

Characterization of a Tn5 Pre-cleavage Synaptic Complex

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Protein catalyzed DNA rearrangements typically require assembly of complex nucleoprotein structures. In transposition and integration reactions, these structures, termed synaptic complexes, are mandatory for catalysis. We characterize the Tn5 pre-cleavage synaptic complex, the simplest transposition complex described to date. We identified this complex by gel retardation assay using short, linear fragments and have shown that it contains a dimer of transposase, two DNA molecules, and is competent for DNA cleavage in the presence of Mg^{2+} . We also used hydroxyl radical footprinting and interference techniques to delineate the protein-DNA contacts made in the Tn5 synaptic and monomer complexes. All positions (except position 1) of the end sequence are contacted by transposase in the synaptic complex. We have determined that positions 2-5 of the end sequence are specifically required for synaptic complex formation as they are not required for monomer complex formation. In addition, in the synaptic complex, there is a strong, local distortion centered around position 1 which likely facilitates cleavage.

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Introduction

DNA transposition involves sophisticated DNA cleavage and joining reactions that lead to various DNA rearrangements such as insertions, deletions and inversions. In order to avoid aberrant and potentially detrimental DNA cleavages by transposase (for example, cleavages that occur at only one transposon end), formation of a complex nucleoprotein structure, termed a synaptic complex, is a prerequisite to the cleavage reactions. Synaptic complexes are ubiquitous through all transposition and integration reactions. Mu and Tn10 synaptic complexes are the best characterized for transposition systems to date (Sakai *et al.*, 1995; Mizuuchi *et al.*, 1992). Here, we describe the Tn5 synaptic complex, the simplest transposition complex characterized so far.

Tn5 is a prokaryotic, composite transposon consisting of two inverted insertion sequences, IS50L

and IS50R, that flank a region of antibiotic resistance determinants. IS50R encodes for the 476 amino acid transposase, the protein catalyzing all the steps in Tn5 transposition. In addition, each IS50 element is bracketed by two 19 bp end sequences, the outside end (OE) and the inside end (IE), that serve as specific binding sites for transposase (Tnp) (reviewed by Berg, 1989; Reznikoff, 1993; Reznikoff *et al.*, 1999). Here, we use the mosaic end sequence (ME), an end sequence that is a hyperactive hybrid of the OE and IE (Figure 1; Zhou *et al.*, 1998). *In vitro* Tn5 transposition only requires hyperactive transposase, EK54/LP372 Tnp, transposon DNA defined by two inverted 19 bp end sequences, Mg^{2+} and target DNA (Goryshin & Reznikoff, 1998).

The current model for Tn5 transposition is a multistep process (Figure 2). First, transposase binds to the 19 bp end sequences of the transposon. We believe that transposase binds to its end sequence as a monomer forming the monomer complex referred to later. The monomer complex consists of one molecule of transposase bound to a single end sequence (in this paper, the mosaic end (ME) sequence). The monomer complex is the first Tnp-DNA complex formed in transposition. The

Abbreviations used: OE, outside ends; IE, inside ends; Tnp, transposase; ME, mosaic end; PEC, paired ends complex; DEBC, double end break complex.

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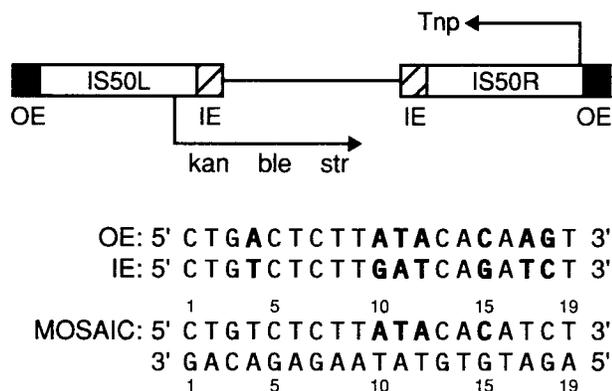


Figure 1. The structure of the Tn5 transposon. Tn5 is composed of two insertion sequences, IS50L and IS50R. IS50R encodes for Tn5 transposase (Tnp). Each insertion sequence is flanked by 19 bp end sequences termed outside end (OE) and inside end (IE). The black box represents the OE and the striped box represents the IE. The OE and IE differ by seven bases (shown in bold). A hyperactive, hybrid end sequence, the mosaic end (ME) was used in this study. The bold letters in the ME sequence are outside end sequences while the plain letters are inside end sequences. The 5' to 3' strand (or top strand) is the non-transferred strand and the 3' to 5' strand (or bottom strand) is the transferred strand. The nucleotides in both strands of the mosaic end sequence are numbered. Base-pair 1 is adjacent to the blunt end cleavage site. The first strand nick generates a 3'OH on nucleotide 1G.

N-terminal domain of transposase has been shown to be responsible for this initial binding step (Zhou & Reznikoff, 1997; Weinreich *et al.*, 1994; de la Cruz *et al.*, 1993). Upon transposase binding, the end sequence DNA is bent approximately 36-48° towards the major groove as determined by phasing analysis (York & Reznikoff, 1997). According to a circular permutation assay, the center of the bend is at position 2 of the end sequence (York & Reznikoff, 1997).

Subsequent to the binding step, the end sequences are brought together *via* transposase dimerization to form a synaptic complex. The C-terminal domain of transposase is required for this dimerization (Steiniger-White & Reznikoff, 2000; Weinreich *et al.*, 1994; Mahnke Braam & Reznikoff, 1998). Once a synaptic complex has been formed, the transposon is excised from the donor DNA by flush double strand breaks precisely at the end sequences (Goryshin & Reznikoff, 1998). All the catalytic reactions occur in the context of a synaptic complex. Throughout the transposition reaction, conformational changes are occurring in the form of nucleoprotein transitions. The resulting nucleoprotein complexes are also termed synaptic complexes. The synaptic complex we describe here is the first synaptic complex formed, the pre-cleavage synaptic complex.

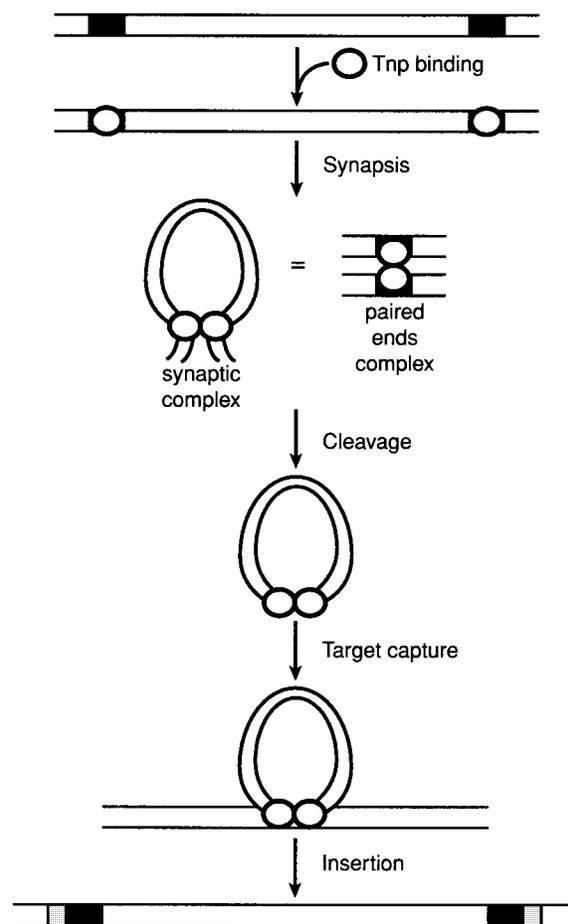


Figure 2. A model of Tn5 transposition. First, Tnp (circle) binds monomerically and bends the transposon end sequences (black rectangles). Then, the end sequences are brought together *via* Tnp dimerization to form a synaptic complex. The synaptic complex is equivalent to the paired ends complex (PEC) characterized in this paper. Once the synaptic complex is formed, the transposon can be cleaved free. The cleaved transposon captures target DNA and inserts the transposon *via* one-step transesterification reactions. The 9 bp duplication flanking the insert is shown as a gray box.

The mechanism of cleavage involves a hairpin intermediate (Bhasin *et al.*, 1999). The first step is a 3' hydrolytic nick by transposase at the end sequence. The free 3'OH attacks the 5' end forming a hairpin at the transposon end and releasing the flanking donor DNA. In order to undergo strand transfer, the hairpin needs to be resolved. Hydrolytic cleavage of the hairpin frees a 3'OH for strand transfer. Strand transfer occurs *via* one step transesterifications in which the released 3'OH groups attack phosphodiester bonds in the target DNA in a staggered fashion (Mizuuchi & Adzuma, 1991; Mizuuchi, 1992). For Tn5, the staggered attack leads to nine bp duplications of the target sequence flanking the integrated transposon (Berg, 1989). The catalytic core of transposase containing the

conserved DDE motif (Davies *et al.*, 1999) is responsible for cleavage and strand transfer in *trans*. Since catalysis has been found to occur in *trans*, meaning transposase bound to one end sequence catalyzes cleavage at the other end sequence (Naumann & Reznikoff, 2000), it is clear that a synaptic complex which brings both transposon ends together is a necessary prerequisite to cleavage.

Using short, radiolabeled DNAs containing a single mosaic end, we have identified and characterized the Tn5 paired ends complex (PEC). This paired ends complex is equivalent to a synaptic complex (Sakai *et al.*, 1995). The difference is that the two 19 bp end sequences exist on the same DNA molecule in a synaptic complex, whereas the two 19 bp end sequences exist on separate molecules in a PEC (Figure 2). Our results demonstrate that this PEC is competent for cleavage. This active PEC contains only a dimer of transposase and two DNA molecules. Utilizing hydroxyl radical footprinting and interference techniques, we have performed a detailed analysis of the DNA requirements and contacts made in the PEC and the Tnp-ME monomer complex. We did this in order to differentiate between initial binding contacts and contacts only required for PEC formation. The monomer complex is formed by using a C-terminally truncated transposase, EK54 Δ 369 (lacking 107 amino acid residues from the C terminus), that has been shown to bind as a monomer (York & Reznikoff, 1996). Monomer binding of full length Tnp has never been detected in gel retardation assays, presumably due to inhibition between the N and C termini of Tnp (Davies *et al.*, 1999; Mahnke Braam *et al.*, 1999) and possible instability of the complex (York & Reznikoff, 1996).

Results

The Tn5 PEC contains two DNA molecules and two transposase molecules

We have identified a PEC (equivalent to the synaptic complex) in Tn5 transposition using gel retardation assays. Upon incubating short, radiolabeled mosaic end DNAs with EK54/LP372 Tnp and electrophoresing the binding reactions through native polyacrylamide gels, a single retarded band was seen (Figure 3(a), lanes 2-4 and 6-8). We hypothesized that this band represented the Tn5 paired ends complex. To verify that this complex contained two DNA molecules, we performed a gel retardation assay in which we mixed two different length ME DNAs (85 bp and 60 bp; both containing 20 bases of donor DNA) with EK54/LP372 Tnp. The complex formed with the 60 bp DNA substrate has a specific mobility in a native polyacrylamide gel (Figure 3(a), lanes 2-4). The complex formed with the longer DNA substrate, 85 bp, migrates slower in the native polyacrylamide gel (Figure 3(a), lanes 6-8). If the complexes contain two DNA molecules, mixing both the

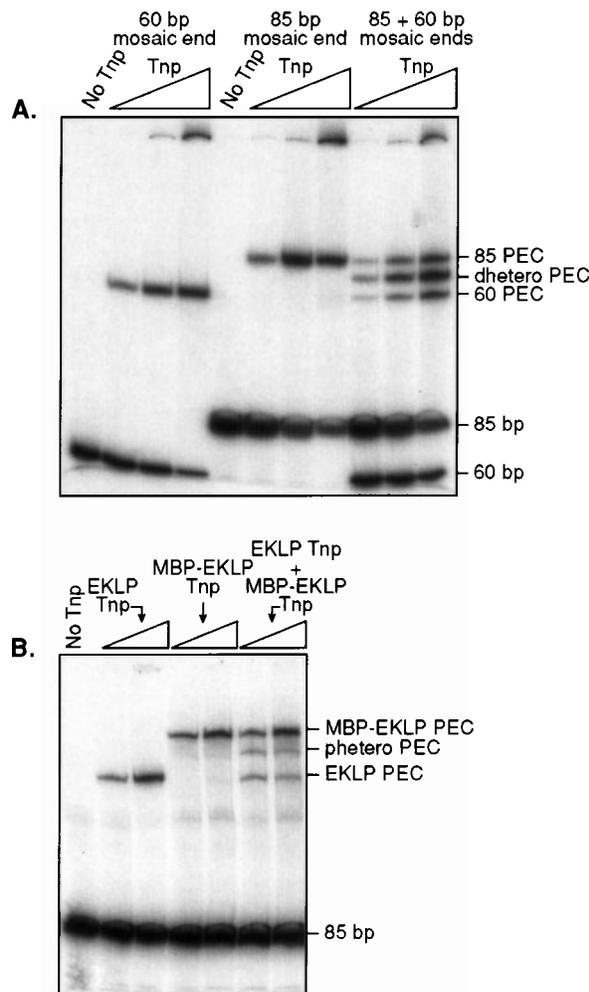


Figure 3. The Tn5 paired ends complex (PEC) consists of two DNA molecules and two transposase molecules. (a) We have identified the Tn5 PEC using a gel retardation assay. Short, radiolabeled, ME DNA fragments (60 bp and 85 bp; 5.3 nM) were incubated with EK54/LP372 Tnp (25-100 nM) at 30°C for three hours and electrophoresed through a 5% native polyacrylamide gel. The last three lanes contain two different length mosaic end DNAs (85 bp and 60 bp) mixed with EK54/LP372 Tnp. The fastest of the three retarded bands is a PEC containing two short DNA fragments (60 bp), while the slowest band is a PEC containing two long DNA fragments (85 bp). The middle band (dheteroPEC) is a heteroPEC containing both a short and a long DNA fragment (60 bp + 85 bp). The appearance of the dheteroPEC shows that the retarded bands contain two DNAs. Also notice that aggregation in the wells occurs upon increased Tnp addition. (b) Radiolabeled ME DNA (85 bp; 5.3 nM) was incubated with EK54/LP372 Tnp (EKL Tnp; 53 kDa, 12.5-25 nM) and/or a maltose binding protein fusion of EK54/LP372 Tnp (MBP-EKL Tnp; 98 kDa, 100-250 nM) at 30°C for three hours and electrophoresed through a native 5% polyacrylamide gel. The last two lanes represent reactions containing both EKL Tnp and MBP-EKL Tnp. The fastest of the three retarded bands contain two monomers of EKL Tnp, while the slowest retarded band contains two monomers of MBP-EKL Tnp. The middle band contains one monomer of EKL Tnp and one monomer of MBP-EKL Tnp (pheteroPEC). The appearance of the pheteroPEC shows that there is a dimer of Tnp in the PEC.

60 bp and 85 bp DNA substrates with EK54/LP372 Tnp should lead to formation of a new complex. This new complex would contain one 60 bp DNA molecule, one 85 bp DNA molecule, and would exhibit an intermediate mobility between the 60 bp and 85 bp complexes. Upon mixing the short (60 bp) and long (85 bp) DNA fragments with EK54/LP372 Tnp, we detected three complexes including a new complex with an intermediate mobility (dheteroPEC, Figure 3(a), lanes 9-11). The fastest of the three bands is a PEC containing two short DNA fragments (60 bp). This PEC has a mobility equivalent to the PEC formed with only a 60 bp ME DNA (compare the fastest retarded band in lane 11 with the retarded band in lane 4). The slowest band is a PEC containing two long DNA fragments (85 bp) and has a mobility equivalent to the PEC formed with only a 85 bp ME DNA (compare the slowest retarded band in lane 11 with the retarded band in lane 8). The middle retarded band migrates in between the 85 bp and 60 bp PECs; therefore, we conclude that it is a DNA hetero-PEC containing one short and one long DNA fragment (60 bp and 85 bp). The appearance of the DNA hetero-PEC shows that each retarded band contains two DNA molecules.

Once we identified the Tn5 PEC, we wanted to determine the stoichiometry of transposase in the complex. To determine this, we incubated a mosaic end DNA with two different molecular mass transposases, EK54/LP372 Tnp (53 kDa) and an N-terminal maltose-binding-protein fusion to EK54/LP372 Tnp (MBP-EKLP; 98 kDa). The PEC formed with EK54/LP372 Tnp has a specific mobility in a native polyacrylamide gel (Figure 3(b), lanes 2 and 3). The PEC formed with the larger MBP-EKLP Tnp migrates slower in the native polyacrylamide gel (Figure 3(b), lanes 4 and 5). If the PECs contain two molecules of transposase, mixing both the EK54/LP372 Tnp and the MBP-EKLP Tnp with DNA should lead to formation of one new complex. This new complex would contain one EK54/LP372 Tnp and one MBP-EKLP Tnp, and would exhibit an intermediate mobility between the EK54/LP372 PEC and the MBP-EKLP PEC. If the PECs contain a tetramer of Tnp, three new intermediate mobility complexes would be detected, each containing one, two, or three molecules of MBP-EKLP. Upon mixing the two different molecular mass Tnps with mosaic end DNA, we detected three complexes including only one new complex of intermediate mobility (pheteroPEC, Figure 3(b), lanes 6 and 7). The fastest of the three retarded bands contain two monomers of EK54/LP372 Tnp. This PEC has a mobility equivalent to the PEC formed only with EK54/LP372 Tnp (compare the fastest of the retarded bands in lane 7 with the retarded band in lane 3). The slowest retarded band contains two monomers of MBP-EKLP Tnp and has a mobility equivalent to the PEC formed with only MBP-EKLP Tnp (compare the slowest retarded

band in lane 7 with the retarded band in lane 5). The middle retarded band migrates in between the EK54/LP372 Tnp and MBP-EKLP Tnp PECs; therefore, we conclude that it contains one monomer of EK54/LP372 Tnp and one monomer of MBP-EKLP Tnp. The appearance of only one intermediate mobility complex indicates that there is a dimer of Tnp in the PEC.

The interpretations described above are correct if Tnp exists as a monomer or an unstable dimer in solution. Wild-type Tnp was found to be a monomer in solution by gel filtration analysis (Mahnke Braam *et al.*, 1999). In addition, preliminary molecular mass determination of the PEC using Ferguson analysis gave a stoichiometry of 1.6 Tnp molecules/PEC (data not shown). And finally, the crystal structure of a Tn5 synaptic complex contains only a dimer of Tnp (Davies *et al.*, 2000).

The PEC is an intermediate competent for cleavage

In order to determine whether the PEC we identified is a true, active intermediate in Tn5 transposition, we tested its ability to proceed with the cleavage reaction. The PECs shown in Figure 3(a), lane 11 (85 PEC, dheteroPEC, 60 PEC), were isolated from the native polyacrylamide gel. The gel slices were soaked in a buffer lacking Mg^{2+} or in a buffer containing 10 mM Mg^{2+} acetate for five minutes at 37°C. The DNA was eluted from these gel slices and electrophoresed through a denaturing 10% (w/v) polyacrylamide gel (Figure 4(a)). Lanes 1-3 show that the PECs were unmetabolized when no Mg^{2+} was present. In the presence of Mg^{2+} , roughly 85% of the PECs were metabolized into cleavage products (hairpin, released transposon and released donor DNA) after only five minutes (Figure 4(a), lanes 4-6). The 85 bp PEC gave rise to a 65 bp transposon, a 20 bp donor DNA and a 130 base hairpin, while the 60 bp PEC gave rise to a 40 bp transposon, a 20 bp donor DNA and a 80 base hairpin.

Previously, we described a hairpin intermediate in Tn5 transposition (Bhasin *et al.*, 1999), but we did not distinguish between a hairpin formed in *cis* or in *trans*. A schematic depicting *cis* and *trans* hairpin formation and the resulting products is shown in Figure 4(b). A hairpin is formed in *cis* when the 3'OH released upon first strand nicking attacks the opposite phosphodiester bond (5' end) on its own end sequence, whereas a hairpin is formed in *trans* when the released 3'OH attacks the 5' end of the partner end sequence in the PEC. A *trans* hairpin is formed in IS911 transposition, and it leads to formation of a circle intermediate (Ton-Hoang *et al.*, 1998). By using the dheteroPEC (containing one end (60 bp) carrying a 40 bp transposon and another end (85 bp) carrying a 65 bp transposon) as a cleavage substrate, we were able to differentiate between *cis* and *trans* hairpin formation. The *cis* hairpin formation on the 60 bp end gave rise to a hairpin of 80 bases on a denatur-

ing gel, while *cis* hairpin formation on the 85 bp end gave rise to a hairpin of 130 bases on a denaturing gel (Figure 4(a), lane 4-6). *Trans* hairpin formation between these two ends would lead to a hairpin of 105 bases on a denaturing gel. We detected strong signals for the 80 and 130 base hairpins, but detected no signal for the 105 base hairpin (Figure 4(a), lane 6); therefore, we conclude that hairpin formation within the same transposon end (*cis*) is the predominant mechanism for hairpin formation in Tn5 transposition.

Analysis of the protein-DNA interactions in the paired ends and monomer complexes

The missing nucleoside assay is a hydroxyl radical interference assay which probes the specific contacts a protein makes with the base moieties of its DNA-binding site (Dixon *et al.*, 1991; Bashkin & Tullius, 1993). Hydroxyl radicals gap DNA by attacking the deoxyribose moiety in DNA causing deletion of the sugar and base (a nucleoside), while the phosphates are left intact. The gapped DNA is mixed with Tnp, and the free and bound DNAs are separated on a native polyacrylamide gel. DNA molecules that are gapped at bases that are required for the Tnp-DNA interaction cannot be bound by the protein and remain with the free DNA during a gel retardation assay. The free and bound DNAs are recovered from the complexes and electrophoresed through a denaturing polyacrylamide gel. On the denaturing gel, bases that are required for the protein-base interaction of interest are detected as reductions in the bound DNA lane and enhancements in the free DNA lane. It is interesting that some bases are enhanced in the bound lane. DNA molecules that are gapped at these positions are bound better, probably due to increased flexibility induced by the loss of a base stack. Bases that are enhanced in the bound DNA lane could indicate areas of structural perturbations in the DNA. One caveat of this experiment is that the removal of a nucleoside could possibly alter the structure of the DNA as to inhibit protein binding (Noel & Reznikoff, 2000).

We used the missing nucleoside assay to probe the Tnp-ME interactions in the paired ends complex, as well as in the monomer complex. A true monomer complex consisting of one full length Tnp molecule and one DNA molecule has never been detected by gel retardation for Tn5. A monomer complex can be created by using a C-terminally truncated Tnp, EK54Δ369 Tnp (York & Reznikoff, 1996). In addition, we performed the missing nucleoside analysis on both DNA strands. The non-transferred strand was radioactively labeled on the 5' end, whereas the 3' end of the transferred strand was radioactively labeled.

On the transferred strand in the PEC, nucleosides 5 to 19 of the mosaic end are required for PEC formation (Figure 5(a)). The strongest requirements are nucleosides 5 to 9 and 13 to 17, whereas

nucleosides 10, 11, 12, 18 and 19 have a slightly weaker requirement. On the other hand, nucleosides flanking the ME are not required for PEC formation, but rather their removal results in enhanced PEC formation. Removal of nucleosides centered around the cleavage site, 3 to -2 (the minus sign denotes bases in the donor DNA), and removal of nucleosides on the transposon end of DNA (21-30) result in enhancements in PEC formation. On the non-transferred strand, nucleosides 4-19 are required, whereas removal of nucleosides 1 to -3 result in enhancements in PEC formation (Figure 5(b)).

Fewer nucleosides are required for monomer complex formation. On the transferred strand, only nucleosides 8-19 are required (Figure 5(a)). As seen for the PEC, nucleosides 10, 11, 12, 18 and 19 have a slightly weaker requirement for monomer complex formation. Also, in the monomer complex, removal of many nucleosides in the donor DNA (-1 to -15) results in weak enhancement in binding. On the non-transferred strand, nucleosides 6-20 are required, although nucleosides 6 and 20 are required less. Again, removal of many nucleosides (5 to -3 and -5 to -10) results in enhanced binding (Figure 5(b)).

We also performed hydroxyl radical footprinting on the PECs and monomer complexes. Hydroxyl radical footprinting differs from the missing nucleoside experiment in that the DNA is cleaved by hydroxyl radicals after it has been bound by protein. Hydroxyl radical footprinting probes the contacts between a protein and the sugar-phosphate backbone of its DNA binding site rather than with the base moieties (Dixon *et al.*, 1991; Bashkin & Tullius, 1993). In essence, hydroxyl radical footprinting gives a high resolution picture of where the protein is "sitting" on the DNA. Cleaved free DNA is compared to cleaved bound DNA on a denaturing polyacrylamide gel. Nucleotides that are diminished in the bound DNA (compared to the free DNA) are nucleotides that the protein binds and protects from hydroxyl radical cleavage. Nucleotides that are enhanced in the bound DNA are nucleotides that are more susceptible to attack by hydroxyl radicals, presumably due to a structural change in the DNA upon protein binding.

On the transferred strand of the PEC, nucleotides 5-8 and 13-17 of the ME are protected by transposase, with nucleotides 13 and 17 only weakly protected (Figure 6(a)). On the other hand, nucleotides centered around the cleavage site, positions 3 to -2, and nucleotides on the transposon end (21-30) are enhanced in the bound DNA. On the non-transferred strand, nucleotides 2-9 and 16-19 are protected (Figure 6(b)). No enhancements are seen on this strand.

In the monomer complex, fewer nucleotides are protected. On the transferred strand, nucleotides 8, 9 and 13-17 are protected, although nucleotides 8 and 9 are protected weakly (Figure 6(a)). A long enhancement is seen in the

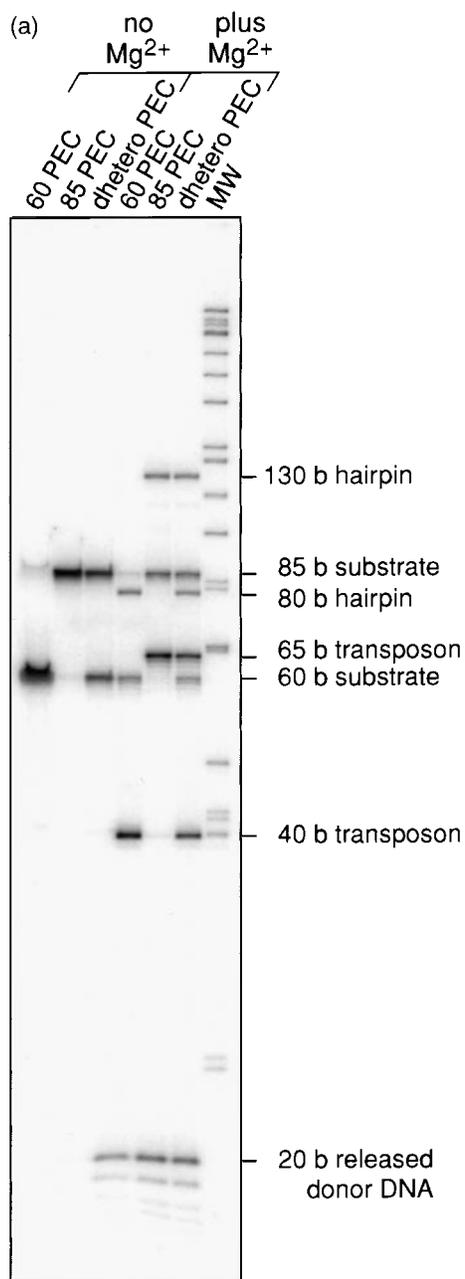


Figure 4 (legend opposite)

donor DNA from nucleotides 1 to -15. On the non-transferred strand, nucleotides 6-19 are protected (Figure 6(b)). Nucleotides 7-9 and 16-19 are protected quite strongly, while nucleotides 6 and 10-15 are less protected. Nucleotides 4 to 1 and -6 to -10 are enhanced in the bound DNA.

It is interesting that the footprint and missing nucleoside pattern for the transferred strand of the PEC are quite similar, except that positions 9 and 13 have a stronger effect in the missing nucleoside experiment than in the footprint. This is also seen

for the monomer complex. However, on the non-transferred strand, the footprints and missing nucleoside patterns differ. The most dramatic difference is seen at nucleotides 10-15; the missing nucleoside assay shows that these nucleosides are required for PEC and monomer complex formation, but hydroxyl radical footprinting shows that these nucleotides are not protected at all or are protected minimally. In addition, removal of nucleosides 1 to -3 results in enhancements in binding, whereas no enhancements are seen for the footprint (Figure 7).

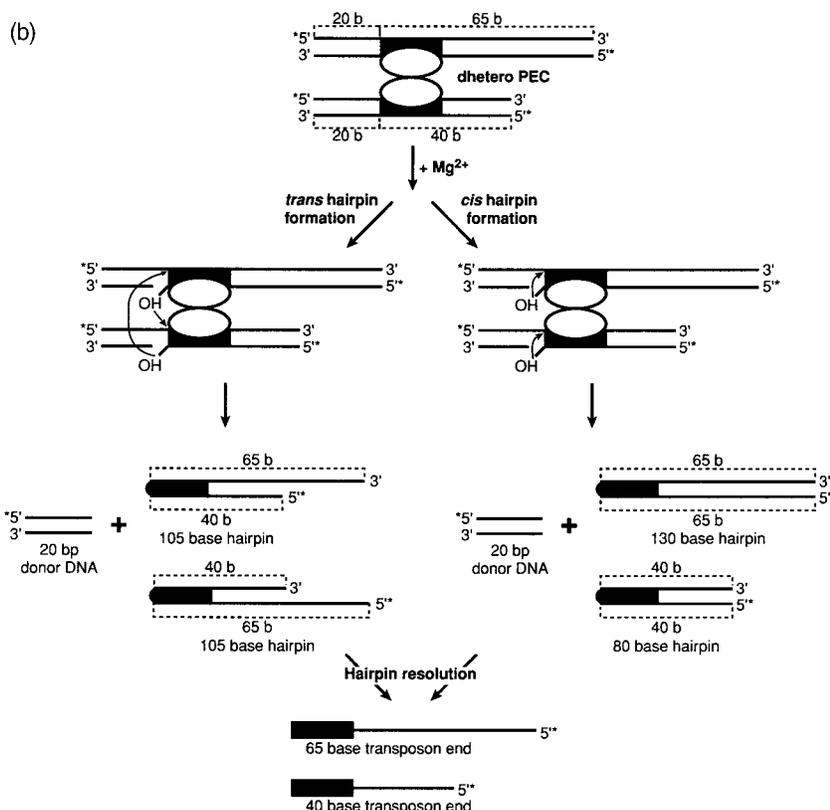


Figure 4. Paired ends complexes (PECs) are transposition intermediates that can be cleaved by Tnp in the presence of Mg^{2+} . (a) The PECs (85 PEC, dheteroPEC, 60 PEC) shown in Figure 3(a) were isolated from the native polyacrylamide gel. The gel slices were soaked in a 20 mM Hepes (pH 7.5), 100 mM potassium glutamate buffer or in a 20 mM Hepes (pH 7.5), 100 mM potassium glutamate, 10 mM magnesium acetate buffer for five minutes at 37°C. Then, the gel slices were rinsed with water and the DNA eluted in 10 mM Tris-HCl (pH 7.9), 10 mM EDTA 1% SDS. The DNA was recovered and loaded on a 15% denaturing gel. Cleavage products (donor backbone, released transposon and hairpin intermediate) were detected when the PECs were incubated with Mg^{2+} (lanes 4-6). Thus, PECs are intermediates that can be cleaved in Mg^{2+} . (b) This schematic depicts *cis* and *trans* hairpin formation. At the top, a dheteroPEC (see Figure 3(a)) is shown. The mosaic end is represented as a black rectangle and Tnp is represented as a white oval. One DNA molecule contains 20 bases of donor DNA and 65 bases of transposon DNA, whereas the second DNA molecule contains 20 bases of donor DNA and 40 bases of transposon DNA. Both DNA molecules are radiolabeled (label is shown as an asterisk) at their 5' ends. A hairpin is formed in *cis* when the 3'OH released upon first strand nicking attacks the opposite phosphodiester bond (5' end) on its own end sequence, whereas a hairpin is formed in *trans* when the released 3'OH attacks the 5' end of the partner end sequence in the paired ends complex. The *cis* hairpin formation on the 60 bp and 85 bp ends gives rise to 80 base and 130 base hairpins, respectively. The *trans* hairpin formation between these two ends should give rise to a 105 base hairpin. The 105 base hairpin is not detectable in this assay ((a), lane 6), therefore we conclude that *cis* hairpin formation is the predominant mechanism.

Discussion

The Tn5 PEC is an intermediate in Tn5 transposition

The data presented here characterizes a pre-cleavage intermediate in Tn5 transposition, the Tn5 PEC. The PEC is equivalent to the synaptic complex (Figure 2). Using ME DNAs of different lengths and transposases of different molecular masses in gel retardation assays (Figure 3(a) and (b)), we have shown the stoichiometry of the Tn5 PEC to be two DNA molecules and two transposase molecules. We have also shown that this complex is competent for cleavage. In the presence of

Mg^{2+} , roughly 85% of the PECs were metabolized into cleavage products (hairpin, released transposon and donor DNA) after only five minutes (Figure 4).

We determined the stoichiometry using DNA molecules containing 20 bp of flanking donor DNA. One can imagine that more flanking donor DNA could allow for accommodation of more molecules of Tnp. If more molecules of Tnp were accommodated, we believe that their role would be architectural. We have shown that only two molecules of Tnp present in PECs are required for catalysis. This stoichiometry is reflected in the recently determined crystal structure for the Tn5 double end break complex (DEBC) (Davies *et al.*, 2000).

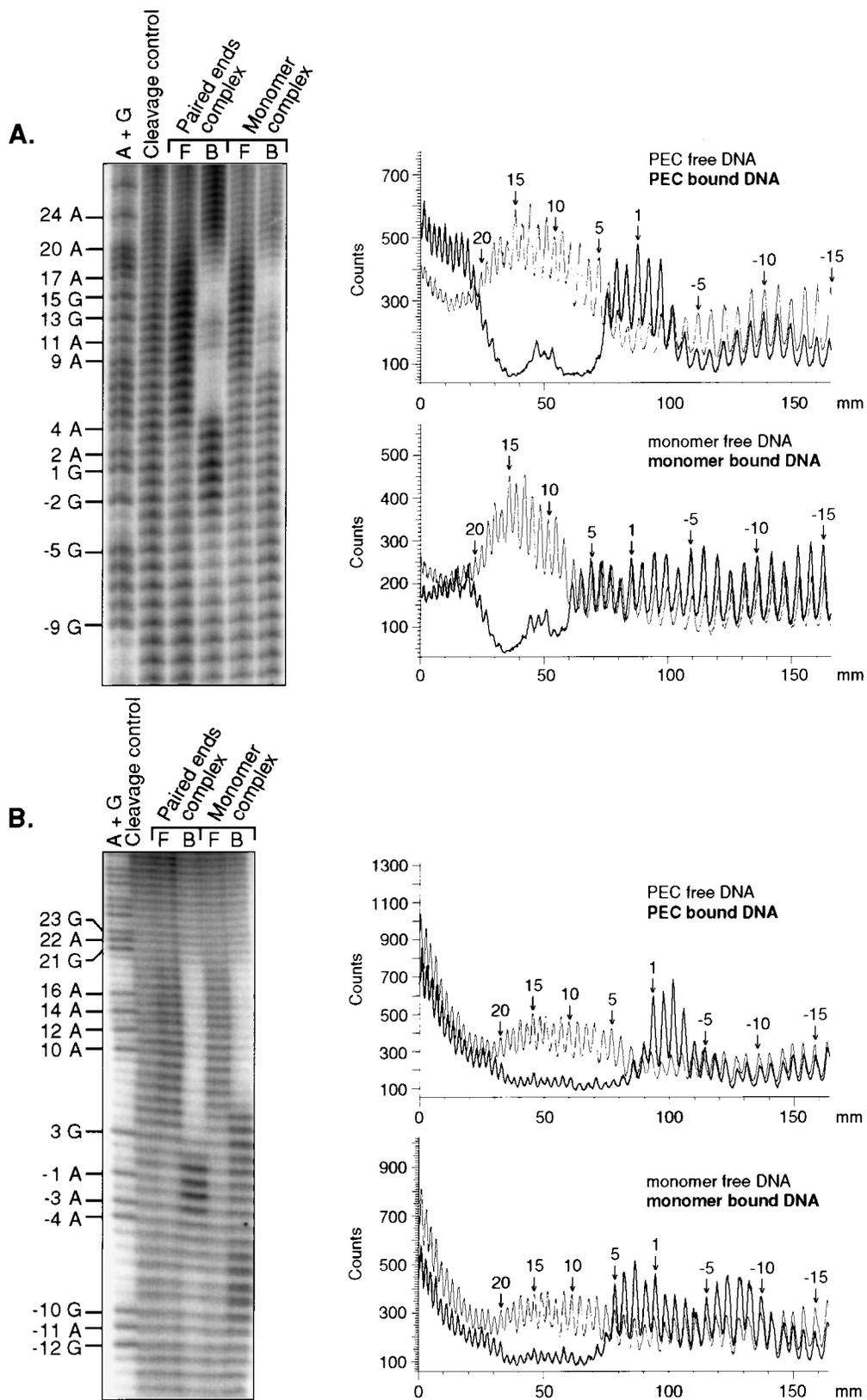


Figure 5 (legend opposite)

Interpretation of transposase-mosaic end DNA contacts

Here, we use the terms base and nucleoside to refer to contacts seen in the missing nucleoside experiment. We use the term nucleotide to refer to contacts seen in the footprinting experiment.

PEC

The hydroxyl radical footprinting and interference data provide insight into the end sequence DNA requirements for PEC formation. First, the end sequence DNA is not regular B-form DNA. The DNA centered around the cleavage site (position 1), positions 3 to -3 on the transferred strand and 1 to -3 on the non-transferred strand, is distorted. The nature of the distortion cannot be assessed by these assays, but is consistent with phasing analysis determining a bend of 36-48° toward the major groove (York & Reznikoff, 1997). According to a circular permutation assay, the center of the bend is at position 2 of the end sequence and the angle of distortion is 80° (York & Reznikoff, 1997). Since circular permutation assays for other distortions in addition to bending, different structural perturbations such as partial denaturation may also be occurring. This distortion is also consistent with the X-ray crystal structure of the Tn5 DEBC. In this structure, the DNA is frayed at positions 1 and 2 of the end sequence (Davies *et al.*, 2000).

The footprinting and interference data for both transferred and non-transferred DNA strands combined show that essentially all the ME sequence positions (except position 1) of the PEC are in contact with transposase either through base-specific interactions or through backbone (sugar-phosphate) interactions (Figure 7), although to varying degrees. Position 1 is immediately adjacent to the cleavage site, and therefore we would expect position 1 to be in contact with Tnp. The footprinting and interference data imply that there are no Tnp contacts with position 1, but imply that position 1 is in a distorted region of the DNA. Since position 1 is distorted, any contacts position 1 makes with Tnp are probably masked by enhancements caused by the DNA deformation. Positions 2 and 3 appear to make weak contacts with Tnp. This is also probably due to their location adjacent to the DNA distortion. The crystal structure of the Tn5 DEBC also

shows extensive transposase interactions with most of the bases and phosphates (except positions 18 and 19) of the end sequence (Davies *et al.*, 2000).

The footprinting and interference data also show that transposase interacts with both strands asymmetrically. There are three distinct regions of asymmetry. (1) On the non-transferred strand, nucleotides 2-4 are protected and base 4 is required, whereas on the transferred strand, nucleotide 4 has no effect and nucleotides 2 and 3 are in a region of distortion. (2) Bases 10, 11, 12, 18 and 19 appear to be only weakly required on the transferred strand, whereas the requirement is strong on the non-transferred strand. In addition, nucleotides 18 and 19 are protected on the non-transferred strand, but not on the transferred strand. (3) Nucleotides 13, 14 and 15 are protected on the transferred strand, but not on the non-transferred strand.

Another noteworthy feature is that for most bases which appear to be required, their corresponding nucleotides (sugar-phosphate backbones) are protected. This is not surprising, since bases are attached to and in close proximity to the sugar-phosphate backbone. We also see bases which are required, but whose corresponding nucleotides are not protected. This occurs with bases 10-15 of the non-transferred strand. This result can be explained in a few ways. (1) Tnp can contact bases 10-15 in the end sequence DNA through a groove of the DNA such that the sugar-phosphate backbone is not contacted. In support of this, in the crystal structure of the Tn5 DEBC, transposase contacts only the bases (no phosphate interactions) of nucleotides 10-13 of the end sequence (Davies *et al.*, 2000). (2) Bases 10-15 of the non-transferred strand may be playing a structural role in the DNA. Recall that the missing nucleoside experiment involves removing a nucleoside (sugar and base) from the DNA, and removal of a nucleoside could possibly alter the structure of the DNA as to inhibit protein binding. Consistent with this is previous hydroxyl radical interference data with heterodimers (one piece of DNA bound by a full length Tnp dimerized with an N-terminally truncated Tnp that cannot bind DNA) of WT and EK54 Tnp which indicated that bases 10-12 were not required for binding, but mutational analysis showed that bases 10-12 had an effect on overall transposition frequency (Zhou *et al.*, 1998). Moreover, the two explanations are not mutually exclusive. Bases

Figure 5. Missing nucleoside (or hydroxyl radical interference) assay. (a) Missing nucleoside assay of the transferred strand (see the legend to Figure 1) in the PEC and monomer complex. The monomer complex is generated using EK54Δs369 transposase. Nucleotides and their positions in the ME sequence are labeled. The A + G lane represents the Maxam-Gilbert A + G marker lane. F stands for free DNA and B stands for bound DNA. Reductions in the bound lane indicate bases which are required for complex formation. Enhancements in the bound lane indicate areas of structural perturbations in the DNA. Quantitation curves are adjacent to the gel. Positions are numbered on the curves. Bound DNA curves are shown in bold whereas free DNA curves are plain. They were generated using Molecular Dynamics ImageQuant software. (b) Missing nucleoside assay of the non-transferred strand (see the legend to Figure 1) in the paired ends and monomer complexes. Quantitation curves are adjacent to the gel.

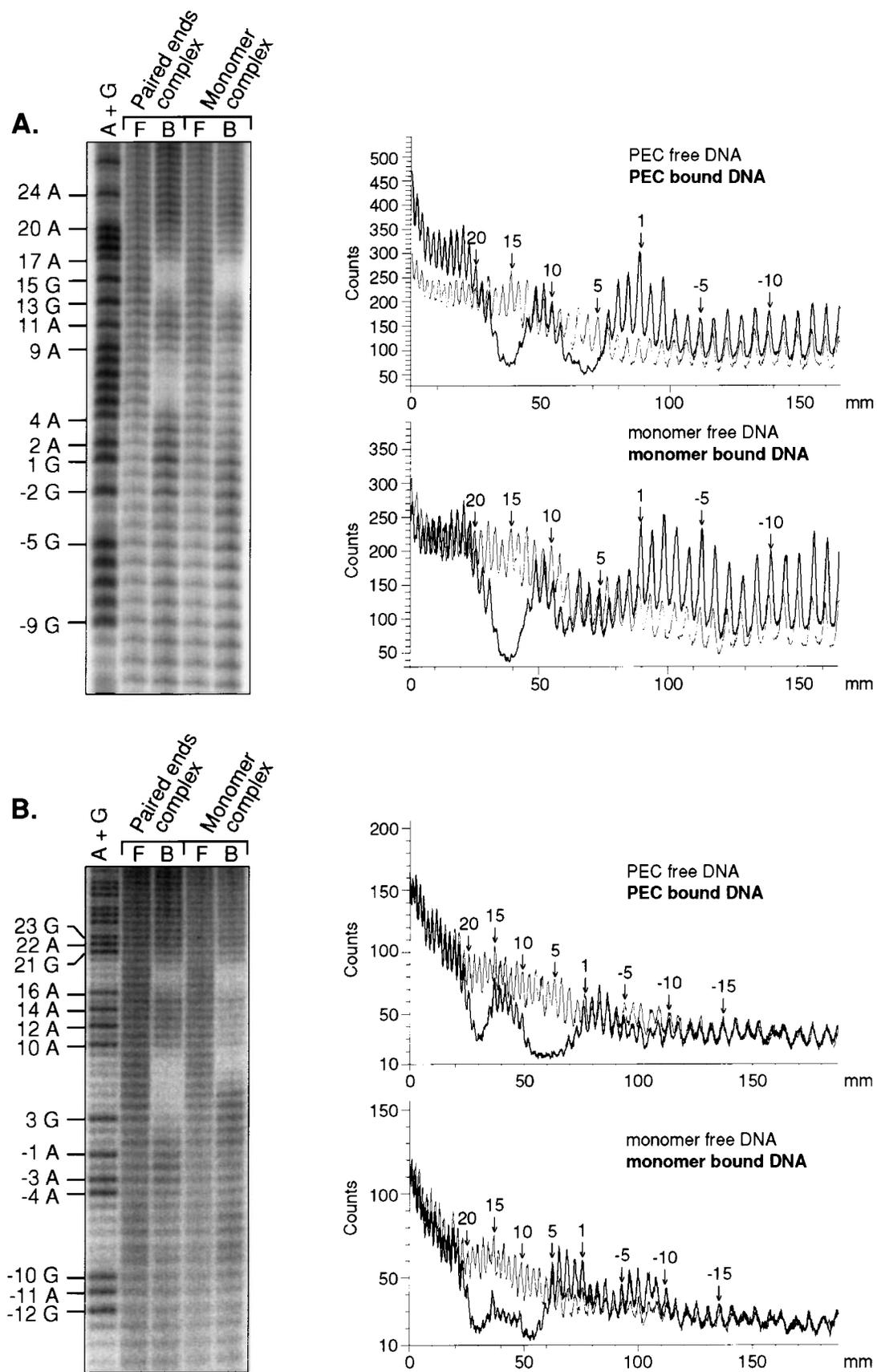


Figure 6 (legend opposite)

10-15 may both contact transposase directly as well as affect the structure of the end sequence.

Comparison with monomer complex

Although the monomer complex displays many of the same base requirements and protections that are seen in the PEC, there is a clear difference in nucleotide usage near the cleavage site (Figure 7). In the monomer complex, there are no transposase contacts on either strand at nucleotides 1-5, whereas there are transposase contacts at nucleotides 2-5 on the PEC. There are a few possibilities for this difference. (1) Amino acid residues in the C-terminal 107 amino acid residues of Tnp make contacts with nucleotides 2-5 of the end sequence, but the monomer complex lacks these amino acids. (2) The second Tnp molecule in the PEC makes contacts with nucleotides 2-5. The monomer complex does not have a second Tnp molecule. This option is favored in light of recent biochemical data showing that Tnp catalyzes cleavage and strand transfer in *trans* (Naumann & Reznikoff, 2000). In addition, the crystal structure of the Tn5 DEBC also shows many *trans* Tnp-DNA contacts from positions 1-7 (Davies *et al.*, 2000). Moreover, these two possibilities are not mutually exclusive.

Comparing the monomer and PEC footprint and interference data allows initial binding contacts to be distinguished from PEC contacts. In addition, the missing nucleoside and footprinting experiments assay contacts made during different steps in transposition. In hydroxyl radical footprinting, the DNA is cleaved after the PEC or monomer complexes have been formed, thus giving a snapshot of backbone contacts made by Tnp in the PEC or monomer complexes. In contrast, in the missing nucleoside experiment, the DNA is cleaved prior to the addition of protein. For the monomer complex, only the initial Tnp-binding step is being assayed. For the PEC, products of two steps in transposition are being assayed: (1) the initial Tnp binding step and (2) PEC formation. Consequently, if a nucleoside required for initial binding is removed, initial binding is inhibited and thereby, PEC formation is also inhibited. Since the missing nucleoside experiment has been performed on the monomer complex as well, these two steps can be distinguished. We presume that nucleosides required for both monomer and PEC formation are nucleosides which are required for the initial bind-

ing step. Nucleosides required for PEC formation and not for monomer complex formation are nucleosides specifically necessary for the second step, PEC formation. Nucleosides 3-5 on the non-transferred strand and nucleosides 5-7 on the transferred strand are specifically necessary for PEC formation, whereas nucleosides 6-19 on the non-transferred strand and nucleosides 8-18 on the transferred strand are required for the initial binding step. The footprinting data correlates with this interpretation. Non-transferred strand nucleotides 2-5 and transferred strand nucleotides 5-7 are protected in the PEC, but not in the monomer complex. Conformational changes occurring between the initial binding step and PEC formation can make interpretations more complicated. For example, transferred strand nucleosides 10-15 are required for the initial binding step, but are not protected in the PEC. Perhaps, after the initial binding step and before PEC formation, a conformational change occurs which inhibits transposase interactions with these nucleotides.

Another difference between the monomer complex and PEC footprint and interference data suggests that a conformational change occurs in the DNA in the transition from monomer complex to PEC. There is a long enhancement starting at nucleotide 5 and extending far into the donor DNA (to nucleotide -15) in the monomer complex, whereas the enhancement in the PEC is local and centered around the cleavage site. The PEC enhancement only extends to -3 in the donor DNA. The monomer complex more closely resembles the missing nucleoside pattern previously seen for a heterodimer complex of Tnp bound to DNA (Jilk *et al.*, 1996). Like the monomer complex, the heterodimer interference pattern has a long enhancement in the donor DNA and bases 1-5 are not required. It is important to note that the monomer and heterodimer complexes are catalytically inert, whereas the paired ends complex is primed for catalysis.

Correlation with mutational analyses

The hydroxyl radical footprinting and interference data on the PEC are consistent with previous mutational analyses of the outside and inside ends. The mutational analyses demonstrated that every nucleotide in the end sequences has an effect on the overall transposition frequency. All point

Figure 6. Hydroxyl radical footprinting. (a) Hydroxyl radical footprinting of the transferred strand (see the legend to Figure 1) in the paired ends and monomer complex. The monomer complex is generated using EK54Δ369 transposase. Nucleotides and their positions in the ME sequence are labeled. The A + G lane represents the Maxam-Gilbert A + G marker lane. F stands for free DNA and B stands for bound DNA. Reductions in the bound lane indicate bases which are protected in the complex. Enhancements in the bound lane indicate areas of structural perturbations in the DNA. Quantitation curves are adjacent to the gel. Positions are numbered on the curves. Bound DNA curves are shown in bold whereas free DNA curves are plain. They were generated using Molecular Dynamics ImageQuant software. (b) Hydroxyl radical footprinting of the non-transferred strand (see the legend to Figure 1) in the paired ends and monomer complexes. Quantitation curves are adjacent to the gel.

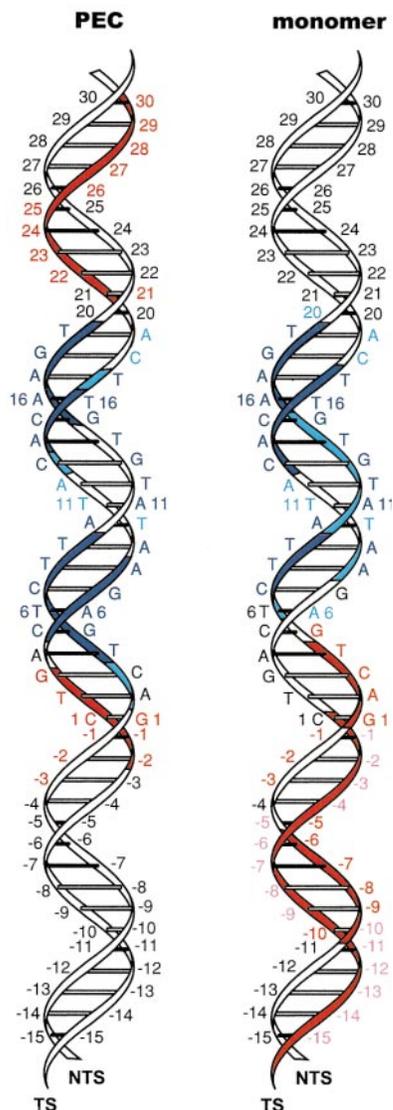


Figure 7. Summary of the missing nucleoside and hydroxyl radical footprinting data for the PEC and monomer complex. TS stands for transferred strand and NTS stands for non-transferred strand. Nucleotides of the mosaic end sequence are shown. Nucleotides 1, 6, 11 and 16 are also numbered. Nucleotides flanking the mosaic end sequence are only numbered. Nucleotides numbered with a minus sign (–) are bases in the flanking donor DNA. Missing nucleoside data are represented on the nucleotide letters and numbers. Red letters and numbers represent bases which are enhanced in the bound lane. Pink letters and numbers represent weak enhancements in the bound lane. Blue letters and numbers represent bases which are diminished in the bound lane. Light blue letters and numbers represent weak diminishments in the bound lane. Black letters indicate bases which have no effect. Hydroxyl radical footprint data are represented on the DNA helix. Red areas on the helix represent nucleotides which are enhanced in the bound lane. Blue areas on the helix represent nucleotides which are protected by transposase. Light blue areas on the helix represent weaker protections. White areas on the helix represent nucleotides which have no effect. In short, blue helix, letters and numbers are interpreted as transposase contacts whereas red helix, letters and numbers are interpreted as regions of distortion.

mutants decreased transposition, although to varying degrees (Phadnis & Berg, 1987; Makris *et al.*, 1988; Berg & Howe, 1989). The PEC footprinting and interference data presented here show that all the positions of the mosaic end (except position 1) have a role in paired ends complex formation. Previously, we showed that nucleotide 1 on the transferred strand is required for efficient hairpin formation (Bhasin *et al.*, 1999).

Mutational analyses of Tn5 (IS50), Tn10 (IS10) and IS903 inverted repeats have suggested that insertion sequence (IS) inverted repeats have a similar domain structure. The inner part of the inverted repeat houses the primary binding domain. This includes positions 6-9 and 13-19 for Tn5 (Jilk *et al.*, 1996), positions 6-13 for Tn10 (Huisman *et al.*, 1989) and positions 7-16 for IS903 (Derbyshire & Grindley, 1992). The terminal positions (positions 1-3) are believed to be necessary for a post-binding step such as cleavage. It has been demonstrated for Tn10 that positions 1-3 affect the step between complete cleavage and strand transfer. Positions 1 and 2 have also been shown to affect a step after primary binding and prior to complete cleavage (Haniford & Kleckner, 1994). The data presented here demonstrate that positions 2-5 are required for Tn5 synaptic complex formation. This extends the structure of the inverted repeats to include a domain for synaptic complex formation located in between the binding and cleavage domains. Since inverted repeats appear to be organized in a similar way, it is likely that synapsis domains will be revealed in other systems.

Comparison with Tn10 and Mu synaptic complexes

The Tn5 PEC is the simplest pre-cleavage complex characterized to date requiring only hyperactive transposase and the 19 bp mosaic end sequence. In contrast, formation of the Tn10 PEC requires the host protein IHF, and IHF substitutes for the lack of supercoiling on short, linear fragments (Sakai *et al.*, 1995; Chalmers *et al.*, 1998). The Mu pre-cleavage stable synaptic complex (SSC or type 0 complex) is even more complex. Formation of the Mu stable synaptic complex typically requires supercoiled donor DNA containing the Mu A binding sites and the enhancer element, divalent metal ion and the host proteins HU and IHF (Mizuuchi *et al.*, 1992).

The stoichiometry of transposase in these complexes has been determined. Like the Tn5 paired ends complex, the Tn10 paired ends complex also contains a dimer of transposase (M. Junop, S. Wardle, D. Haniford & W. Yang, personal communication). In contrast, the Mu transposase synaptic complexes contain a tetramer of MuA transposase (Mizuuchi *et al.*, 1992; Baker & Mizuuchi, 1992), although only two molecules of MuA transposase are directly involved in catalysis (Namgoong & Harshey, 1998). It appears that a requirement for

only two transposase active sites to perform the cleavage and strand transfer steps is common.

An in depth analysis of protein-DNA interactions in Mu synaptic complexes has also been undertaken. Since the MuA-transposase-binding sites differ greatly in length, sequence and number from the Tn5-transposase-binding sites, we cannot compare Tn5 and Mu transposase-end sequence interactions. It is interesting that DNA distortions adjacent to the cleavage site in both Tn5 and Mu synaptic complexes have been detected. In the Mu type 0 complex, distortions adjacent to the cleavage site were detected by potassium permanganate footprinting and P₁ nuclease cleavages (Wang *et al.*, 1996). In addition, in the Mu type 1 synaptic complex, hydroxyl radical footprinting was used to detect a distortion two to three positions from the cleavage site (Lavoie *et al.*, 1991). We also detected distortions centered around the cleavage site (positions -3 to 3) using hydroxyl radical footprinting and interference techniques. It is likely that distorting the DNA (by bending and/or melting the DNA) adjacent to the cleavage site facilitates the cleavage step in transposition and integration reactions.

In conclusion, the simplicity of the Tn5 synaptic complex makes further investigations of transposase-DNA interactions amenable.

Materials and Methods

DNA substrates

DNA substrates were 5' end-labeled using T4 polynucleotide kinase (New England Biolabs) and [γ -³²P]ATP (Amersham, Redivue) unless stated otherwise. Unincorporated nucleotides were removed using the QIAquick nucleotide removal kit (QIAGEN). Oligonucleotides were annealed in 20 mM Tris-HCl (pH 7.5), 50 mM NaCl by heating at 80°C for two minutes and then cooling the reaction to room temperature. DNA was quantitated using various methods: spectroscopy, gel electrophoresis along with known mass markers (PCR markers; Promega), or according to DNA recovery estimates in the QIAquick nucleotide removal kit protocol.

The 60 bp ME (the ME is shown in bold-face letters) DNA substrate (40 base transposon and 20 base donor DNA) was made by annealing two oligonucleotides, 5'-CTCAGTTCGAGCTCCCAACTG**TCTCTTATACA**CATCTTGAGTGAGTGAGCATGCATGT-3' and 5'-ACATGCATGCTCACTCAAGATGT**GTATAAGAGACAGTGTGGGAGCTCGAACTGAG-3'**.

An 85 bp ME (the ME is shown in bold-face letters) DNA substrate (65 base transposon and 20 base donor DNA) was made by annealing two oligonucleotides, 5'-CTCAGTTCGAGCTCCCAACTG**TCTCTTATACA**CATCTTGAGTGAGTGAGCATGCATGCTCGCTGAGGTCGCCTCGTGAAGAAG-3' and 5'-ATTCTTCAGCGCAGACCTCAGCGACATGCATGCTCACTCAAGATGT**GTATAAGAGACAGTGTGGGAGCTCGAACTGAG-3'**.

Another 85 bp ME (the ME is shown in bold-face letters) DNA substrate (40 base transposon and 45 base donor DNA) was used only in the footprinting and inter-

ference assays (Figures 5 and 6). It was made by annealing two oligonucleotides, 85/45ME top: 5'-GCCAAGCTTGCATGCCTGCAGGTCGCTCAGTTCGAGCTCCC AACACTG**TCTCTTATACACATCTTGAGTGAGTGAG**CATGCATGT-3' and 85/45ME bottom: 5'-ACATGCATGCTCACTCAAGATGT**GTATAAGAGACAGTGTGGGAGCTCGAACTGAGCGACCTGCAGGCATGCAAGCTTGGC-3'**. All oligonucleotides were from Integrated DNA Technologies, PAGE-purified.

Protein purification

EK54/LP372 Tnp was purified as described (Bhasin *et al.*, 1999). The expression vector pRZI369 was used to purify EK54 Δ e369 Tnp. pRZI369 was constructed as follows. Bases corresponding to amino acid residues 301 to 369 of Tnp were amplified by PCR from plasmid pRZI10300. pRZI10300 was constructed (by L.Mahnke) as follows. The *Xba*I-*Sal*II Tnp fragment from pRZ7075 (Wiegand & Reznikoff, 1994) was cloned into the large *Xba*I-*Sal*II fragment from pBAD18 (Guzman *et al.*, 1995) to create pRZI10250. The hyperactive EK54 and LP372 mutations were then introduced by substituting the *Xba*I-*Bgl*III Tnp fragment from pRZPET2 (Goryshin & Reznikoff, 1998) into the large *Xba*I-*Bgl*III fragment of pRZI10250. The PCR was done using Pfu DNA polymerase (Stratagene), an internally annealing primer and a *Kpn*I tailed primer (Integrated DNA Technologies). The D369 PCR product was then digested with *Nhe*I and *Kpn*I (New England Biolabs) and substituted into the large *Nhe*I-*Kpn*I fragment of pGRTYB35 (Bhasin *et al.*, 1999). EK54 Δ e369 Tnp was purified by the same method used to purify EK54/LP372 transposase.

The maltose binding protein fusion of EK54/LP372 Tnp (MBP-EKLP Tnp) was purified using the pMAL Protein Fusion and Purification System (New England Biolabs). The Tnp gene was cloned into the vector pMAL-c2XSapI (made by L.Wood). This vector has a *Sap*I restriction site on the linker ATGAGAAGAGC which was introduced into the *Xmn*I digest of the vector pMAL-c2X (New England Biolabs). The original *Sap*I site in pMAL-c2X was removed by digestion with *Sap*I and *Tth*111I, a fill-in reaction and ligation. PMAL-c2XSapI was digested with *Sap*I and *Sal*II, and the large fragment was ligated to the 1.5 kb *Bsp*HI-*Sal*II fragment of pGRTEMP2 (Goryshin & Reznikoff, 1998) to form pGRMALS2. For the MBP-EKLP Tnp purification, pGRMALS2 was transformed into the strain TB1 (New England Biolabs). Cells grown to an A₆₀₀ of 0.5 were induced with 0.1 mM IPTG (final concentration) at 30°C for three hours, harvested and resuspended in column buffer (20 mM Tris-HCl (pH 7.5), 0.3 M NaCl, 1 mM EDTA, 10% (v/v) glycerol) containing a protease inhibitor cocktail (Roche). After cells were sonicated and centrifuged, the crude extract was loaded on an amylose column. The column was then washed with column buffer (and protein was eluted with column buffer plus 10 mM maltose according to New England Biolabs recommendations).

Formation of PECs and gel retardation assays

Short, radiolabeled ME DNA fragments were mixed with EK54/LP372 Tnp and/or a maltose binding protein fusion of EK54/LP372 Tnp in a 20 mM Hepes (pH 7.5), 100 mM potassium glutamate buffer (final concentrations) in a total reaction volume of 15 μ l. The binding reactions were incubated at 30°C for various times (see

Figure legends). Gel-loading buffer (0.25% (w/v) bromophenol blue, 0.25% (w/v) xylene cyanol FF, 30% (v/v) glycerol) was added and the samples were electrophoresed at 15 V/cm and at 4°C through 5% (w/v) native polyacrylamide gels (acrylamide/bis-acrylamide, 19:1). Protein and DNA concentrations are indicated in Figure legends.

Cleavage of the PECs

The PECs were isolated from a native polyacrylamide gel. Each gel slice was cut in half. One half was soaked in a 20 mM Hepes (pH 7.5), 100 mM potassium glutamate buffer for five minutes at 37°C while the other half was soaked in a 20 mM Hepes (pH 7.5), 100 mM potassium glutamate, 10 mM magnesium acetate buffer for five minutes at 37°C. The gel slices were then rinsed with water, and the DNA was eluted in 10 mM Tris-HCl (pH 7.9), 10 mM EDTA, 1% (w/v) SDS overnight at 4°C. The eluates were concentrated and the buffer exchanged using Amicon microcon YM-10 centrifugal filter devices (Millipore). Equal volumes of formamide loading buffer were added to the recovered DNAs, the samples were boiled for five minutes and electrophoresed through a denaturing 15% polyacrylamide gel.

Missing nucleoside assay and hydroxyl radical footprinting

The 85/45 ME top oligonucleotide was 5' end-labeled using T4 polynucleotide kinase (New England Biolabs) and [γ -³²P]ATP (Amersham, Redivue) whereas the 85/45 ME bottom oligonucleotide was 3' end-labeled using Terminal Transferase (New England Biolabs) and [γ -³²P]ddATP (Amersham). The individually labeled oligo nucleotides were then purified from a 10% denaturing gel. Bands were detected by autoradiography, excised and eluted overnight at 4°C in 20 mM Tris-HCl (pH 7.4), 1 mM EDTA, 0.2 M NaCl. The DNAs were recovered from the eluates using Elutip-d[®] Minicolumns (Intermountain Scientific). The ethanol precipitation was done in the presence of 20 μ g of glycogen. The labeled, purified oligonucleotides were annealed to their cold, complementary strands as described above. The annealed DNA was gapped using hydroxyl radicals. The hydroxyl radicals needed for cleavage of the DNA were generated by the Fenton reaction (Dixon *et al.*, 1991). Fifty microliters of DNA (7 pmol) were treated with 5 mM ferrous ammonium sulfate (Aldrich), 10 mM EDTA and 0.01% H₂O₂ (final concentrations; reagents were prepared in degassed water) at room temperature for two minutes. The reactions were stopped with 11.4 mM thiourea and 0.4 mM EDTA (final concentrations), and the DNA was ethanol precipitated in the presence of glycogen and resuspended in TE. This gapped DNA (53 nM) was incubated with EK54/LP372 Tnp (220 nM, 440 nM) at 30°C for two to three hours to form paired end complexes. This DNA was also incubated with EK54 Δ 369Tnp (missing 107 amino acids from the C terminus; 165 nM, 330 nM) at 30°C for approximately one minute to form monomer complexes. These binding reactions were then electrophoresed on a native 5% polyacrylamide gel. The free and bound DNAs were excised from the gel and the DNAs were eluted in 20 mM Tris-HCl (pH 7.4), 1 mM EDTA, 0.2 M NaCl overnight at 4°C. The DNAs were recovered from the eluate using Elutip-d[®] minicolumns and ethanol precipitated in the presence of glycogen. The DNAs were resus-

pended in formamide loading buffer, boiled and electrophoresed through denaturing 10% polyacrylamide gels.

Hydroxyl radical footprinting was performed just as the missing nucleoside assay described above, except that the DNA was treated with hydroxyl radicals after being bound by transposase.

Quantitation

All gels were visualized by phosphorimaging and quantitation was done using Molecular Dynamics ImageQuant software.

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