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Functional Characterization of Arginine 30, Lysine 40, and Arginine 62 in Tn*5* Transposase*

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Three N-terminal basic residues of Tn*5* transposase, which are associated with proteolytic cleavages by *Escherichia coli* proteinases, were mutated to glutamine residues with the goal of producing more stable transposase molecules. Mutation of either arginine 30 or arginine 62 to glutamine produced transposase molecules that were more stable toward *E. coli* proteinases than the parent hyperactive Tn*5* transposase, however, they were inactive *in vivo*. *In vitro* analysis revealed these mutants were inactive, because both Arg^{30} and Arg^{62} are required for formation of the paired ends complexes when the transposon is attached to the donor backbone. These results suggest Arg^{30} and Arg^{62} play critical roles in DNA binding and/or synaptic complex formation. Mutation of lysine 40 to glutamine did not increase the overall stability of the transposase to *E. coli* proteinases. This mutant transposase was only about 1% as active as the parent hyperactive transposase *in vivo*; however, it retained nearly full activity *in vitro*. These results suggest that lysine 40 is important for a step in the transposition mechanism that is bypassed in the *in vitro* assay system, such as the removal of the transposase molecule from DNA following strand transfer.

Transposases move transposons from one genomic location to another by either a conservative (cut and paste) or a replicative mechanism. Although Tn*5* uses the simpler cut and paste mechanism of transposition, there are still multiple steps involved (see Fig. 1 below). Genetic engineering of Tn*5* transposase to generate a hyperactive form (EKLP)[1] and the optimization of the transposon end sequences allow many of the individual steps of the Tn*5* transposition mechanism to be studied *in vitro* (1–6). The hyperactive EKLP form of Tn*5* transposase contains the mutations E54K, M56A, and L372P (4). The optimized mosaic transposon end sequence (ME) is a hybrid between the bases found in the inside end (IE) and outside end (OE) sequences of the IS*50* transposon (7).

The first steps of the transposition mechanism are binding of the two end sequences of the transposon to two transposase molecules and dimerization of these transposase molecules to form a synaptic complex (see Fig. 1*A* below) (8, 9). Transposase cleaves the transposon from the donor backbone by a multistep reaction. This cleavage involves *trans* catalysis in which the transposon end held by one transposase molecule is cleaved by the second transposase molecule (10). In the first step of the cleavage reaction, the transposase in the presence of Mg^{2+} catalyzes a 3′-hydrolytic nick at position +1 of the transposon end that results in a free 3′-OH. This reactive group attacks the phosphodiester bond on the opposite strand to form a hairpin intermediate (11). The transposase then catalyzes the resolution of the hairpin by a second hydrolytic cleavage reaction. Cleavage of the two ends of the transposon is not a concerted process, because single end cleavage products have been observed (4).

Next, target DNA is captured by Tn*5* transposase and the transposon is inserted into the target. Transposon insertion involves a catalyzed attack of the 3′-OH groups of the cleaved transposon on the phosphodiester bonds of the target DNA. This attack is staggered leading to 9-bp gaps. After repair, these are observed as duplications of the target sequence flanking the transposon. For this repair step to occur, the transposase, which binds to the DNA very tightly, must be removed. In the *in vitro* assays, the transposase is removed either by phenol-chloroform extraction or incubation with 0.5% SDS at 68 °C (4). The mechanism of this step *in vivo* is not known for Tn*5* transposase. The IS*903* and bacteriophage Mu systems are the only transposition systems in which transposase removal has been studied (12, 13). In both of these systems, a chaperone-mediated step is involved. In the Tn*5* transposition system, the removal of the transposase probably requires proteins other than transposase (2).

In the Tn*5* transposition mechanism, synaptic complex formation, transposon cleavage, target capture, and strand transfer into target DNA *in vitro* only require end sequence DNA, target DNA, the transposase, and Mg^{2+}. In other transposition systems, additional transposon-encoded and/or host proteins are required for the transposition reaction such as the activator protein MuB for Mu transposition, the DNA integration host factor for Tn*10* transposition, and TnsC for Tn*7* transposition (14, 15).

Truncation of the N-terminal end of Tn*5* transposase prevents binding of the transposon end sequences and converts the transposase molecules into inhibitors of the transposase reaction (16). N-terminal truncation products are generated by proteolysis or by the use of an alternative promoter whose mRNA has a translation initiation site corresponding to methionine 56 of the transposase molecule (16, 17). To prevent syn-

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[1] The abbreviations used are: EKLP, hyperactive form of Tn*5* transposase with the mutations M56A, E54K, and L372P; bp, base pair(s); DBB, donor backbone; DTT, dithiothreitol; IE, inner end sequence of Tn*5* transposon; ME, mosaic end sequence of transposon; OE, outer end sequence of Tn*5* transposon; P1, amino acid residue on the N-terminal side of a proteolytic cleavage site; P1′, amino acid residue on the C-terminal side of a proteolytic cleavage site; PEC, paired ends complex; PAGE, polyacrylamide gel electrophoresis; X-gal, 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside; PCR, polymerase chain reaction.

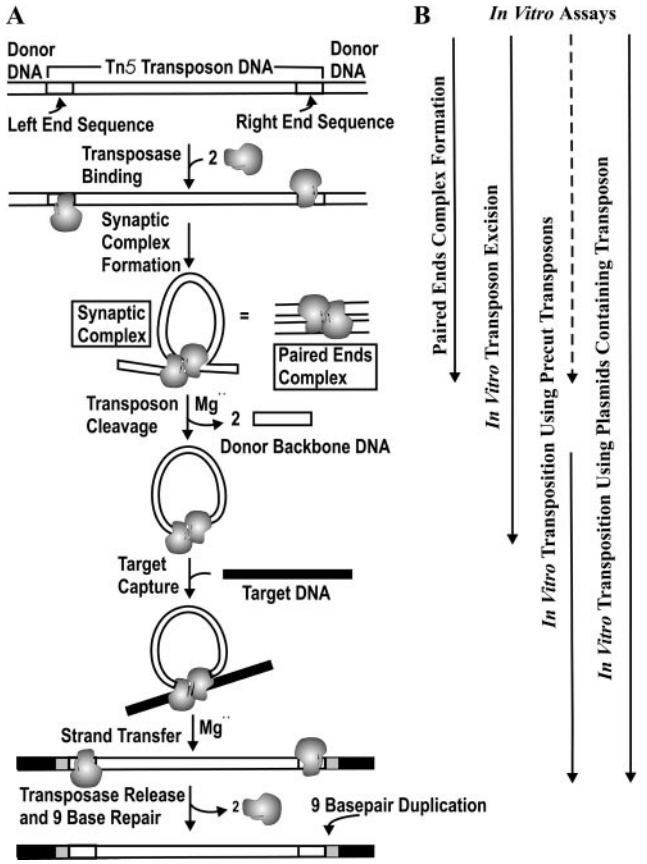

FIG. 1. *A*, model of Tn*5* transposition mechanism. The N-terminal domain plus several residues in the catalytic domain of two Tn*5* transposase molecules bind monomerically to the transposon end sequences. The two bound transposase molecules form synaptic complexes in which the C-terminal ends of the two transposases dimerize (9). The catalytic domain of the transposase molecule bound to the left end is positioned to cleave the transposon end on the right end, and the transposase bound on the right end is positioned to cleave the left transposon end (10, 21). In the presence of Mg^{2+}, the donor backbone DNA is cleaved via a mechanism involving a hairpin intermediate (11). Target DNA is captured and the transposon is inserted into the captured DNA. This step results in nine unpaired bases at each end of the transposon (2). The transposase molecules are removed, and the 9-bp gaps are repaired producing 9-bp duplications. *B*, *in vitro* transposition assays used to characterize Tn*5* transposase mutants. Paired ends complex formation examines the binding of transposases to transposon end sequences present on short pieces of DNA with and without donor backbone. The *in vitro* transposon excision assay tests these steps plus the ability of the transposases to cut the transposon from a plasmid. The *in vitro* transposition reaction using precut transposons bypasses the transposon cleavage step to test the effect on a mutation of target capture and strand transfer. The *in vitro* transposition reaction using plasmids containing transposons tests the transposition reaction through strand transfer complex formation. For quantification of the *in vitro* transposition reaction, the transposase molecules are removed with SDS or chloroform-phenol treatment and the naked DNA is transformed into *E. coli.* Repair of the 9-bp gaps occurs *in vivo*. Acquisition of antibiotic resistance and presence of the transposon are used for quantification of the reaction.

thesis directed by the alternative promoter, methionine 56 is mutated to an alanine to ensure full-length expression of the transposase. This, however, does not prevent proteolysis of the full-length transposase to form three stable N-terminal truncation products (6). These act as transposase inhibitor molecules and react with full-length transposase to form nonproductive complexes (18). Three truncation products result from *Escherichia coli* proteinase cleavage: $\alpha 1$ from cleavage of the Arg^{30}-Leu^{31} bond, $\alpha 2$ from cleavage of the Lys^{40}-Tyr^{41} bond and β from cleavage of the Arg^{62}-Phe^{63} bond of Tn*5* transposase. Arg^{30}, Lys^{40}, and Arg^{62} in the N-terminal DNA binding domain were selected for mutation to genetically engineer more stable forms of Tn*5* transposase. The three basic residues were individually mutated to glutamine, a residue that potentially can interact with DNA directly through a hydrogen bond or indirectly through interaction with a water molecule.

Here we show the stabilization of Tn*5* transposase against proteolytic degradation by *E. coli* enzymes by mutation of Arg^{30} to Q and Arg^{62} to Q but not Lys^{40} to Q. We identify two DNA binding residues (Arg^{30} and Arg^{62}) in Tn*5* transposase that are required to form stable complexes with transposon end sequences attached to donor backbone. We also demonstrate that mutation of another N-terminal basic residue, Lys^{40} to Q, does not significantly alter synaptic complex formation, transposon excision, target capture, or strand transfer using *in vitro* assays. This mutation, however, affects a step in the transposition mechanism that occurs *in vivo* not probed by the *in vitro* assays such as the removal of the transposase from the DNA.

EXPERIMENTAL PROCEDURES

Mutation of Tn5 Transposase—The plasmid pRZPET2, which contains the hyperactive Tn*5* transposase gene (EKLP), with the mutations E54K, M56A, and L372P (4), was used as a template to individually mutagenize residues Arg^{30}, Lys^{40}, and Arg^{62} in the N-terminal DNA binding region of the transposase to Q. The following PAGE purified oligonucleotide primers were obtained from Integrated DNA Technologies: R30Q FORWARD, TCGCCGTACTGCCCAATTGGTTAACGTCGCCGCCCAATT-3′; R30Q REVERSE, 5′-TTAACCAATTGGGCAGTACGGCGAGGATCACCCAGCGCC-3′; K40Q FORWARD, 5′-GCCCAATTGGCACAATATTCTGGTAAATCAATAACCATC-3′; K40Q REVERSE, 5′-ACCAGAATATTGTGCCAATTGGGCGGCGACGTTAACCAA-3′; R62Q FORWARD, 5′-GGCGCTTACCAATTTATCCGCAATCCCAACGTTTCTGCC-3′; PET21 TPASE FORWARD, 5′-CGACTCACTATAGGGGAATTGTGAGCGG-3′; and PET21 TPASE REVERSE 607, 5′-CTCATCATGCTGCCCATGCGTAACCGG-3′.

The R30Q (pRZPET2-R30Q) and the K40Q (pRZPET2-KQ40) mutants were constructed by PCR using the template pRZPET2 and *pfu* DNA polymerase (Stratagene) to produce two overlapping products that contained the desired mutation and *Xba* and *Not*I cleavage sites. For the R30Q mutant, the two overlapping PCR products were obtained using the R30Q REVERSE primer and the PET21 TPASE FORWARD primer for the first product and the R30Q FORWARD primer and the PET21 TPASE REVERSE 607 primer for the second product. For the K40Q mutant, the two overlapping PCR products were obtained using the K40Q REVERSE primer and the PET21 TPASE FORWARD primer for the first product and the K40Q FORWARD primer and the PET21 TPASE REVERSE 607 primer for the second product. The respective products for each mutation were purified on agarose gels and extracted using the QIAquick PCR gel extraction kit (Qiagen). The overlapping products were mixed with the PET21 TPASE FORWARD and TPASE REVERSE 607 primers, and PCR was carried out. The products were gel-purified and digested with *Xba* and *Not*I (Promega), and the enzymes and small digestion products were removed using a PCR purification kit (Qiagen). The plasmid pRZPET2 was digested with the same two enzymes, and the plasmid minus the *Xba-Not*I fragment was gel-purified. The mutated *Xba-Not*I fragments were ligated into the cut plasmids. These plasmids were transformed into *E. coli* strain DH5α, grown in Luria Bertani medium (LB) for 1 h, and plated on LB agar containing ampicillin (100 μg/ml, Sigma Chemical Co.).

The R62Q (pRZPET2-R62Q) mutant was constructed using PCR to produce a mega primer containing the mutation, and then this primer was used to produce the mutated *Xba-Not*I fragment for insertion into the digested pRZPET2. The first PCR reaction used the R62Q FORWARD primer and the TPASE REVERSE 607 primer. After gel purification, this product was used in a second PCR reaction as a mega primer with the PET21 TPASE FORWARD primer to produce the full-mutated *Xba-Not*I product. The resulting PCR product was purified and inserted into the digested pRZPET2 plasmid as described above. All three mutant plasmids were manually sequenced using Sequenase Version 2 (Amersham Pharmacia Biotech) and [α-^{32}P]ATP from Amersham Pharmacia Biotech to confirm the presence of the desired mutations and the absence of unwanted mutations.

Papillation Assay—The mutant plasmids and pRZPET2 were isolated from DH5α, transformed into *E. coli* strain MDW320, and plated on Trp^--X-gal-phenyl-β-D-galactoside agar containing ampicillin (100 μg/ml) and tetracycline (15 μg/ml, Sigma). The papillation assay was

carried out as reported by Weinreich *et al.* (16). This assay measures the rate of Tn*5*lac transposition into an actively transcribed gene through the appearance of blue papillae over time.

Mating Out Assay—The mutant plasmids and pRZPET2 were transformed into *E. coli* strain JCM101 containing pOX38-Gen, an F′ plasmid, and pFMA187OOΔ, a plasmid containing a transposon with two outside ends. The mating out assay was performed as previously described using RZ224 as the recipient strain (18). To determine total exconjugates formed in this assay, the bacteria were grown on LB agar containing gentamicin (5 μg/ml, Sigma) and nalidixic acid (20 μg/ml, Sigma). To quantify exconjugates that contain the transposon on the conjugated pOX38-Gen F-factor, the bacteria were grown on LB agar containing gentamicin (5 μg/ml, Sigma), nalidixic acid (20 μg/ml, Sigma), and chloramphenicol (20 mg/ml, Sigma)

Expression of Tn5 Transposase Mutants—The constructs for expressing mutated proteins were generated by swapping the *Xba-Not*I cleaved products containing the codon for glutamine at residues 30, 40, or 62 into the expression vector pGRTYB35 that encodes for the hyperactive EKLP Tn*5* transposase fused at the C-terminal end to the intein-chitin binding domain (11). The plasmids, pRZPET2-R30Q, pRZPET2-K40Q, pRZPET2-R62Q, and pGRTYB35 were digested with the restriction enzymes *Xba* and *Not*I. The mutated *Xba-Not*I fragments and the cleaved pGRTYB35 plasmid were gel-purified, and then the mutated fragments were individually ligated into the cleaved expression vector. These vectors were transformed into *E. coli* strain BL21 DE3 plys. The hyperactive EKLP transposase and the mutated transposases were expressed and then purified on a chitin column (New England BioLabs) in the presence of a proteinase inhibitor mixture (Roche Molecular Biochemicals) as previously reported (11). 50 mM dithiothreitol (DTT) was used to induce intramolecular cleavage of the protein to release the free transposase molecules.

Proteolysis of Transposases by E. coli Proteinases—The mutant and hyperactive transposases were expressed in parallel 1-liter cultures at 23 °C as described above. After 4 h of induction of protein expression with isopropyl-1-thio-β-D-galactopyranoside, all cultures were normalized to an optical density of 0.6 with LB (11). The *E. coli* organisms in 1 liter were pelleted by centrifugation, resuspended, and then sonicated in the presence or absence of the proteinase inhibitor mixture. The transposase molecules were isolated as described above. The C-terminal portion of proteolysis products bound to the chitin column through the C-terminal intein-chitin binding domain. The transposase portion of the fusion protein was released upon treatment with DTT. The N-terminal portions of proteolysis products were lost in the purification process. The isolated molecules were separated by SDS-PAGE, stained using Sypro orange (Molecular Probes), and visualized on a FluoroImager (Molecular Dynamics).

Limited Proteolysis of Transposases by Thermolysin—To remove the EDTA and the DTT present in the elution buffer from the chitin column, the isolated transposases were diluted three times in 50 mM Tris buffer, pH 7.0, containing 2 mM $CaCl_2$ and then concentrated using a Centricon 30 (Millipore) at 4 °C. The mutant transposases and the hyperactive EKLP transposase (15 μg) were incubated with thermolysin (100 ng, Calbiochem) at 37 °C. Samples were taken at 0, 5, and 15 min and immediately placed into SDS-PAGE sample buffer containing DTT and boiled for 3 min. The cleaved samples were separated on 12% SDS-polyacrylamide gels and then stained with Coomassie Brilliant Blue.

Paired Ends Complex Formation—Six PAGE-purified oligonucleotides were obtained from Integrated DNA Technologies: 60-bp oligonucleotides containing the mosaic end sequence (*underlined*) plus 21 bp of the transposon beyond the end sequence and 20 bp of the donor backbone, Top 5′-CTCAGTTCGAGCTCCCAACACTGTCTCTTATACACATCTTGAGTGAGTGAGCATGCATGT-3′ and Bottom 5′-ACATGCATGCTCACTCACTCAAGATGTGTATAAGAGACAGTGTTGGGAGCTCGAACTGAG-3′; 40-bp oligonucleotides containing the mosaic end sequence plus 21 bp of the transposon beyond the end sequence, Top 5′-CTCAGTTCGAGCTCCCAACACTGTCTCTTATACACATCT-3′ and Bottom 5′- AGATGTGTATAAGAGACAGTGTTGGGAGCTCGAACTGAG-3′; and 20-bp oligonucleotides containing 20 bp of the donor backbone Top 5′-CTCAGTTCGAGCTCCCAACA-3′ and Bottom 5′-TGTTGGGAGCTCGAACTGAG-3′. The 5′-ends of the oligonucleotides were end-labeled using T4 polynucleotide kinase (New England BioLabs) and [γ-^{32}P]ATP (Amersham Pharmacia Biotech, Redivue). Unincorporated nucleotides were removed with a nucleotide removal kit (Qiagen). The oligonucleotides were annealed in 20 mM Tris-HCl, pH 7.5, containing 50 mM NaCl by heating at 80 °C for 2 min and then by slowly cooling to room temperature. Four different labeled transposon forms were constructed by annealing the following oligonucleotides: 60-bp DBB, [5′-^{32}P]ATP top and bottom 60-bp oligonucleotides; 40-bp precut transposon, [5′-^{32}P]ATP top and bottom 40-bp oligonucleotides; 60-bp 3′ Nick, [5′-^{32}P]ATP top 60-bp oligonucleotide plus bottom [5′-^{32}P]ATP 40-bp oligonucleotide and unlabeled bottom 20-bp oligonucleotide; and 60-bp 5′ Nick, [5′-^{32}P]ATP bottom 60-bp oligonucleotide plus unlabeled top 40-bp oligonucleotide and top [5′-^{32}P 20-bp oligonucleotide.

The 5′-^{32}P-labeled mosaic end DNA fragments (28 nM) were mixed individually with the mutant or hyperactive EKLP transposases (400 nM) in 20 mM HEPES, 100 mM potassium glutamate buffer and incubated at 30 °C for 3 h. The reaction mixtures were immediately mixed with native gel loading buffer (0.25% bromphenol blue, 0.25% xylene cyanol FF, 30% glycerol in H_2O), and the products were separated on a 5% native polyacrylamide gel. The bands were visualized using a PhosphorImager (Molecular Dynamics).

In Vitro Transposon Excision—pGRST2, a plasmid that contains a 1.3-kb transposon with the kanamycin gene flanked by the mosaic ends, was used for *in vitro* assays as previously reported (4, 17). The hyperactive transposase and the three mutant transposases (200 nM) were individually incubated with pGRST2 (34 nM) in transposition buffer (0.1 M potassium glutamate, 25 mM Tris acetate, pH 7.5, 10 mM magnesium acetate, 50 μg/ml bovine serum albumin, 0.5 mM β-mercaptoethanol, 2 mM spermidine, 100 μg/ml tRNA, 50 mM NaCl) at 37 °C for 1 h. *Xma*I (Promega) was added, and the reactions were incubated for an additional hour at 37 °C. The transposases and restriction enzymes were removed by chloroform-phenol extraction followed by ethanol precipitation. The products were resuspended in H_2O and analyzed on 1.5% agarose gels. The gels were stained with SYBR green (Molecular Probes) and visualized using a FluoroImager (Molecular Dynamics).

Precut Transposon Insertion into Target DNA—pGRPK7876, containing a 1.8-kb transposon, which includes the Tn*5* mosaic end sequences and the R6Kγori and a *kan*^{R} gene from Tn*903*, was cleaved with *Pvu*II to remove donor DNA (3). The resulting transposon was gel-purified as given above. The precut transposons (60 nM) were incubated for 2 h with the mutant or the hyperactive Tn*5* transposases (400 nM) and the target DNA (pUC19, 60 nM) in 20 μl of transposition buffer. Following incubation, SDS was added to 0.5%, and the tubes were further incubated at 68 °C for 15 min to dissociate the transposases from the DNA. The reaction mixtures were placed on 0.05-μm VM nitrocellulose membranes (Millipore, Bedford, MA) suspended on the surface of deionized water to remove the SDS and protein dissociated from the DNA. After 2 h, 3 μl of the dialyzed mixture was electroporated into DH5α cells using standard procedures. *Kan*^{R}-*Amp*^{R} colonies containing pUC19 (Amp^R) with the transposon (Kan^R) were quantified, and the presence of the transposon was confirmed by analysis of the size of the plasmids on agarose gels.

In Vitro Transposition Using the Intact Plasmid pGRPK7876—This assay was the same as given above for the precut transposon assay with the exception that uncleaved pGRPK7876 was used. In this assay the transposase must cleave the transposon from the donor backbone prior to insertion into pUC19.

In Vitro Transposition Using Intact Plasmids with Outside and Mosaic End Sequences—Two plasmids that differ only by the presence of the outside end sequences in pRZTL1 and the mosaic end sequences in pRZTL4 (4, 7) were used for *in vitro* transposition reactions in which the transposon ends are inserted within the transposon-forming nested deletions and inversions of the transposon (1). The transposon contains a promoterless tetracycline-resistant gene that can be expressed upon insertion of the gene in the correct orientation in a unit of transcription within the transposon.

Either pRZTL1 or pRZTL4 (20 nM) were incubated with the hyperactive or mutant transposases (300 nM) in 10 μl of transposition buffer at 37 °C for 2 h. The reaction mixture was treated with 0.5% SDS at 68 °C for 15 min and dialyzed against water, and 2 μl of the dialyzed preparation was electroporated into DH5α cells as given above. The numbers of tetracycline-resistant colonies from three independent experiments were averaged.

Proteolysis of the Transposase in the Strand Transfer Complex—Annealed 5′-^{32}P-labeled 40-bp mosaic ends containing oligonucleotides (2.8 nM) were incubated in the presence of pUC19 (6 nM) and either the K40Q mutant transposase or the hyperactive EKLP transposase (20 nM) in 10 μl of transposition buffer without bovine serum albumin for 2 h at 37 °C. Either *N*-tosyl-L-phenylalanine chloromethyl ketone trypsin (10–1000 nM, Sigma) or proteinase K (6 μM, Sigma) were added to the samples and further incubated for 15 min at 37 °C. The reaction was stopped by the addition of native gel loading buffer containing *N*-tosyl-L-lysine chloromethyl ketone (4 mM, Sigma), and the samples were immediately separated on a 1% agarose gel. The DNA bands were visualized using ethidium bromide. The gel was carefully dried under

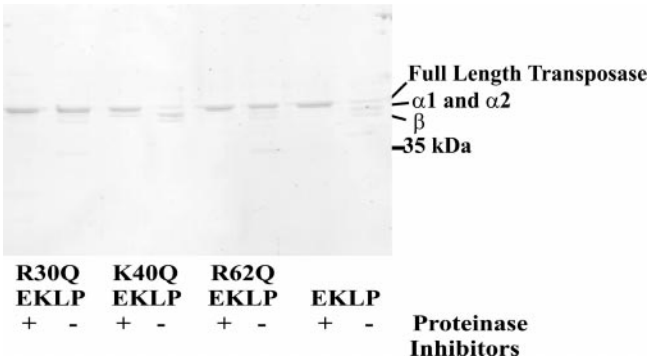

FIG. 2. **Tn*5* transposase N-terminal mutations R30Q and R62Q but not K40Q stabilize against cleavage by *E. coli* proteinases.** The hyperactive Tn*5* transposase (*EKLP*) construct and those containing R30Q, K40Q, or R62Q mutations were expressed in *E. coli* as a fusion protein with an intein-chitin binding domain fused to the C-terminal end of the transposase molecules. The cells were sonicated in the presence (+) or absence (−) of proteinase inhibitors. The solubilized proteins were isolated on a chitin affinity column, and the transposase was released by an intramolecular cleavage in the presence of DTT. The eluted proteins were separated by SDS-PAGE (10%), and the bands were stained with Sypro orange and visualized using a PhosphorImager. $\alpha1 = \Delta$ 1–30 amino acids of Tn*5* transposase; $\alpha2 = \Delta$ 1–40 amino acids of Tn*5* transposase; $\beta = \Delta$ 1–62 amino acids of Tn*5* transposase.

vacuum onto nitrocellulose. The bands on the dried gel were visualized using a PhosphorImager and quantified using the ImageQuaNT program (Molecular Dynamics).

RESULTS

Stability of Tn5 Transposase Mutants—With the goal to produce more stable forms of Tn*5* transposase, three basic Tn*5* transposase residues (Arg^{30}, Lys^{40}, and Arg^{62}), present at the P1 positions (N-terminal side) of the sites cleaved by *E. coli* proteinases during purification (6), were individually mutated to glutamine residues by site-directed mutagenesis. The hyperactive EKLP Tn*5* transposase was used as the parent form because its *in vitro* activity allows dissection of the transposition reaction mechanism (4, 17). The R30Q and R62Q mutants were much more stable than either the parent EKLP or the K40Q mutant transposases toward the proteinases present in *E. coli* (Fig. 2). The EKLP transposase preparation isolated in the absence of proteinase inhibitors contained two stable bands of proteolytic products in addition to full-length transposase. Previous studies showed the α band contains transposase molecules cleaved after Arg^{30} ($\alpha1$) and Lys^{40} ($\alpha2$), and the β band contained transposase molecules cleaved after Arg^{62} (6). In contrast to the R30Q and the R62Q mutants, the intact K40Q transposase mutant was sensitive to proteolysis like the EKLP transposase (Fig. 2). It was degraded to two stable products with more of the α band than was observed for the EKLP transposase. This probably represents the $\alpha1$ product, because the $\alpha2$ cleavage site is mutated in the K40Q mutant. The R30Q and R62Q mutants were very stable with small amounts of the α and β products detected. The β product in the R62Q preparation is probably due to cleavage at Arg^{65}, a nearby positively charged amino acid. In addition, a small amount of a 35-kDa product was observed for both the R30Q and R62Q mutant transposases. The identity of this product is not known.

The increased overall stability of the R30Q and R62Q transposase mutants suggests not only that the peptide bond next to the mutated residue is protected against cleavage but also that the N-terminal region of these mutants may be less flexible and/or less exposed to solvents. Either of these possibilities would lead to an overall decrease in susceptibility to proteolysis by proteinases that degrade at sites other than the mutated basic amino acids. To test this hypothesis, the three mutants and the parent EKLP transposase were subjected to limited

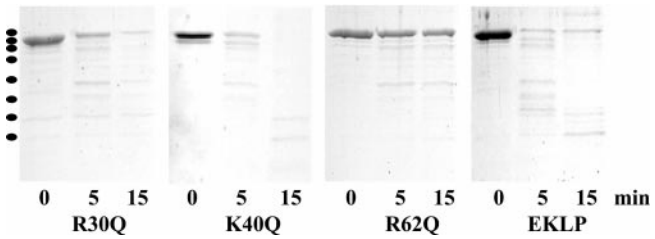

FIG. 3. **The R62Q mutation, but not the R30Q or K40Q mutations, stabilizes Tn*5* transposase against thermolysin cleavage.** The hyperactive (*EKLP*) Tn*5* transposase and the R30Q, K40Q, and R62Q transposase mutants were incubated with thermolysin at a ratio of 150:1. Samples were removed at 0, 5, and 15 min and immediately placed in SDS sample buffer and boiled. The degradation products were separated by SDS-PAGE (10%), and the products were visualized with Coomassie Brilliant Blue.

proteolysis by thermolysin, a metalloproteinase that cleaves preferentially at peptide bonds with the hydrophobic residues, Leu, Phe, Ile, or Val, in the P1′-site (C-terminal side of the cleavage site) but that can also accommodate Met, His, Tyr, Ala, Asn, Ser, Thr, Gly, Lys, Glu, or Asp at the P1′-position (19). The R62Q mutant was much more stable than the parent EKLP transposase (Fig. 3), however, similar products were observed for both molecules, suggesting that this mutation alters the flexibility or solvent accessibility of the N-terminal end of the transposase but not the overall conformation. The R30Q transposase mutant was slightly more stable over the first 5 min of degradation with thermolysin than the parent EKLP transposase, but similar products were formed for the two transposases. By 15 min, similar amounts of both transposases were degraded. The K40Q mutant transposase was degraded at a similar initial rate to that of the parent EKLP with similar degradation products, indicating similar conformations and flexibilities. These results suggest that the overall conformations of the mutant transposases were similar but the accessibility of the N-terminal region of R62Q mutant and, to some extent, the R30Q mutant to the proteinases may be decreased.

In Vivo Transposition—Two *in vivo* transposition systems were used to test the activity of the three mutants relative to the parent hyperactive EKLP transposase. The papillation assay was used as a qualitative initial screen of activity. Multiple papillae per colony were observed for both the EKLP and K40Q transposases but not for the R30Q and R62Q mutants within 7 days. Although multiple papillae were observed, the initial papillae on the K40Q mutant colonies were observed 8–12 h after those for the EKLP colonies, and the numbers of papillae per colony were fewer for the K40Q mutants than for the EKLP colonies.

For a more quantitative assay, the *trans*-mating out assay was used to compare the *in vivo* transposition frequencies. No activity over background was noted for the R30Q and R62Q mutants (Table I, *column 1*), suggesting the mutations prevent one or more of the required steps of transposition. The K40Q mutant transposase was about 100× less active than the parent EKLP transposase, indicating the mutation alters but does not knock out transposition activity.

In Vitro Paired Ends Complex Formation by K40Q Mutant Tn5 Transposase—To explore the step or steps in the transposition mechanism altered by the K40Q mutation, the activity of this mutant was compared with the hyperactive parent EKLP Tn*5* transposase using *in vitro* assays (Fig. 1*B*). The first assay used an electrophoretic mobility shift assay to determine the ability of the K40Q mutant transposase to form synaptic complexes (paired ends complexes) with three forms of the transposon encountered by the transposase at various steps of the

TABLE I

Effect of R30Q, K40Q, and R62Q mutations of Tn5 transposase on in vivo and in vitro transposition frequencies

Transposase	*In vivo*		*In vitro*		*In vitro*	
	Transposition frequency by the mating out assay using pFMA 18700 with OE[a,b,e]	Relative frequency[c]	Transposition frequency/μg transposase using pRZTL-1 with OE[b,d,e]	Relative frequency[c]	Transposition frequency/μg transposase using pRZTL-4 with ME[b,d,e]	Relative frequency[c]
EKLP	$1.5 \pm 0.3 \times 10^{-5}$	1	$2.5 \pm 0.6 \times 10^{4}$	1	$2.6 \pm 0.9 \times 10^{5}$	1
R30Q	0.0	0	0.0	0	0.0	0
K40Q	$1.9 \pm 0.2 \times 10^{-7}$	0.013 ± 0.002	$2.8 \pm 1.2 \times 10^{4}$	1.1 ± 0.47	$1.7 \pm 1.1 \times 10^{5}$	0.66 ± 0.44
R62Q	0.0	0	0.0	0	0.0	0

[a] *In vivo* transposition frequencies were measured by *trans* mating out assays and were calculated by dividing the exconjugates containing transposons by the total number of exconjugates.

[b] Transposition frequencies are averages of three to four independent experiments. Standard deviations are given.

[c] Relative frequencies are calculated using the transposition frequency of the parent EKLP Tn*5* transposase as equal to 1.

[d] *In vitro* transposition frequency/μg transposase = number of tetracycline-resistant colonies obtained in which the promoterless tetracycline resistance gene located in the transposon is placed by transposition in the correct orientation of a unit of transcription.

[e] OE = outside end sequence; ME = mosaic end sequence.

transposition process and a control form of the transposon not associated naturally with the transposon (Fig. 4*A*). The transposon forms are 1) the normal substrate form composed of transposon end sequences attached on one end (+19) to transposon sequences and on the other end (+1) to donor backbone (60 bp); 2) the first intermediate in the transposon cleavage step, a transposon with a 3′-nick in the transferred strand between the +1 position of the transposon end sequence and the donor backbone (60-bp 3′-nick); 3) the cleaved transposon (40 bp); and 4) a control for the 3′-nicked transposon form, a 5′-nicked transposon with a nick in the nontransferred strand between the +1 position of the transposon end sequence and the donor backbone (60 bp 5′-nick). This last form is used as a control to determine whether any differences observed between the intact and nicked forms of the transposon-containing donor backbone are due to differences in the flexibility of the DNA or whether the differences are due to specific interactions between the DNA and the transposases.

The K40Q mutant was equally active as the EKLP mutant in the formation of paired ends complexes with DNA with donor backbone sequences (Fig. 4*B*, 60 bp, compare *lanes 14* and *18*) or without donor backbone sequences (Fig. 4*B*, 40 bp, compare *lanes 11* and *15*). In addition, both forms of the transposase formed paired ends complexes equally well with DNA containing nicks in the backbone either at the natural position for formation of the hairpin intermediate (Fig. 4*B*, 60-bp 3′-nick, compare *lanes 12* and *16*) or on the opposite strand (Fig. 4*B*, 60-bp 5′-nick, compare *lanes 13* and *17*). This suggests the K40Q mutation does not alter DNA binding nor synaptic complex formation.

In Vitro Excision of Transposons by K40Q Mutant Tn5 Transposase—The next step in the transposition reaction, the cleavage of the transposon from donor backbone, was compared between the K40Q mutant and the parent EKLP transposases using pGRST2 that contains a 1.3-kb transposon with mosaic ends (Fig. 5*A*). To characterize the excision step, the products were digested with *Xmn*I that asymmetrically cleaves the donor backbone. This allows visualization of cleavage of the transposon at one end as well as cleavage at both ends. Analysis of the products following digestion revealed that both the K40Q mutant and parent EKLP forms of the transposase produced the same single left end cut product (3.2 kb) and right end cut product (2.0 kb) with slightly higher amounts for the R40Q mutant than for EKLP transposase (Fig. 5*B*). Both forms of the transposase produced the same double-transposon end cleavage product (1.3 kb) at similar amounts, as well as the same excised donor backbone products (right, 1.9 kb; left, 0.7 kb) with slightly higher amounts for the EKLP transposase that for the R40Q mutant.

In Vitro Transposition by K40Q Mutant Tn5 Transposase—With the knowledge that the K40Q mutant can bind the transposon end sequences and cleave the transposon from donor backbone, two additional assays (Fig. 1*B*) were used to quantify the transposase activities of the K40Q mutant and the parent

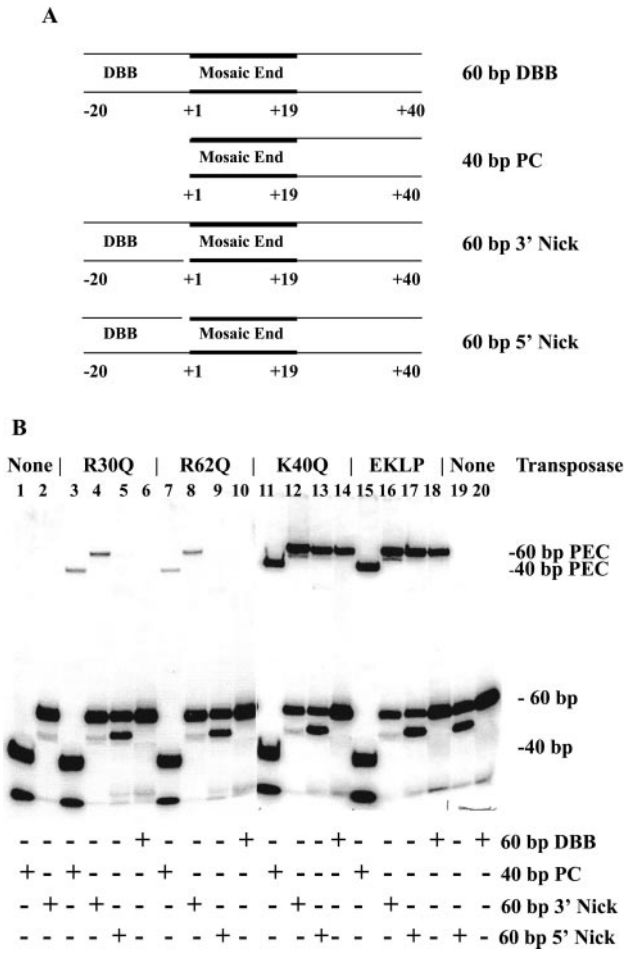

FIG. 4. **The ability of the R30Q and R62Q but not K40Q Tn*5* transposase mutants to form paired ends complexes (*PEC*) is impaired.** *A*, transposon forms. The 60-bp DBB is a 40-bp transposon containing the mosaic end sequence (+1 to +19) with 20 bp of donor backbone (*DBB*) attached (−20 to −1). The 40-bp PC is the excised transposon or the precut transposon (*PC*). The 60-bp 3′-nick is the natural intermediate in the transposon cleavage reaction with a nick on the *bottom* (transferred) *strand*. The 60-bp 5′-nick is a control with the nick on the *top* (nontransferred) *strand*. *B*, paired end complex formation. Purified hyperactive (*EKLP*) transposase and the R30Q, K40Q, and R62Q transposase mutants were incubated for 3 h at 30 °C with 5′-^{32}P-labeled annealed oligonucleotides containing mosaic ends given in *A*. The paired ends complex (*PEC*) products were separated on 5% native polyacrylamide gels, and the labeled bands were visualized using a PhosphorImager. The paired ends complexes were composed of two molecules of transposase and two DNAs as previously reported (8).

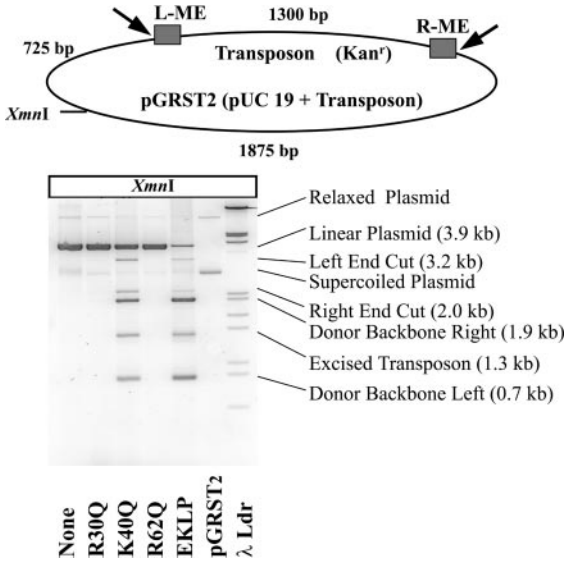

FIG. 5. **The Tn*5* transposase mutant K40Q, but not the R30Q and R62Q transposase mutants, retains *in vitro* transposon cleavage activity.** *Top*, the plasmid pGRST2 contains a 1.3-kb transposon with the coding sequence for the kanamycin (kan) resistance gene and mosaic end sequences at both the left and right ends (*L-ME* and *R-ME*). The mosaic end sequences are a hybrid between the inside and outside end sequences present in transposons found in Tn*5*. The sizes of potential cleavage products are given. *Bottom*, purified hyperactive (EKLP) transposase and the R30Q, K40Q, and R62Q transposase mutants were incubated for 1 h at 37 °C and then digested for 1 h with *Xmn*I. The DNA was extracted with phenol-chloroform and separated on agarose gels. The DNA was stained with SYBR Green II and visualized using a FluoroImager. Transposition products and their sizes are indicated. λ*Ldr* = Lambda DNA/*Eco*RI + *Hin*dIII markers. *Arrows* are shown at the positions of transposase cleavage of the transposon.

EKLP transposase. In these assays, the transposition reaction was carried out *in vitro* by the transposase, and then the transposase was dissociated by treatment of the DNA·protein complexes with SDS and heating. The protein-free DNA was transformed into *E. coli* for quantification of the transposition events as antibiotic resistant colonies. The number of transposition events per microgram of transposase was similar for both the R40Q mutant and the parent EKLP transposases when either the precut transposon (Table II, *columns 3* and *4*) or the plasmid pGRPK7876 was used as the source of the transposon for insertion into pUC19 (Table II, *columns 1* and *2*). These results demonstrate the K40Q mutant transposase is as active as the parent EKLP transposase in these *in vitro* assays.

Because these *in vitro* assays use transposons with mosaic end sequences and the *in vivo* assays use transposons with outside end sequences, the 100× difference observed in the transposition frequencies of the K40Q mutant *in vitro* compared with *in vivo* could be due to the end sequences used. To test this, two transposon-containing plasmids were used that differed only by the presence of outside end sequences in pRZTL1 and mosaic end sequences in pRZTL4. No significant differences were noted in transposition frequencies between the K40Q mutant and the hyperactive EKLP parent transposase with either form of the transposons; however, the transposition frequency using mosaic ends was about 10× greater than using outside ends for both transposases (Table I, *columns 3–6*). Based on these data, the 100× difference in activity between these two transposase molecules observed in the *in vivo* mating out assay is due to a process that occurs *in vivo* not measured by the *in vitro* assay.

Proteolytic Cleavage of the K40Q Mutant Tn5 Transposase from the DNA following Strand Transfer—One step that must occur in the *in vivo* transposition reaction and is bypassed in the *in vitro* assays is the removal of the transposase molecule from the transposon·target DNA complex. In the *in vitro* assays, the transposases are removed by incubating with 0.5% SDS at 68 °C followed by dialysis. A possible mechanism for the removal of transposase from DNA after the strand transfer reaction *in vivo* is direct proteolysis. To test whether the strand transfer complexes formed with the K40Q mutant transposase are more stable to proteolysis than those formed with the parent EKLP transposase, *in vitro* transposition was carried out using 40 bp of precleaved DNA containing the mosaic end sequence and pUC19 as the target DNA. The complexes formed were subjected to trypsin cleavage, a proteinase that cleaves proteins at lysine and arginine residues, to mimic the cleavage observed for the *E. coli* proteinase(s) (6). The K40Q mutant and the parent EKLP transposases were equally susceptible to trypsin cleavage (Fig. 6). This suggests that the decrease in transposition activity of the K40Q mutant *in vivo* is not due to increased stability of the transposase·DNA complex toward direct proteolysis at basic residues. This, however, does not rule out a difference in other mechanisms of removal of the transposase from DNA such as the interaction of the transposase with a chaperone (13).

In Vitro Analysis of R30Q and R62Q Mutant Transposases—Unlike the K40Q mutant transposase, the R30Q and R62Q mutant transposases were inactive *in vivo* (Table I, *columns 1* and *2*). Because the thermolysin degradation products of these two mutants and the parent EKLP transposases were similar (Fig. 3), the overall conformation of the mutants probably is not changed. This would suggest the active site is intact but the two mutations, which are in the N-terminal domain associated with DNA binding (6), alter the ability of the mutant transposases to either bind DNA and/or form synaptic complexes. To test these possibilities, *in vitro* transposition assays (Fig. 1*B*) were used to compare the properties of the R30Q and R62Q mutant transposases to the parent EKLP transposase.

In Vitro Paired Ends Complex Formation by R30Q and R62Q MutantTn5 Transposases—The ability of the R30Q and R62Q mutant transposases to form paired ends complexes with DNA was compared with that of the parent EKLP transposase (Fig. 4*B*). The two mutants did not form paired ends complexes with the 60-bp DNAs (Fig. 4*B*, *lanes 6* and *10*) that contained donor backbone plus the mosaic end sequence and a small amount the transposon. The mutants did form complexes with the 40-bp DNAs that do not contain donor backbone (Fig. 4*B*, *lanes 3* and *7*). Although the two mutants formed complexes, a substantially lower amount was formed than with the parent EKLP transposase (Fig. 4*B*, compare *lanes 3* and *7* with *lane 15*), suggesting a difference in either the binding affinity for the DNA or the stability of the complexes.

To further characterize this binding, DNA was tested that mimics the natural nicked DNA intermediate formed in the first step of the transposon cleavage reaction (Fig. 4*B*, 60-bp 3′-nick). The R30Q and R62Q mutant transposases were able to assemble paired ends complexes with this 3′-nick form of the DNA (Fig. 4*B*, *lanes 4* and *8*) but not with DNA containing the nick in the nontransferred strand (Fig. 4*B*, 60-bp 5′-nick, *lanes 5* and *9*). The 3′-nicked form of the 60-bp DNA was utilized with a similar efficiency as the 40-bp precut transposon form. This demonstrates that the two mutations prevent the binding of transposons attached to donor backbone unless the natural 3′-nick is present. Furthermore, because complexes were not formed with the 60-bp DNA with a 5′-nick, the interaction between the mutant transposases and the 60-bp DNA with the 3′-nick is probably due to specific interactions and not to an increase in flexibility of the DNA.

In Vitro Transposition Reaction of R30Q and R62Q Mutant

TABLE II

Comparison of the effect of R30Q, K40Q, and R62Q mutations of Tn5 transposase on in vitro transposition frequencies using transposons with and without donor backbone

Transposase	With DBB		Without DBB	
	Transposition frequency/μg transposase using pGRPK7876[a,c]	Relative frequency[c]	Transposition frequency/μg transposase using precut transposon pGRPK7876[a,d]	Relative frequency[c]
EKLP	$4.9 \pm 0.1 \times 10^5$	1	$7.8 \pm 2.6 \times 10^5$	1
R30Q	0.0	0.0	$2.0 \pm 0.7 \times 10^5$	0.25 ± 0.09
K40Q	$3.4 \pm 0.2 \times 10^5$	0.71 ± 0.051	$8.7 \pm 3.5 \times 10^5$	1.12 ± 0.45
R62Q	0.0	0.0	$0.11 \pm 0.09 \times 10^5$	0.014 ± 0.011

[a] Transposition frequencies are averages of three to four independent experiments. Standard deviations are given.
[b] Relative frequencies are calculated using the transposition frequency of the parent EKLP Tn*5* transposase as equal to 1.
[c] *In vitro* transposition frequency/μg transposase = number of colonies obtained containing the transposon excised from pGRPK7876 by the transposase and inserted into pUC19/μg transposase.
[d] *In vitro* transposition frequency/μg transposase = number of ampicillin-kanamycin-resistant colonies obtained containing the precut transposon from pGRPK7876 inserted into pUC19/μg transposase.

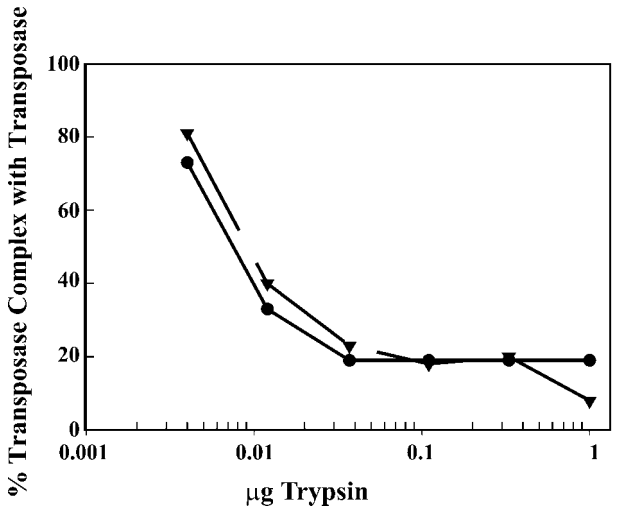

FIG. 6. **The K40Q Tn*5* transposase mutation does not alter the trypsin susceptibility of the final *in vitro* transposition product.** Final transposition products were produced by incubation of the hyperactive (EKLP) and the K40Q mutant transposase with 5′-^{32}P-labeled 40-bp annealed oligonucleotides containing the transposon mosaic end sequence (see Fig. 5A) and pUC19 as the target DNA. The products were subjected to trypsin degradation (4 ng to 1 μg) for 15 min at 37 °C and separated on an agarose gel, and the products were visualized using a PhosphorImager. ● = R40Q transposase complex; ▼ = EKLP transposase complex.

Tn5 Transposases—Because the R30Q and R62Q mutant transposases are predicted to have similar overall conformations and can form synaptic complexes in the absence of donor backbone, the mutants would not be expected to excise transposons from plasmids but would be expected to insert precut transposons into donor DNA. Neither of these mutants excised the transposon from pGRST2 (Fig. 5) nor produced antibiotic-resistant colonies diagnostic for transposition using plasmids containing transposons with either mosaic or outer end sequences (Table I, *columns 3–6* and Table II, *columns 1* and *2*). However, both mutants were able to insert precut transposons into pUC19 *in vitro*, albeit with lower efficiencies, 25% for R30Q and 1.4% for R62Q, than for the parent EKLP transposase (Table II, *columns 3* and *4*). These results suggest that target capture and strand transfer are not affected by the R30Q and R62Q mutations and the major effect of these mutations is on the interaction of the transposases with the transposon end sequences attached to donor DNA.

DISCUSSION

R30Q and R62Q Tn5 Transposase Mutants Are More Stable Than EKLP Transposase but Are Defective in Synaptic Complex Formation—Because N-terminal-truncated Tn*5* transposase molecules form inactive heterodimers with full-length transposase and act as inhibitor molecules (17, 18), mutations of the transposase at known proteolytic cleavage sites (6) were expected to stabilize the protein toward proteolysis and increase *in vivo* transposition. Mutation of either Arg^{30} to Q or Arg^{62} to Q in Tn*5* transposase increased the stability of the transposase toward *E. coli* proteinases but completely inhibited *in vivo* transposition. Limited proteolysis using thermolysin, a general proteinase that prefers hydrophobic amino acids at the P1′-site, generated similar proteolysis products with these two mutants and the parent EKLP transposase, suggesting the overall conformations of the three molecules are similar. The ability of the mutants to catalyze *in vitro* transposition using precut transposons further supports the similarity of the overall conformations of the two mutant and the EKLP transposases. The R62Q mutant was more stable to thermolysin digestion than the R30Q mutant or the parent EKLP transposases, suggesting the N-terminal portion of the molecule of the R62Q mutant may not be as flexible as the other two forms of the transposase or the N-terminal end of the molecule forms a tighter complex with the C-terminal end (16, 20). The difference in thermolysin susceptibility of the R62Q mutant from the R30Q mutant may be related to the 18× difference in the *in vitro* transposition activity between the R30Q and R62Q mutants when the precut transposon from pGR7876 was used.

Overall, mutations of Arg^{30} and Arg^{62} in the Tn*5* transposase molecule affect the transposition mechanism in a similar manner. Both mutants form synaptic complexes with precut transposons and 3′-nicked transposons containing donor backbone in the paired ends complex assay but form fewer complexes than the parent EKLP transposase. Neither mutant forms synaptic complexes with transposons containing donor backbone or 5′-nicked transposons containing donor backbone. These similarities suggest both residues may be important for formation and/or stabilization of the DNA·protein complex. This conclusion is supported by previous experiments showing transposon end sequences protected Arg^{30}-Leu^{31} and Arg^{62}-Phe^{63} of the transposase from trypsin cleavage (6) and by the recently solved crystal structure of ELKP Tn*5* transposase complexed with Tn*5* transposon outside end sequences in the form of a paired ends complex (21).

Both Arg^{30} and Arg^{62} in the N-terminal domain interact directly with DNA (Fig. 7). Although Arg^{30} is found on the second turn of helix 2, and Arg^{62} is near the C-terminal end of helix 4, these two residues are close in space. In fact, these amino acids both interact with guanidine 13 of the transferred strand. The NH1 of Arg^{62} forms hydrogen bonds with N7 of guanidine 13 of the transferred strand (Fig. 7*A*) and the NH2 and NH1 of Arg^{30} bind directly or indirectly through a water molecule to the phosphate of this residue (Fig. 7*B*).

At first glance, the requirement of both Arg^{30} and Arg^{62} for

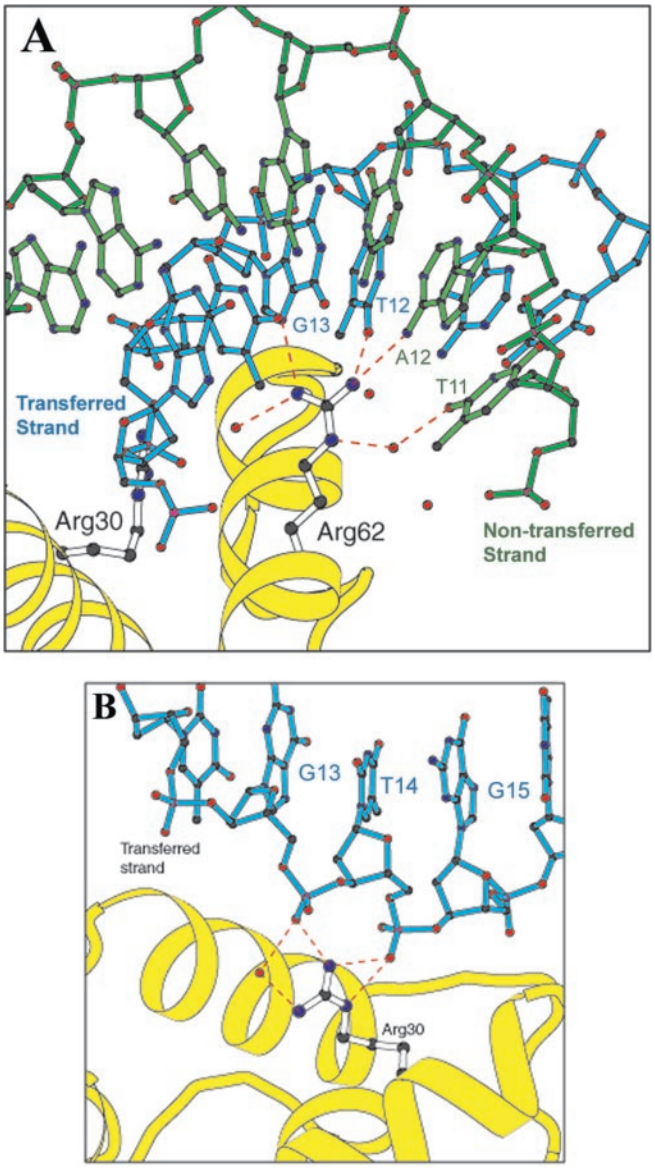

FIG. 7. **Interaction of arginines 30 and 62 of Tn*5* transposase with DNA in a synaptic complex.** *A*, the DNA of the synaptic complex is bent at 41° between positions 11 and 12 (21). The guanidinium group of Arg^{62} forms multiple hydrogen bonds directly or indirectly through a water molecule on both sides of the DNA bend. *B*, the guanidinium group of Arg^{30} interacts with backbone phosphate groups of the transferred strand of DNA through ionic and hydrogen bonds.

efficient binding of the transposon to Tn*5* transposase is surprising, considering the large number of contact residues between the transposon end sequences and the transposase (21). In addition to Arg^{30} and Arg^{62}, six residues, Arg^{26}, Arg^{27}, Ser^{45}, Lys^{54}, Gln^{57}, and Glu^{58}, of the N-terminal domain of the transposase and three distant residues, Arg^{342}, Glu^{344}, and Asn^{348}, directly bind to the transposon ends at positions +4 to +17 (21). In the synaptic complex, 15 additional residues, Thr^{99}, Arg^{210}, Tyr^{237}, Gln^{243}, Lys^{244}, Arg^{253}, Trp^{298}, Tyr^{319}, Arg^{322}, Lys^{330}, Lys^{333}, Thr^{334}, Ser^{438}, Lys^{439}, and Ser^{445}, of the second transposon molecule also bind to the transposon end DNA at positions +1 to +7.

The importance of individual specific interactions between the transposase and the transposon has been demonstrated by missing nucleoside experiments (7, 8, 22). Removal of individual nucleosides at positions +4 to +19 of the top nontransferred strand or +5 to +19 of the bottom transferred strand of the mosaic end sequences results in dramatic decreases in synaptic complex formation of EKLP transposase to the DNA (8). A single amino acid mutation in a DNA binding residue of the transposase probably affects binding of the transposon at least to the same degree as a missing nucleoside because, in both cases, critical interactions between the transposon end sequences and the transposase are altered or missing.

Although the R30Q and R62Q mutations are not expected to alter the formation of the respective α-helixes, closer examination of the interactions of Arg^{30} and Arg^{62} of the transposase molecule with DNA in the synaptic complex (2) suggests how mutation of either of these residues to glutamine results in the dramatic effects on transposition. Arg^{62} forms four hydrogen bonds with bases at positions 11, 12, and 13 in the major groove of the outer end sequence (Fig. 7*A*). The observed 41° bend in the DNA between positions 11 and 12 of the transposon end probably requires the positive charge on Arg^{62}. Mutation of Arg^{62} to the noncharged shorter glutamine residue not only disrupts the bonds between residue 62 and the DNA but likely also alters the multiple interactions between the DNA and the transposase that are dependent on the presence of the bend. The interactions most affected probably are those between the DNA and the other binding residues on helix 4, Lys^{54}, Glu^{58}, and Gln^{57} (21).

Arg^{30} forms one hydrogen bond through a water molecule and three distinct ionic bonds with two of the backbone phosphates at positions 13 and 14 of the transferred strand (Fig. 7*B*). Substitution of glutamine for this arginine results in the loss of the positive charge and the ability of the amide group on the side chain to interact with the DNA backbone because of the shorter side chain. The absence of the four bonds between the transposase and the DNA may influence the binding of the additional residues Arg^{26} and Arg^{27} on helix 2 to the outer end sequence DNA. Arg^{27} forms an ionic bond with the phosphate backbone at the 5′-side of guanidine 15 of the transferred strand (21). Arg^{26} forms hydrogen bonds with the O_2 of thymidine 16 of the transferred strand and the N3 of adenosine 17 of the nontransferred strand. It is possible that these three residues bind DNA in a synergistic manner. If true, the loss of the interaction of one of these amino acids with the DNA may alter the binding of the second two amino acids.

Transposase mutations at residues Arg^{30} and Arg^{62} prevent synaptic complex formation when donor backbone is attached at position +1 of the transferred strand but allow complex formation, albeit at reduced levels, when DNA is precut or has a 3′-nick. A possible explanation may be that a substantial DNA bend is found at or near the −1/+1 cleavage site when the donor backbone is present in the transposase·DNA complex (22, 23). Presumably, this bend is required for DNA binding when donor backbone is present. The R30Q and R62Q mutations may decrease the affinity of the transposase for the DNA sufficiently so that it may not be able to grip the DNA tightly enough to induce the bend, and thus binding cannot occur at all. In the absence of donor backbone DNA, binding can occur in the absence of this bend. The 3′-nick (which occurs as a natural intermediate) may facilitate proper bend formation, whereas the 5′-nick may not.

K40Q Tn5 Transposase Mutation Affects a Step in the Transposition Mechanism Not Measured by in Vitro Transposition Assays—The third mutation, K40Q, affects *in vivo* transposition to a much greater extent than *in vitro* transposition. There are steps that may be affected by the mutation *in vivo* but are not tested *in vitro*. These include *in vivo* stability of the transposase, binding of potential accessory molecules to the Tn*5* transposase·DNA complex not required *in vitro*, or the removal of the transposase following insertion of the transposon into target DNA. *In vivo* stability of the K40Q mutant transposase

is probably similar to that of EKLP transposase, because similar amounts of the two intact transposases were recovered from the *E. coli* expression system.

The K40Q mutation may alter the interaction of the transposase·DNA complex with a binding molecule that is important for *in vivo* transposition but is not required in the *in vitro* transposition assays. This is supported by the surface location of R40 in the transposon end·transposase synaptic complex structure within a groove between the N-terminal DNA binding site and the C-terminal end of the transposase molecule (21). The mutation could alter the binding of molecules such as topoisomerase I or a chaperone molecule involved in the removal of transposase from the strand transfer complex. Topoisomerase I copurifies with Tn*5* transposase, binds to the N-terminal end of the transposase, and can increase transposition rates (24). A more intriguing possibility is the interference by the K40Q mutation of the binding of a chaperone involved in removal of the transposase from the strand transfer complex. This step is bypassed in the *in vitro* system by the removal of the transposase by either heating in the presence of SDS or by phenol-chloroform extraction prior to transformation into *E. coli* for quantification of the transposition reaction.

Nothing is known about the mechanism by which the Tn*5* transposase is removed from the strand transfer complex *in vivo*; however, a chaperone and a protease may be required as shown for the bacteriophage Mu and the IS*903* transposition systems (12, 13). Mu transposase (MuA) is removed and degraded by the ClpX·ClpP chaperone·proteinase complex (13). The IS*903* transposase is degraded by Lon (12), a protein that contains a chaperone domain at the N-terminal end, a proteinase domain on the C-terminal end and is homologous to ClpX·ClpP (25). The mutation of Lys^{40} to Q of Tn*5* transposase may alter the ability of the sensor, and substrate discrimination domains of the Clp chaperones or protease Lon to recognize, bind, and remove the transposase. Experiments are ongoing to test this hypothesis.

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