

Mutational Analysis of the Base Flipping Event Found in Tn5 Transposition*

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Brandon Ason and William S. Reznikoff‡

From the Department of Biochemistry, University of Wisconsin, Madison, Wisconsin 53706

This work identifies novel structure-function relationships between Tn5 transposase (Tnp) and its DNA recognition sequence. The Tn5 Tnp-DNA co-crystal structure revealed the protein-DNA contacts of the post-cleavage complex (Davies, D. R., Goryshin, I. Y., Reznikoff, W. S., and Rayment, I. (2000) *Science* 289, 77–85). One of the most striking features of this complex is the rotation of thymine 2 (T2) away from the DNA helix and into a pocket within the Tnp. This interaction appears similar to the “base flipping” phenomenon found in many DNA repair enzymes such as T4 endonuclease V and uracil DNA glycosylase (Roberts, R. J., and Cheng, X. (1998) *Annu. Rev. Biochem.* 67, 181–198). To study the biochemical significance of this phenomenon, we mutated the Tnp residues proposed to be involved in stabilizing this interaction and removed the T2 nucleotide to examine which steps in the transposition reaction require T2-Tnp interactions. From this work, we have determined that stacking interactions between T2 and Tnp are critical for efficient transposition *in vitro*. In addition, our results suggested that T2-Tnp interactions facilitate hairpin formation and hairpin resolution primarily through base stacking and that T2 plays a role in the alignment of the transposon DNA for strand transfer.

Mobile DNA elements share many similar characteristics. The proteins responsible for movement of these elements manifest common mechanistic features and contain considerable structural similarities in their catalytic domains. In the Tn5 system, Tn5 transposase (Tnp)¹ functions as a homodimer and catalyzes all of the steps involved in the movement of Tn5 from one location to another within a genome (3–5).

The model for Tn5 transposition is illustrated in Fig. 1. First, it is believed that each Tnp monomer binds to two separate recognition sequences, forming the initial contacts. The monomers then dimerize to form the synaptic complex (6). This complex brings the end sequences proximal to one another. In the synaptic complex, each Tnp monomer binds to both end sequences. The first set of contacts (cis contacts) are maintained predominately between the N-terminal domain and the

middle of one end sequence, whereas the second set of Tnp-DNA contacts (trans contacts) are formed primarily between the catalytic domain and the other end sequence (1). This complex is a prerequisite to cleavage, since residues near the trans Tnp-DNA contacts are responsible for strand cleavage (1, 7). The cleavage reaction begins as Tnp nicks the transferred DNA strand at the donor DNA-transposon junction, adjacent to position 1 of the end sequence, leaving a 3' OH group. This nick occurs through the nucleophilic attack by an activated water molecule coordinated by Mg²⁺. The 3' OH group on the transferred strand in turn acts as a nucleophile to cleave the complementary nontransferred DNA strand, forming a hairpin at the transposon end and releasing the donor DNA. Hydrolysis of the hairpin by another activated water molecule, coordinated in the same Tnp monomer, resolves the hairpin and results in the formation of transposon DNA competent for integration (8). The cleaved transposon DNA remains in a nucleoprotein complex called the post-cleavage synaptic complex. This complex binds to the target DNA, forming the target capture complex (9). The transposon DNA contains free 3' OH moieties on the transferred strands that attack the phosphodiester backbone of the target DNA in a staggered transesterification reaction termed strand transfer. A 9-bp stagger for the strand transfer step creates two complementary 9-base single-stranded DNA regions flanking the Tnp insertion. These single-stranded DNA regions are repaired by the cell DNA replication machinery, creating a 9-bp duplication in the target DNA (10).

The Tn5 Tnp-outside-end co-crystal structure was the first structural model solved for a reaction intermediate for any mobile DNA element (Fig. 2). This structure provided insight into the interactions of Tnp with DNA after the release of the transposon from the donor DNA and before integration into the new site (1). At this step, the Tnp points the ends of each strand in the DNA helix toward one another, giving the appearance of a DNA hairpin, a known intermediate of the preceding cleavage step. This distortion of the end of the DNA recognition sequence includes the rotation of the second nucleotide on the non-transferred strand (T2) away from the DNA helix and into a pocket within Tnp. This rotation of T2 is structurally similar to the base-flipping event described for many DNA repair enzymes. The Tnp-DNA structure corresponds to other flipped base co-crystal structures in that these structures contain aromatic amino acid residues in the binding pocket that stabilize the rotation of the base through base stacking (2). DNA base flipping is increasingly being reported in the literature, and it has been proposed to be a general feature of many DNA-metabolizing enzymes including recombinases and polymerases (11). The Tn5 Tnp structure is the first example of this phenomenon found in either transposases or integrases.

In this work, we addressed the significance of this feature for the transposition mechanism. We were particularly interested in determining if this flipped base influenced transposon cleav-

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‡ To whom correspondence should be addressed: Dept. of Biochemistry, University of Wisconsin-Madison, 433 Babcock Dr., Madison, WI 53706. Tel.: 608-262-3608; Fax: 608-262-3453; E-mail: reznikoff@biochem.wisc.edu.

¹ The abbreviations used are: Tnp, Tn5 transposase; Abasic, a d-spacer in place of thymine 2, removing the base at position 2; bp, base pair(s); cis, initial transposase DNA contacts; DEB, double end break; Hp, hairpin; Nick, strand nicking; nt, nucleotide; trans, the second set of transposase DNA contacts; T2, thymine 2.

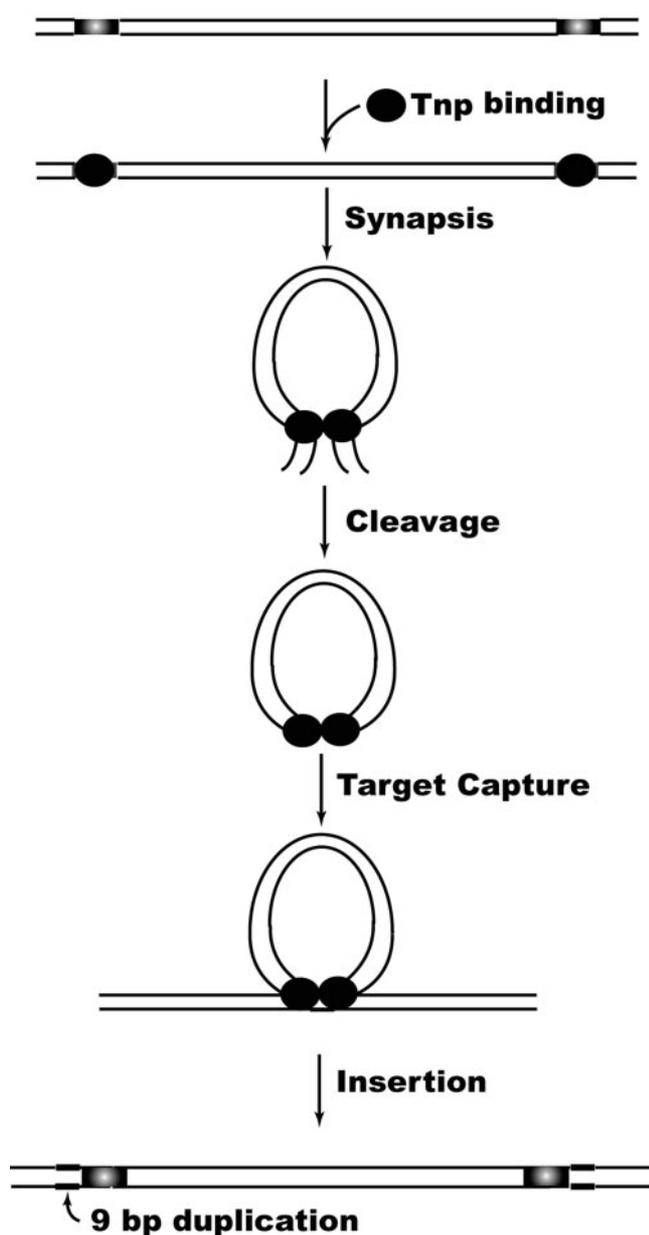


FIG. 1. **The molecular model for Tn5 transposition.** Tn5 transposition is believed to be initiated by Tnp monomers binding to two recognition sequences. The monomers subsequently dimerize, forming the synaptic complex. Double strand cleavage occurs at the ends of the recognition sequences, releasing the transposon from the donor DNA. Then, the post-cleavage synaptic complex binds to a site for insertion through a process termed target capture. Strand transfer then occurs with a 9-bp staggered attack into the insertion site and, with the aid of the replication machinery, generates a 9-bp duplication.

age and integration. We chose a mutational analysis, disrupting contacts between T2 and Tnp, to determine the role of base flipping in Tn5 transposition. In the Tn5 system, T2 was proposed to be stabilized away from the helix through stacking interactions with tryptophan 298 and a hydrogen bond to tyrosine 237 (Fig. 2) (1). Results are shown for protein mutations that alter the stacking interactions by changing the tryptophan to either a phenylalanine (Tnp W298F) or an alanine (Tnp W298A) and removal of the hydrogen bond by changing the tyrosine to a phenylalanine (Tnp Y237F). We have also examined the effect an abasic DNA substrate has on the transposition process by removing T2, generating an abasic site (Abasic). All of these changes were analyzed for their effects on the *in*

in vitro transposition reaction and for their effects on the individual steps of the transposition mechanism; that is, synapsis, cleavage, and strand transfer. Together, these experiments are intended to determine the role of this flipped base in the Tn5 system.

EXPERIMENTAL PROCEDURES

DNA Substrates—The short oligonucleotides used for these experiments were provided by Integrated DNA Technology. One μmol of each oligonucleotide was annealed to its complement by heating to 96 °C for 1 min followed by a decrease in temperature at 0.1 °C/s to 4 °C in 10 mM Tris-HCl, pH 8, and 10 mM NaCl. DNA substrates were monitored with ^{32}P . Double-stranded DNA fragments were 5' end-labeled using T4 polynucleotide kinase (New England Biolabs) and [γ - ^{32}P]ATP (Amersham Biosciences, Inc.). The labeled DNA was purified using the QIAquick nucleotide removal kit (Qiagen) and quantitated using gel electrophoresis and comparison to known molecular mass standards (Promega).

The sequence of the mosaic end, 60-bp DNA substrate was 5'-CTC AGT TCG AGC TCC CAA CAC TGT CTC TTA TAC ACA TCT TGA GTG AGT GAG CAT GCA TGT-3' and its complement. The sequence of the Abasic 60-bp DNA substrate was 5' CTC AGT TCG AGC TCC CAA CAC XGT CTC TTA TAC ACA TCT TGA GTG AGT GAG CAT GCA TGT 3', where X denotes the abasic site (d-spacer¹). The Abasic DNA was annealed to its complement that contained an A opposite the abasic site.

The sequence of the pre-cleaved mosaic end 40-bp DNA substrate was 5'-CTG TCT CTT ATA CAC ATC TTG AGT GAG TGA GCA TGC ATG T-3' and its complement. The sequence of the pre-cleaved Abasic 40-bp DNA substrate was 5'-CXG TCT CTT ATA CAC ATC TTG AGT GAG TGA GCA TGC ATG T-3'.

The sequence of the mosaic end hairpin was 5'-ACA TGC ATG CTC ACT CAC TCA AGA TGT GTA TAA GAG ACA GCT GTC TCT TAT ACA CAT CTT GAG TGA GTG AGC ATG CAT GT-3'. The sequence of the Abasic hairpin was 5'-ACA TGC ATG CTC ACT CAC TCA AGA TGT GTA TAA GAG ACA GCT XTC TCT TAT ACA CAT CTT GAG TGA GTG AGC ATG CAT GT-3', where X denotes the abasic deoxyribose sugar with an A opposite the abasic site.

Protein Purification and Mutagenesis—All Tnp mutant constructs were derived from the hyperactive Tnp background, E54K, M56A, and L372P. The E54K, M56A, and L372P mutant has an increased transposition frequency *in vitro* and is referred to as the positive control throughout this manuscript. Site-directed mutagenesis was performed using the QuikChange site-directed mutagenesis kit (Stratagene) and pGRTYB35 as the template plasmid. All protein mutants were purified as described previously (12). All constructs were sequenced to verify the absence of secondary mutations.

Synaptic Complex Assay—The synaptic complexes in this assay consist of two DNA fragments, 60 bp in length, each containing one mosaic end recognition sequence flanked by 20 bp of donor DNA and 21 bp of transposon DNA (Fig. 3A). The synaptic complexes were formed by incubating 300 nM Tnp with 20 nM DNA at 37 °C for 1 h in binding buffer (25 mM HEPES, pH 7.5, 100 mM potassium glutamate, 0.5 mM β -mercaptoethanol, 10 $\mu\text{g}/\text{ml}$ tRNA, and 0.25 mg/ml bovine serum albumin). After a 1-h incubation, a 10- μl aliquot of each reaction was mixed with 2 μl of 6 \times loading dye (Promega) and electrophoresed on a 10% native polyacrylamide gel at 300 V. The gels were subsequently dried, and DNA bands were visualized using a PhosphorImager.

In Vitro Transposition, Strand Cleavage, Hairpin Resolution, and Strand Transfer Assays—*In vitro* transposition and strand cleavage assays were carried out using the 60-bp DNA fragments. Hairpin resolution and strand transfer assays used the 80-base DNA hairpin and the 40-bp DNA substrates, respectively (Fig. 3A). All of these assays involved the incubation of 20 nM DNA with 300 nM Tnp at 37 °C in cleavage buffer (25 mM HEPES, pH 7.5, 100 mM potassium glutamate, 0.5 mM β -mercaptoethanol, 10 mM magnesium acetate, 10 $\mu\text{g}/\text{ml}$ tRNA, and 0.25 mg/ml bovine serum albumin). Reactions were stopped by removing 8- μl aliquots and mixing with an equal volume of 25 mM EDTA. The quenched reaction mixtures were subsequently phenol/chloroform-extracted and ethanol-precipitated. Pellets were resuspended in 4 μl of H_2O and 1 μl of FEXB (80% formamide, 10 mM EDTA, 1 mg/ml xylene cyanol, and 1 mg/ml bromophenol blue). *In vitro* transposition and strand transfer samples were loaded onto a 1% agarose gel. The gels were run at 7.3 V/cm for 1.5 h. Strand cleavage and hairpin resolution samples were boiled for 5 min and loaded onto a 12% denaturing polyacrylamide gel. The gels were run at 30 W for 45 min and subsequently dried. DNA bands in all assays were visualized using a

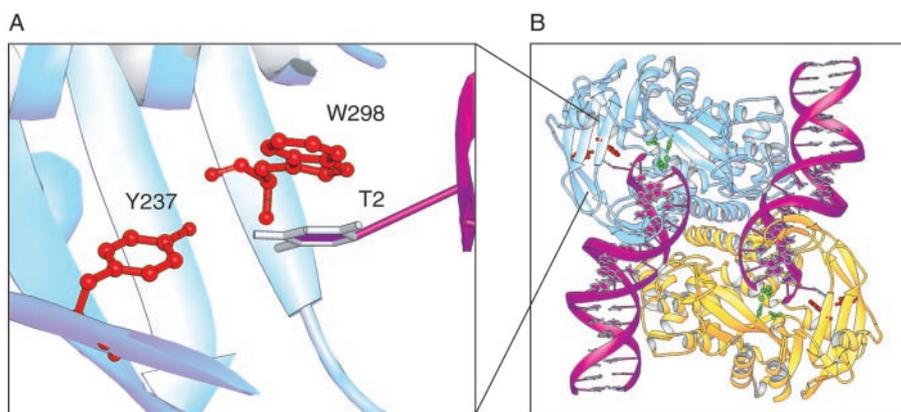


FIG. 2. **T2-Tnp interactions.** The Tn5 Tnp-outside-end co-crystal structure is shown. A, a close up view of T2-Tnp interactions illustrating the stacking interactions between Tnp Trp-298 and T2 as well as the hydrogen bond between Tnp Tyr-237 and T2. To examine how destabilization of these interactions affected transposition, stacking interactions were altered by removing T2, generating an abasic site (Abasic) or mutating the tryptophan to either an alanine (Tnp W298A) or a phenylalanine (Tnp W298F), and the hydrogen bond was disrupted by mutating the tyrosine to a phenylalanine (Tnp Y237F). B, this view illustrates the location of T2 in relation to the remainder of the recognition sequence and the Tnp. The residues involved in catalysis (Asp-97, Asp-188, and Glu-326) along with one of the two metals coordinated in catalysis (green) and the residues proposed to stabilize T2 (Trp-298 and Y237) (orange) are shown.

PhosphorImager. All assays were repeated a minimum of four times for each mutation.

Quantitation—Quantitation was performed using ImageQuant (Molecular Dynamics) software analysis of images obtained from a PhosphorImager phosphor screen. The intensity of each DNA fragment for synaptic complex formation, strand cleavage, and hairpin resolution assays was determined by measuring the percent intensity of each DNA fragment to the total intensity of all DNA fragments per lane. The percent incorporation for each DNA fragment in the *in vitro* transposition and strand transfer assays was determined by comparing the intensity of the sample to the intensity of the completed control reaction.

RESULTS

Stacking between T2 and Tnp Is Required for *in Vitro* Transposition—Each protein mutation and the Abasic DNA substrate were analyzed for their effects *in vitro* to determine whether the mutations that have been proposed to destabilize the flipped base alter the degree of *in vitro* transposition. Tn5 *in vitro* transposition was analyzed by monitoring the incorporation of radiolabeled transposon DNA into an unlabeled circular target. Short DNA fragments, 60 bp in length and each containing one Tnp recognition sequence, were used in this reaction (Fig. 3). The reaction was monitored by the incorporation of radiolabel from the transposon DNA into an unlabeled target DNA over time.

Results from these experiments are illustrated in Fig. 4. These results indicated that all mutations reduced the amount of transposition *in vitro* compared with the control. The largest reductions were seen with either the Tnp W298A mutant or the Abasic mutant recognition sequence; both reduced the level of transposition to ~1% of the transposition level for the control. Together, these results suggested that stacking interactions between Tnp Trp-298 and T2 play an important role in the transposition process. T2-Tnp interactions did not show an absolute requirement for either stacking to Tnp Trp298 specifically or hydrogen bonding to Tnp Tyr-237, since only modest reductions were seen with the Tnp W298F mutation and the Tnp Y237F mutation.

T2-Tnp Interactions Produced Slight Variations in Synaptic Complex Formation—Results from the *in vitro* transposition reactions suggested that mutations, which destabilized T2 in the flipped conformation, affected one or more steps of the transposition mechanism. To investigate if the reduction in transposition was due to a defect in synaptic complex formation, each mutation was analyzed for its effect on synapsis. The reactions were carried out in the absence of Mg^{2+} , thereby

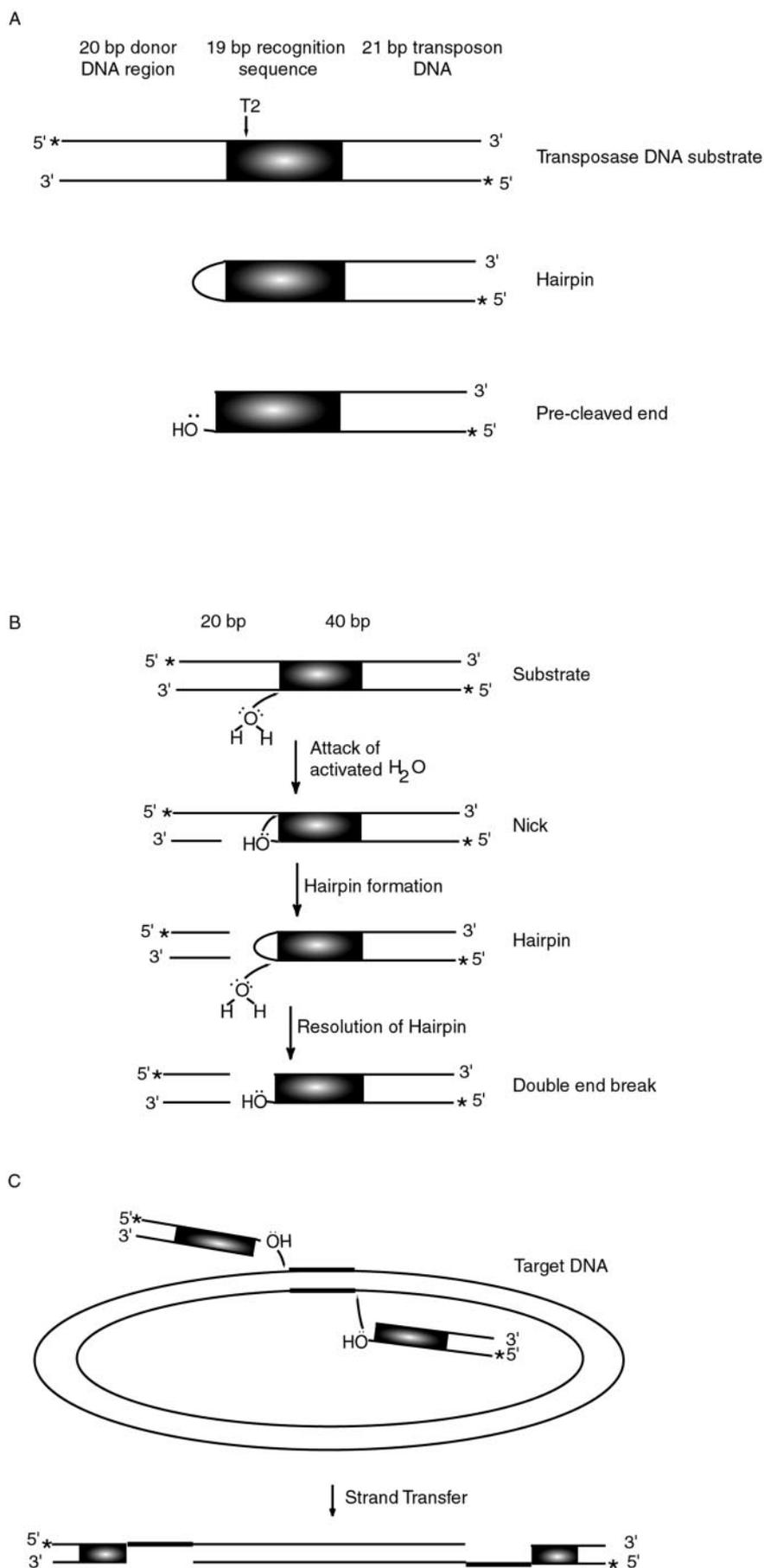
blocking cleavage and the remaining steps of the transposition process.

Results examining the impact of these mutations on synaptic complex formation are shown in Fig. 5. This assay indicated that only slight changes occurred in the amount of synaptic complexes formed for the protein mutations and the Abasic DNA substrate when compared with the control. There was a slight decrease in the amount of synaptic complexes formed for the Tnp W298A mutant, and there were slight increases for the other mutations, with the largest increase observed for the Abasic DNA substrate. This result with an abasic site at position 2 correlates with missing nucleoside data (8). These data indicated that removal of the second nucleoside enhanced transposase binding. It was proposed that removal of this nucleoside enhanced synaptic complex formation because it facilitated DNA bending. Likewise, the increase observed with Abasic DNA may also be due to an increase in the flexibility of the DNA strand. Because none of these results for synaptic complex formation can account for the reduction seen in the *in vitro* transposition assay, we believe that T2-Tnp interactions must influence another step or steps of the mechanism.

T2-Tnp Interactions Influenced Strand Cleavage—The mutations, which were designed to destabilize T2 in the flipped conformation, were shown to reduce the level of *in vitro* transposition with a minimal effect on synaptic complex formation. Therefore, we hypothesized that these mutations affected one or more of the subsequent steps in transposition, strand cleavage and strand transfer. To test the effect of the protein mutations and the Abasic DNA substrate on strand cleavage, the 60-bp radiolabeled DNA fragments, each containing one Tnp recognition sequence with or without a base at position 2, were incubated with either Tnp or the Tnp mutants in buffer containing Mg^{2+} , and the cleavage products were purified and separated on a denaturing polyacrylamide gel. Because both ends of the 60-bp substrate were labeled, we were able to follow the intermediates of the cleavage reaction (Fig. 3). The first step, strand nicking, was apparent through the disproportionate appearance of the 40-nucleotide (nt) fragment. The next step, hairpin formation, can be visualized by the appearance of both the 80- and 20-nt products. Complete cleavage can be seen with the appearance of equal intensities for the 40- and 20-nt products.

Results from the cleavage assays are shown in Fig. 6. These results illustrated that no single mutation completely blocked cleavage in the Tn5 system. However, results with the Tnp W298A mutation revealed that removal of the T2-Tnp stacking

FIG. 3. DNA structures and chemical steps within the Tnp assays. In each structure the 5' ends are labeled (*asterisks*). *A*, the transposon DNA fragment is 60 bp in length and contains the 19-bp transposase recognition sequence flanked by a 20-bp donor and a 21-bp transposon DNA region. This fragment is used in the synapsis, strand cleavage, and *in vitro* transposition assays. The hairpin consists of the 21-bp transposon DNA and the 19-bp recognition sequence, and it is used as a substrate for the hairpin resolution assay. The pre-cleaved end consists of the 21-bp transposon DNA and the 19-bp recognition sequence, and it is used in the strand transfer assay. *B*, the chemical steps in strand cleavage are shown. The nucleophilic attack by a water molecule within the active site nicks the DNA on the transferred strand. The 3' hydroxyl group generated by the nick acts as a nucleophile to attack the non-transferred strand, forming a hairpin intermediate. The hairpin is resolved by the nucleophilic attack of an additional water molecule within the active site, generating a blunt end cleavage product. Cleavage of the 60-bp DNA substrate produced a radiolabeled 40-nt product at the strand nicking and the double end break steps, whereas the radiolabeled 20-nt product is produced at hairpin formation. *C*, the chemical step in the strand transfer assay is shown. In this reaction, the 3' hydroxyls at the ends are used to attack the target plasmid, incorporating the radiolabel into the linearized plasmid.



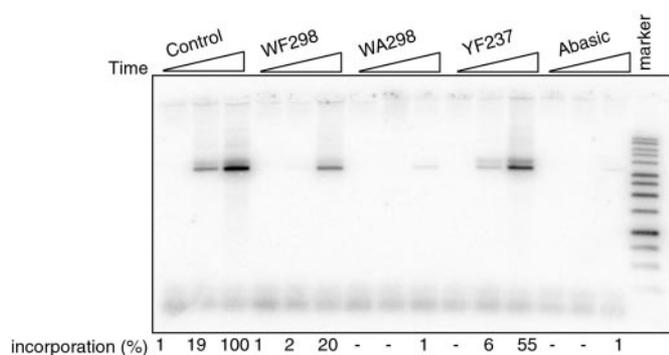


FIG. 4. **In vitro transposition.** Short radiolabeled DNA fragments, 60 bp, each containing one Tnp recognition sequence, were incubated with Tnp and a large unlabeled circular target DNA. *In vitro* transposition was monitored by visualizing the incorporation of radiolabel into the unlabeled target over time. Each reaction was stopped at 5, 40, and 120 min. The percent intensities, relative to the control, of the time points for each mutation and the Abasic DNA substrate is indicated below the respective lanes. The *top band* represents the reaction where only one end sequence is incorporated into the target DNA. This product consists of a relaxed circular labeled plasmid. The *bottom band* represents the reaction where both end sequences are being incorporated. This product consists of a linear labeled plasmid.

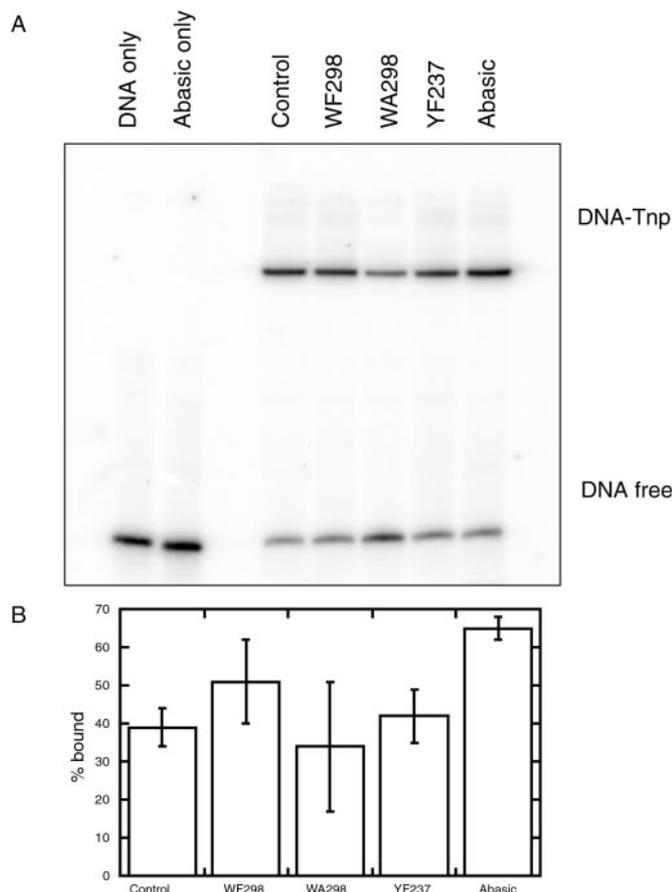


FIG. 5. **Synapsis assay.** A 60-bp DNA fragment was used to analyze how each mutation affected the degree of synaptic complex formation. *A*, synapsis was monitored by the shift generated from the binding of transposase to the radiolabeled DNA. Each shifted complex is composed of two transposase proteins bound to two DNA fragments (7). *B*, the percent of slower mobility DNA complexed with transposase for each mutation is shown. The average of four or more experiments is shown along with the S.D.

interactions impeded the reaction after strand nicking. This was observed by the greater abundance of the 40-nt Nick/DEB product compared with the 20-nt Hp formation/DEB product.

One would expect an equal abundance of these two species for the completed reaction, as observed for the control. This suggested that stabilization of the flipped base through stacking interactions was important for the step after strand nicking, hairpin formation, since a block at hairpin formation would lead to an accumulation of the nicked DNA product. This effect was also seen to a lesser extent with the Tnp W298F mutation. The results with the Abasic DNA did not show this effect. An accumulation of the 40-nt Nick/DEB product was not observed for the Abasic DNA substrate. Therefore, specific T2-Tnp interactions did not facilitate hairpin formation. However, removal of the base may have aided this step. For this hypothesis to be valid, one would expect that the Abasic DNA substrate would rescue the effect seen with the Tnp W298A mutant (see below).

Although the Abasic DNA substrate was able to form the DNA hairpin, it appeared that this DNA substrate reduced the amount of DNA hairpin resolved into blunt-ended cleavage products, as seen by the increase in intensity of both the 80- and 20-nt fragments compared with the 40-nt fragment. This suggested that contacts between T2 and Tnp aided in hairpin resolution. In this situation, we expect that Tnp would less efficiently resolve DNA hairpins containing an abasic site at this position (see below).

Rescue of the Tnp W298A Mutant with Abasic DNA—Strand cleavage results suggested that movement of T2 away from the DNA helix and not specific contacts to T2 aided in hairpin formation. For further support of this hypothesis, we tested the Tnp W298A ability to cleave the Abasic DNA. Results from this experiment illustrated that the accumulation of the 40-nt Nick/DEB product observed for Tnp W298A did not occur when the Abasic DNA substrate was used as a substrate for Tnp W298A (Fig. 7). These results indicated that the T2 base was required for the accumulation of excess nicked product for Tnp W298A. Because Abasic DNA rescued this mutant, these results demonstrated that the accumulation of excess nicked product seen for Tnp W298A was due to a reduced ability to remove the base from the control DNA helix through stacking interactions. Together, these data provided additional support for a mechanism in which the removal of the T2 base, not specific contacts to the T2 base, aided in hairpin formation.

T2 Aids in Hairpin Resolution—The cleavage experiments with the Abasic DNA substrate suggested that T2 facilitates hairpin resolution, as observed by the accumulation of the 80-nt and the 20-nt fragments compared with the 40-nt fragment. For further support, we tested the ability of Tnp to cleave a DNA hairpin with and without a nucleotide at position 2. Results from these experiments revealed that removal of T2 reduced the ability of transposase to react with the hairpin, leading to half the accumulation of cleavage product with an Abasic DNA substrate compared with the control (Fig. 8). These results provided further support for T2 playing a direct role in hairpin resolution.

T2-Tnp Interactions Influenced Strand Transfer—*In vitro* transposition results underscored the significance of stacking interactions between T2 and Tnp Trp-298 for transposition based on the reduction observed for both Tnp W298A and Abasic DNA. Both reduced transposition levels in this assay to 1% of the control. However, strand cleavage results indicated that disruption of stacking interactions hindered cleavage, but they did not sufficiently block this step of the mechanism to account for the level of reduction seen for transposition *in vitro*. Therefore, we speculated that T2 also played a role in the last step catalyzed by Tnp, strand transfer. The strand transfer reaction was isolated from the preceding steps through the use of pre-cleaved DNA fragments (Fig. 3A).

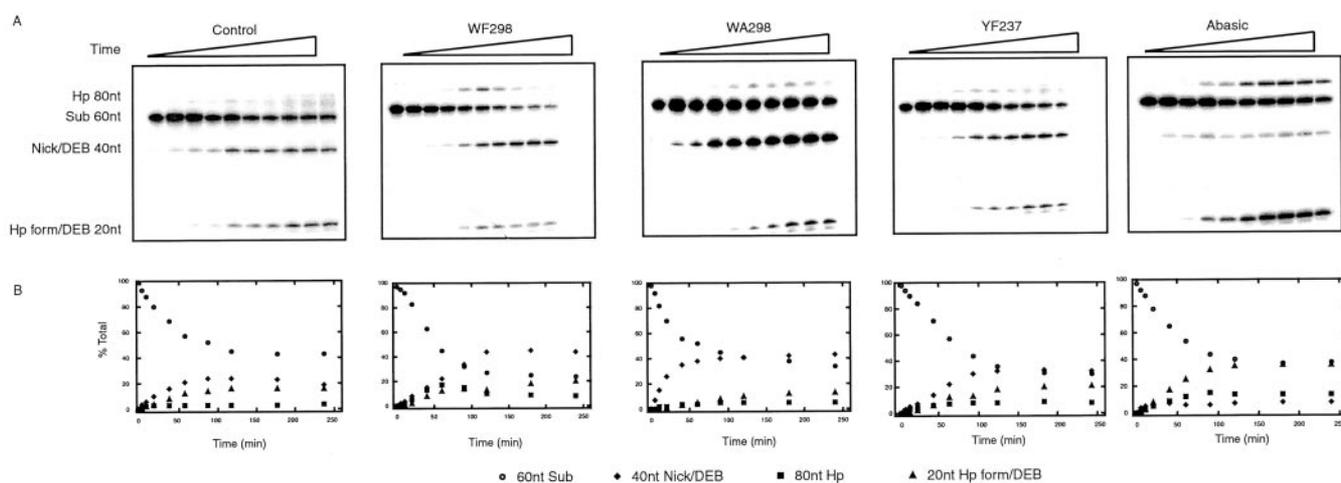


FIG. 6. **Strand cleavage.** The ability to proceed through the cleavage step was assessed for each protein mutation and the Abasic DNA substrate. Reactions were carried out under synapsis conditions with the addition of magnesium. *A*, reactions were stopped by the addition of an equal volume of 25 mM EDTA. The samples were extracted with phenol/chloroform, precipitated with ethanol and then loaded onto a 12% denaturing polyacrylamide gel. The sizes for the substrates, intermediates, and reaction products are indicated, where *Sub* represents the DNA substrate, *Hp* the hairpin intermediate, *Nick/DEB* the product of the initial strand nicking as well as the double end break, and *Hp form/DEB* the product of hairpin formation and the double end break. *B*, a graphical representation of the time course is shown.

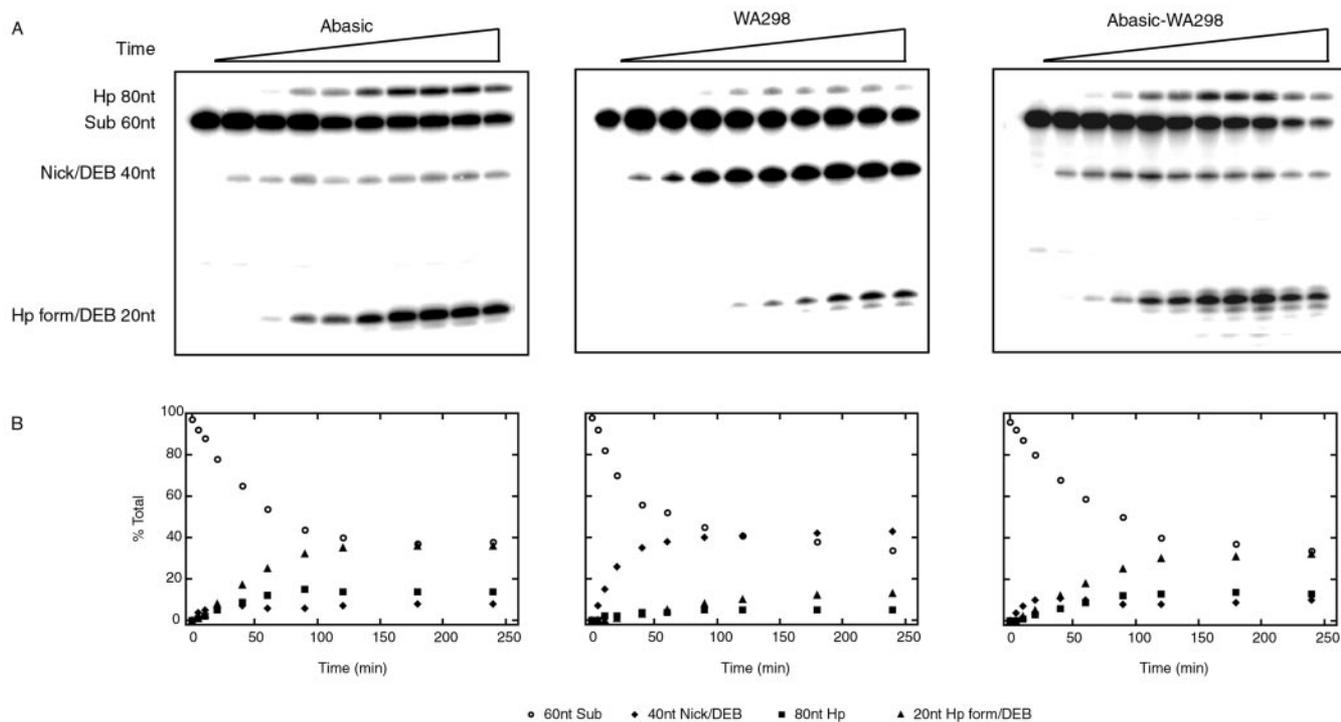


FIG. 7. **Tnp W298A rescue experiment.** To provide support for the Tnp W298 role in removing T2 out of the DNA helix for hairpin formation, we analyzed the cleavage pattern of Tnp W298A with and without a nucleotide at position 2. Reactions were carried out and treated as previously described for the cleavage experiments. The nomenclature is also the same as the ones used in Fig. 6. *B*, the graphical representation of the time course.

Results from these experiments revealed that each protein mutation and the Abasic DNA substrate reduced the level of strand transfer when compared with the control (Fig. 9). Although no single mutation completely blocked strand transfer, all of the mutants reduced the level of strand transfer by 63% or greater, with the greatest reduction seen with Tnp W298A. It is important to note that post-cleavage synaptic complex formation was not affected for any of these mutations except Tnp W298A. This mutation exhibited a slight reduction in the level of synaptic complexes formed (data not shown). Therefore, the level of strand transfer for this mutation is a combination of a reduction in the ability to form pre-cleaved synaptic complexes and the ability to perform strand transfer.

DISCUSSION

The results from this work have identified the steps within the transposition mechanism that are influenced by T2-Tnp interactions. The rotation of T2 away from the DNA helix allows for a higher specificity of interaction between T2 and Tnp, since this conformation exposes the nucleotide to a larger surface within Tnp. This increase in specificity for T2-Tnp interactions would favor tighter control over the location of the end of the non-transferred strand during the reaction. In this manuscript, we have identified that T2-Tnp interactions aid in the cleavage and strand transfer steps of the reaction. How these interactions facilitate these processes are discussed below.

Stacking Interactions Facilitate Hairpin Formation and Hairpin Resolution—Interactions between Tnp and T2 itself did not facilitate hairpin formation, but removal of the DNA base from the helix, either by flipping or an abasic site, acted as

a stimulus for hairpin formation. This was supported by two observations. First, the nicked DNA product accumulated during strand cleavage for the Tnp W298A mutant. Second, the Tnp W298A mutant was able to form the DNA hairpin with the Abasic DNA substrate at higher levels than with the control DNA substrate.

Stacking between Tnp Trp-298 and T2 would presumably aid in hairpin formation through the distortion within the DNA helix generated by the interaction of Tnp Trp-298 with T2. This distortion would facilitate forming the DNA hairpin by removing T2 from the helix, providing space for the necessary tight turn along the phosphate backbone and relieving strain. A flipped base at this position is one of several ways that have been observed to relieve the strain induced by such sharp turns along the phosphate backbone, and it appears that Tnp Trp-298 in the Tn5 system facilitates such a mechanism (13). Mutations made on the analogous tryptophan residue in the Tn10 system exhibited a similar result, where substitution of the tryptophan by either leucine or valine led to an accumulation of the nicked DNA product (14). Therefore, this result was not limited to the Tn5 system, and thus, it may be a general phenomenon for other mobile DNA elements that transpose via a hairpin intermediate.

Although T2 is not required for hairpin formation, it appeared to play a role in hairpin resolution. This was shown by the accumulation of the hairpin intermediate for the cleavage reaction using the Abasic DNA substrate. Also, in the hairpin resolution experiment the Abasic DNA reacted half as well as the control. Presumably, a base was needed at this position for proper alignment of the phosphate backbone, metals, and water molecules within the active site, whereas the strand nicking and hairpin formation steps did not require this level of contact with the non-transferred strand.

T2-Tnp Contacts Facilitate Strand Transfer—Every mutation intended to perturb T2-Tnp contacts reduced the amount of strand transfer. Based on the level of reduction seen for all of the mutations, this step held the highest specificity for interaction when compared with the other steps that were analyzed, and it suggested that these interactions facilitated strand transfer with higher specificity for interactions between T2 and Tnp than the preceding steps. It is interesting to note that T2 is located on the non-transferred strand and that throughout the Tn5 mechanism none of the nucleophiles responsible for catalysis are located on the non-transferred strand. These results suggest that the non-transferred strand aids the last catalytic step of the transposition mechanism. At this step, three DNA helices must come into contact with two Tnp active sites that carry out all of the catalytic steps throughout the mechanism. Therefore, the crowded nature of this region during strand transfer would necessitate tight control over the

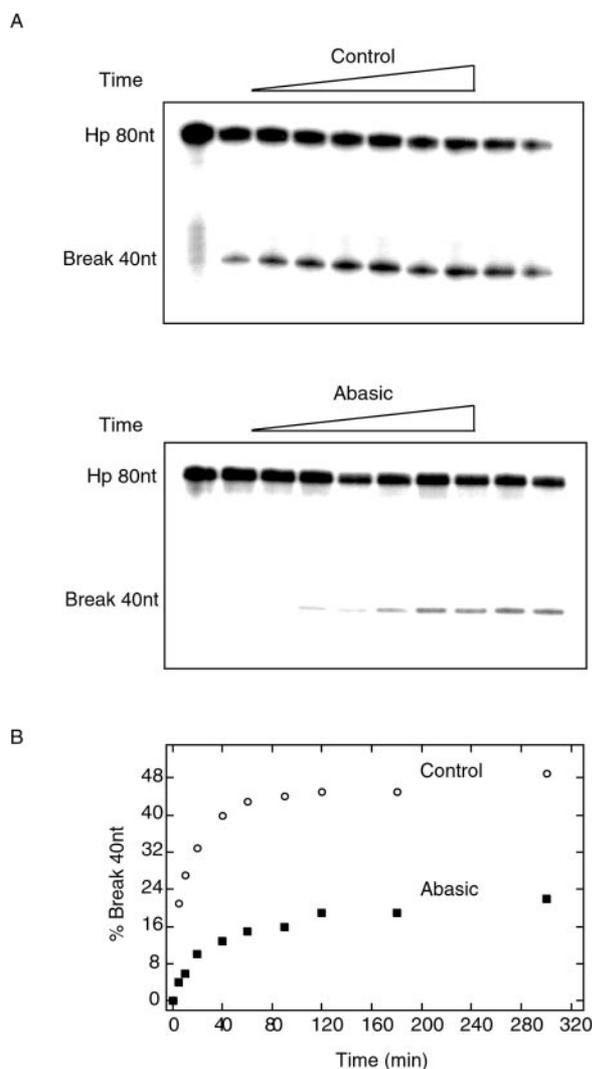
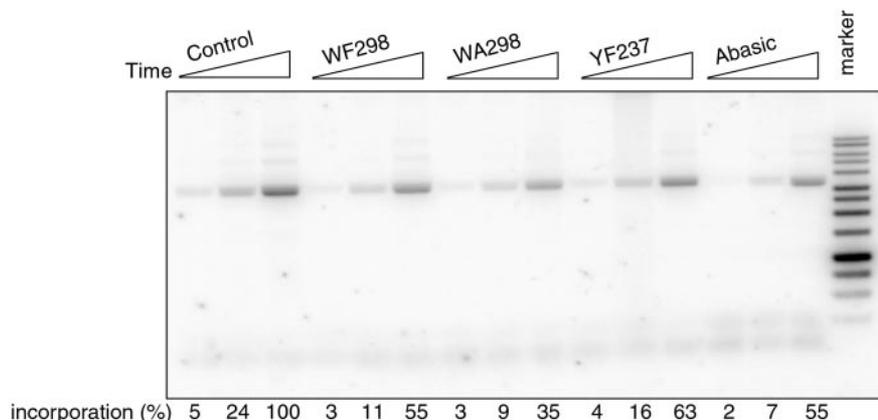


FIG. 8. **Hairpin resolution.** The ability of Tnp to resolve the DNA hairpin with or without a base at position 2 was measured to provide support for the role of T2 during the hairpin resolution step. *A*, reaction conditions and analysis were performed as described for the cleavage experiments. The nucleotide length of the DNA hairpin (*Hp*) and the cleavage product (*Break*) are shown. *B*, a graphical representation comparing the accumulation of cleavage products for the two DNA substrates is shown.

FIG. 9. **Strand transfer.** A 40-bp pre-cleaved DNA substrate was used to isolate strand transfer from the preceding steps in transposition. Strand transfer was monitored as described in "Experimental Procedures." Each reaction was stopped at 5, 40 and 120 min. The percent incorporation relative to the positive control for the final time point is listed in each lane, respectively.



location of each DNA helix. Tnp-DNA high specificity contacts would be one possible solution to control the location of the DNA strands. This has been previously characterized in the Tn10 system where mutations at the ends of the Tn10 recognition sequence of the non-transferred strand have been shown to block strand transfer (15). In addition, previous mutations in Tn5 Tnp that disrupted contacts to the non-transferred strand have also shown a reduction in strand transfer ability, providing further support for the generality of this feature.²

Model for T2 in the Flipped Conformation—This manuscript has discussed the role of stabilization of the flipped base in Tn5 transposition. Because the nature of T2-Tnp contacts appeared to change from the early steps of the mechanism to the subsequent steps, it allows us to speculate on where within the mechanism base flipping occurs. Existing structural information provides evidence for this event in the post-cleavage synaptic complex. This structure is situated between the two chemical steps, hairpin resolution and strand transfer. Hairpin resolution is the first chemical step that is affected by the removal of the thymine base. Beginning with this step, the Abasic DNA affected this and all subsequent steps (Fig. 8). We speculate that at these steps, the base is flipped away from the DNA helix and into the pocket within Tnp. In these latter steps, T2 presumably acts to position the transposon DNA in the proper orientation for catalysis during the late stages of transposition.

Working backwards through the preceding steps, we believe that T2 is also flipped away from the helix at hairpin formation. At hairpin formation, T2-Tnp interactions removed the base from the DNA helix presumably to relieve strain. Whether base flipping is initiated at a step earlier than hairpin formation is not as clear. During synapsis and strand nicking, contacts to T2 did not appear to play a significant role, since no significant effect is observed by any of these mutations at either of these steps. Synaptic complex formation has been previously reported to distort the DNA helix from position -3 to 2 (8, 16). However, based on the lack of evidence for T2-Tnp interactions, we believe that this distortion is not dependent on T2-Tnp interactions, and it does not resemble the T2 rotation found in the post-cleavage synaptic complex.

In conclusion, contacts between T2 and Tnp facilitate both

cleavage and strand transfer. The greatest reductions in strand cleavage and strand transfer were observed for both the Tnp W298A mutation and the Abasic DNA substrate. For both changes, the combined reductions in strand cleavage and strand transfer appear, at least qualitatively, to be cumulative. The combined reduction for these two steps caused the significant drop in transposition *in vitro*.

From these experiments, a clearer picture has emerged in regard to the significance of flipping T2 in transposition. To date, Tn5 is the only transposition or integration system known to flip a nucleotide during its mechanism. It is highly probable that this event is conserved throughout this family of proteins, and based on the similarity in chemical mechanisms between Tn5 and Tn10 transposases, it is likely that the role of the flipped base is identical for these two systems. Furthermore, the importance of the flipped base for the hairpin mechanism suggests that this phenomenon may be expanded to include all mobile DNA elements that undergo a hairpin intermediate.

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