THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 277, No. 20, Issue of May 17, pp. 17623–17629, 2002
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Printed in U.S.A.

Tn*5* Transposase Active Site Mutants*

Received for publication, January 23, 2002, and in revised form, March 1, 2002
Published, JBC Papers in Press, March 4, 2002, DOI 10.1074/jbc.M200742200

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Tn*5* transposase (Tnp) is a 53.3-kDa protein that is encoded by and facilitates movement of transposon Tn*5*. Tnp monomers contain a single active site that is responsible for catalyzing a series of four DNA breaking/joining reactions at one transposon end. Based on primary sequence homology and protein structural information, we designed and constructed a series of plasmids that encode for Tnps containing active site mutations. Following Tnp expression and purification, the active site mutants were tested for their ability to form protein-DNA complexes and perform each of the four catalytic steps in the transposition pathway *in vitro*. The results demonstrate that Asp-97, Asp-188, and Glu-326, visible in the active site of Tn*5* crystal structures, are absolutely required for all catalytic steps. Mutations within a series of amino acid residues that are conserved in the IS4 family of transposases and retroviral integrases also impair Tnp catalytic activity. Mutations at either Tyr-319 or Arg-322 reduce both hairpin resolution and strand transfer activity within protein-DNA complexes. Mutations at Lys-333 reduce the ability of Tnps to form protein-DNA complexes, whereas mutations at the less strongly conserved Lys-330 have less of an effect on both synaptic complex formation and catalytic activity.

Tn*5* transposase (Tnp)[1] is a catalytic protein that is responsible for movement of the Tn*5* transposon, a 5.8-kb segment of DNA that is defined by two 19-bp inverted terminal repeats termed outside end (OE) (for a review see Ref. 1). Movement of the transposon occurs via a conservative transposition mechanism that involves the complete excision of the transposon from the donor DNA prior to insertion into a new location (Fig. 1). This excision from the donor and insertion into the target involves four catalytic steps at each end of the transposon (2, 3). These reactions take place within a protein-DNA complex termed a synaptic complex. The synaptic complex consists of two DNA end sequences and two molecules of transposase (4, 5). Tnp is unable to promote catalytic reactions in the absence of a synaptic complex because the active site of the transposon monomer that binds to one end of the transposon is responsible for catalytic reactions on the other transposon end or *in trans* (5, 6).

Tnp contains a single active site (5, 7). The active site is responsible for carrying out four DNA breaking/joining reactions (Fig. 1). In both the initial 3′ nicking step and the hairpin resolution step, a water molecule acts as a nucleophile to hydrolyze a DNA–phosphate bond. In the hairpin formation and strand transfer steps, the 3′-hydroxyl group of the transferred strand end acts as a nucleophile.

Within the active site is a triad of acidic residues termed the DDE motif. This motif is believed to occur in all transposase and retroviral integrase proteins. The function of this triad is to coordinate divalent metal ions that are required for catalysis (8–11). Some transposases and retroviral integrases contain a pair of lysine residues that follow the DDE glutamate (E*XXX*K*XXX*K). Studies performed on HIV integrase have demonstrated that these two basic residues are important for catalytic activity and can be cross-linked to DNA ends (12). The DDE glutamate residue and the second lysine in Tnp make up part of the YREK signature motif that is conserved at the primary sequence level among all members of the IS4 family of transposases (including Tn*5*) (13). Data from various *in vivo* and *in vitro* assays of mutants in this region have been reported for transposase proteins from Tn*10* (14–17) and Tn*903* (18). It is still not clear, however, what role these residues play in catalysis and why their conservation has been absolute among related transposases that otherwise have little sequence identity.

In vivo transposition of Tn*5* occurs at a very low frequency, probably because high rates of transposition would be detrimental to the survival of the cell. The low level of transposition is in part due to the very low level of activity in the protein. This low level of activity has been increased by point mutagenesis (19–22) and increased even further by combining multiple mutations (6, 23). A hyperactive transposase double mutant that contains the mutations E54K and L372P (EK/LP) is able to promote transposition of OE defined DNA segments *in vitro*. The transposition rate facilitated by Tnp EK/LP can further be increased by replacing the OE DNA end sequences with a 19-bp site termed mosaic end (ME). This end sequence is an artificially constructed end that differs from the OE at three positions and results in a 10–50-fold increase in transposition rate when used in conjunction with the E54K Tnp mutation (24).[2] This combination of Tnp EK/LP and ME-flanked DNAs has resulted in the development of an efficient series of gel shift assays (4) and catalytic assays (2, 23) used to study the transposition process. Tn*5 in vitro* assays have also been used to study C-terminally truncated forms of Tnp EK/LP and full-length Tnp EK/LP with point mutations added to either the N or C terminus (26, 27).

In this paper we describe the analysis of mutant Tnps that contain the hyperactive mutations E54K and L372P as well as

* This work was funded by National Institutes of Health Grant GM50692 (to W. S. R.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ Supported in part by National Institutes of Health Training Grant GM08349.

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[1] The abbreviations used are: Tnp, transposase; ME, mosaic end DNA sequence; OE, outside end DNA sequence; PEC, paired ends complex; EK/LP, hyperactive transposase double mutant that contains the mutations E54K and L372P; HIV, human immunodeficiency virus.

[2] M. Steiniger-White, A. Bhasin, S. Lovell, I. Rayment, and W. S. Reznikoff, submitted for publication.

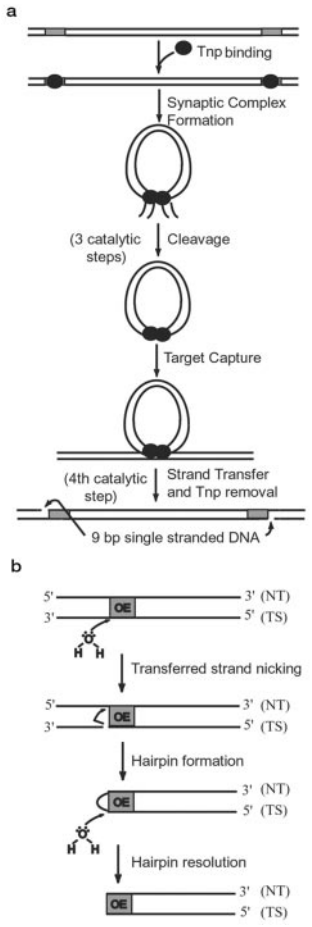

FIG. 1. *a*, conservative "cut and paste" transposition. Tnp monomers bind to each DNA end sequence and then associate to form the synaptic complex. Within this complex Tnp catalyzes blunt-ended cleavage of the transposon by a 3-step mechanism. The excised transposon then undergoes target capture. Tnp then catalyzes the fourth step in the reaction (*Strand Transfer*), covalently linking the 3′ ends of the transposon into target DNA in a staggered fashion. *b*, 3-step cleavage mechanism. Tnp activates a water molecule that attacks the 3′ end of the transferred strand DNA (*TS*). The exposed hydroxyl then acts as a nucleophile to attack the 5′ phosphate of the nontransferred DNA strand (*NT*) to form a hairpin intermediate. Tnp then utilizes another water molecule to relieve the hairpin, resulting in blunt-ended, cleaved DNA ends.

an additional amino acid change in the active site. Alanine substitutions were introduced at seven evolutionarily conserved amino acids. Additionally, conservative amino acid mutations were constructed at three of these positions based on structural information. Following protein purification, the active site mutant Tnps were characterized by gel shift assays for defects in synaptic complex formation and subsequently tested for the ability to promote each of the four catalytic steps in the transposition pathway. Results show that Tyr-319 and Arg-322 are important for hairpin resolution and strand transfer, whereas Lys-333 is necessary for formation of synaptic complexes. Alanine substitutions at any of the DDE motif residues are catalytically inactive at all reaction steps without greatly impairing synaptic complex formation.

EXPERIMENTAL PROCEDURES

Media and Reagents—All bacterial cultures for cloning, protein purification, and purification of DNA substrates were grown in Luria broth (28). Ampicillin was purchased from Sigma and added to plates or liquid medium at 100 μg/ml. Restriction enzymes and T4 DNA ligase were purchased from New England Biolabs and Promega Corporation. *Taq* DNA polymerase was purchased from Promega. *Pfu* polymerase was purchased from Stratagene. Oligonucleotides used for site-directed mutagenesis came from either Research Genetics or Integrated DNA Technology. Some sequencing was performed using Sequenase 2.0 (United States Biochemical) and [α-^{32}P]dATP purchased from Amersham Biosciences. Other sequencing was performed using a PerkinElmer Big Dye kit with the aid of the University of Wisconsin Madison Biotechnology Center.

Site-directed Mutagenesis—The construction of plasmid pGRTYB35, which encodes Tnp EK/LP as a C-terminal fusion to intein and chitin binding domains, was described previously (2). Construction of pGRTYB35 (E326A) was performed by site-directed mutagenesis using the method of Martin *et al.* (29) as described previously (6). Plasmids pGRTYB35 (Y319A), pGRTYB35 (R322A), and pGRTYB35 (K333A) were constructed in the same manner. Plasmids pGRTYB35 (D97A), pGRTYB35 (D188A), pGRTYB35 (R322K), pGRTYB35 (R322Q), pGRTYB35 (K330A), pGRTYB35 (K330R), and pGRTYB35 (K333R) were constructed by site-directed mutagenesis using the single end overlap procedure (30). All constructs were confirmed by DNA sequence analysis.

Bacterial Strains—Expression of Tnp mutants for purification was performed in *Escherichia coli* strain ER2566 ($F^-\lambda^-$ *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* [ϕ*80 dlac*Δ(*lacZ*)*M15*) as a host strain.

Purification of Protein and Preparation of DNA Substrates—Purification of Tnp EK/LP as well as the active site mutant forms was performed using the IMPACT T7 system from New England Biolabs. The concentration of all Tnp proteins was determined using the method of Bradford (25).

Plasmid pGRPP2 and target plasmid pUC19 were purified from bacterial lysates by use of the Qiafilter plasmid Mega kit from Qiagen. Purified, supercoiled monomers were isolated from a 1% agarose gel using a Qiaquick gel purification kit (Qiagen).

Paired ends complex (PEC) and catalytic reaction substrates were prepared via ^{32}P labeling of the 5′ ends of oligonucleotides with T4 DNA kinase and annealing of the appropriate oligonucleotides (2, 4). For PEC formation (uncleaved) and 3′ nicking assays, the following oligonucleotides were used (ME shown in bold): 5′-CTCAGTTCGAGCTCCCAACA**CTGTCTCTTA TACACATCT**TGAGTGAGTGAGCATGCATGT-3′ (nontransferred strand) and 5′-ACATGCATGCTCACTCACTCA**AGATGTGTATAAGAGACAG**TGTTGGGAGCTCGAACTGAG-3′ (transferred strand). For PEC formation (pre-nicked) and hairpin formation assays, the nontransferred strand was the same as above, and the transferred strand oligonucleotide was replaced by the following two oligonucleotides: 5′-TGTTGGCAGCTCGAACTGAG-3′ (donor backbone compliment) and 5′-ACATGCATGCTCACTCACTCA**AGATGT GTATAAGAGACAG**-3′ (ME and transposon DNA compliment). For PEC formation (hairpin) and hairpin resolution assays, the substrate was a single 50-mer: 5′-CACGTGTTG**ACATGTGTATAAGAGAC<u>AGCT</u>GTCTCTTATACACATGT**CAA (hairpin loop underlined). For the PEC (precleaved) and strand transfer assays the following two oligonucleotides were annealed: 5′-**CTGTCTCTTATACACATCTTGAGTGAGTGAGCA TGCATGT**-3′ (nontransferred strand) and 5′ -**ACATGCATGCTCACTCACTCA AGATGTGTATAAGAGACAG**-3′ (transferred strand).

In Vitro Transposition Reactions—All catalytic reactions were performed in 20-μl reaction volumes containing 94 nM Tnp, 0.1 M potassium glutamate, 25 mM Tris acetate, pH 7.5, 50 μg/ml bovine serum albumin, 0.5 mM β-mercaptoethanol, and 100 μg/ml tRNA. Plasmid substrate pGRPP2 (3621 bp) was added to a concentration of 4.7 nM, and short DNA substrates were added to a concentration of 1 nM. For the strand transfer assay, target plasmid pUC19 (2686 bp) was added to 14 nM. Reactions were incubated at 37 °C for 1 h.

Sample Preparation for Agarose Gel Electrophoresis—SDS was added to reaction products to a concentration of 0.2%, and samples were incubated at 68 °C for 5 min to remove Tnp from the DNA. After the addition of loading dye, 5-μl aliquots were loaded onto either a 1.5% agarose gel (for the plasmid reaction) or a 1% agarose gel (for the strand transfer reaction) and electrophoresed in 1× Tris acetate buffer (28) at 15 V/cm followed by staining with ethidium bromide and visualization by UV illumination. For the strand transfer assay, the gel was subsequently dried on nitrocellulose and visualized by autoradiography.

Sample Preparation for Denaturing Gel Electrophoresis—After incubation at 37 °C, each 20-μl sample underwent phenol/chloroform extraction to remove transposase. The DNA was then ethanol-precipitated, dried, and resuspended in 1× formamide dye. After boiling for 3 min, 2 μl of each sample was loaded onto a prewarmed 15% denaturing polyacrylamide gel and electrophoresed at 35 watts constant power (2).

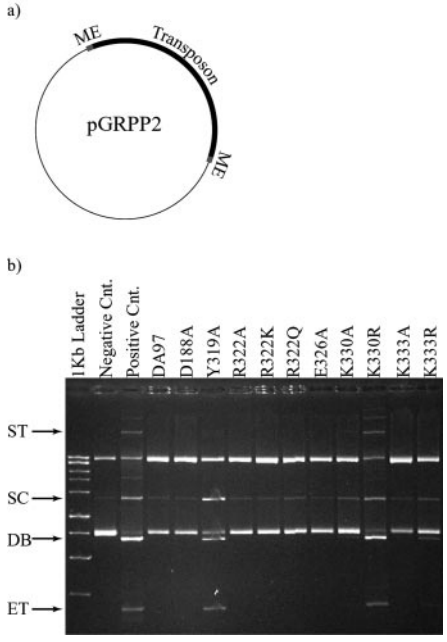

FIG. 2. ***In vitro* transposition activity of Tnp mutants with transposon containing plasmids.** *a*, plasmid pGRPP2 contains a transposon defined by ME sequences. *b*, incubation with Tnp in the presence of Mg^{2+} at 37 °C results in conversion of plasmid into cleavage and strand transfer products. After treatment to remove Tnp from DNA (see "Experimental Procedures"), the resulting DNA products are electrophoresed on a 1.5% agarose gel and visualized by ethidium bromide staining and UV illumination. *ET*, excised transposon; *DB*, donor backbone; *SC*, single end cleavage; *ST*, strand transfer of ET into unreacted plasmid.

The gel was then dried and visualized using a PhosphorImager.

PEC Formation Assays—PEC assays contained 9.4 nM Tnp, 1 nM DNA, 0.1 M potassium glutamate, 25 mM Tris acetate, pH 7.5, 50 μg/ml bovine serum albumin, 0.5 mM β-mercaptoethanol, and 100 μg/ml tRNA in a total volume of 20 μl. Reactions were incubated at 37 °C for 1 h prior to the addition of glycerol loading dye. Samples were electrophoresed in a 7% nondenaturing polyacrylamide gel at 300 V and otherwise handled as described previously (4).

Quantitation—All quantitation was performed using ImageQuant software from Molecular Dynamics. All experiments were reproduced at least three times. The reported data are from one typical experiment.

RESULTS

In Vitro Reactions with Plasmid Substrates—The ability of each of 11 different catalytic mutant forms of Tnp EK/LP to promote transposition of ME defined transposons from plasmid substrate (pGRPP2) was tested *in vitro* (Fig. 2). Incubation of plasmid DNA with Tnp EK/LP (*Positive Cnt*.) resulted in conversion of plasmid substrate into cleavage products (single end cleavage (*SC*), donor backbone (*DB*), excised transposon (*ET*)) as well as strand transfer products (*ST*). With two exceptions, the active site mutant Tnps had little or no activity. However, the activity of Tnp K330R appeared close to that of Tnp EK/LP. It cleaved the plasmid substrate efficiently and formed strand transfer products. The catalytic activity of the second exception, Tnp Y319A, was clearly altered. It formed cleavage products less efficiently than the positive control (reduced donor backbone signal) and also accumulated excised transposons. This indicates that the amino acid mutation Y319A in the active site of Tnp reduces the rate at which the transposon is cleaved from the donor DNA and does not convert these excised transposons into strand transfer products. It is not possible by this assay to determine whether the excised transposon ends contain resolved or unresolved DNA hairpins. The mutants K330A and K333R were able to promote formation of a low, yet detectable amount of reactions products.

Short Fragment Analysis—The transposition activity of Tnp can be studied in more detail using short, radiolabeled DNA fragments that contain ME sequences. Incubation of these DNAs with Tnp in the absence of divalent metal ions results in the formation of protein-DNA complexes consisting of two molecules of Tnp and two molecules of DNA. These complexes, termed paired ends complexes, are equivalent to transposon synaptic complexes and can be visualized by native polyacrylamide gel electrophoresis. Incubation of short, radiolabeled DNAs with Tnp in the presence of divalent metal ions results in Tnp-mediated cleavage of substrate DNA. Denaturing polyacrylamide gel analysis of products allows for the stepwise visualization of cleavage reactions. Pre-nicked and hairpin DNA intermediates can also function as cleavage substrates, thus allowing hairpin formation and hairpin resolution to be assayed without the requirement that mutant Tnps can catalyze previous steps in the cleavage mechanism. Additionally precleaved DNA substrate can be used to test the ability of Tnp to promote strand transfer into unlabeled plasmid target.

PEC Assays—Tnp active site amino acid residues contact DNA ends near the site of cleavage. Therefore amino acid mutations in this area can effect PEC formation. The efficiency of each Tnp mutant to form PECs with a short, radiolabeled 60-bp oligonucleotide containing an embedded ME sequence was tested by gel shift assay (Fig. 3). Incubation of Tnp EK/LP (*Positive Cnt*.) with DNA substrate in buffer lacking Mg^{2+} resulted in the formation of PECs with retarded migration on a native polyacrylamide gel. Mutations at either amino acid 322 or 333 strongly impaired the formation of this complex, indicating that R322 and K333 are important for PEC formation. Tnp K330A and Tnp K330R are both reduced in the ability to form PECs but less severely. The remaining mutants, including mutations in the DDE motif (D97A, D188A, and E326A) have a less than 2-fold effect on PEC formation.

Additional PEC assays were performed on pre-nicked, hairpin, and precleaved DNA substrates (data not shown; Table I). In all assays, Tnp K333A is at least 5-fold reduced for PEC formation. A conservative amino acid mutation of this residue to arginine, which conserved the side chain positive charge, is also strongly impaired for PEC formation using all substrates. In contrast, substitution of arginine 322, which reduced PEC formation with uncleaved substrate, has only a small effect on PEC formation with either hairpin or precleaved DNA ends. Additionally, the PEC defect of Tnp K330A with uncleaved substrate is not observed with pre-nicked, hairpin, or precleaved DNA. Therefore the role of these amino acid residues in stabilizing the PEC changes at different reaction stages.

PEC complexes visualized by gel shift may or may not be competent for cleavage. In the following cleavage assays, all reactions contained equal amounts of Tnp and substrate end DNA. The values that are obtained are expressed as both activity values normalized to Tnp EK/LP and also as values that are adjusted to the normalized PEC formation data. The PEC adjusted values can be used to determine whether formed complexes are competent for cleavage (= 1) or if formed complexes are reduced in catalytic competence (<1).

3′ Nicking of Transposon Ends—The substrate used in this assay consisted of a double-stranded 60-bp oligonucleotide that was radiolabeled on the 5′ end of the transferred strand. Introduction of a nick by Tnp results in a labeled 40-mer that can be distinguished from the unreacted 60-mer on a denaturing gel (Fig. 4*a*). Nicked molecules that undergo hairpin formation are visualized as labeled 80-bp products that move more slowly. Resolution of these hairpins then results in a labeled 40-bp oligonucleotide. The amount of DNA that undergoes transferred strand nicking (at least) can therefore be determined by

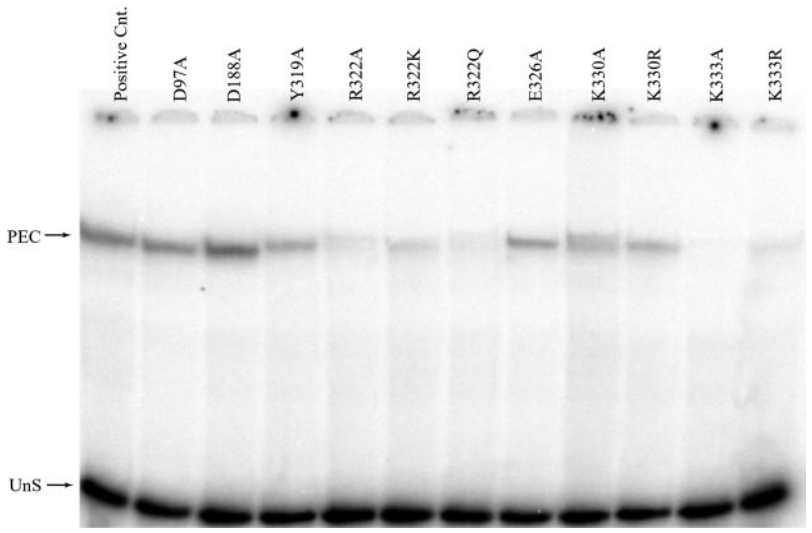

FIG. 3. **PEC formation of Tnp mutants.** The efficiency of each mutant Tnp to form PECs with short, labeled oligonucleotide substrate was determined by gel shift assays. Incubation of Tnp with uncleaved DNA substrate results in the formation of PEC complexes that migrate more slowly in a nondenaturing polyacrylamide gel. The results of incubation with uncleaved substrate are shown. Identical assays were also performed with pre-nicked, hairpin, and precleaved DNA substrates. *UnS*, unshifted DNA substrate; *PEC*, paired ends complex.

TABLE I
Normalized PEC formation with active site mutants

Data for uncleaved (Unclvd) substrate are from gel shown in Fig. 3. The remaining data are from similar experiments performed with pre-nicked (PreNck), hairpin (HP), or precleaved (Preclvd) DNA substrates (data not shown). All values normalized to % signal shifted with Tnp EK/LP (uncleaved = 8.9%; prenicked = 26.6%; hairpin = 36.8%; precleaved = 50.5%).

	D97A	D188A	Y319A	R322A	R322K	R322Q	E326A	K330A	K330R	K333A	K333R
Unclvd	1.09	1.31	0.51	0.06	0.16	0.06	0.61	0.31	0.42	0.0	0.04
PreNck	1.37	0.38	0.22	0.17	0.17	0.29	0.47	1.8	0.49	0.07	0.09
HP	1.3	1.0	0.36	0.57	0.69	0.49	0.93	1.8	1.6	0.2	0.23
Preclvd	1.3	0.72	0.46	0.58	0.87	0.66	0.4	1.6	1.0	0.06	0.22

TABLE II
Normalized catalytic activity of active site mutants

HForm, hairpin formation; HRes, hairpin resolution; ST, strand transfer.

	D97A	D188A	Y319A	R322A	R322K	R322Q	E236A	K330A	K330R	K333A	K333R
3′Nick	0	0	0.7	0.3	0.2	0.1	0	0.15	1.6	0	0.14
HForm	0	0	0.54	0.1	0.4	0	0	0.8	0.7	0.1	0.04
HRes	0	0	0.15	0.02	0.04	0.02	0	1.7	0.6	0.02	0.04
ST	0	0	0	0	0	0.01	0	0.4	0.6	0	0

TABLE III
PEC adjusted catalytic activity of active site mutants

	D97A	D188A	Y319A	R322A	R322K	R322Q	E326A	K330A	K330R	K333A	K333R
3′Nick	0	0	1.4	5.0	1.25	1.7	0	0.48	3.8	0	3.5
HForm	0	0	2.5	0.59	2.4	0	0	0.44	1.4	1.4	0.44
HRes	0	0	0.42	0.04	0.06	0.04	0	0.94	0.38	0.10	0.17
ST	0	0	0	0	0	0.02	0	0.25	0.6	0	0

quantitating the percentage of total labeled DNAs that migrate on a denaturing gel at both the 40-mer and 80-mer positions (Table II). Consistent with the results of the plasmid-based assay, only Tnp K330R and Tnp Y319A are able to nick the uncleaved transposon substrate efficiently. In both the positive control and Tnp K330R reactions, the greater part of the signal is in the 40-mer position, because at this time point all cleavage products have undergone all three cleavage steps (2) (time course not shown). In contrast, the majority of the Tnp Y319A product is present as hairpin DNA.

The 3′ nicking activity level of Tnp Y319A is similar to its PEC level, and therefore the 3′ nicking activity of Tnp Y319A PECs is close to 1 (Table III). Tnp K330R, in contrast, was impaired for PEC formation by >2-fold, whereas the 3′ nicking activity was higher than that for Tnp EK/LP. Therefore the 3′ nicking activity of Tnp K330R PECs is increased relative to that of Tnp EK/LP. The PEC adjusted catalytic values for Tnp R322A and Tnp K333R indicate that both have increased catalytic activity within formed PECs relative to the wild-type active site. However, given the low rate of PEC formation with each of these Tnp mutants, it is possible that these adjusted values are artificially high.

Hairpin Formation—The catalytic mutant forms of Tnp EK/LP were tested next for the ability to convert 3′ nicked substrate DNA into DNA hairpins (Fig. 5*a*). In this assay, hairpin formation is observed directly without the prerequisite Tnp catalyzed nicking of the 3′ end of the transferred strand. Tnp catalyzed hairpin formation of the pre-nicked substrate releases the labeled 5′ end of the nontransferred strand to produce a labeled 20-mer that can be seen on a denaturing acrylamide gel (Fig. 5*b*). As indicated by the 3′ nicking experiment, both Tnp K330R and Tnp Y319A are able to facilitate hairpin formation. In addition, Tnp K330A, which had low activity with uncut substrate, efficiently forms hairpins with the pre-nicked substrate. Noticeable activity is also evident for Tnp R322K and Tnp K333A.

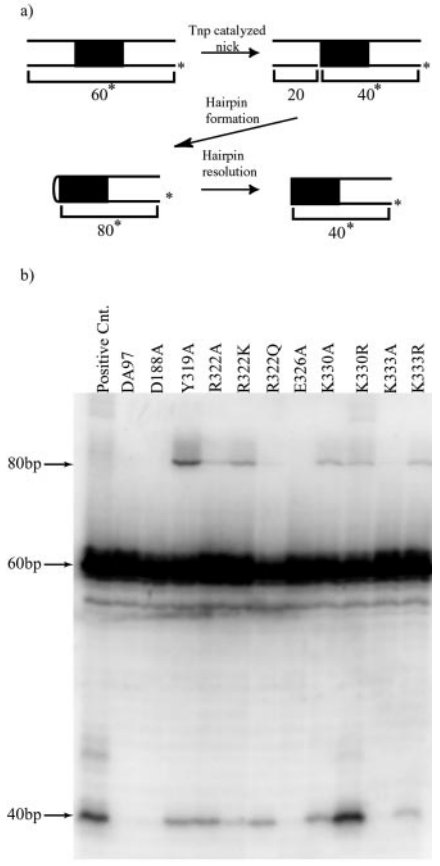

FIG. 4. **DNA end cleavage of labeled 60-mer oligonucleotides by Tnp mutants.** Tnp catalyzed transferred strand nicking results in a labeled 40-mer that migrates faster through a denaturing polyacrylamide gel. DNA ends undergoing Tnp catalyzed hairpin formation results in a labeled 80-mer that migrates more slowly than the unreacted DNA. Hairpins that are resolved by Tnp restore the break on the transferred strand, and the labeled band migrates as a 40-mer.

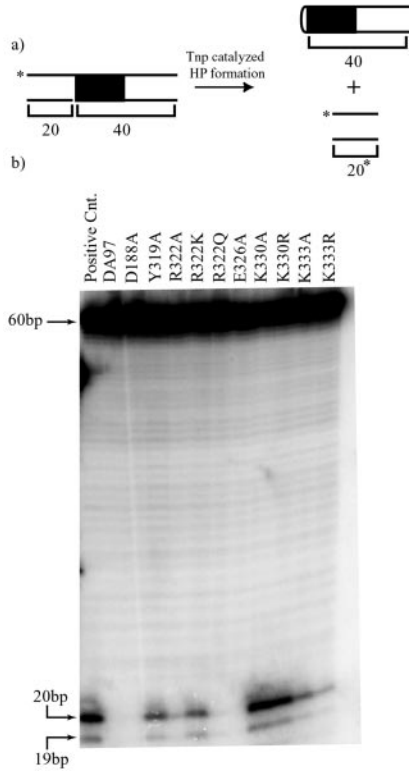

FIG. 5. **Hairpin formation of pre-nicked substrates.** Tnp catalyzed hairpin formation results in a labeled 20-mer. Tnp occasionally forms imprecise hairpins that result in a labeled 19-mer.

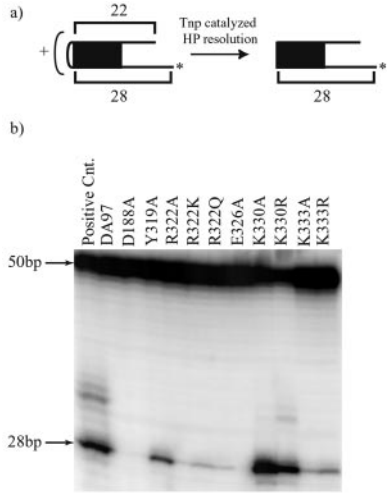

FIG. 6. **Hairpin resolution.** Unresolved DNA hairpins migrate as an 50-mer on a denaturing polyacrylamide gel. Tnp catalyzed hairpin resolution results in a labeled 28-mer.

PEC-adjusted data (Table III) show that for both Tnps Y319A and R322K the deficiency in catalytic activity is due to loss of PEC formation efficiency and that the formed PECs undergo hairpin formation, respectively, at 2.5 and 2.4 times the rate of Tnp EK/LP. It is unclear why these amino acid substitutions increase the rate of hairpin formation. Although Tnp K330A forms hairpins at nearly the same rate as Tnp EK/LP (0.8), the efficiency with which PEC complexes undergo hairpin formation is still decreased, as the high normalized catalytic rate is due to the fact that this mutant forms approximately double the amount of PEC as Tnp EK/LP.

Hairpin Resolution—Hairpin resolution was assayed directly by incubating Tnps with DNA hairpin substrate (Fig. 6*a*). The substrate consists of a single 50-bp oligonucleotide annealed onto itself to form a double-stranded DNA molecule with a hairpin. Tnp catalyzed resolution of hairpin DNA results in a labeled 28-bp fragment. Hairpin resolution is strongly impaired by all mutants except Tnp K330A and Tnp K330R (Fig. 6*b*). Tnp Y319A is reduced by 8-fold. However some of this reduction in catalytic activity is due to PEC deficiencies.

Tnp R322A, Tnp R322K, and Tnp R322Q are nearly devoid of hairpin resolution activity despite the fact that PEC formation was only slightly affected with these mutant Tnps. Therefore Arg-322 is specifically required for hairpin resolution and cannot be mutated to another amino acid without loss of hairpin resolution activity. This is in contrast to 3′ nicking and hairpin formation results where all mutants were impaired for PEC formation and one mutation (R322A for 3′ nicking and R322K for hairpin formation) yielded some level product formation.

Strand Transfer—The strand transfer activity of each catalytic mutant Tnp was then compared with that of Tnp EK/LP. A double-stranded 40-bp substrate consisting of the precleaved ME sequence and donor backbone was incubated in the presence of divalent metal ions with a plasmid DNA target and the appropriate Tnp (Fig. 7*a*). Tnp catalyzed strand transfer of labeled oligonucleotide substrate into unlabeled target plasmid results in either linearized plasmid with two oligonucleotides inserted (double-ended strand transfer) or nicked plasmid with a single labeled oligonucleotide inserted (single-ended strand transfer). It should be noted that the binding of target DNA is a necessary prerequisite for strand transfer activity and that for simplicity we use the term "strand transfer" to refer to the

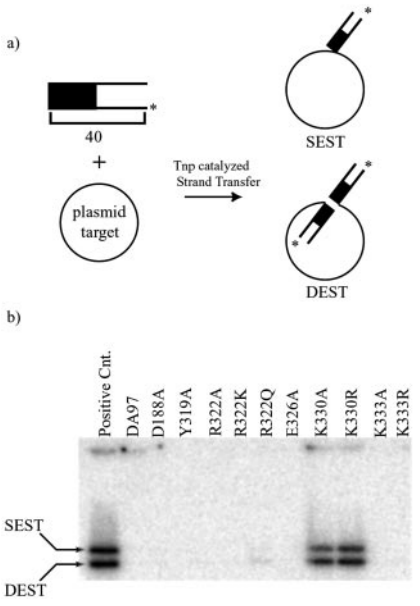

FIG. 7. **Strand transfer.** Labeled oligonucleotides with pre-cut MEs can be inserted into unlabeled plasmid target DNA by Tnp. Following treatment of reactions to remove Tnp from the DNA (see "Experimental Procedures"), the reactions are electrophoresed on a 1% agarose gel, dried onto nitrocellulose, and imaged by autoradiography. *a*, if both DNA ends undergo strand transfer, the product is a labeled plasmid that is linearized. Insertion of only one DNA end results in a relaxed plasmid covalently linked to one labeled DNA end. *b*, the positive control (Tnp EK/LP) and mutant Tnps K330A and K330R are able to catalyze strand transfer. Tnp R322Q was able to catalyze a small amount of strand transfer, detectable in the intensified image.

process of target DNA binding followed by the catalytic strand transfer reaction.

Reaction products were treated with SDS and heat to remove transposase from the DNA and were subsequently loaded on a 1% agarose gel, electrophoresed, dried, and visualized by autoradiography (Fig. 7*b*).

Both mutants Tnp K330A and K330R were active for strand transfer in this assay. Of the remaining nine mutant Tnps, only Tnp R322Q gave any detectable activity (0.01). Although Tnp K333A and Tnp K333R are defective in PEC formation, Tnp Y319A and the Tnps with mutations at Arg-322 are only mildly inhibited in PEC formation with precleaved DNA ends and yet are unable to catalyze strand transfer. Therefore both Tyr-319 and Arg-322 are required for strand transfer activity.

DISCUSSION

DDE Motif—A central feature of transposase proteins as well as retroviral integrases is the presence of a triad of acidic amino acid residues, called the DDE motif, located in the active site. The DDE motif is responsible for binding of divalent metal ions in the active site. This motif in Tn*5* transposase, as indicated by crystallographic information, and consists of Asp-97, Asp-188, and Glu-326. In the current study we analyzed the effects of alanine substitutions at each of these three positions. The catalytic data reveal that substitution of alanine at any of these three positions abolishes the catalytic activity of Tnp at all four chemical steps while not strongly impairing PEC formation. In a crystallographic structure of Tnp EK/LP complexed with OE in a precleaved complex, a manganese ion is shown coordinated by Asp-97 and Glu-326, and a later structure has revealed the presence of a second metal ion coordinated by Asp-97 and Glu-188.[3] Because mutation of either

[3] S. Lovell, I. Goryshin, W. S. Reznikoff, and I. Rayment, submitted for publication.

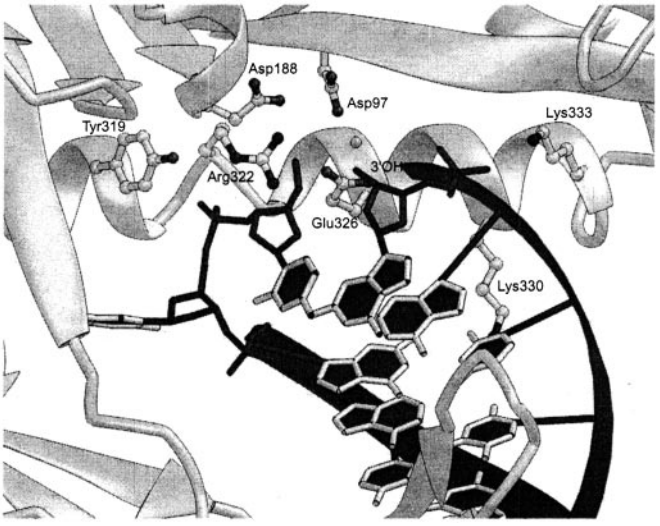

FIG. 8. **Crystal structure.** Tyr-319 and Arg-322 contact the nontransferred strand phosphate backbone between bases 1 and 2. Mutation of either residue affects hairpin resolution and strand transfer. Mutation of Lys-333, which contacts the phosphate backbone of the transferred strand of DNA, impairs the ability to form synaptic complexes with all DNA substrates. Mutation of Lys-330 has less drastic effects on Tnp activity.

Asp-188 or Glu-326 directly affects only one metal ion binding site, the results suggest that both metal ions are required for each of the four catalytic steps.

Tyr-319—Mutation of Tyr-319 to alanine in the active site of Tnp mildly reduces PEC formation with all DNA substrates. The catalytic activity of this mutant is relatively unimpaired for 3′ nicking and hairpin formation, whereas the hairpin resolution step is strongly impaired and strand transfer activity is undetectable. The PEC-adjusted catalytic data reveal that the 3′ nicking and hairpin formation steps of formed PECs are increased relative to Tnp with a native active site. The efficiency of the hairpin resolution step for formed PECs, although only mildly reduced relative to Tnp EK/LP (0.42), is more than 5-fold less than the efficiency of hairpin formation. This explains why hairpin DNA accumulates when an uncleaved DNA substrate is used (Fig. 4*b*).

Tnp Y319A is unable to catalyze strand transfer into target DNA. When incubated with plasmid substrate the mutant protein acts as a restriction enzyme, cleaving the plasmid into transposon and donor backbone fragments. The Tnp EK/LP:OE cocrystal structure of the preintegration complex shows that the hydroxyl group of Tyr-319 contacts the phosphate between the first and second base pair of the nontransferred strand of the transposon end. These contacts may be crucial for the movement of the nontransferred strand of DNA, which must occur to allow the target DNA access to the active site.

It has been reported previously that a tyrosine-to-serine replacement at the analogous amino acid residue in the IS4 relative Tn*10* Tnp blocks hairpin formation and results in the accumulation of nicked ends (17). Although the phenotype of this serine substitution differs from the results reported here for Tnp Y319A, it is not inconsistent with the idea that replacement of the conserved tyrosine can result in incorrect positioning of the nontransferred strand in the site.

Arg-322—Three different amino acid substitutions were constructed and tested at position 322; lysine (charge conservation), glutamine (structure conservation), and alanine. With uncleaved or pre-nicked substrate, the main defect of these proteins is their inability to efficiently form synaptic complexes. This result was not surprising given that an arginine-to-alanine mutant of Tn*10* Tnp has previously been reported to be unable to nick DNA ends

or promote strand transfer of precleaved ends (15). Additionally mutation of the analogous arginine in Tnp from Tn*903* results in loss of *in vivo* transposition activity (18).

With hairpin or precleaved substrate, the synaptic complex formation defects for these mutants were less apparent. In the hairpin resolution and strand transfer assays, all three Tnp mutants with substitutions at position 322 were extremely impaired for hairpin resolution and strand transfer activity. The Tnp EK/LP:OE co-crystal structure of the preintegration complex shows that, like the hydroxyl group of Tyr-319, both the Nϵ and NH_2 of the arginine side chain are in contact with the phosphate between positions 1 and 2 of the nontransferred strand. The data suggest that these contacts are crucial for correct orientation of hairpin DNA in the active site and subsequently the movement of the nontransferred strand out of the active site, to allow correct binding of target DNA and strand transfer.

Lys-330 and Lys-333—In addition to the IS4 conserved YREK signature, Tn*5* transposase also contains a second lysine residue, Lys-330, that, along with Glu-326 and Lys-333, forms a motif that is conserved among many retroviral integrases and bacterial transposases. Although the glutamate and second lysine are absolutely conserved, the internal amino acid in the motif is not. This residue is in many cases positively charged and is never a negatively charged amino acid.

Mutations of amino acid 333 of Tnp from lysine to alanine drastically impaired the ability of transposase to form PECs with all DNA end substrates. A more conservative change at this position, Tnp K333R (charge conservation), was also highly defective for PEC formation at all steps. This lysine residue has been shown by crystallography to make a phosphate contact between nucleotides 1 and 2 of the transferred strand of OE. It is therefore tempting to assume that this contact is present in all reaction steps and is critical for the formation of stable synaptic complexes.

Mutation of the more weakly conserved Lys-330 to either an uncharged alanine or a positively charged arginine had less drastic effects on transposase activity. Tnp K330R was not strongly impaired for PEC formation or catalytic activity at any step in the transposition pathway. Tnp K330A had poor activity *in vitro* with plasmid DNA substrate. However a more detailed analysis of its activity at individual catalytic steps revealed that only the initial 3′ nicking activity was strongly defective. Furthermore, the low activity at this reaction step is due to a combination of mild defects in both complex formation and catalytic activity.

In the case of Tn*10* Tnp, a mutation of the Lys-330 analog (Arg-297) was isolated as a dominant negative mutant (16). This is consistent with our results suggesting that substitution at this position may inhibit Tnp function. The major discrepancy between results reported here for Tn*5* Tnp and those reported previously for Tn*10* Tnp occurs at the conserved Lys-333 (Lys-299 in Tn*10* Tnp) (15). Although the Tn*5* mutants are clearly defective in complex formation, an alanine substitution for Tn*10* Tnp has been reported to not only form synaptic complexes but also promote double-ended cleavage of transposon ends. Therefore this conserved residue may be playing a different mechanistic role in each Tnp.

Model—Tnps mutated at either amino acid 319 or 322 are catalytically defective for both hairpin resolution and strand transfer activities. In a crystallographic structure of Tnp complexed with precleaved DNA, the amino acid side chains of these residues contribute three contacts with the phosphate between bases 1 and 2 of the nontransferred strand (Fig. 8). PEC-normalized hairpin resolution of all three tested substitutions at position 322 were reduced ~20-fold, whereas Tnp Y319A was reduced >2-fold. This indicates that stabilization of the hairpin via these contacts is crucial for correct orientation of the hairpin in the active site.

Tnp Y319A, Tnp R322A, Tnp R322K, and Tnp R322Q, with the exception of the low level activity of Tnp R322Q, were unable to catalyze strand transfer. Once the cleavage reactions are completed, the nontransferred strand of DNA must be removed from the active site in order to accommodate binding of target DNA followed by attack of a phosphate bond by the 3′ OH of the transferred strand. It is likely that protein-DNA contacts between the side chains of Tyr-319 and Arg-322 and the phosphate bond of the nontransferred strand play a crucial role in the removal of the nontransferred strand and correct binding of target DNA into the active site for catalytic attack via the transferred strand 3′ OH.

Mutations at the conserved Lys-333 resulted in impaired PEC formation with uncleaved, pre-nicked, hairpin, and precleaved DNA ends. Crystallographic information reveals that in the precleaved protein-DNA complex, this residue contacts the DNA phosphate between positions 1 and 2 of the transferred strand (Fig. 8). The transferred strand of the DNA end must remain in the active site throughout the transposition process, and it is not surprising that a protein contact that is important in stabilizing the strand near the DNA end yields a consistent phenotype through all catalytic steps.

Acknowledgments—We thank Doug Davies and Scott Lovell for help in creating Fig. 8.

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