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Site-Directed Mutagenesis Studies of Tn*5* Transposase Residues Involved in Synaptic Complex Formation▽†

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Transposition (the movement of discrete segments of DNA, resulting in rearrangement of genomic DNA) initiates when transposase forms a dimeric DNA-protein synaptic complex with transposon DNA end sequences. The synaptic complex is a prerequisite for catalytic reactions that occur during the transposition process. The transposase-DNA interactions involved in the synaptic complex have been of great interest. Here we undertook a study to verify the protein-DNA interactions that lead to synapsis in the Tn*5* system. Specifically, we studied (i) Arg342, Glu344, and Asn348 and (ii) Ser438, Lys439, and Ser445, which, based on the previously published cocrystal structure of Tn*5* transposase bound to a precleaved transposon end sequence, make *cis* and *trans* contacts with transposon end sequence DNA, respectively. By using genetic and biochemical assays, we showed that in all cases except one, each of these residues plays an important role in synaptic complex formation, as predicted by the cocrystal structure.

Transposition is the movement of a defined DNA segment, a transposon, from one location to another within the same or different DNA molecules. Tn*5*, which is a 5.8-kbp prokaryotic transposon, consists of two inverted terminal repeats, IS*50*L and IS*50*R, that flank the genes encoding for antibiotic resistance (Fig. 1A) (5). IS*50*R carries the gene for Tn*5* transposase (Tnp), the only protein catalyzing Tn*5* transposition steps (13). Each IS*50* element is flanked by two inverted 19-bp end sequences (ESs) (12), called the outside end (OE) and the inside end (IE), that are recognized by Tnp during the transposition process (Fig. 1) (4). During transposition, the transposon is completely excised from donor DNA and inserted into a target DNA. This movement is carried out through a conservative "cut-and-paste" mechanism (8), which is explained and shown schematically in Fig. 2. The transposition process is mediated by three factors: Tnp, a transposon (which is bracketed by two inverted ESs), and Mg^{2+} ions. Tnp is critical in many transposition steps, including binding to the transposon DNA ESs (24), bringing the two ESs together via formation of a protein homodimer that results in synaptic complex formation, cleaving the DNA adjacent to the ESs, and inserting the transposon into a target DNA (3, 7, 8, 20–23).

The synaptic complex is a key intermediate in Tn*5* transposition. This is because all catalysis occurs in *trans*, with the Tnp monomer that initially bound to one ES performing the catalytic events on the ES-donor backbone (DBB) border that was initially bound by the other Tnp monomer. Throughout our studies we used a previously described hyperactive mutant (E54K, M56A, L372P) of Tnp, called EK/LP, as a control (9, 11, 21, 29, 31). The previously published cocrystal structure of EK/LP bound to the OE ES (Fig. 3) (generated from PDB ID 1MUH) predicts the existence of multiple Tnp-ES DNA contacts both in *cis* and in *trans* (9). These contacts are presumed to play key roles in stabilizing the synaptic complex and in establishing detailed ES DNA conformations necessary for catalysis. Thus, confirming the existence and role of each of these proposed Tnp-ES DNA contacts is of considerable interest.

Two Tnp-ES DNA contact regions (involving *cis* contacts between the ES and residues 342 through 348 and *trans* contacts between the ES and residues 438 through 445) have not been previously studied in detail. Therefore we undertook an effort to study those residues that, based on the cocrystal structure, seemed to be critical in synapsis and therefore in the transposition process. Specifically, we studied two groups of these residues, (i) Arg342, Glu344, and Asn348 and (ii) Ser438, Lys439, and Ser 445, which make *cis* and *trans* contacts with the ES, respectively (Fig. 3). Using site-directed mutagenesis, these residues were individually mutated to alanine. Several in vivo and in vitro assays were then used to investigate which of these residues are important for the Tn5 transposition process and, furthermore, which steps of the transposition process are affected by each mutation.

Our results show that, as expected from the cocrystal structure of Tnp bound to the OE ES (9), R342A, N348A, S438A, K439A, and S445A show impaired or no transposition activity both in vivo and in vitro and that these defects are due to impaired synaptic complex formation. E344A proved to have enhanced hyperactivity, due mainly to its increased ability to form synaptic complexes. The enhanced hyperactive property of E344A was explained through a fresh analysis of the crystal structure.

MATERIALS AND METHODS

Mutation of Tnp. All the mutations were generated using the Stratagene QuikChange multi-site-directed mutagenesis system. *Pfu Turbo* (Stratagene) was used for DNA polymerization. The mutations were generated using plasmid pGRTYB35 (4), carrying the gene for EK/LP (28, 31). This plasmid allows for the expression of a C-terminal Tnp-intein fusion product that can bind to chitin beads and facilitate the protein purification. The polyacrylamide gel electro-

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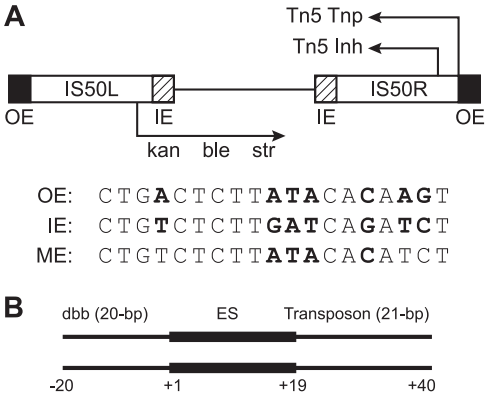

FIG. 1. (A) Tn*5* transposon structure. Tn*5* consists of two nearly identical insertion sequences (IS*50*L and IS*50*R). IS*50*L carries the genes encoding kanamycin (kan), bleomycin (ble), and streptomycin (str) resistances. IS*50*R encodes Tnp and its inhibitor (Inh). Each IS*50* is bracketed by two 19-bp ESs, called the OE and IE, that define the ends of the transposable element at which Tnp acts. The OE (in vivo assays) and ME (in vitro assays) were used in our studies. The base pairs of the ME that are shown in bold are from the OE sequence, and the others are from the IE sequence. (B) The 60-bp transposon substrate consists of 20 bp of DBB, 19 bp of ES, and 21 bp of transposon.

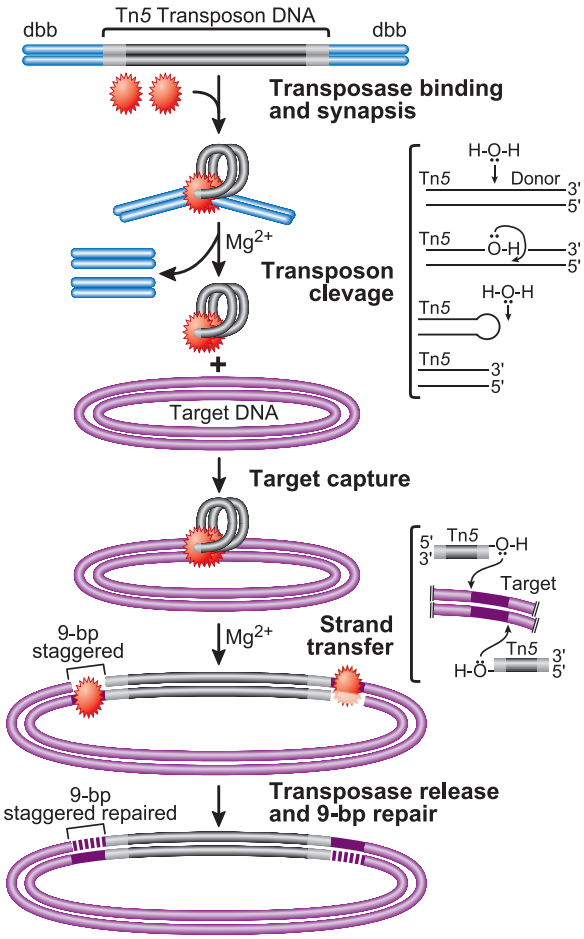

FIG. 2. Schematic model of the Tn*5* "cut-and-paste" transposition mechanism. Two Tnps bind to transposon ESs to form a synaptic complex (1, 24). Each monomer makes contacts with both its own ES (*cis* contacts) and the transposon ES bound to the other monomer (*trans* contacts) (9, 18). The catalytic domain of each Tnp is positioned in such a way as to cleave the transposon end of the other monomer (*trans* cleavage) (9, 18). DNA cleavage to excise the transposon proceeds through a number of steps. Tnp, in the presence of Mg^{2+}, cleaves the DBB via a mechanism that involves a hairpin intermediate and results in a DEB complex (a synaptic complex with two cleaved ends) (4, 5). The chemical steps of the cleavage process are shown for one of the strands only. Following binding of the DEB complex to a nonspecific target DNA, the activated 3′-OH groups at the ends of the transposon perform nucleophilic attacks on the target DNA, accomplishing strand transfer with a 9-bp spacing (23). The Tnps leave the target DNA and the gaps are repaired, producing a 9-bp duplication (for reviews, see references 10, 15, and 21).

phoresis-purified primers and their complements were ordered from Integrated DNA Technologies. The forward primers were as follows: R342A, 5′-GCAGGAGCCGACAGGCAAGCCATGGAGGAGCCGG-3′; E344A, 5′-CGAGAGGCAACGCATGGCGGAGCCGGATAATCTGG-3′; N348A, 5′-CATGGAGGAGCCGGATGCCCTGGAGCGGATGGTC-3′; S438A, 5′-GGGCGGTTTTATGGACGCCAAGCGAACCGG-3′; K439A, 5′-GGTTTTATGGACAGCGCCCGAACCGGAATTGCC-3′; and S445A, 5′-CCGGAATTGCCGCCTGGGGCGCCC-3′.

The PCR products were electroporated into TransforMax EC100 electrocompetent *Escherichia coli* (Epicenter Biotechnologies), grown in Luria-Bertani (LB) medium for 1 h, and selected for ampicillin resistance by plating on LB agar plates containing 100 μg/ml ampicillin (Amp^{100}) (Sigma). The DNAs from several single colonies were purified using the QIAprep spin miniprep kit (QIAGEN) and were sequenced to confirm the presence of desired mutations and to ensure fidelity otherwise. Once the sequence was confirmed, the isolate was maintained as a glycerol stock culture at −80°C.

Papillation assay. The in vivo papillation assay was performed as previously described (14). The mutant plasmids were transformed into a tetracycline-resistant *E. coli* strain, called MDW320 (29), which contains a Tn*5*-derived transposon with a promoterless *lacZ*. These cells were then plated on minimal glucose (2 mg/ml)-agar plates containing 0.04 mg/ml X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) (Sigma), 0.25 mg/ml P-Gal (phenyl-β-D-galactoside) (Sigma), Amp^{100}, tetracycline (10 μg/ml), and Casamino Acids (3 mg/ml). The plates were incubated overnight at 37°C, and then single colonies from EK/LP and different mutants were replica plated onto the same types of plates in order to give each colony the same resources. These plates were then incubated for 3 to 5 days at 30°C to allow production of blue papillae. The number and rate of production of blue papillae on the colonies from different mutants were judged by eye and compared with EK/LP. In order to produce a darker blue color, plates were stored overnight at 4°C prior to being photographed.

Protein expression. All proteins were expressed and purified as previously described (24). The plasmids were transformed into *E. coli* expression strain ER2566 (New England Biolabs) and plated on LB agar plates containing Amp^{100}. Plates were incubated overnight at 37°C to grow single colonies. Six ml LB with Amp^{100} was inoculated with a single colony and grown by shaking at 37°C to an optical density at 600 nm (OD_{600}) of 0.6. Two milliliters of this culture was harvested (centrifuged for 10 min at 4,000 rpm at room temperature) and resuspended in 2 ml fresh LB medium. One hundred milliliters of LB containing Amp^{100} was inoculated with this 2-ml starter culture and grown by shaking at 250 rpm at 37°C to an OD_{600} of 0.6. This culture was stored overnight at 4°C (this step can be eliminated if desired). The next day, for the final growth, 20 ml of this 100-ml culture was harvested (10 min at 4,000 rpm at 4°C), and the pellet was resuspended in 20 ml of fresh LB, which was used to inoculate 1 liter of LB containing Amp^{100}. The culture was grown by vigorous shaking (250 rpm) at 37°C to an OD_{600} of 0.25. Growth was continued by shaking the culture at 250 rpm at room temperature to an OD_{600} of 0.6. At this point cells were induced using 1 mM isopropyl-β-D-1-thiogalactopyranoside (IPTG) and grown for 1 h. Cells were harvested by centrifugation at 4°C for 30 min at 4,000 rpm. The pellet was washed with 20 ml of phosphate-buffered saline (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.7 mM KH_2PO_4) and stored overnight at −20°C.

Protein purification. The pellet was resuspended in 20 ml ice-cold $HEG_{0.7}$ buffer (20 mM HEPES [pH 7.5], 1 mM EDTA, 10% glycerol, and 0.7 M NaCl). The suspended cells were lysed by sonication on ice and centrifuged for 30 min at 12,000 rpm at 7°C. The supernatant containing Tnp protein was added to 2.5 ml chitin beads (New England Biolabs), which were preequilibrated with $HEG_{0.7}$ buffer. The mixture of chitin beads and lysate was shaken very gently for 1.5 h at 4°C to let intein-Tnp bind to the beads. The mixture was then transferred into a 1-ml QIAGEN polypropylene column and allowed to flow through the column.

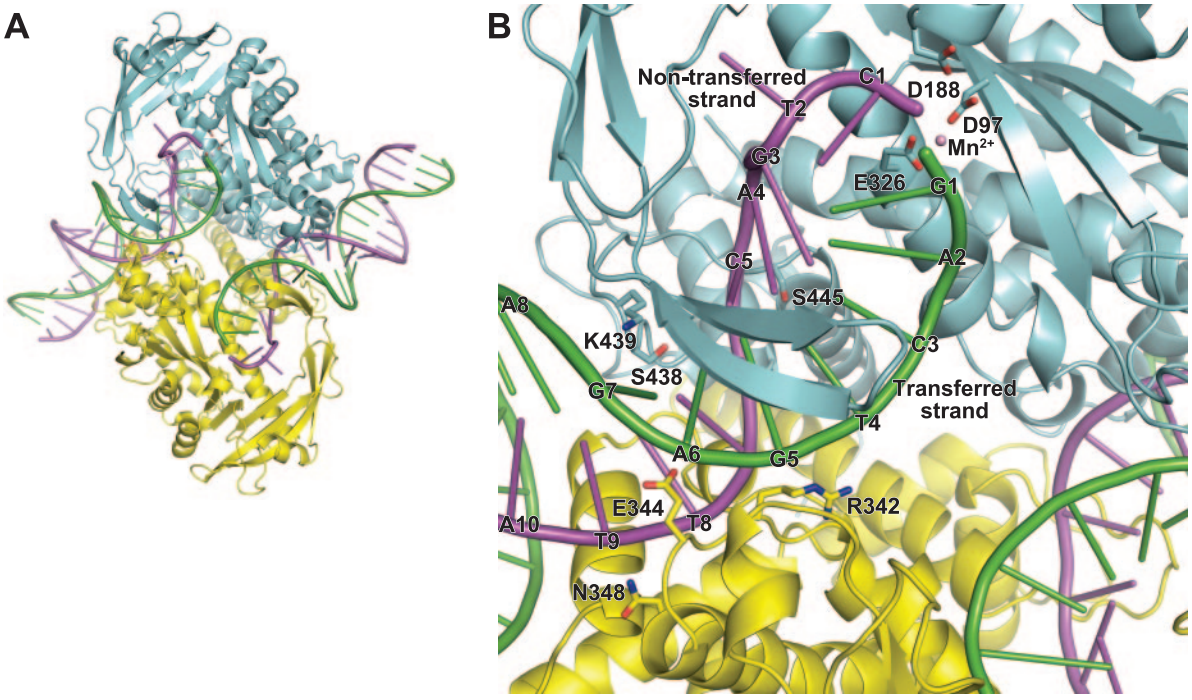

FIG. 3. (A) Structure of Tn*5* Tnp dimer bound to two OE sequences (PDB ID 1MUH) (9). Transferred and nontransferred strands are shown in green and purple, respectively. (B) Arg342, Glu344, and Asn348 and Ser438, Lys439, and Ser445 make *cis* and *trans* contacts with OE DNA, respectively. Mn^{2+} in the active site coordinates to Asp97, Asp188, Glu326 (DDE motif) and 3′-OH of the transferred strand. The DDE motif is a common catalytic triad of acidic residues among transposases and retroviral integrases, which provides a binding site for Mg^{2+} ions (9). Arg342 and Asn348 have water-mediated interactions with backbone phosphates of transferred and nontransferred strands, respectively. Glu344 has proposed base-specific contacts with G7 of the transferred strand and T8 and T9 of the nontransferred strand. Ser438 has a base-specific contact with A8 of the transferred strand and C7 of the nontransferred strand. Lys439 has a base-specific contact with G7 of the transferred strand. Ser445 has a water-mediated interaction with a backbone phosphate of the nontransferred strand. All these contacts are with the base pairs located in the major groove of ES DNA.

The column was washed with 3 column volumes of $HEG_{0.7}$ buffer. To cleave the protein, the column was washed with 3.5 ml of $HEG_{0.7}$ containing 50 mM hydroxylamine. Then column was closed and left overnight at 4°C in order to allow for hydroxylamine cleavage of the Tnp from chitin beads. The next day, Tnp was eluted using 1 ml of $HEG_{0.7}$ buffer. Eluted solutions were later analyzed for protein contents using the standard Bradford assay (6) and sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

Oligonucleotide substrates. Two types of oligonucleotides were used: (i) the 60-bp "full-length" substrate containing the 19-bp mosaic end (ME) (a hyperactive hybrid ES) (underlined) flanked by the 20-bp DBB and 21 bp of transposon DNA (Fig. 1) (5′-GGCCACGACACGCTCCCGCGCTGTCTCTTATACACATCTTG AGTGAGTGAGC TGCATGT-3′) and (ii) the 40-bp "precleaved" substrate, which is identical to the 60-bp substrate but lacks the DBB (5′-CTGTCTCTTATA CACATCTTGAGTGAGTGAGCATGCATGT-3′). The oligonucleotides and their complements were purchased from Integrated DNA Technologies. The 5′ ends of both strands in the 60-bp substrate were labeled with 6-carboxyfluorescein to facilitate detection of the oligonucleotides in the subsequent assays. For the 40-bp substrate, only the 5′ end of the transferred strand was fluorescein labeled to avoid steric interactions between the 5′ end of the nontransferred strand and residues in the catalytic core. Since during the cleavage step a phosphate group is generated at the 5′ end of the nontransferred strand, this end was phosphorylated in the 40-bp substrate. Substrates were annealed (final concentration of 1 μM in a buffer containing 10 mM NaCl and 10 mM Tris-HCl, pH 7.5) using a thermocycler, where substrates were heated to 95°C for 1 min and then cooled to 4°C at 0.1°C/s.

PEC formation assay. The paired-end complex (PEC) formation ability of each of the mutants with respect to that of EK/LP was assessed essentially as previously described (26). In order to form PECs, various amounts of purified Tnps were mixed with 60-bp substrates (final concentration, 100 nM) in a transposition buffer lacking Mg^{2+} (500 mM potassium glutamate, 125 mM HEPES [pH 7.5], 2.5 mM β-mercaptoethanol, 200 μg/ml tRNA, and 1.25 mg/ml bovine serum albumin). Twenty microliters of reaction mixture was prepared for EK/LP and each of the mutants. Reaction mixtures were incubated at 37°C for 1.5 h to form the complexes (most of the PECs form within an hour [data not shown]). PEC formation was stopped by adding 5 μl of blue/orange 6× glycerol loading dye (Promega) to each reaction mixture, and tubes were stored at −20°C. PECs were separated from unbound substrates by electrophoresis on a 7% native polyacrylamide gel (300 V, 1.5 h, 4°C). The gels were scanned using a Typhoon 9410 variable-mode imager to detect the positions of the fluorescence-labeled oligonucleotides. For each protein, the PEC formation ability was measured at its optimum concentration (Tnp concentration that gave the plateau value of PECs, 500 nM). For all mutant Tnps and EK/LP, PECs and unbound substrate were separated on the same gel to compare their PEC formation abilities at the same time. The PEC assay was performed in triplicate for each protein.

Cleavage assay. The PECs were formed with the 60-bp substrate as described above. After 1.5 h, a 20-μl aliquot was removed, added to 5 μl of 6× loading dye, and immediately stored at −20°C. This was called the zero time point. Magnesium acetate (final concentration of 10 mM) was added to provide the necessary Mg^{2+} ions for catalysis. Then, 20-μl samples were taken at 0.75, 2, 4, 6, 8, 10, 15, 20, 30, 45, and 60 min. Time point samples were added to 5 μl 6× loading dye and immediately stored at −20°C. The cleavage products were separated on a 7% native polyacrylamide gel (300 V, 2 h, 4°C). The gel was scanned using a Typhoon imager to detect the positions of the 60-bp substrates. The bands corresponding to PEC, single-end break (SEB) (the synaptic complex containing one cleaved and one uncleaved end), and double-end break (DEB) (the synaptic complex with both ends cleaved) (5) complexes were quantified relative to each other using ImageQuant Total Lab software. The percentages of PECs, SEBs, and DEBs were plotted versus time to compare the cleavage behavior of each mutant with that of EK/LP.

Strand transfer assay. The PECs were formed with the "precleaved" oligonucleotide (40 bp), as described above for PEC formation assay. After 1.5 h, a 20-μl aliquot was removed, added to 5 μl 6× loading dye, and stored at −20°C. This was called the zero time point. Magnesium acetate (10 mM final concentration) and supercoiled pUC19 (8 nM final concentration) were added, at the same time, in order to allow the target (pUC19) capture and initiation of strand

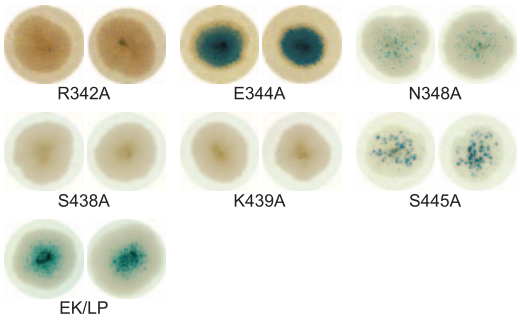

FIG. 4. Results of in vivo papillation assay for the mutant and the control (EK/LP) Tnps. R342A, S438A, and K439A have no transposition activity. S445A and N348A show impaired activity, and E344A shows enhanced hyperactivity compared to EK/LP.

transfer. Samples (20 μl) were then taken at 0.5, 1, 2, 3, 4, 5, 10, 15, 20, 30, 45, and 60 min. Time point samples were added to a mixture of 5 μl 6× loading dye and 5 μl 1% sodium dodecyl sulfate and immediately stored at −20°C, to denature Tnp and therefore break the Tnp-DNA complex in order to stop the reaction at the specific time point. The single-end strand transfer (SEST) (relaxed pUC19) and double-end strand transfer (DEST) (linear pUC19) products were separated on a 2% agarose gel (46 V, 3.5 h). The gel was scanned with the fluorescence channel (526 nm) of a Typhoon imager to detect the positions of the 40-bp substrate. The bands corresponding to SEST were quantified using ImageQuant Total Lab software. Subsequently, the values of fluorescence units (FU) corresponding to each SEST product were normalized to the largest amount of SEST product obtained for each protein in each reaction. The normalized values of the observed FU were plotted versus time to compare the strand transfer catalytic behavior of each mutant with respect to that of EK/LP. After gels were scanned with the fluorescence channel, they were stained with ethidium bromide and scanned with the ethidium bromide channel (610 nm) of the Typhoon imager to visualize the positions of the nonlabeled DNAs (relaxed, linear, and supercoiled pUC19).

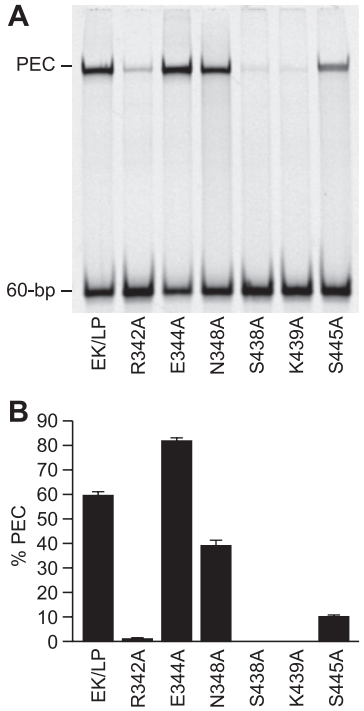

FIG. 5. (A) PEC formation with the control (EK/LP) and the mutant Tnps. PECs and the unbound 60-bp substrates are marked. (B) Percentage of PECs formed by each protein, which is measured as the amount of ES-containing oligonucleotide in PECs divided by the total amount of ES-containing oligonucleotide in each reaction. These results show that R342A, S438A, and K439A form insignificant amounts of PECs. N348A and S445A show impaired activities, and E344A demonstrates enhanced hyperactivity in PEC formation compared to EK/LP. Error bars indicate standard deviations.

RESULTS

The previous crystal structure of Tn*5* Tnp bound to a double-stranded oligonucleotide containing the recognition ES (Fig. 3A) (generated from PDB ID 1MUH) (9) depicts Tnp residues that are in close contact with the ES and therefore are presumed to be involved in DNA binding. Based on these structural data, we undertook a genetic study of the importance of several of these residues in the different steps of Tn*5* transposition. Using site-directed mutagenesis, Arg342, Glu344, and Asn348 (*cis* contacts) and Ser438, Lys439, and Ser 445 (*trans* contacts) (Fig. 3B) were individually mutated to alanine. In vivo and in vitro assays were used to investigate the impact of each of these mutations on the Tn*5* transposition process.

Papillation assay. To study the in vivo transposition efficiency of each mutant, an in vivo transposition assay, called the papillation assay, was performed (14). In this assay the competence of a Tnp to transpose *lacZ* (lacking transcription and translation initiation signals) flanked by ESs is assessed compared to EK/LP (as the control). Plasmids encoding the desired mutants were electroporated into MDW320 *E. coli* cells (29), which contain a plasmid with a promoterless *lacZ* gene within a transposon flanked by ESs, and the cells were plated on glucose minimal agar plates containing P-Gal and X-Gal. The number and rate of blue papillae production in a colony are a measure of the transposition frequency yielding $lacZ^+$ fusions. The results of the papillation assay are shown in Fig. 4. R342A, S438A, and K439A have no detectable transposition activity. S445A and N348A show impaired activity compared to EK/LP. Finally, E344A is more active than the EK/LP control, since not only did it produce more blue papillae than EK/LP, but also the blue papillae in the E344A colonies appeared earlier than those in the EK/LP colonies.

In order to understand why the mutants behave differently from the EK/LP control in the papillation assay, the ability of each mutant to perform various steps of the transposition process was examined.

PEC formation assay. We used an in vitro assay called the PEC formation assay (5) to assess the ability of each mutant to perform synapsis. The substrate for this assay was a 19-bp recognition ES oligonucleotide, attached on one end (+19) to transposon sequences (21 bp) and on the other end (+1) to the DBB (20 bp) (Fig. 1B) (27). Tnp homodimerizes to form a PEC, or in other words, a PEC consists of two Tnp molecules bound to two 19-bp ES-containing oligonucleotides.

The PEC formation ability of the proteins was measured by an electrophoretic mobility shift (gel shift) assay. PECs and unbound substrates for the mutant Tnps and EK/LP were separated on the same gel to compare their PEC formation abilities at the same time. The typical results of these assays are shown in Fig. 5A. The efficiency of PEC formation for each protein was measured by the percentage of PECs formed by each protein, which is measured as the amount of ES-containing oligonucleotide in PECs divided by the total amount of

ES-containing oligonucleotide in each reaction. The PEC formation assay was repeated at least three times for each protein to ensure the reproducibility of the results. Figure 5B shows a bar graph representing the average values of percent PEC formation for each protein. As the error bars show, the PEC formation data are highly reproducible for all Tnps.

These results demonstrate that R342A, S438A, and K439A, which are inactive in the papillation assay, form insignificant amounts of PECs. N348A and S445A, which had impaired activity in the papillation assay, also show impaired activities in PEC formation compared to EK/LP. Finally, the data strongly suggest that E344A, which had an enhanced hyperactive phenotype in the papillation assay, also demonstrated enhanced hyperactivity in PEC formation compared to EK/LP. Therefore, the PEC formation results correlate well with the results of the in vivo papillation assay (see Discussion).

Cleavage assay. As shown in Fig. 2, after synaptic complex formation, in the presence of a divalent cation such as Mg^{2+}, a three-step reaction occurs to cleave transposon DNA free from the adjacent DBB. To investigate whether the mutants that allow measurable formation of PECs also affect the cleavage steps, catalyzed by Tnp, we carried out an assay similar to the PEC formation assay, but after formation of the complexes, magnesium acetate was added to allow catalysis to occur. A schematic representation of the cleavage reaction after PEC formation is shown in Fig. S1A in the supplemental material. Figure S1B to F in the supplemental material shows the gel analyses of cleavage assays for EK/LP and its mutants that were studied in this work. The percentages of PECs, SEBs, and DEBs were plotted against time to show the course of the cleavage process for each mutant (see Fig. S1F to H in the supplemental material). Among the three mutations of residues that form *cis* contacts, R342A did not form enough PECs to be studied by cleavage assay, and among those that form *trans* contacts, S438A and K439A did not form enough PECs to be studied by cleavage assay.

The data presented in Fig. S1 in the supplemental material indicate that mutants E344A, N348A, and S445A have cleavage catalytic properties similar to those of the control Tnp (EK/LP).

Strand transfer assay. To investigate whether our mutations affect the last step of transposition, an in vitro assay called the strand transfer assay was used. In this assay, instead of the 60-bp substrate, which needs to be cleaved to release the transposon (40 bp) from the DBB, annealed 40-bp ES-containing oligonucleotides were used. In this way, we made sure that the observed results are due only to the strand transfer and not the cleavage step.

Figure S2A in the supplemental material shows a schematic representation of the strand transfer. The strand transfer products (SEST and DEST), were analyzed by gel electrophoresis on a 2% agarose gel (see Fig. S2B to F in the supplemental material). To compare the strand transfer catalytic behaviors of the mutants with that of EK/LP, the FU corresponding to DEST products were quantified for each Tnp and normalized against the highest value of DEST formed by that Tnp. These normalized FU values were plotted versus time (see Fig. S2G in the supplemental material). The graphs show that mutants E344A, N348A, and S445A have very similar behavior in strand transfer properties to the control. Thus, these data strongly suggest that the mutations studied here do not affect the strand transfer catalytic properties of Tnp.

DISCUSSION

Synaptic complex formation is a key step in Tn*5* transposition, since all transposition-related catalytic events can occur only in the context of this dimeric protein-DNA arrangement. That is, all reactions occur in *trans* in that the Tnp subunit that initially bound to one ES DNA performs catalysis on the ES DNA bound to the other subunit (*trans* catalysis) (18). It is likely that most other transposition systems manifest the same property. A key feature of the synaptic complex is the multiplicity of Tnp-ES DNA contacts. The published cocrystal structure predicts the existence of five different Tnp regions that make ES contacts; two regions form *cis* contacts, and three regions form *trans* contacts (9). Three of these regions have been extensively studied in other work (2, 5, 16, 17, 19, 25, 27, 30, 31). In this communication we present analyses of the remaining two regions (the *cis* contact region from residue 342 to 348 and the *trans* contact region from residue 438 to 445) and verify, through genetics and biochemistry, that these regions play an important role in synaptic complex formation.

Mutants R342A, S438A, and K439A showed no transposition activity in the in vivo papillation assay (Fig. 4). The effect of these mutations can be entirely attributed to an inhibition of the first step in transposition (PEC formation) (Fig. 5). Therefore, these results suggest that Arg342, Ser438, and Lys439 are critical residues in synaptic complex formation by Tn*5* Tnp in vivo.

The E344A mutant showed enhanced hyperactivity in the in vivo papillation assay (Fig. 4). Studying different steps of the transposition process for this mutant showed a significant increase in the PEC formation ability of this mutant compared to EK/LP but no effects on the DNA cleavage or strand transfer steps. Therefore, the data strongly suggest that the enhanced hyperactivity of E344A compared to EK/LP is merely due to its enhanced PEC formation ability. Since replacing this bulky residue (Glu344) with an alanine leads to enhanced hyperactivity of E344A, one can assume that Glu344 has steric interactions and/or repulsive contacts with the ES DNA. Interestingly, looking closely at the interactions of Glu344 and the ES (in the structure of Tnp bound to ES, PDB ID 1MUH) shows that $O\varepsilon^1$ of Glu344 is located within 3.32 and 2.97 Å of C^4 and C^5 of T9, in the major groove of the nontransferred strand, respectively (Fig. 6) (9). This is likely to cause steric interactions between Glu344 and the ES, and therefore these observations clearly explain the observed enhanced hyperactivity of E344A in papillation and PEC formation assays. It should be noted that the available cocrystal structure of Tnp bound to ES (9) shows the conformation of ES and Tnp after synaptic complex formation and cleavage of the DBB and not upon synaptic complex formation. However, both structural and biochemical data strongly suggest that Glu344 has steric contact with the ES upon synaptic complex formation. Another way to consider this phenomenon is to posit that the DNA flexibility that would be enhanced by the glutamate-to-alanine change is important for synaptic complex formation, whereas the previously proposed specific contacts for Glu344 are not so important. Moreover, we can also conclude from our results that the

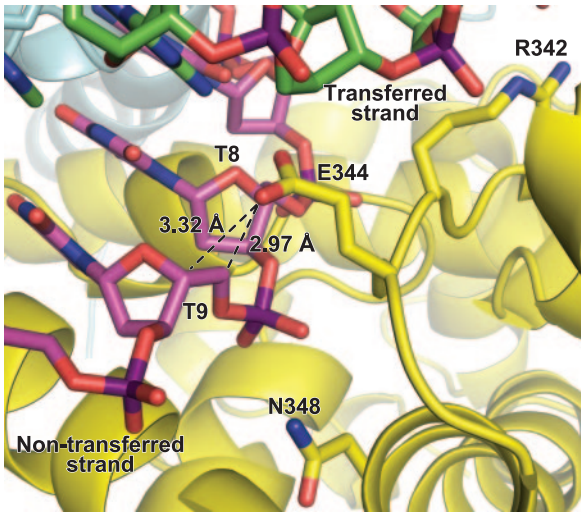

FIG. 6. Oε[1] of Glu344 is located within 3.32 and 2.97 Å of C^4 and C^5 of T9 located in the major groove of nontransferred strand, respectively, which will cause steric interactions between Glu344 and the ES.

previously proposed specific contacts for Glu344 are also not important for any of the other steps in Tn*5* transposition. Finally, the enhanced hyperactivity of E344A suggests another mechanism by which the wild-type Tnp is suboptimal in order to down-regulate transposition activity in vivo.

The S445A and N348A mutants showed impaired activity in the in vivo papillation assay. Studying the transposition steps for these mutants showed that these mutants have impaired activity in PEC formation; however, they behave similarly to EK/LP in cleavage and strand transfer assays. These results suggest that the hypoactivity of these mutants in the in vivo papillation assay must be merely due to their impaired ability to form PECs (and synapses) with ES DNA.

Tn*5* is a model system for studying transposition and may also be useful in understanding related recombinant processes. The individual steps of Tn*5* transposition have been well characterized. However, our knowledge regarding the protein-DNA interactions leading to synapsis has been incomplete. This work has provided a better understanding of the protein-DNA interactions leading to Tn*5* synapsis.

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REFERENCES

1. **Adams, C. D., B. Schnurr, D. Skoko, J. F. Marko, and W. S. Reznikoff.** 2006. Tn5 transposase loops DNA in the absence of Tn5 transposon end sequences. Mol. Microbiol. **62:**1558–1568.
2. **Ason, B., and W. S. Reznikoff.** 2002. Mutational analysis of the base flipping event found in Tn5 transposition. J. Biol. Chem. **277:**11284–11291.
3. **Berg, D. E., and M. M. Howe.** 1989. Mobile DNA. American Society for Microbiology, Washington, DC.
4. **Bhasin, A., I. Y. Goryshin, and W. S. Reznikoff.** 1999. Hairpin formation in Tn5 transposition. J. Biol. Chem. **274:**37021–37029.
5. **Bhasin, A., I. Y. Goryshin, M. Steiniger-White, D. York, and W. S. Reznikoff.** 2000. Characterization of a Tn5 pre-cleavage synaptic complex. J. Mol. Biol. **302:**49–63.
6. **Bradford, M. M.** 1976. Rapid and sensitive method for quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. **72:**248–254.
7. **Craig, N. L.** 2002. Mobile DNA II. ASM Press, Washington, DC.
8. **Curcio, M. J., and K. M. Derbyshire.** 2003. The outs and ins of transposition: from mu to kangaroo. Nat. Rev. Mol. Cell Biol. **4:**865–877.
9. **Davies, D. R., I. Y. Goryshin, W. S. Reznikoff, and I. Rayment.** 2000. Three-dimensional structure of the Tn5 synaptic complex transposition intermediate. Science **289:**77–85.
10. **Goryshin, I. Y., J. A. Miller, Y. V. Kil, V. A. Lanzov, and W. S. Reznikoff.** 1998. Tn5/IS50 target recognition. Proc. Natl. Acad. Sci. USA **95:**10716–10721.
11. **Goryshin, I. Y., and W. S. Reznikoff.** 1998. Tn5 in vitro transposition. J. Biol. Chem. **273:**7367–7374.
12. **Johnson, R. C., and W. S. Reznikoff.** 1983. DNA-sequences at the ends of transposon Tn5 required for transposition. Nature **304:**280–282.
13. **Johnson, R. C., and W. S. Reznikoff.** 1984. Role of the IS50R proteins in the promotion and control of Tn5 transposition. J. Mol. Biol. **177:**645–661.
14. **Krebs, M. P., and W. S. Reznikoff.** 1988. Use of a Tn5 derivative that creates LacZ translational fusions to obtain a transposition mutant. Gene **63:**277–285.
15. **Mizuuchi, K., and K. Adzuma.** 1991. Inversion of the phosphate chirality at the target site of Mu-DNA strand transfer—evidence for a one-step transesterification mechanism. Cell **66:**129–140.
16. **Naumann, T. A., and W. S. Reznikoff.** 2002. Tn5 transposase active site mutants. J. Biol. Chem. **277:**17623–17629.
17. **Naumann, T. A., and W. S. Reznikoff.** 2002. Tn5 transposase with an altered specificity for transposon ends. J. Bacteriol. **184:**233–240.
18. **Naumann, T. A., and W. S. Reznikoff.** 2000. Trans catalysis in Tn5 transposition. Proc. Natl. Acad. Sci. USA **97:**8944–8949.
19. **Peterson, G., and W. Reznikoff.** 2003. Tn5 transposase active site mutations suggest position of donor backbone DNA in synaptic complex. J. Biol. Chem. **278:**1904–1909.
20. **Reznikoff, W. S.** 1993. The Tn5 transposon. Annu. Rev. Microbiol. **47:**945–963.
21. **Reznikoff, W. S.** 2003. Tn5 as a model for understanding DNA transposition. Mol. Microbiol. **47:**1199–1206.
22. **Reznikoff, W. S.** 2006. Tn5 transposition: a molecular tool for studying protein structure-function. Biochem. Soc. Trans. **34:**320–323.
23. **Reznikoff, W. S., A. Bhasin, D. R. Davies, I. Y. Goryshin, L. A. Mahnke, T. Naumann, I. Rayment, M. Steiniger-White, and S. S. Twining.** 1999. Tn5: a molecular window on transposition. Biochem. Biophys. Res. Commun. **266:**729–734.
24. **Steiniger, M., C. D. Adams, J. F. Marko, and W. S. Reznikoff.** 2006. Defining characteristics of Tn5 transposase non-specific DNA binding. Nucleic Acids Res. **34:**2820–2832.
25. **Steiniger, M., J. Metzler, and W. S. Reznikoff.** 2006. Mutation of Tn5 transposase beta-loop residues affects all steps of Tn5 transposition: the role of conformational changes in Tn5 transposition. Biochemistry **45:**15552–15562.
26. **Steiniger-White, M., A. Bhasin, S. Lovell, I. Rayment, and W. S. Reznikoff.** 2002. Evidence for "unseen" transposase-DNA contacts. J. Mol. Biol. **322:**971–982.
27. **Twining, S. S., I. Y. Goryshin, A. Bhasin, and W. S. Reznikoff.** 2001. Functional characterization of arginine 30, lysine 40, and arginine 62 in Tn5 transposase. J. Biol. Chem. **276:**23135–23143.
28. **Weinreich, M. D., A. Gasch, and W. S. Reznikoff.** 1994. Evidence that the cis preference of the Tn5 transposase is caused by nonproductive multimerization. Genes Dev. **8:**2363–2374.
29. **Weinreich, M. D., L. Mahnke-Braam, and W. S. Reznikoff.** 1994. A functional-analysis of the Tn5 transposase—identification of domains required for DNA-binding and multimerization. J. Mol. Biol. **241:**166–177.
30. **Zhou, M., A. Bhasin, and W. S. Reznikoff.** 1998. Molecular genetic analysis of transposase-end DNA sequence recognition: cooperativity of three adjacent base-pairs in specific interaction with a mutant Tn5 transposase. J. Mol. Biol. **276:**913–925.
31. **Zhou, M., and W. S. Reznikoff.** 1997. Tn5 transposase mutants that alter DNA binding specificity. J. Mol. Biol. **271:**362–373.