Tn5 Transposase Active Site Mutations Suggest Position of Donor Backbone DNA in Synaptic Complex*

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Tn5 transposase (Tnp), a 53.3-kDa protein, enables the movement of transposon Tn5 by a conservative mechanism. Within the context of a protein and DNA synaptic complex, a single Tnp molecule catalyzes four sequential DNA breaking and joining reactions at the end of a single transposon. The three amino acids of the DDE motif (Asp-97, Asp-188, and Glu-326), which are conserved among transposases and retroviral integrases, have been shown previously to be absolutely required for all catalytic steps. To probe the effect of active site geometry on the ability to form synaptic complexes and perform catalysis, single mutations at each position of the DDE motif were constructed. The aspartates were changed to glutamates, and the glutamate was changed to an aspartate. These mutants were studied by performing *in vitro* binding assays using short oligonucleotide substrates simulating the natural substrates for the synaptic complex formation and subsequent transposition steps. The results indicate that the aspartate to glutamate mutations restrict synaptic complex formation with substrates resembling the natural transposon prior to transferred strand nicking. This suggests a structural model in which the donor backbone DNA, prior to nicking, occupies the same space that is invaded by the longer side chains present in the aspartate to glutamate mutants. Additionally, catalytic assays support the previous proposal that the active site coordinates two divalent metal ions.

Tn5 transposase (Tnp)[1] is the protein responsible for the mobility of the Tn5 transposon (1). Movement of the transposon occurs via a conservative "cut and paste" mechanism that is mediated by Tnp and 19-bp inverted terminal repeats. Transposition involves the complete excision of the transposon from donor DNA followed by insertion into a new location (Fig. 1). The excision and insertion involve four catalytic steps at the transposon ends: transferred strand nicking, hairpin formation, hairpin resolution, and strand transfer into target DNA (2). These reactions occur within a protein-DNA complex, termed a synaptic complex, consisting of two Tnp molecules and two end sequences (3, 4). Single Tnp molecules bound to single end sequences are not catalytically competent, thus restricting the transposition reactions to synaptic complexes (4, 5).

The naturally isolated Tn5 transposon transposes at a very low frequency, most likely because frequent transposition would be detrimental to the survival of the cell, constituting a selective disadvantage to a highly active transposon. The low level of transposition can be attributed to both the low activity of the Tnp protein and the suboptimal 19-bp end sequences. The low level of protein activity has been increased by the introduction of two point mutations, E54K and L372P (5, 6). This mutant is termed EK/LP and is the standard for this and other studies of Tn5 Tnp. The rate of transposition is further increased by replacing the naturally occurring end sequences with a 19-bp site termed the mosaic end (ME) (7). The combination of Tnp EK/LP and ME sequences has allowed the development of an efficient series of *in vitro* assays used to study the individual steps of the transposition process via gel shift (3) and catalytic assays (2, 6, 8, 9).

Each Tnp molecule possesses a single active site, responsible for carrying out all four catalytic steps, at a single 19-bp end (4, 10). In the first and third steps, transferred strand nicking and hairpin resolution, a water molecule acts as a nucleophile to hydrolyze a DNA phosphate bond, whereas in the second and fourth reactions, hairpin formation and strand transfer, the 3′-hydroxyl group of the transferred strand acts as the nucleophile (Fig. 1). The active site contains a triad of acidic residues (Asp-97, Asp-188, and Glu-326) termed the DDE motif. This motif is believed to be conserved among all transposases and retroviral integrases. The DDE residues serve to coordinate divalent metal ions that are required for catalysis (11–14). A previous study has suggested that Asp-97 and Glu-326 contribute to the coordination of one metal ion, whereas Asp-97 and Asp-188 coordinate a second ion (15). All three of the DDE residues are required for all four catalytic steps of transposition (9).

In this study point mutations were introduced at each of the DDE residues in the EK/LP background, creating three separate single mutants. These mutations consist of changing the two aspartates to glutamates and the glutamate to an aspartate, yielding D97E, D188E, and E326D. These alterations were chosen because they leave the catalytically essential carboxyl groups in place at each of the three positions, while slightly altering their spatial location within the active site. By studying the ability of these mutants to form paired ends complexes (PECs), the equivalent of synaptic complexes with short oligonucleotide substrates, we were able to predict the spatial position assumed by the nontransposon DNA adjacent to the transposon end sequences, termed donor backbone (dbb), in synaptic complexes prior to the first catalytic step of transposition, transferred strand nicking. Additionally, use of these mutants in catalytic studies lends support to the previous proposal that the DDE motif serves to coordinate two divalent metal ions in the active site (15). To our knowledge, no studies of similar chemically conservative mutations of the DDE motif
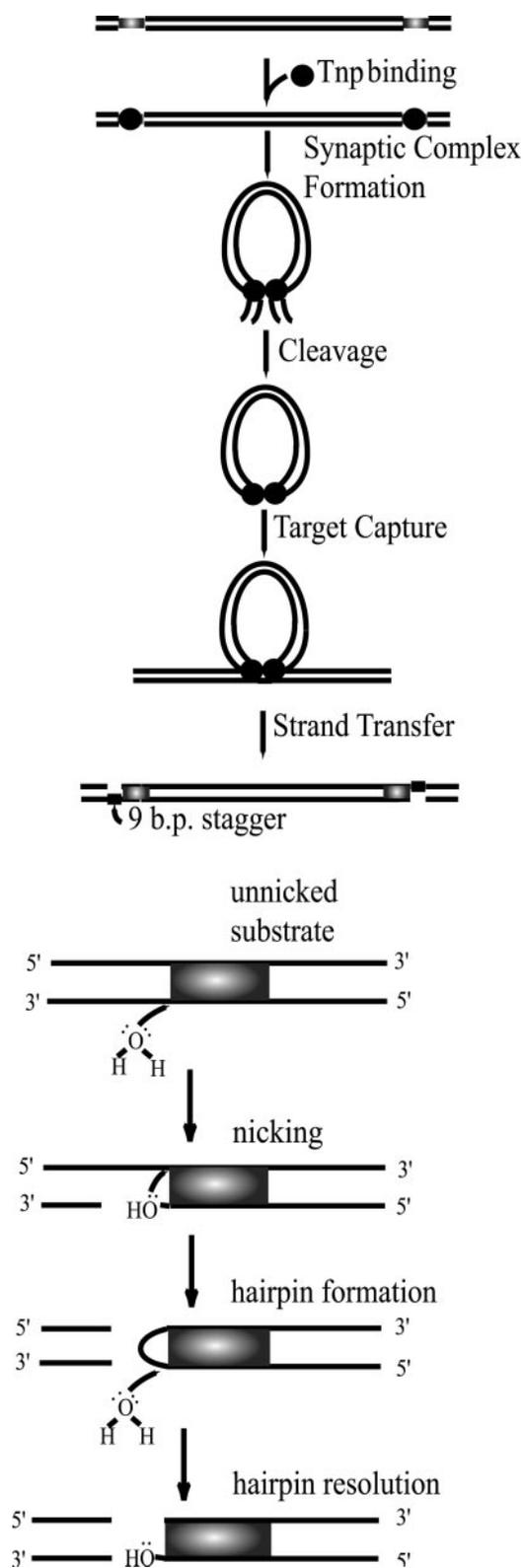
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[1] The abbreviations used are: Tnp, transposase; EK/LP, mutations E54K and L372P; ME, mosaic end; PEC, paired ends complex; dbb, donor backbone.

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in other transposases or retroviral integrases have been reported in the literature.

EXPERIMENTAL PROCEDURES

Cultures—All bacterial cultures for cloning and protein purification were grown in Luria broth (16). Ampicillin was purchased from Sigma and added to agar or liquid media at 100 μg/ml. Restriction enzymes were purchased from New England Biolabs and Promega. T4 DNA ligase was purchased from Promega. *Pfu* polymerase was purchased from Stratagene. T4 DNA polynucleotide kinase was purchased from New England Biolabs. Oligonucleotides were purchased from Integrated DNA Technology. Sequencing was performed using the PerkinElmer "Big Dye" kit with the aid of the University of Wisconsin-Madison Biotechnology Center.

Site-directed Mutagenesis—The plasmid pRZPET2, which contains the hyperactive EK/LP Tn5 *tnp* gene (6), was used as a template for site-directed mutagenesis using the single end overlap procedure (17). PCR products bearing the desired mutations were cloned into pRZ-PET2. D97E was cloned at the *Xba*I and *Not*I sites, D188E was cloned at the *Not*I and *Nhe*I sites, and E326D was cloned at the *Nhe*I and *Hind* III sites. The constructs for protein expression were created by swapping the mutated regions from the pRZPET2 derived plasmids into the expression vector pGRTYB35, which encodes for the EK/LP Tn5 Tnp as a carboxyl-terminal fusion to intein and chitin binding domains as described previously (2). The pRZPET2 plasmids containing the D97E, D188E, and E326D mutations were digested with *Bgl*II, and the overhangs filled in with *Pfu* followed by digestion with *Xba*I. The fragments containing the mutated *tnp* genes were cloned into the pGRTYB35 *Sma*I and *Xba*I sites to yield pWSR5021 (D97E), pWSR5022 (D188E), and pWSR5018 (E326D). The presence of mutations was confirmed by DNA sequencing.

Bacterial Strains—Expression of Tnp mutant proteins for purification was performed in *Escherichia coli* strain ER2566 (*F*[λ−]*fhuA2 [lon] ompT lacZ::T7 gene1 gal sulA11 Δ(mcrC-mrr)114::IS10 R(mcr-73:: miniTn10-TetS)2 R(zgb-210::Tn10)(TetS) endA1 [dcm]*) (New England Biolabs). All plasmid propagation utilized DH5α (*endA1 hsdR17(r*$_k^-$ *m*$_k^+$*) glnV44 thi-1 recA1 gyrA (Nal*[r]*) relA1 Δ(lacIZYA-argF)U169 deoR [φ80 dlacΔ(lacZ)M15]*) as a host strain.

Purification of Proteins and Preparation of DNA Substrates—Purification of Tnp EK/LP as well as mutant proteins was performed using the IMPACT T7 system from New England Biolabs. The concentrations of proteins were determined following the method of Bradford (18) using a BSA standard. Protein yields were between 1 and 2 mg of Tnp/liter of cells. The purity of the Tnp preparations was determined by SDS-PAGE analysis and Coomassie staining. No bands other than that for Tnp were observed. It has previously been shown that this method of protein purification gives very little nuclease contamination (2). Substrates for PEC and catalytic assays were prepared via ^{32}P labeling of the 5′-ends of oligonucleotides with T4 DNA polynucleotide kinase followed by annealing of appropriate oligonucleotides (2, 3) and subsequent cleaning up using a Qiagen nucleotide removal kit.

The following oligonucleotides were used for the unnicked PEC formation and 3′ nicking assays (ME shown in bold): 5′-CTCAGTTCGA-GCTCCCAACA**CTGTCTCTTATACACATCT**TGAGTGAGTGAGCA-TGCATGT-3′ (nontransferred strand) and 5′-ACATGCATGCTCACTC-ACTCA**AGATGTGTATAAGAGACAG**TGTTGGGAGCTCGAACTGA-G-3′ (transferred strand).

For prenicked PEC formation and hairpin formation assays, the nontransferred strand was as shown above, and the transferred strand oligonucleotide was replaced by the following two oligonucleotides: 5′-TGTTGGCAGCTCGAACTGAG-3′ (donor backbone compliment) and 5′-ACATGCATGCTCACTCACTCA**AGATGTGTATAAGAGACAG**-3′ (ME and transposon DNA compliment).

The following oligonucleotide was used for hairpin PEC formation and hairpin resolution assays: 5′-CACGTGTTG**ACATGTGTATAAG-AGACAGCTGTCTCTTATACACATGT**CAA (hairpin loop underlined).

The following oligonucleotide, annealed to the 40-base oligonucleotide listed above as the transferred strand of the prenicked substrate, constitutes the precleaved substrate used for PEC formation and strand transfer assays: 5′-**CTGTCTCTTATACACATGT**TGAGTGAGTGAG-CATGCATGT-3′.

In Vitro Catalytic Reactions—All catalytic reactions were performed in 10-μl reaction volumes containing 235 nM Tnp, 0.1 M potassium glutamate, 25 mM HEPES, pH 7.5, 50 μg/ml bovine serum albumin, 1 mM β-mercaptoethanol, 1 mM MnCl$_2$, and 75 μg/ml tRNA. DNA substrates were added to a concentration of 25 nM. Reactions were incu-

FIG. 1. *A*, conservative "cut and paste" transposition. Tnp monomers bind to each DNA end sequence and then associate to form the synaptic complex. Within this complex Tnp catalyzes the three steps that cleave the transposon out of donor backbone DNA: transferred strand nicking, hairpin formation, and hairpin resolution. The excised transposon then undergoes target capture followed by the catalytic strand transfer step, leading to the covalent linkage of the 3′-ends of the transposon to target DNA in a staggered fashion. *B*, three-step cleavage mechanism. Tnp activates a water molecule that attacks the 3′-end of the transferred strand. The exposed hydroxyl then acts as a nucleophile to attack the 5′-phosphate of the nontransferred strand to form a hairpin. Tnp then uses another water molecule to resolve the hairpin, resulting in blunt-end cleavage.

bated at 37 °C for 1 h prior to denaturing gel electrophoresis.

Sample Preparation for Denaturing Gel Electrophoresis—Each sample underwent phenol/chloroform extraction to remove transposase. The DNA was then ethanol-precipitated, dried, and resuspended in 1× formamide dye. After samples were boiled for 3 min, 5 μl of each sample was loaded onto a prewarmed 15% denaturing polyacrylamide gel and electrophoresed at 28 W constant power (2). The gel was then dried and visualized with a PhosphorImager. Radiolabeled DNA was quantitated using ImageQuant software from Amersham Biosciences. Catalytic activity is presented as the percentage of total counts per lane running at the positions expected for reaction products. All values represent the average of three independent reactions.

PEC Formation Assays—PEC assays were performed in 10-μl reaction volumes containing 235 nM Tnp, 0.1 M potassium glutamate, 25 mM HEPES, pH 7.5, 50 μg/ml bovine serum albumin, 1 mM β-mercaptoethanol, and 75 μg/ml tRNA. DNA substrates were added to a concentration of 25 nM. Reactions were incubated at 37 °C for 1 h prior to addition of glycerol loading dye. Samples were electrophoresed in an 8% native polyacrylamide gel at 300 V and otherwise handled as described previously (3) prior to drying and visualization with a PhosphorImager. Radiolabeled DNA was quantitated using ImageQuant software. PEC formation is presented as the percentage of total counts per lane shifted as a result of decreased PEC mobility. All values represent the average of three independent reactions.

RESULTS

Three single mutants were constructed in the EK/LP background, a hyperactive version of Tnp containing mutations E54K and L372P. These mutations were made at the catalytically critical DDE motif residues. The substitution mutations (D97E, D188E, and E326D) are chemically conservative, differing by only one carbon atom in the length of the side chain. Using these mutants, which have slightly perturbed active site geometry, we were able to probe the spatial requirements for formation of paired ends complexes and catalytic activity in Tn5 transposition. The ability of each of the three active site mutants to form PECs with oligonucleotides simulating the natural substrate at all steps of transposition and to catalyze the three reactions comprising cleavage of the transposon from donor backbone (dbb) DNA was tested *in vitro*.

It is possible to examine the individual steps of the transposition process in isolation by approximating the naturally occurring DNA substrates at different stages of the transposition mechanism using short radiolabeled oligonucleotides containing the 19-bp ME sequence, which is recognized by Tnp and defines the end of the transposon. Incubation of DNA substrates with Tnp in the absence of divalent metal ions, required for catalytic activity, allows the formation of PECs containing two DNA molecules and two Tnp molecules (3). These PECs are analogous to the synaptic complexes that are the context of transposition. The PECs can be distinguished from free DNA by the decreased mobility of complexes in native polyacrylamide electrophoresis. Because coordination of divalent metal ions is required for catalytic activity, PEC assembly represents the ability of Tnp to form the nucleoprotein complexes within which the transposition reactions occur but not the reactions themselves. Incubation of Tnp with the same radiolabeled oligonucleotides in the presence of divalent metal ions results in Tnp-mediated processing of substrate DNA. Denaturing polyacrylamide electrophoresis of the products allows an analysis of the catalytic reactions (2).

Unnicked Substrate—An unnicked substrate analogous to naturally occurring Tn5 DNA, containing both transposon DNA with an embedded ME sequence and dbb DNA, was used to investigate PEC formation and transferred strand nicking. This 60-bp substrate was radiolabeled with ^{32}P at the 5'-end of the transferred strand. PEC formation was investigated by incubating the unnicked substrate with Tnp and determining the percentage of DNA shifted on a native gel because of decreased PEC mobility (Fig. 2). Two of the mutants, D97E and
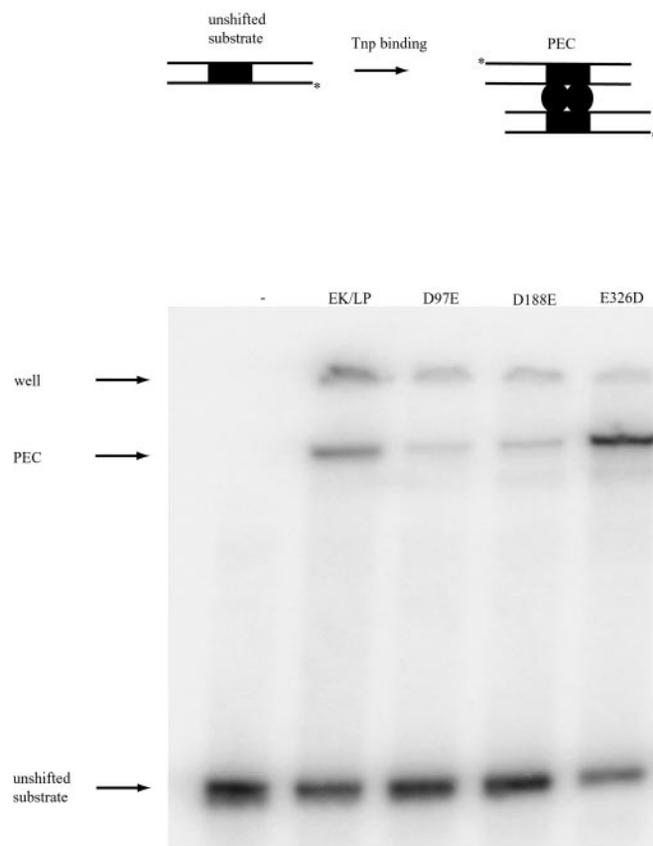

FIG. 2. **PEC formation assay with unnicked substrate**. The efficiency of each mutant to form PECs with short radiolabeled oligonucleotides was determined by gel shift assays. Incubation of Tnp with unnicked DNA substrate results in the formation of PECs that have decreased mobility in a native polyacrylamide gel. The diagram (*top*) graphically depicts the complexes that are formed, and an example is shown (*bottom*) of the shifts produced by the three DDE mutants, EK/LP, and a no-protein negative control (−) as visualized on a native gel.

D188E, show a marked decrease in the ability to form PECs with the unnicked substrate. Without replacing the chemical group at either position, a carboxyl, the change from aspartate to glutamate in both mutants has the effect of increasing the length of the respective amino acid side chains by one additional carbon atom. This extends the carboxyl group by about 1.5 angstroms. Both of these mutants show a severalfold decrease in the ability to bind the unnicked substrate. D97E forms PECs with the unnicked substrate 30% as efficiently as EK/LP, whereas D188E forms PEC only 22% as well. The opposite change in mutant E326D, which withdraws the carboxyl group by one carbon atom by replacing the glutamate with an aspartate, shows no decrease in PEC formation ability and actually seems to increase preference for the unnicked substrate, forming PECs 170% as efficiently as EK/LP (Table I).

The activity of the first catalytic step of transposition, transferred strand nicking, was investigated by incubating the unnicked substrate with Tnp and Mn^{2+}. Introduction of a nick by Tnp produces a labeled 40-mer. Those nicked molecules that undergo hairpin formation result in a labeled 80-mer. Finally, those hairpins that are resolved produce a labeled 40-mer (Fig. 3). Therefore, the amount of DNA that undergoes transferred strand nicking can be determined by quantitating the percentage of total DNA that migrates on a denaturing gel at both the 40-mer and 80-mer positions. The D97E mutant shows no ability to nick the substrate, whereas both D188E and E326D show decreased nicking, performing the reaction 9 and 34% as

TABLE I
PEC formation with DDE mutants

The ability of DDE mutants to form PECs was tested with un-nicked, pre-nicked, hairpin, and pre-cleaved substrates. Values represent the percentage of total counts/lane shifted because of decreased PEC mobility. All values are the average of three independent reactions. Standard deviations are shown in superscript. The numbers in parentheses refer to PEC formation as a percentage of EK/LP PEC formation.

	EK/LP	D97E	D188E	E326D
Un-nicked	$28^{2.5}$ (100)	$8^{1.2}$ (30)	6^0 (22)	47^3 (170)
Pre-nicked	62^5 (100)	$77^{1.5}$ (125)	$67^{3.1}$ (108)	$81^{6.7}$ (131)
Hairpin	$29^{4.2}$ (100)	51^4 (176)	$41^{3.8}$ (140)	68^5 (236)
Pre-cleaved	$36^{4.5}$ (100)	43^{18} (121)	$53^{21.5}$ (149)	32^3 (89)

TABLE II
Catalytic reactions with DDE mutants

The ability of the DDE mutants to catalyze the three DNA cleavage steps of transposition was tested. Values represent reaction products as a percentage of total counts/lane. All values are the average activity of three independent reactions. Standard deviations are shown in superscript. The numbers in parentheses refer to activity as a percentage of EK/LP activity.

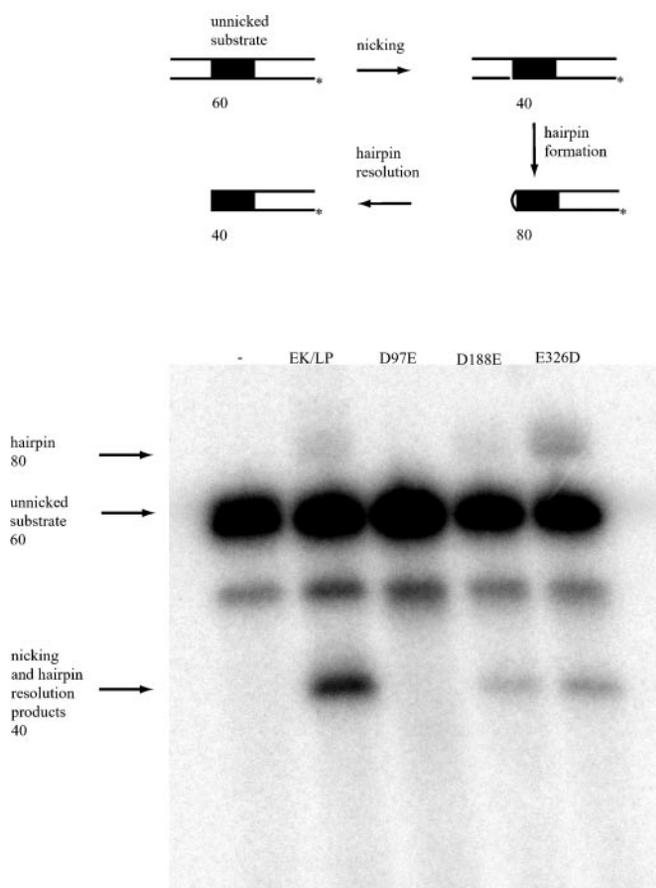|	EK/LP	D97E	D188E	E326D
Nicking	31^5 (100)	0^0 (0)	$3^{0.6}$ (9)	$10^{1.2}$ (34)
Hairpin formation	44^2 (100)	$3^{0.6}$ (8)	8^1 (18)	$16^{1.2}$ (37)
Hairpin resolution	$51^{6.6}$ (100)	0^0 (0)	$16^{1.5}$ (32)	$14^{1.5}$ (27)

FIG. 3. **Transferred strand nicking assay.** The ability of each mutant to catalyze the first step of transposition, transferred strand nicking, was assayed by incubating Tnp with unnicked DNA substrates in the presence of Mn^{+2}. Nicking produces a labeled 40-mer, whereas the following two steps, hairpin formation and resolution, produce a labeled 80-mer and 40-mer, respectively. Adding the amount of substrate that migrates as 40 and 80 mers on a denaturing polyacrylamide gel accounts for all of the substrate that underwent transferred strand nicking. The diagram (*top*) graphically depicts the labeled oligonucleotides that are present at each reaction step, and an example is shown (*bottom*) of the reaction with the DDE mutants, EK/LP, and a no-protein negative control (−) as visualized on a denaturing gel.

efficiently as EK/LP (Table II). It should be noted that a substantial part of the D188E decreased nicking ability is likely because of its impaired ability to form PECs with the unnicked substrate.

Prenicked Substrate—The same assays were performed with a prenicked substrate that simulates the substrate for the second catalytic step of the mechanism, hairpin formation. The prenicked substrate differs from the unnicked substrate only in that the transferred strand is replaced by two oligonucleotides, to simulate the transferred strand nick, and that the DNA is radiolabeled at the 5′-end of the nontransferred strand rather

than the transferred strand (Fig. 4). All three of the mutants were able to form PECs with high efficiency (Table I). The introduction of a nick appears to greatly relax constrains on PEC formation. In catalytic assays, those molecules that are processed into hairpins produce a labeled 20-mer, which when quantitated via denaturing polyacrylamide electrophoresis accounts for all hairpin formation. None of the three mutant proteins facilitates efficient hairpin formation. E326D carries out this reaction best, with activity reduced to 37% of EK/LP, followed by D188E with 18%, and finally D97E with 8% (Table II).

Preformed Hairpin Substrate—The same assays were performed with preformed hairpin substrate. A 50-mer self-complementary DNA molecule, radiolabeled at the 5′-end, was used as the preformed hairpin substrate (Fig. 4). All three mutants show excellent ability to form PECs with the preformed hairpin (Table I). In catalytic assays, resolution of the hairpin releases a labeled 28-mer, which when quantitated accounts for all hairpin resolution. D188E and E326D show a severalfold decrease in hairpin resolution activity, 32 and 27% of EK/LP, whereas D97E is completely defective at this step (Table II).

Precleaved Substrate—The final substrate used in this study was a precleaved molecule that simulates the substrate used for the final step of transposition, strand transfer. This 40-bp substrate, containing transposon DNA and ME sequence but no dbb DNA, was labeled at the 5′-end of both strands (Fig. 4). All three mutants formed PECs with high efficiency (Table I). None of these mutant proteins was able to promote transposition in a qualitative *in vivo* assay (data not shown).

DISCUSSION

The DDE motif (residues 97, 188, 326) has been shown by crystallographic studies to coordinate the divalent metal ions necessary for the catalytic action of Tn5 Tnp (4, 15). It has also been shown that all three residues are required for every catalytic step of transposition. Replacing any of them with an alanine (9) or switching them to their amide equivalents, aspartate to asparagine and glutamate to glutamine (data not shown), destroys all activity. Although the DDE residues are required for catalysis, they are not required for PEC formation. None of the alanine or amide substitutions substantially depresses PEC formation. In this study we analyzed the effects of substituting glutamate for Asp-97 and Asp-188, while changing Glu-326 to an aspartate. The only difference between aspartate and glutamate is an additional carbon in the glutamate side chain. This should amount to an ~1.5-angstrom difference in the position of the catalytically important carboxyl group in any of the three single mutants. D97E and D188E should result in a slightly deeper insertion of the carboxyl into the active site at the altered residue, whereas in the case of E326D the carboxyl should be withdrawn by a similar amount. The obvious consequence of these changes is to increase crowding in the D97E and D188E
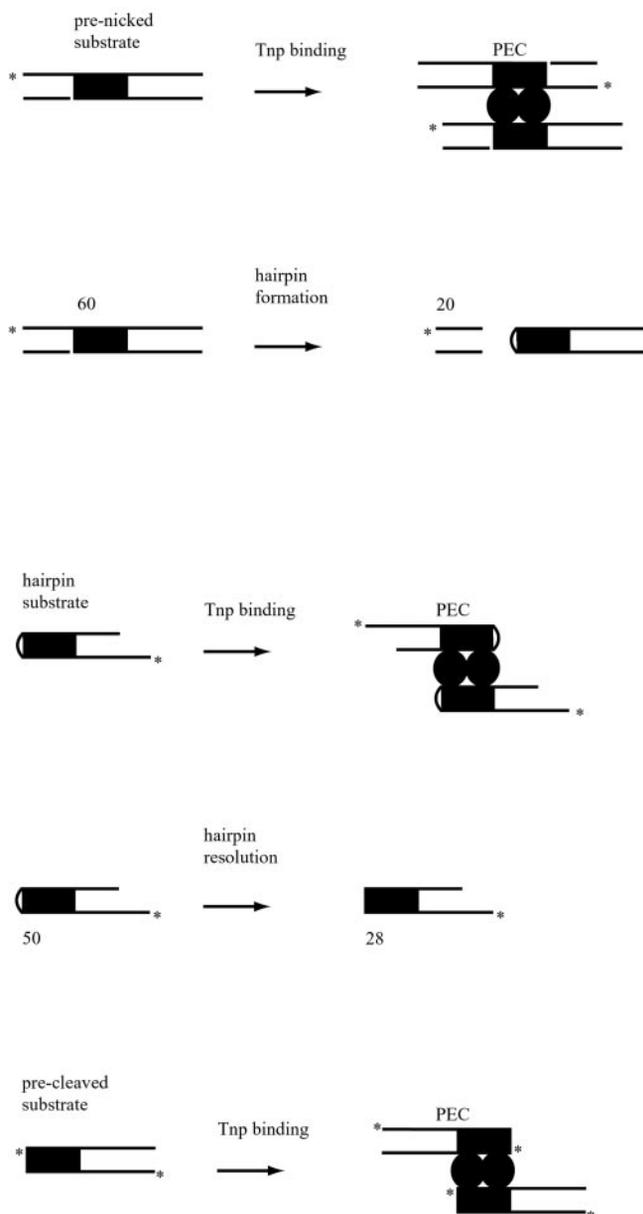
FIG. 4. **PEC formation with prenicked, hairpin, and precleaved substrates, catalytic hairpin formation, and hairpin resolution assays.** Diagrams graphically depict the formation of PECs with prenicked, hairpin, and precleaved DNA substrates, the radiolabeled products produced during the catalytic hairpin formation, and hairpin resolution assays.

FIG. 5. **Tn5 active site mutations.** A previous Tn5 crystal structure (15) is depicted with the D97E, D188E, and E326D mutations superimposed in *red* over the naturally occurring residues in *green*. Tnp is depicted in *blue*, DNA from the 19-bp terminal repeat is shown as *gray bases* and *purple ribbons*, and Mn^{2+} ions as *dark blue spheres*. The glutamate mutations at positions 97 and 188 impair PEC formation with unnicked substrate. All three mutations impair transferred strand nicking, hairpin formation, and hairpin resolution, with D97E showing the most severe inhibition in all three cases.

mutants, while providing more space in the E326D mutant.

Crystallographic studies reveal the position of DNA substrate in the active site. However, all existing structural data depicts Tnp complexed with precut transposon DNA or with DNA containing only one base of dbb DNA. Consequently, we have no knowledge about the position of dbb DNA in synaptic complexes. By allowing a comparison of how these mutations perturb PEC formation, these experiments suggest the position of dbb DNA in synaptic complexes prior to transferred strand nicking.

Both of the mutations that increase side chain length of the altered residue, D97E and D188E, dramatically impair PEC formation with the 60-bp unnicked substrate, which resembles the natural substrate for synaptic complex formation, and for the first catalytic step, transferred strand nicking. Not surprisingly, the E326D mutant, in which the side chain is withdrawn f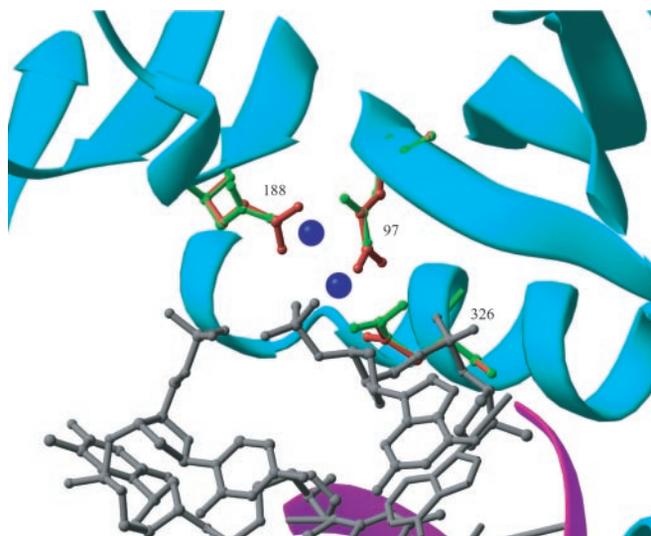rom the active site, forms PECs proficiently. This suggests that the interference with PEC formation by D97E and D188E is a result of increased crowding. This observation makes it appear likely that unnicked dbb DNA is positioned such that it experiences steric interference from the mutationally extended side chains present in these two proteins.

Other possible explanations, such as a general decrease in ability to interact with DNA, seem unlikely in light of the undiminished ability of these mutants to bind prenicked, hairpin, and precleaved substrates. Both D97E and D188E bind prenicked and hairpin substrates at high levels. The prenicked substrate has the same number and sequence of base pairs as the unnicked substrate. The only difference between these two substrates is the introduction of a transferred strand nick by annealing two oligonucleotides to the nontransferred strand instead of one. The introduction of the nick should have the effect of making the DNA more flexible. Increased flexibility, which could allow the dbb to avoid steric conflict introduced by either of the mutated side chains, is consistent with the observation that PEC formation is unimpaired with the prenicked substrate for all three of the mutants. The unhampered ability of all three mutants to form PECs with the hairpin and precleaved substrates, which have no dbb DNA, is also consistent with the suggestion that it is steric interactions between the dbb and the mutated residues at positions 97 and 188 that impair PEC formation with the unnicked substrate.

The possibility that the mutated 97 and 188 residues clash sterically with dbb DNA suggests a model in which the dbb DNA of the unnicked substrate normally occupies space that is invaded by the extended side chains in D97E and D188E. Fig. 5 depicts the active site with the three DDE mutations overlaid on a Tn5 crystal structure (15). This graphically shows the differences in side chain length between the unaltered DDE residues and those in the mutant proteins. The figure shows the mutated side chains in specific orientations, whereas in reality they can rotate and potentially occupy space differently than depicted. Consequently, all of the possible orientations may be responsible for the observed interference with dbb DNA. Because there is only a limited amount of space that the mutated side chains can occupy, the possibilities for the loca-

tion of dbb DNA are also constrained to the space that can be occupied by the longer glutamate side chains but not the shorter aspartate side chains at positions 97 and 188. This indicates that dbb DNA lies directly adjacent to the active site prior to transferred strand nicking.

A relative wealth of structural information is available for Tn5. Recent crystallographic evidence indicates that the DDE residues coordinate two divalent metal ions to the active site (15). The results of the catalytic reactions performed as a part of this study support this two metal model. Although all three mutations to the DDE motif retain the carboxyl that is critical for metal ion coordination, it is reasonable to expect that slightly altering their spatial positions in the active site would decrease the efficiency with which they perform this function, which is what was observed. None of the mutants is as catalytically competent as EK/LP at the nicking, hairpin formation, or hairpin resolution steps, suggesting that the ions are appropriately coordinated for these reactions less frequently in the mutant proteins. According to the two-metal model, Asp-97 is involved in the coordination of both metal ions, whereas Asp-188 and Glu-326, each assisting Asp-97 with a different ion, are responsible for only one. If this model is correct and Asp-97 is required for the coordination of two metal ions, it is likely that any change that perturbs its function would be more detrimental to catalytic activity than a similar change to one of the residues that is involved only in coordination of one metal ion. Of the three catalytic steps tested, D97E shows no activity at the nicking and hairpin resolution steps and a level of hairpin formation activity that is drastically reduced from EK/LP and significantly lower than that of either of the other two mutants. Both of the other mutations show activity, although reduced from EK/LP, at the nicking, hairpin formation, and hairpin resolution steps. The elimination of function at the nicking and hairpin resolution steps and the relatively more severe reduction of function at the hairpin formation step caused by the D97E mutation supports the model of an active site that coordinates two divalent metal ions.

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