incubation of Tnp EKΔ369 with small, radiolabeled substrates containing Tnp recognition ESs results in monomer complexes containing one molecule of Tnp bound to one DNA substrate molecule. When separated on a native polyacrylamide gel, these complexes are shifted relative to the substrate DNA.

Incubation of three concentrations of Tnp EKΔ369 with non-nicked substrates containing the ME (see Figure 1(c), lanes 1–3) results in 32%, 45%, and 70% of the substrate being shifted into a monomer complex. Incubation of these same concentrations of Tnp EKΔ369 with non-nicked substrates containing the OE (see Figure 1(c), lanes 5–7) results in 33%, 47%, and 60% of the substrate being shifted into a monomer complex. Because similar amounts of each substrate are shifted, we conclude that more efficient monomer binding cannot be the cause of the eightfold increase in *in vitro* activity with the ME.

PEC formation with OE and ME

Tnp binds the OE and the ME equally well as a monomer. To determine if the ME is more efficient than the OE in the next step of transposition, PEC formation was assayed. Briefly, if full-length Tnp is incubated with a small radiolabeled substrate containing a favorable recognition ES, a PEC results. A PEC consists of dimerized Tnp molecules each bound to both substrate molecules. When these complexes are separated on a native polyacrylamide gel, they are shifted relative to free substrate.

Incubation of three concentrations of Tnp with the non-nicked substrate containing the ME (see Figures 1(d), lanes 1–4 and 2(a)) results in 9%, 23%, and 35% of the substrate being shifted into PECs. Incubation of these same concentrations of Tnp with the non-nicked substrate containing the OE (see Figure 1(d), lanes 5–8) results in non-detectable (ND), 1%, and 3% of the substrate being shifted into PECs. This represents a tenfold increase in PEC formation on the ME, and indicates that the ME forms PECs more efficiently than the OE. This increase in PEC formation on the ME is consistent with the eightfold increase in *in vitro* transposition activity observed for the ME (see above).

Comparison of PEC formation on ME-4A, ME-17/18, OE, and ME

From the previous experiments, we can conclude that the ME forms PECs more efficiently than the OE. To determine which base-pairs (position 4, 17, or 18) are responsible for this increase, PEC formation on the ME, OE, and two mutant ends (see Table 1) was compared. Mutant end ME-4A is the ME with the base-pair at position 4 changed to the corresponding base-pair in the OE. Mutant

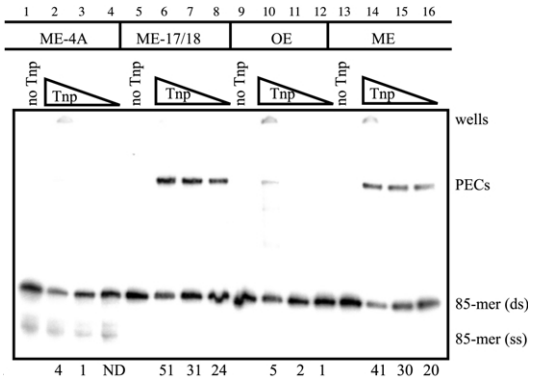

Figure 3. The OE defect in PEC formation is caused by the base-pair at position 4. The non-nicked substrate DNAs ME-4A, ME-17A/18G, OE and ME were incubated with Tnp in a buffer without Mg^{2+} and then electrophoresed on a native polyacrylamide gel to visualize PECs. The PECs are shifted relative to non-nicked substrate DNAs and a small amount of single-stranded non-nicked substrate is seen. The percentage of substrate complexed by Tnp is indicated. This experiment shows that mutation of the base-pair at position 4 has a profound affect on PEC formation, while changing the bases at positions 17 and 18 does not inhibit PEC formation. This shows that the base-pair at position 4 impairs PEC formation on the OE.

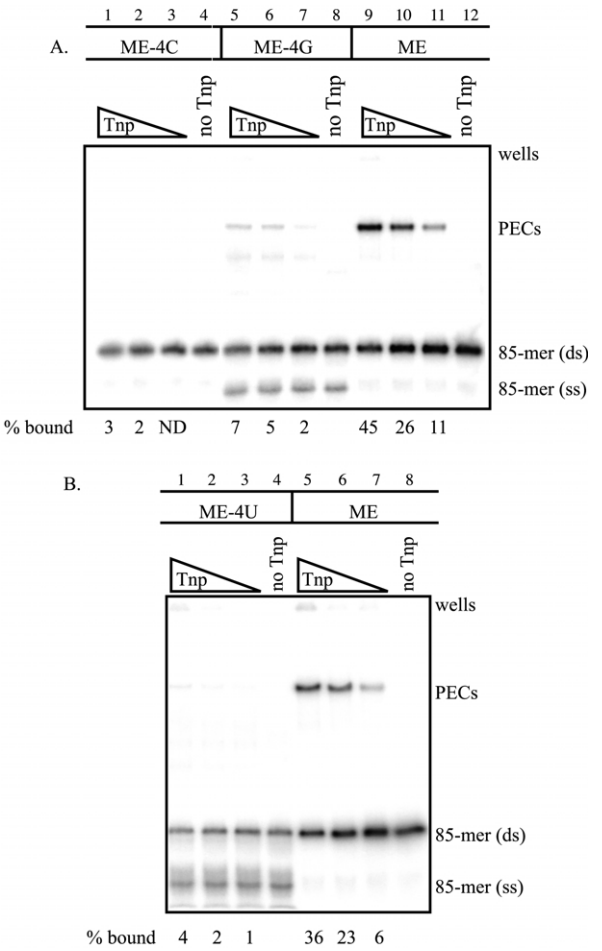

Figure 4. Contacts between Tnp and position 4 are critical for efficient PEC formation. (a) The non-nicked substrate DNAs ME, ME-4C and ME-4G were incubated with Tnp in a buffer without Mg^{2+} and then electrophoresed on a native polyacrylamide gel to visualize the PECs. The PECs are shifted relative to unbound non-nicked substrate DNAs and a small amount of single-stranded, non-nicked substrate is seen. The percentage of substrate complexed by Tnp is indicated. This experiment shows that stronger stacking interactions between bases 3 and 4 cannot be a cause of decreased PEC formation on the OE, and that the orientation of the N6 and O4 of the A–T base-pair of the OE may be important for PEC. (b) Non-nicked substrate DNAs ME-4U and ME were incubated with Tnp in a buffer without Mg^{2+} and then electrophoresed on a native polyacrylamide gel to visualize the PECs. The PECs are shifted relative to the non-nicked substrate DNAs and a small amount of single-stranded, non-nicked substrate is seen. The percentage of substrate complexed by Tnp is indicated. This experiment shows that an interaction between Tnp and the C5-Me of the non-transferred strand T4 is critical for efficient PEC formation.

end ME-17A/18G is the ME with base-pairs at positions 17 and 18 changed to the corresponding base-pairs in the OE.

The PEC formation assay was performed as described above. Incubation of three concentrations of Tnp with the non-nicked ME substrate resulted in 20%, 30%, and 41% (see Figure 3, lanes 13–16) of the substrate being shifted into PECs. Incubation of Tnp with the non-nicked OE substrate resulted in 1%, 2%, and 5% (see Figure 3, lanes 9–12) of the substrate being shifted into PECs. Incubation of three concentrations of Tnp with the non-nicked ME-4A substrate resulted in ND, 1%, and 4% of the substrate being shifted into PECs (see Figure 3, lanes 1–4), while incubation of the same concentrations of Tnp with the non-nicked ME-17A/18G substrate resulted in 24%, 31%, and 51% of the substrate being shifted into PECs (see Figure 3, lanes 5–8). The ME-17A/18G and the ME substrates form tenfold more PECs than both the ME-4A and the OE substrates. From this experiment, we can conclude that the

Table 2. *In vivo* transposition frequencies determined by the mating out assay

Substrate name[a]	*In vivo* transposition frequency[b]
ME	6.3×10^{-4}
OE	5.0×10^{-5}
ME-4A	5.3×10^{-5}
ME-17A/18G	6.5×10^{-4}

The mating out assay was used to quantify *in vivo* transposition frequencies for the following mutant end sequences.

[a] See Table 1, and Materials and Methods.

[b] The *in vivo* transposition frequency is the ratio of exconjugants that have a transposon on their conjugated pOX38-Gen *F*-factor to total exconjugants that may or may not contain a transposon on the conjugated pOX36-Gen *F*-factor.

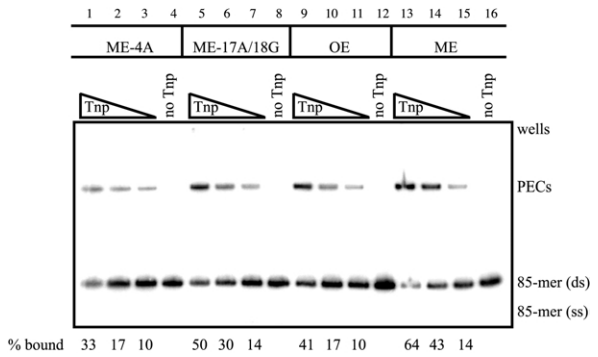

Figure 5. Specific contacts to the base-pair at position 4 are important only for transposition steps preceding transferred strand nicking. The nicked substrate DNAs ME-4A, ME-17A/18G, OE and ME were incubated with Tnp in a buffer without Mg^{2+} and then electrophoresed on a native polyacrylamide gel to visualize the PECs. The PECs are shifted relative to unbound nicked substrate DNAs and a small amount of single-stranded substrate is seen. The percentage of substrate complexed by Tnp is indicated. This experiment shows that specific contacts to the base-pair at position 4 are important only for transposition steps preceding transferred strand nicking.

hyperactivity of the ME is not related to the base-pairs at positions 17 and 18, but is due to the orientation of the A–T base-pair at position 4.

In vivo transposition frequency of ME, OE, ME-4A, and ME-17A/18G

In vivo transposition frequency on recognition ESs ME, OE, ME-4A, and ME-17A/18G was measured using a mating out assay (see Table 2). This assay measures the frequency of transposition onto an *F*-factor and is quantified by comparing the number of exconjugants having a transposon on their *F*-factor to the total number of exconjugants.

In this system, the ME has a transposition frequency of 6.3×10^{-4}. The OE has a transposition frequency of 5.0×10^{-5}. When position 4 of the ME is changed to the corresponding base-pair of the OE (ME-4A), the transposition frequency is 5.3×10^{-5}. When positions 17 and 18 of the ME are changed to the corresponding base-pairs of the OE (ME-17A/18G), the transposition frequency is 6.5×10^{-4}. Both the OE and the ME-4A have transposition frequencies that are about tenfold lower than either ME or ME-17A/18G. These results are consistent with *in vitro* results and, collectively, these data support a role for the position 4 base-pair in the transposition mechanism.

PEC formation on substrates containing alternate base-pairs at position 4

As shown, reversing the orientation of the base-pair at position 4 of the ME (ME-4A) to correspond with the OE is detrimental to PEC formation. What happens if the base-pair at position 4 is changed to G–C? The substrate ME-4G refers to a non-nicked substrate having a G–C base-pair with the G on the non-transferred strand. ME-4C refers to a non-nicked substrate having a G–C base-pair with the C on the non-transferred strand (see Table 1).

To address this, the PEC formation assay was performed as described above. Incubation of three concentrations of Tnp with the non-nicked ME substrate results in 11%, 26%, and 45% of the substrate shifted into PECs (see Figure 4(a), lanes 9–12). Incubation of three concentrations of Tnp with the non-nicked ME-4C substrate results in ND, 2%, and 3% of the substrate shifted into PECs (see Figure 4(a), lanes 1–4), while incubation of Tnp with the non-nicked ME-4G results in 2%, 5%, and 7% of the substrate shifted into PECs (see Figure 4(a), lanes 5–8). From the results of this experiment, we conclude that, although the ME-4G substrate forms some PECs, a G–C base-pair cannot substitute adequately for the A–T (with T on the non-transferred strand) base-pair of the ME.

Comparison of PEC formation on ME-4U and ME

To determine if the C5-Me group on T4 of the non-transferred strand of the ME is important for PEC formation, a deoxyuridine base (U) was placed at this position. This oligonucleotide was annealed to the ME transferred strand oligonucleotide to give a U–A base-pair resulting in the non-nicked substrate ME-4U (see Table 1). The PEC formation assay was performed as described above. Incubation of three concentrations of Tnp with non-nicked ME substrate results in 6%, 23%, and 36% of the substrate being shifted into PECs (see Figure 4(b), lanes 5–8), while incubation of Tnp with the non-nicked ME-4U substrate results in 1%, 2%, and 4% of the substrate being shifted into PECs (see Figure 4(b), lanes 1–4). These data show that the C5-Me group of the non-transferred strand T4 of the ME is essential for efficient PEC formation.

Determination of PEC formation on nicked substrates

The step of transposition following PEC formation is nicking of transferred strand DNA between +1 of the recognition ES and −1 of the flanking donor DNA. We next wanted to determine if contacts to the base-pairs at positions 4, 17 and 18 were important after completion of the nicking step. To do this, nicked substrates that were identical with the substrates used for other PEC formation assays (see above) except that they had a nick in the transferred strand between +1 of the recognition ES and −1 of the flanking donor DNA, were designed. Annealing two separate transferred strand oligonucleotides to one

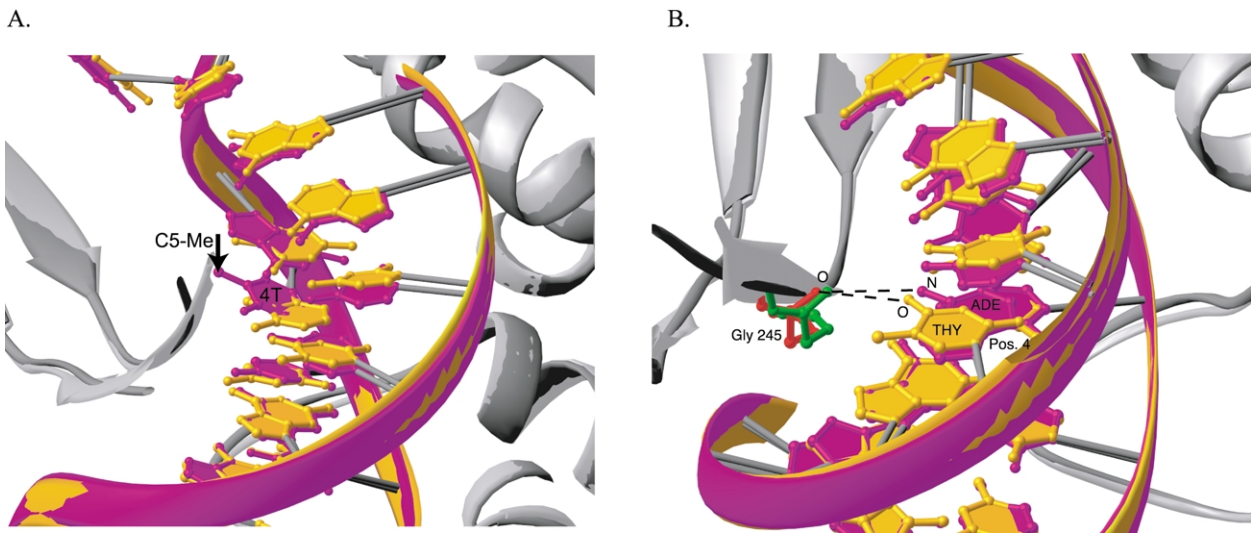

Figure 6. Overlay of Tnp-OE and Tnp-ME post-cleavage PEC co-crystal structures. Because few differences are seen between the Tnp-OE and Tnp-ME post-cleavage PEC co-crystal structures, only an overlay focused on position 4 of the ES, is shown here. OE DNA is shown in purple, while the ME DNA is shown in yellow. (a) This Figure emphasizes the 5-Me of the non-transferred strand T4 of the ME protruding into the major groove. Also, the proximity of 5-Me to the anti-parallel β-sheet between Ser240 and Lys260 is seen. (b) The overlay is rotated approximately 180° relative to that in (a). This Figure shows the potential repulsive interaction between O4 of the OE transferred strand T4 and the backbone O of Gly245 (shown in green). A potential attractive interaction occurs between N6 of the ME transferred strand A4 and the backbone O of Gly245 (shown in red).

non-transferred strand oligonucleotide created this nick (see Materials and Methods).

The PEC assay was performed as above. Incubation of three concentrations of Tnp with the nicked ME substrate resulted in 64%, 43%, and 14% of the substrate being shifted into PECs (see Figure 5, lanes 13–16), while incubation of the nicked OE substrate resulted in 41%, 17%, and 10% of the substrate being shifted into PECs (see Figure 5, lanes 9–12). The nicked OE substrate binds about 1.5-fold less well than the nicked ME substrate. Incubation of the same concentrations of Tnp with nicked ME-17A/18G substrate resulted in 50%, 30%, and 14% of the substrate being shifted into PECs (see Figure 5, lanes 5–8), while incubation of the nicked ME-4A substrate resulted in 33%, 17%, and 10% being shifted into PECs (see Figure 5, lanes 1–4). The nicked ME-17A/18/G substrate binds comparably to the nicked ME substrate, while the nicked ME-4A substrate binds twofold less well. Thus, nicking the DNA substantially relieves the OE position 4 defect in PEC formation.

Determination of the Tnp-ME co-crystal structure

The post-cleavage PEC co-crystal structure of Tnp and the OE was has been determined.[15] To aid in understanding the functional differences between the OE and the ME, the post-cleavage PEC co-crystal structure of the ME and Tnp was solved. No additional specific contact is seen between Tnp and the base at position 4 (see Figure 6). Also, the conformation of the DNA backbones and Tnp in these structures shows very little variation.

One potential *trans* contact between the transferred strand base at position 4 and Tnp is interesting. In the Tnp-OE post-cleavage PEC co-crystal structure, O4 of the transferred strand 4T and the backbone O of Gly245 are 3.65 Å apart, resulting in a potential repulsive interaction. In the Tnp-ME post-cleavage PEC co-crystal structure, because the orientation of the position 4 base-pair is reversed, the backbone O of Gly245 now has the potential to interact with N6 of the transferred strand A, resulting in a potential attractive interaction.

Discussion

The OE is defective in PEC formation

Tn*5* transposition requires binding of the Tnp to transposon recognition ESs. A wild-type Tn*5* recognition ES, the OE, is suboptimal *in vivo*. This inefficiency ensures that overall transposition activity in the cell remains low, thereby allowing survival of the transposon. The OE, is not an efficient end because only 3.5% dbb release results *in vitro* after a 30 minute incubation at 37 °C (see Figure 2(b)). Previously, a more hyperactive ES was isolated by mutagenesis of the seven non-identical base-pairs between the OE and the IE. This hybrid sequence, the ME, is eightfold more efficient, resulting in 28.4% dbb release when incubated with Tnp *in vitro* (see Figure 2(b)). These data are interesting because the sequences of the OE and the ME differ

at only three positions; 4, 17, and 18. Also, it is only the orientation of the same A–T or G–C base-pair that changes at these positions. To begin to understand the basis for the functional differences between these ends, the specific step of transposition affected by the three base-pair changes was determined.

Binding of monomeric Tnp to recognition ESs is the first step of transposition. Comparison of Tnp EKΔ369 binding to the OE and the ME reveals nearly equal EKΔ369 binding to both ends. This implies that the base-pair differences between OE and ME affect the first step of transposition only minimally and that the major difference in *in vitro* transposition activity on the OE and the ME cannot be due to differences in monomer binding alone.

The next step of transposition is synaptic complex or PEC formation. The data showing that ME forms tenfold more PECs than the OE following a 1.5 hour incubation are consistent with the hypothesis that, because the OE cannot form PECs efficiently, *in vitro* transposition activity on this end is decreased greatly. We cannot test directly whether the chemical steps of transposition are affected by the base-pair differences between the OE and the ME, because very little PEC formation is seen on the OE following any length of incubation (data not shown). Therefore, we cannot correlate the rate of cleavage with the formation of PECs. But, while the base-pair differences between the OE and the ME may cause subtle differences in chemical mechanism, the differences in trasposition frequency are similar to the differences in PEC formation.

The OE defect in PEC formation is caused by the base-pair at position 4

When substrates ME-4A, ME-17A/18G were tested for PEC formation (see Table 1), both the ME and ME-17A/18G formed PECs five- to tenfold better than the OE or ME-4A following a 1.5 hour incubation. Also, a transposon flanked by the ME-4A recognition ES had an eightfold lower *in vivo* transposition frequency than a transposon flanked by the ME-17A/18G sequence. These data indicate that the base-pair at position 4 is solely responsible for more efficient PEC formation and for hyperactivity on the ME. It should be noted that the greater percentage bound seen for substrate ME-17A/18G (when compared to ME) was seen consistently, especially at higher concentrations of Tnp (see Figure 4, compare lanes 5 and 13), but this phenomenon was not examined further in this study.

To ensure that substrate ME-4A was not defective in binding monomeric Tnp, this substrate was incubated with Tnp EKΔ369. ME-4A and ME bound equally well to Tnp EKΔ369 (data not shown), indicating that substrate ME-4A is defective in PEC formation but not in binding monomeric Tnp.

Contacts between Tnp and position 4 are critical for efficient PEC formation

Because no base-specific contact between the base-pair at position 4 and Tnp is seen in the Tn5-ES post-cleavage PEC co-crystal structures (see Figure 6) and conclusions from previous studies on this position are inconsistent, many hypotheses for the role of this base-pair were possible. A severe bend of approximately 30° is known to occur near position 1 upon PEC formation.[17] Therefore, stacking interactions between position 4 and positions 3 and 5 might be important for PEC formation. Second, specific contacts between Tnp and the amine and carbonyl functional groups of the position 4 base-pair may be required for efficient PEC formation. This requirement may constrain the orientation of these groups within the base-pair. Third, the crystal structures reveal one interesting potential *trans* contact between the transferred strand base at position 4 and Gly245 of Tnp that may affect PEC formation. In the ME-Tnp post-cleavage PEC co-crystal structure, N6 of the transferred strand A4 may interact with the backbone O of Gly245 (see Figure 6(b)), aiding in PEC formation. In the OE-Tnp co-crystal structure, O4 of the transferred strand T4 may repel the same backbone O of Gly245, inhibiting PEC formation. Unfortunately, the effect of specific contacts to N6 of A and O4 of T of the position 4 base-pair and the effect of repulsion between O4 or O6 of the transferred strand and the backbone O of Gly245 are indistinguishable on the basis of the experiments shown. Finally, there may exist a specific contact between the C5-Me group of the non-transferred strand T4 and Tnp that is required for efficient PEC formation.

To begin testing these hypotheses, PEC formation on substrates ME-4C, and ME-4G (see Table 1) was compared to PEC formation on the ME (see Figure 4(a)). From this experiment, we can make several conclusions. First, because a pyrimidine cannot be substituted for a pyrimidine (a C cannot be substituted for a T on the non-transferred strand), altered stacking interactions between bases 3 and 4 of the non-transferred strand cannot be a cause of increased PEC formation on the ME. Second, because C and A both have an amine group at C4 and C6, respectively, while T and G have a carbonyl group at C4 and C6, respectively, we can conclude that the orientation of the amine and carbonyl groups within the base-pair may be important for PEC formation. If the base with the amine group is on the non-transferred strand (ME-4C or ME-4A) and the base with the carbonyl group is on the transferred strand, almost no PEC formation is seen. If the orientation is reversed (ME or ME-4G), PEC formation is seen, although sixfold lower with the ME-4G than with the ME. Because PEC formation is decreased sixfold on the ME-4G substrate, we can conclude that the orientation of the amine and the carbonyl groups within the base-pair cannot

be the only reason for decreased PEC formation on the OE. Third, these data indicate that the potential repulsive interaction between O4 of the position 4 transferred strand T in the OE and the backbone O of Gly245 may have an important effect on PEC, because no PEC formation is seen when a G (O6) is placed at this position (ME-4C). Therefore, it cannot be excluded that this interaction inhibits PEC formation severely. But, the potential attraction between N6 of the position 4 transferred strand A in the ME and the backbone O of Gly245 is not solely responsible for increased PEC formation, because a sixfold decrease in PEC results when a C is placed at this position (ME-4G). Finally, a substrate having U at the non-transferred strand T4 position inhibited PEC formation. These data indicate that an interaction between Tnp and the C5-Me group is essential for efficient PEC formation.

Specific contacts to the base-pair at position 4 are important only for transposition steps preceding transferred strand nicking

The co-crystal structures do not reveal any contacts between Tnp and position 4 of the recognition ES. Therefore, the contact required to the C5-Me group of the non-transferred strand T4 must be lost following PEC formation. To determine how many steps of transposition require the contact between Tnp and the C5-Me group of the non-transferred strand T4, substrates that simulated completion of the first catalytic step, transferred strand nicking, were tested for PEC formation (see Figure 5). The result of this experiment indicates that the contact to the C5-Me group of the non-transferred strand T4 is required only for initial PEC formation and is not required to maintain the PEC.

The functional difference between the nicked substrates and the non-nicked substrates is the flexibility of the DNA. The nicked substrates bend more easily than non-nicked substrates.[18] This explains the overall increase in binding of the nicked ME substrate compared to the non-nicked ME substrate (compare 65% bound at the highest concentration of Tnp for the nicked substrate to 35–40% bound at the highest concentration of Tnp for the non-nicked substrate). While there is a slight decrease in binding to mutant nicked substrates (1.5-fold on the OE substrate and twofold on the ME-4A substrate), the fold decrease in binding is much less when compared to the non-nicked substrates. The remaining discrepancy in binding may be due to orientation of the A–T base-pair at position 4 in the OE and 4A (see above) or due to the repulsion of O4 of the transferred strand T at position 4 and the backbone O of Gly245. These data indicate that a conformational change eliminating specific contacts to the base-pair at position 4 must occur between the onset of PEC formation and the completion of transferred strand nicking.

Model to explain the role of the position 4 base-pair

The C5-Me group of the non-transferred strand T4 is critical for efficient PEC formation. Also, either the repulsion of O4 of the transferred strand base at position 4 with the backbone O of Gly245 or the orientation of the carbonyl and amine functional groups within the position 4 base-pair are important for PEC formation. But, the essential contact to the C5-Me group is not important for maintaining the complex following transferred strand nicking.

These data can be explained with the following model. To form a PEC, a Tnp monomer bound to an ES must orient the +1 cleavage site in the active site of another Tnp monomer bound to an ES. We hypothesize that this process can begin efficiently only if O4 or N6 of the base-pair at position 4 are oriented favorably so they can make specific hydrogen bonds to Tnp or if no repulsive interaction between the backbone O of Gly245 and O4 of the transferred strand base exists. If the ES is not repelled by unfavorable interactions, the C5-Me group of the non-transferred strand T4 then interacts in *trans* specifically with Tnp to allow the ES DNA to bend into the correct confirmation for transferred strand nicking.

It is unclear which Tnp residues interact with the C5-Me group of the non-transferred strand T4. In both Tnp-ES post-cleavage PEC co-crystal structures, a segment of anti-parallel β-sheet that protrudes from the catalytic domain between Ser240 and Lys260 interacts with base-pairs 3–6 of the ES. This region of Tnp is hypothesized to clamp the PEC together following initial interaction of the two ES bound monomeric Tnps. Whether a potential interaction between the anti-parallel β-sheet and the C5-Me group of the non-transferred strand T4 is transient or whether it exists in the final PEC conformation is unknown. If Tnp interacts with the C5-Me group in the final PEC conformation, a conformational change must remove this specific contact following transferred strand nicking. We hypothesize that the requirement for a specific contact to the C5-Me group of T4 is lessened when nicked substrates are used because the increased flexibility of these substrates compensates for the specific interaction.

Significance of the specific contact to the C5-Me group on the non-transferred strand T4

The nature of the specific contact between Tn*5* Tnp and the C5-Me group on the non-transferred strand T4 is unknown. The contact could involve either a CH–O hydrogen bond between the C5-Me group and a polar or charged amino acid residue or hydrophobic van der Waals interactions between the C5-Me group and a non-polar residue. Several examples of non-classical CH–O hydrogen bonds contributing to the stability of protein–DNA complexes or to protein recognition of target DNA

sequences have been published.[19,20] Also, the importance of van der Waals interactions in protein–DNA recognition have been documented for interaction of the glutocorticoid receptor DNA-binding domain with its DNA recognition sequence.[21–23]

Recent work in this laboratory suggests that mutation of Glu to Val at position 58 of Tn*5* Tnp (Tnp sC7v2.0) improves recognition of methylated IE DNA by creating favorable hydrophobic interactions between the N6-Me group of adenine and Val58. Also, steric hindrance caused by the large glutamate residue is removed.[24] But, the interaction between Val58 and the N6-Me group is not required for binding, since Tnp sC7v2.0 also functions well *in vivo* with non-methylated IEs. Therefore, the specific contact between Tn*5* Tnp and the C5-Me group on the ME non-transferred strand T4 discussed here represents the only known interaction of this type to be required for Tn*5* Tnp-ES recognition. Also, no other hydrophobic interaction or non-classical CH–O hydrogen bond is known to be required for recognition of ESs in any other transposition systems.

Materials and Methods

DNA substrates

All oligonucleotides were PAGE-purified from Integrated DNA technologies, Inc. (IDT). Sequences of the substrate oligonucleotides are as follows. Non-transferred strand (85-mer): *5′ GCCAAGCTTGCATGCCTGCAGGTCGCTCAGTTCGAGCTCCCAACACTGNCTCTTATACACANNTTGACTGAGTGAGCATGCATGT 3′, transferred strand (85-mer): *5′ ACATGCATGCTCACTCACTCAANNTGTGTATAAGAGNCAGTGTTGGGAGGCTCGAACTGAGCGACCTGCAGGCATGCAAGCTTGGC 3′, nicked dbb (45-mer): 5′ TGTTGGGAGCTCGAACTGAGCGACCTGCAGGCATGCAAGCTTGGC 3′, and nicked transferred strand (40-mer): 5′ ACATGCATGCTCACTCACTCAAGATGTGTATAAGAGNCAG 3′. N indicates a position where modified or alternate bases were substituted to investigate properties of these positions (see Table 1). An asterisk (*) indicates the location of the radioactive label. The recognition end sequenced within each oligonucleotide is underlined.

Two types of DNA substrates were used for *in vitro* experiments. The first was constructed by annealing non-transferred strand and transferred strand oligonucleotides having complementary sequences. These substrates are referred to as non-nicked substrates. The second was constructed by annealing the nicked dbb oligonucleotide and a nicked transferred strand oligonucleotide to the complimentary non-transferred strand oligonucleotide. These substrates are referred to as nicked substrates. See Figure 1(a) for the general structure of the oligonucleotide substrates.

DNA substrate preparation

Non-nicked substrates were prepared for use in binding experiments as follows: 10 μg of each oligonucleotide was annealed in 20 mM Tris–HCl pH (7.5), 20 mM NaCl in a total reaction volume of 50 μl by heating to 97 °C for ten seconds and then allowing the reaction to cool 0.1 deg. C/second to 4 °C. Each substrate was then 5′ end-labeled with [γ-^{32}P]ATP (Amersham) using bacteriophage T4 polynucleotide kinase. Extraneous nucleotides were removed from the reaction using a Nucleotide Removal kit (Qiagen) and each substrate was eluted in 30 μl of TE buffer (20 mM Tris–HCl (pH 7.5), 1 mM EDTA).

Nicked substrates were prepared for use in binding experiments as follows. The non-transferred strand oligonucleotide was first labeled with [γ-^{32}P]ATP using T4 polynucleotide kinase. The T4 polynucleotide kinase was then heat killed and the concentration of NaCl was adjusted to 20 nM. A nicked dbb oligonucleotide and a nicked transferred strand oligonucleotide were then annealed to the complementary, labeled non-transferred oligonucleotide. Extraneous nucleotides were removed and each substrate was eluted in TE buffer.

A small aliquot of each labeled oligonucleotide was visualized on a native 8% polyacrylamide gel and quantified by comparison to standards of known concentration.

Plasmids

All DNA manipulations were performed in *E. coli* strain DH5α. All PCR primers were PAGE-purified from IDT. Two ES mutants were cloned into pRZTL1[13] for *in vivo* analysis. pRZTL4A (constructed by A.B.) and pRZTL17A/18G (constructed by M.S.-W.) were made by amplifying transposon DNA from pRZTL1 by PCR using a *Hin*dIII-tailed primer with base mismatches to either position 4 (pRZTL4) or positions 17 and 18 (pRZTL17A/18G). The PCR product was digested with *Hin*dIII and ligated to the large fragment of *Hin*dIII-digested pRZTL1. Both plasmids were sequenced to determine proper mutation of the ESs. Previously constructed plasmids, pRZTL1 (containing a transposon with OEs) and pRZTL4 (containing a transposon with MEs) were used for *in vivo* analysis.[13]

In vitro substrate plasmids pKJ1 and pKJ4 were constructed by Kristen Jansen as follows. Plasmids pRZTL1 and pRZTL4 were cut with *Hin*dIII, resulting in 1300 bp fragments containing OEs or MEs, respectively. The fragments containing OEs and MEs were then ligated to *Hin*dIII-cut pUC19 to create pKJ1 and pKJ4, respectively.

In vivo transposition assay

In vivo transposition frequencies were determined by a mating out assay similar to that described previously.[4] RZ212 donor cells containing an F-factor, pOX38-Gen,[25] a plasmid expressing Tnp (pGRPET2),[10] and plasmid pGRTL1, pGRTL4, pRZTL4A, or pRZTL417A/18G (see above) were grown overnight at 37 °C; 40 μl of overnight culture was then mixed with 40 μl of overnight culture of recipient cells, 14R525.[13] After seven hours of gentle shaking at 37 °C, aliquots of cells were plated on LB agar containing gentamycin (5 mg/ml) and nalidixic acid (20 mg/ml) and on LB agar containing gentamycin (5 mg/ml), nalidixic acid (20 mg/ml) and chloramphenicol (20 mg/ml). The ratio of colonies growing on gentamycin, nalidixic acid and chloramphenicol to colonies growing on gentamycin and nalidixic acid is interpreted as the transposition frequency. The mating out assay was repeated three times for each mutant ES.

In vitro transposition assay

In vitro transposition efficiency for OE and ME was determined as described.[4] pKJ1 or pKL4 (see above) and purified hyperactive Tnp[9] were incubated in transposition buffer containing Mg^{2+}. The Tnp was removed from the substrate DNA using SDS and heat. Reaction products were then separated by agarose gel electrophoresis and visualized by staining with ethidium bromide. Control reactions contained all components except Tnp. The percentage donor backbone (dbb) release was calculated.[4]

Monomer binding and paired end complex (PEC) assays

Monomer Tnp binding to each ES mutant was analyzed as follows: 200 nM, 100 nM, and 50 nM C-terminally truncated form of Tnp, Tnp EKΔ369, were incubated with 20 nM non-nicked substrates in buffer G (100 mM potassium glutamate, 25 mM Hepes (pH 7.5), 100 μg/ml of tRNA, 50 μg/ml of BSA, 0.5 mM β-mercaptoethanol) for 1.5 hours at 37 °C. Several concentrations of EKΔ369 were used to ensure consistency over a concentration range. Control reactions contained all of these components except EKΔ369. A portion (3 μλ of each reaction was added to 6 × agarose loading dye and 3 μl was run on a native 8% polyacrylamide gel for one hour. The gel was dried and exposed on a phosphoimager screen for approximately 30 minutes. The percentage bound was calculated using Molecular Dynamics Image Quant Software.

PEC formation was assayed *in vitro* for each ES mutant exactly as monomer binding was tested, except full-length Tnp was used.

Tn*5* Tnp-ME co-crystal structure determination

ME DNA was purchased PAGE-purified from IDT with the following sequence: transferred strand, 5′-GAGATGTGTATAAGAGACAG-3′, non-transferred strand, 5′-CTGTCTCTTATACACATCTC-3′. These oligonucleotides were annealed and then combined with Tn*5* Tnp in a 1:1.1 protein to DNA molar ratio. The mixture was dialyzed overnight against 300 mM KCl, 20 mM Hepes (pH 7.7), 2 mM EDTA. The dialyzed complex was concentrated to approximately 10 mg/ml and used for crystallization. Crystals were obtained by the hanging-drop method from 15% (w/v) PEG 1500, 300 mM sodium tartrate, 50 mM Hepes (pH 8.0) at room temperature. Samples were soaked in growth solution containing 5 mM $MnCl_2$ for 18 hours and frozen in 30% PEG 1500, 300 mM sodium tartrate, 15% (v/v) ethylene glycol, 50 mM Hepes (pH 8.0). Data were collected to 2.9 Å resolution at the Advanced Photon Source, SBC-CAT in Argonne, IL and processed with the HKL2000 software package. A total of 299,225 reflections were collected with 22,984 unique reflections, $R_{merge} = 0.145$.

Structure solution was carried out by molecular replacement using the program EPMR and the Tn*5* Tnp-20-mer OE structure as a search model. The space group was $P6_522$ with a $a = 112.9$ Å, $c = 232.6$ Å. Refinement with CNS (simulated annealing) yielded $R = 21.7\%$ and $R_{free} = 27.1\%$. The coordinates have been deposited in the RCSB Protein Data Bank and have the following accession number: 1MM8.

Acknowledgements

We thank Kristen Jansen for construction of some plasmids used in this study, Kelly Winterberg, Brandon Ason, Greg Peterson, and Robert White for helpful discussions, and Barb Schriver for preparing bacterial media and other solutions. This research was supported by National Institutes of Health grant GM50692 and a USDA Hatch Grant.

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Edited by M. Belfort

(Received 23 April 2002; received in revised form 30 July 2002; accepted 9 August 2002)