Carboxyl Terminal Truncations of the Phage Mu Transposase

by

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ABSTRACT

The Phage Mu transposase (MuA) is a 663 amino acid momomeric multidomain protein. A tetramer of MuA catalyzes the replicative transposition of Phage Mu. MuA pairs the ends of the Mu genome, cleaves the donor DNA and subsequently joins the Mu DNA to the target DNA in a concerted cleavage and joining reaction known as strand transfer. A series of truncations deleting 101, 136 and 173 amino acids from the carboxyl terminal end of MuA were constructed, purified and analyzed. As expected the truncation proteins were inactive when assayed individually under standard *in vitro* transposition reaction conditions. In the presence of either wild type MuA, or a catalytic site point mutant, MuA D269N/E392Q, the truncation proteins were able to assemble into mixed tetramers that displayed partial catalytic activity. These mixed tetramers were capable of carrying out donor cleavage, and under relaxed reaction conditions, strand transfer. The data suggests that some, but not all, of the multimerization determinants lie in the deleted region of the protein and that the deleted regions also contain a putative nonspecific DNA binding region and catalytic site responsible for donor cleavage.

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For My Dearest Friends
and Family

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INTRODUCTION

Transposition is a mode of genetic recombination that imparts mobility to self contained genetic elements. Transposable genetic elements have been found in a very diverse set of organisms. They range from the bacterial transposons and insertion sequences such as Tn5, Tn7, Tn10(IS10), IS1 and IS3, to *Drosophila* P-elements, to the Ac and En/Spm elements of maize to the mariner family of elements (including the Tc elements of *C.elegans*)(for review see Berg and Howe, 1989; Mizuuchi, 1992; Sandmeyer, 1992). Members of the mariner family of elements have been found in almost every higher organism in which it has been looked for including humans (Robertson and Lampe, 1995). The Phage Mu family of transposing bacteriophages employs transposition for an approximately 50-200 fold expansion of its genome per lytic cycle prior to DNA packaging in a viral coat (Symonds *et al.*, 1987). Packaging of the phage DNA into the viral coat provides a mechanism for its horizontal transmission throughout a bacterial population. Integration of the cDNA of retroviruses such as HIV-1, Avian Sarcoma Virus, and Moloney Murine Leukemia Virus and the long terminal repeat-containing retrotransposons such as the Ty-*copia* family of retroelements share similar chemical pathways with bacterial transposition (Craig, 1995; Engelman *et al.*, 1991). In addition, the integrases of retroviruses share sequence and structural homology with members of the transposase family (Rice and Mizuuchi, 1995), and there are structural similarities between the transposases/integrases and the *E. coli* Holliday junction resolvase RuvC (Ariyoshi *et al.*, 1994) and both *E. coli* and HIV ribonuclease H (reviewed in Yang and Steitz, 1995). Recently there has been strong evidence that the mechanism of V(D)J recombination, which is responsible for development of a mature vertabrate immune system, has an uncanny chemical similarity to transposition (McBlane *et al.*, 1995; van Gent *et al.*, 1996a; van Gent *et al.*, 1996b).

Three Types of Transposition

Most transposases and integrases progress through similar chemical steps when carrying out transposition or integration (Haniford and Chaconas, 1992; Craig, 1995). The enzyme either nicks or fully cuts the element from the donor DNA in a process termed donor cleavage. In all systems that have been analyzed biochemically this cleavage exposes a 3' hyrodoxyl group (which is usually on an adenosine). The cleaved donor then invades the target DNA in a staggered fashion in a reaction termed strand transfer. The strand transfer intermediate is then resolved by either DNA replication through the element or by host encoded DNA repair pathways.

The transposases and integrases can be categorized into three groups based on the mechanism of their recombination process. In nonreplicative transposition, also known as "cut and paste transposition" (see Figure 1, panel A) the element first undergoes double strand DNA cleavage at both ends to liberate the element. The liberated element invariably has hydroxyl moieties at both 3' terminal nucleotides (which are usually adenosines). The ends are then joined to the target DNA molecule (Berg and Howe, 1989; Mizuuchi 1992). Because the invasion occurs in a staggered fashion, small single stranded gaps are produced and get repaired by host encoded DNA repair enzymes. This mode of transposition does not require a replication fork to pass through the transposon, and the transposon does not get duplicated during the transposition process. Tn7 and Tn10 both display this type of transposition.

In replicative transposition (see Figure 1, panel B), the element undergoes single strand donor DNA cleavage at both ends of the element exposing 3' hydroxyl moieties. The 3' hydroxyls are then used as the nucleophiles in the subsequent strand transfer step (Berg and Howe, 1989; Mizuuchi 1992). The three-way junction thus formed can be resolved by DNA replication through the element. The fork can serve as a nascent replication fork on which the DNA replication machinery will form. Replication through the transposon will produce two copies of the

transposon in the same DNA molecule; the product of this reaction is termed a cointegrate. Phage Mu is the best understood member of this class.

Simple integration is displayed by retroviruses and retro-transposons (see Figure 1, panel C) First the RNA genome is reverse transcribed into cDNA. Donor DNA cleavage introduces single strand nicks several (two in HIV) bases from the ends of the cDNA and exposes 3' hydroxyl moieties. This nucleolytic reaction has been termed 3' end processing. The dinucleotide (in HIV) then dissociates from the cDNA . The exposed 3' hydroxyls are used as nucleophiles to attack the target genome in the subsequent strand transfer step (Engelman *et al.,* 1991). The single stranded gaps that result are mended by host encoded DNA repair enzymes. This type of genetic recombination is termed integration. The products of nonreplicative transposition and integration are termed simple inserts, because DNA replication through the element subsequent to strand transfer is not involved in the process. Cointegrate formation, which is the result of replicative transposition, has the added requirement that a DNA replication fork proceed through the element producing two copies of the transposon.

Although replicative transposition, conservative transposition and integration have their biochemical differences, the overall theme of the three processes is remarkably similar (Baker, 1995). Initially there is a donor cleavage event that uncovers a 3' hydroxyl moiety almost exclusively on an adenosine (the penultimate 3' residue is usually a cytosine). The exposed 3' hydroxyl is then used as a nucleophile to invade the target DNA in a staggered fashion in the strand transfer event. The resulting strand transfer products get resolved either by DNA replication through the element, thus forming a cointegrate, or by DNA repair at the ends of the element, thus forming a simple insert. For both the Phage Mu transposase and HIV-1 integrase, the stereochemical course of the phosphoryl transfer reactions have been followed using chiral phosphorothioates at the insertion site in the target DNA. Inversion of chirality of the phosphorothioate during strand transfer suggested that the 3' hydroxyls revealed by donor

cleavage are used as the nucleophiles in strand transfer in both Mu transposition and HIV-1 with a one step in-line phosphotransferase type mechanism (Engelman *et al.,* 1991; Mizuuchi and Adzuma, 1991). Inversion of chirality of the phosphorothioate during donor cleavage in HIV-1 suggested that donor cleavage occurrs without formation of a covalent protein/DNA intermediate and that H_2O acts as the nucleophile (Engelman *et al.,* 1991). These results strongly supported an an inline SN2 type mechanism for the phosphoryl transfer reactions of both donor cleavage and strand transfer.

Given the similarity of the biochemical pathways it is not that surprising that as crystal data has become available, the overall topology of the core domains of the transposase of Phage Mu (Rice and Mizuuchi, 1995), HIV-1 integrase (Dyda *et al.,* 1994), and ASV integrase (Bujacz *et al.,* 1995) are similar, even though sequence homologies are low. The three putative acidic active site residues (the catalytic DDE motif), present in these proteins, and in other members of the transposase/integrase family, was found to be clustered in similar orientations (Rice and Mizuuchi, 1995). In addition the overall topology and clustering of the DDE motif was observed in three other DNA processing enzymes, the Holliday junction resolvase RuvC (Ariyoshi *et al.*, 1994), ribonuclease H from *E. coli* (Yang *et al.,* 1990) and HIV-1 (Davies *et al.,* 1991, reviewed in Yang and Steitz, 1995).

In Vitro Transposition of Phage Mu

The transposase of Phage Mu (MuA) is the best characterized transposase. Replicative transposition of Phage Mu is a highly regulated process. Many cofactors, both proteins and DNA sequences, aid in its regulation. It is important for Phage Mu transposition to be regulated temporally with respect to its lytic cycle in order to insure that prior to viral capsid assembly, there are sufficient properly transposed copies of its genome available for packaging into the capsid. Once transposition initiates, its nucleolytic processes are essentially irreversible. Thus it is

neccessary to insure completion of transposition in order to avoid irreparably damaging host chromosomal rearrangements.

MuA is a 663 amino acid protein with a molecular weight of 75kDa. It is a monomer in solution *in vitro*. MuA reversibly binds as a monomer to several DNA binding sites at the ends of the Mu genome (Allison and Chaconas, 1992). The end sequences required for transposition are comprised of three MuA binding sites each (R1, R2 and R3 on the right end, and L1, L2 and L3 on the left end) (Craigie *et al.*1984) and must be in their natural order for proper binding(Schumm and Howe, 1981; Craigie *et al.,* 1985). The catalytically active form of MuA is a tetramer bound to the two ends of the Mu DNA (Lavoi *et al.,* 1991, Craigie and Mizuuchi, 1987, Surette *et.al* 1987). The active complex is termed a transpososome (Surette *et al.,* 1987). In order to initiate transposition, two host encoded proteins and three DNA elements are neccessary in addition to MuA transposase. They are HU protein, Integration Host Factor (IHF), both sets of end binding sites, and the Internal Activating Sequence (IAS) (Mizuuchi and Mizuuchi, 1989; Leung *et al.,* 1989, Surette *et al.,* 1989). The IAS can be located either on the mini-Mu donor plasmid in its natural position about 950 base pairs from the left end binding sites, or it can be donated by a second piece of DNA (Surette and Chaconas, 1992). Divalent metal ion (either Mg^{2+} or Ca^{2+}) is also neccessary (Mizuuchi *et al.,* 1992). If a supercoiled mini-Mu is used as the donor DNA then IHF can be replaced with a high concentration of HU. In the presence of these cofactors, MuA will assemble into a tetramer on the two ends of the Mu genome (or on the mini-Mu) into a structure called the stable synaptic complex (Mizuuchi *et al.,* 1992). Addition of target DNA, the transpositional activator and ATPase MuB and ATP will lead to the formation of the cleaved donor complex with two single strand nicks being produced at the ends of the Mu genome (Craigie and Mizuuchi, 1987). If MuB is omitted, intramolecular transposition, in which the target of transposition is the donor molecule itself, will be favored. If target DNA is omitted intramolecular transposition will occur. The cleaved donor complex will then continue on to form the strand transfer complex in which the newly exposed 3' hydroxyls attack the target DNA and join the

target strand in a one step concerted phosphotransferase reaction (Mizuuchi and Adzuma, 1991). Ca^{2+} is sufficient for formation of the stable synaptic complex and for strand transfer but is insufficient to support donor cleavage. Mg^{2+} is neccessary for donor cleavage (Mizuuchi *et al.,* 1992).

Domain Structure of MuA

Limited proteolytic digestion of MuA has indicated that it is comprised of three principal domains (Nakayama *et al.,* 1987). Various functions have been ascribed to each of the domains and subdomains (see Figure 2). Domain I contains two subdomains. Domain IA (amino acids 1-76) is responsible for binding the enhancer element IAS (Leung *et al.,* 1989; Mizuuchi and Mizuuchi, 1989). The solution structure for domain IA has been solved by NMR and revealed a winged helix turn helix motif (Clubb *et al.,* 1994). Domain IB (amino acids 77-243) is responsible for sequence specific binding to the Mu end binding sites (Nakayama *et al.,* 1987). The extreme carboxyl terminal subdomain, domain IIIB, is involved in interactions with the transpositional activator protein MuB (Wu and Chaconas, 1994; Baker *et al.,* 1991). It is also involved in interaction with the chaperone ClpX, which disassembles the strand transfer complex (Levchenko *et al.,* 1995).

The overall topology of the MuA core is very similar (almost superimposeable) to the structure of the core domain of HIV-1 integrase (Rice and Mizuuchi, 1995). Domain IIA (amino acids 244-574) contains the conserved catalytic triad DDE motif (Baker and Luo, 1994). Many members of the transposase/integrase family share a conserved acidic aspartate-aspartate-35-glutamate motif (Fayet *et al.*, 1990; Rowland and Dyke, 1990; Kulkosky *et al.*, 1992; Radstrom *et al.*, 1994; Baker and Luo, 1994), with the 35 signifying the usual separation of the aspartate and the glutamate. In MuA, although the separation is 55 amino acids, according to the crystal structure (which contained amino acids 243-574, and resolved amino acids 258-560) there is an extended loop that is not present in HIV-1 integrase that accounts for the extra 20 residues (Rice and Mizuuchi, 1995). The DDE motif has been implicated in coordination of the divalent metal required for

phosphoryl transfer in catalysis (Kulkosky *et al.*, 1992; Engelman and Craigie, 1992). Aspartate 269, aspartate 336 and glutamate 392 have been identified as the members of the putative DDE motif in MuA by *in vitro* (Baker and Luo, 1994; Krementsova *et al.*, 1996) and *in vivo* (Kim *et al.*, 1995) studies. Aspartate 269, aspartate 336, and glutamate 392 were found to be clustered in a potential active site in the crystal structure of the core domain (Rice and Mizuuchi, 1995). Domain IIB which extends from amino acid 490 to amino acid 574, has been proposed to be a nonspecific DNA binding domain due to a large positively exposed patch on the side of the β-barrel (Rice and Mizuuchi, 1995, see Figure 3 for a ribbon model of the core domain).

Baker *et al.* (1993) had previously constructed a carboxyl terminal truncation of MuA in which the carboxyl terminal 89 residues are deleted. This construct, termed MuA1-574, was unable to assemble into tetramers and perform any transposition chemistry under standard *in vitro* reaction conditions. However, when MuA1-574 was mixed with wild type MuA (WT MuA) in standard *in vitro* transposition reactions, MuA1-574 was incorporated into tetramers that contain a mixture of WT MuA and MuA1-574 subunits that displayed partial enzymatic function. At concentrations of WT MuA that were otherwise too low to support transposition, the addition of MuA1-574 enabled mixed tetramers to form, and these complexes were able to carry out donor cleavage. These mixed tetramers, while competent for donor cleavage, did not proceed and catalyze strand transfer, even if provided with a precleaved donor DNA molecule that had been constructed by using a restriction endonuclease to expose the proper 3' hydroxyl moieties. These results suggested that domain IIIA was critical for both tetramer formation and catalysis. Wu and Chaconas have reported both cryptic nuclease and nonspecific DNA binding activities in a 26 residue peptide (residues 575-600) derived from the amino terminal end of domain IIIA (Wu and Chaconas, 1995). The presence of domains IB, IIA and IIIA are all neccessary for tetramer formation and catalytic activity *in vitro* (Baker et.al. 1993), and phage growth *in vivo* (Desmet *et al.*, 1989; Bétermier *et al.*, 1989).

In order to elucidate the function of domain IIB a series of carboxyl terminal truncations throughout this region of the Phage Mu transposase was constructed. The series consists of MuA containing deletions of 101, 136 and 173 residues from the carboxyl terminus and the truncated proteins will be referred to as MuA1-562, MuA1-527 and MuA1-490 respectively. These MuA derivatives were purified to at least 90% homogeneity and analyzed. As expected, the truncation proteins individually all failed to form active tetramers or carry out any transposition chemistry. However, in concert with either WT MuA or a full length catalytic point mutant of MuA, they assembled into mixed tetramers and displayed partial activity. The results indicate that domains IB, IIA, IIB and IIIA are all required for self assembly to form catalytically active transposase. This defect can be overcome by providing the truncation mutants an oligomerization scaffold comprised of full length MuA. In association with this scaffold, the truncated proteins, lacking domain IIB, can donate their catalytic residues to carry out partial transposition biochemistry. Even after relieving the block in assembly by providing an assembly scaffold, the truncation proteins still remain partially defective catalytically. This argues that domains IIB and IIIA, as defined by partial proteolysis, should be combined into a single functional domain.

MATERIALS AND METHODS

Chemicals and Reagents

Tris (Tris(hydroxymethyl)aminomethane) was from Boehringer Mannheim (Indianapolis IN). $MgCl_2$, NaCl, acetic acid, EDTA ([ethylenedinitrilo]-tetraacetic acid), glycerol, ammonium persulfate, methanol, chloroform, sucrose, and potassium phosphate (both mono- and di-basic) were from Mallinckrodt (Paris KY). DTT (1,4-Dithio-DL-threitol) and IPTG (isopropyl-thio-B-D-galactoside) were from Bachem Bioscience (Torrance CA). BSA (bovine serum albumin), heparin, tris-buffered phenol (buffered with 10 mM Tris-HCl, 1 mM EDTA), ampicillin, tetracycline, spermidine, lysozyme (from chicken egg white), PEG-8000 (polyethyleneglycol average molecular weight 8000) and ATP were from Sigma (St. Louis MO). Triton X-100, Ficoll-400 and DMSO (dimethylsulfoxide) were from American Analytical (Natick MA). Restriction enzymes and DNA modifying enzymes were from New England Biolabs (Beverly MA). bromophenol blue and hydroxyapatite were from Bio-Rad (Hercules CA). SeaKem HGT agarose was from FMC BioProducts (Rockland ME). LMP (low melting point) agarose, SDS (sodium dodecylsulfate), Coomassie Brilliant Blue R-250 and ΦX174 RF I (target DNA) were from Gibco BRL (Gaithersburg MD). Ethidium bromide, Sequenase V2.0 and Hepes (N-[2-Hydroxyethyl] piperazine-N'-[2-ethanesulfonic acid]) were from United States Biochemical (Cleveland OH). Deionized water was further purified through a Millipore (Bedford MA) MilliQ Water Purification System. Imobilon-P (nitrocellulose paper) was also from Millipore. Carnation Nonfat Dry Milk was from Nestlé Food (Glendale CA). ECL Western Blotting Reagent and goat anti-rabbit Ig, horseradish peroxidase linked whole antibody (secondary antibody) was from Amersham Life Sciences (Arlington Heights IL). X-OMAT AR film was from Kodak (Rochester NY). Acrylamide:Bis-acrylamide (29:1) and glycine were from Fisher BioTech (Fair Lawn NJ). PMSF (phenylmethylsulfonyl fluoride) was from Pierce (Rockford IL). 5x protein sample buffer contained 30% glycerol, 250 mM Tris-HCl pH 8 at room temperature, 1% SDS, 40 mM DTT, 5 mg/ml bromophenol blue.

Plasmid pKN2 (Adzuma and Mizuuchi, 1988) was digested with restriction enzyme Hae II according to the manufacturer's protocol with a subsequent phenol/chloroform extraction to insure purity. Donor DNA was pSG1 (Baker and Luo, 1994). MuA1-574 was from the same fraction as previously described (Baker *et al.,* 1993). HU, WT MuA and MuA D269N/E392Q were from the same fractions as previously described (Baker *et al.,* 1994). MuB was subcloned and purified by M. Yamauchi as follows. The NdeI-BamHI fragment of pMK109C (Craigie and Mizuuchi, 1985) containing the MuB coding sequence was swapped into the NdeI-BamHI site of pET14b (Novagen, Madison WI), and expressed in HMS174 (Novagen, Madison WI). The cells were lysed in 335 mM Tris-HCl (pH 8 at room temperature), 10% sucrose, 1.4 M NaCl, 72 mg/ml spermidine, 4.6 mM EDTA, 63% ammonium sulfate, 0.28 mg/ml lysozyme. After two freeze thaw cycles the crude lysate was centrifuged in a Beckman (Fullerton CA) SS-34 rotor at 47,800 g for 30 minutes and the supernatant loaded onto a Qiagen (Chatsworth CA) QIA Qia*express* Ni-NTA agarose column. The column was washed first with buffer A (50 mM sodium phosphate pH 7.8, 300 mM NaCl) and then with buffer B (50 mM sodium phosphate pH 6, 800 mM NaCl, 10% glycerol, 10 mM imidazole). MuB was eluted off the column with buffer B plus 250 mM imidazole, and dialized against MuB storage buffer (1M NaCl, 23 mM Hepes pH 7.3 at 250 mM, 20% glycerol, 1 mM DTT). The dialysate was frozen and stored at -80 degrees until use. NovaBlue competent cells (*endA1 hsdR17* (r_{K12}^- m_{K12}^+) *supE44 thi-1 recA1 gyrA96 relA1 lac* [F' *proA*$^+$*B*$^+$ *lacI*^{q}*ZΔM15*::Tn10(Tc^R)]) were from Novagen (Madison WI).

Recombinant Protein Cloning, Expression and Purification

A plasmid expressing MuA1-490 was constructed by site specific mutagenesis of pSKMuA (Baker and Luo, 1994). Deprotected oligonucleotide TB 250 was synthesized by the MIT Biopolymers Lab and had a sequence of 5'-GCTGTTACTGTAAGCCGAGGCGG-3'. TB 250 was phosphorylated in a final reaction volume of 30 μl. The reaction contained 0.43 mM ATP, 5U T4 polynucleotide kinase, 70 mM Tris-HCl (pH 7.6 at 25 degrees), 10 mM $MgCl_2$, 5 mM DTT and 4 nMol TB 250. TB 250 contains a base sequence that alters codon 491 of the WT MuA sequence

into an ochre stop codon. The reaction was incubated at 37 degrees for 45 minutes to allow phosphorylation and then heated to 65 degrees to inactivate the kinase. 0.5 μl of the kinase reaction mixture was added to 200 ng of pSKMuA in a final reaction volume of 10 μl containing 20 mM Tris-HCl (pH 7.4 at room temperature), 20 mM $MgCl_2$, 50 mM NaCl, and heated to 75 degrees for 5 minutes. The reaction was cooled over one hour to room temperature to allow for annealing of the oligonucleotide to the single stranded pSKMuA. Synthesis of the second strand was accomplished by adding 1 μl 10x synthesis mix (5 mM dATP, 5 mM dCTP, 5 mM dGTP, 5 mM dTTP, 10 mM ATP, 100 mM Tris-HCl (pH 7.4 at room temperature), 50 mM $MgCl_2$, 20 mM DTT), 80 U T4 DNA Ligase, and 1.5 U T4 DNA Polymerase. The reaction mixture was incubated on ice for 5 minutes, followed by room temperature for 5 minutes, and 37 degrees for one and a half hours. The reaction was stopped by addition of EDTA to 10 mM. An aliquot was transformed into competent MM294 cells and plated on LB plates containing 100 μg/ml ampicillin and grown overnight at 37 degrees. Potential mutants were picked off the plates with sterile toothpicks, grown in 5 ml LB broth plus 100 μg/ml ampicillin overnight and the plasmid DNA purified via NaOH/SDS minipreps. Double stranded sequencing using Sequenase V2.0 (USB, Cleveland OH) according to the manufacturer's protocol was done to verify the DNA sequence. The stop codon mutation was introduced into expression vector pET3dMuA (Baker and Luo, 1994) by fragment swapping the Bam HI-Asc I fragment. The gene expressing MuA1-562 was constructed by L. Luo by excising the NcoI-BanI fragment from pET3dMuA and ligating it into the the NcoI-BamHI site of pET3dMuA with a BanI-BamHI linker that introduced a stop codon after amino acid 562 forming plasmid pET3dMuA1-562. The sequence of the linkers were 5'-GCACCTTAGG-3' and 5'-GATCCCTAAG-3'. The gene expressing MuA1-527 was constructed by L. Luo by excising the NcoI-TagI fragment from pET3dMuA and ligating it into the the NcoI-BamHI site of pET3dMuA with a TagI -BamHI linker that introduced a stop codon after amino acid 527 forming plasmid pET3dMuA1-527. The sequence of the linkers were 5'-CCGGCTAGG-3' and 5'-GATCCCTAG-3'.

Expression plasmids pET3dMuA1-562, pET3dMuA1-527, pET3dMuA1-490 containing MuA1-562, MuA1-527 and MuA1-490 respectively were each transformed into competent NovaBlue cells (Novagen, Madison WI) according to the manufacturer's protocol. Potential transformants were picked off LB plates containing 100 μg/ml ampicillin and 12 μg/ml tetracycline with sterile toothpicks and grown in 5 ml LB broth containing 100 μg/ml ampicillin and 12 μg/ml tetracycline with constant shaking at 37 degrees. Expression of the proteins was induced by adding IPTG to a final concentration of 0.5 mM when the cell cultures reached an A_{600} of 0.5. Uninduced cultures were grown in parallel to serve as starter cultures for large scale protein purifications. Induction was allowed to continue for one hour at which time the cells were pelleted at 15,000 g for 30 seconds in microfuge tubes, the supernatant culture broth removed and 100 μl of 1x protein sample buffer (6% glycerol, 50 mM Tris-HCl pH 8 at room temperature, 0.2% SDS, 8 mM DTT, 1 mg/ml bromophenol blue) was added. The cell paste was boiled for 3 minutes and loaded directly onto an 8% SDS-PAGE and electrophoresed in Tris-glycine running buffer for 0.9 hours at 140 V. After electrophoresis the gels were stained with Coomassie Brilliant Blue R-250 to visualize the expressed proteins.

For large scale protein purification, one or two liters LB broth containing 100 μg/ml ampicillin and 12 μg/ml tetracycline were inoculated with one or two ml respectively uninduced mid-log cultures from the small scale protein expression assays. The cultures were grown at 37 degrees with shaking until A_{600} was 0.5 and then the proteins were induced by adding IPTG to a final concentration of 0.5 mM. Induction was allowed to continue for one hour at 37 degrees with constant shaking. The broth was then centrifuged in a Beckman (Fullerton CA) JS-4.2 rotor at 4,900 g for 15 minutes to pellet the cells. The supernatant broth was decanted and the cell pellet was resuspended (for each 500 ml broth) in 5 ml cold Tris-sucrose (50 mM Tris-HCl pH8.0 at room temperature, 10% sucrose). For each 10 g of cell paste, 8 ml Buffer A (Tris-sucrose plus 2.5 mM DTT and 12.5 mM EDTA) and 2 ml Buffer B (200 mM Tris-HCl pH 8.0 at room temperature, 1 M NaCl, 102 mg spermidine and 580 mg ammonium sulfate) were added. After

complete cell resuspension, lysozyme was added to a final concentration of 200 μg/ml and the cell paste was then incubated on ice for 30 minutes. PMSF to a final concentration of 0.1 mM was also added to the lysate of MuA1-490. After incubation was complete the cell paste was put through two freeze thaw cycles by repeated freezing in liquid nitrogen and subsequent thawing in a 30 degree water bath. The cell lysate was then centrifuged in a Beckman SS-34 rotor for 40 minutes at 47,800 g and the supernatant saved. Solid ammonium sulfate was slowly added to the supernatant at 4 degrees with constant gentle stirring to bring the final concentration of ammonium sulfate to 30%. Gentle stirring was continued overnight (MuA1-527 required 35% ammonium sulfate before visible cloudiness, and MuA1-490 required a final concentration of 40% ammonium sulfate). The cloudy mixture was then centrifuged in a Beckman SS-34 rotor for 30 minutes at 17,210 g. The supernatant was decanted and the protein pellet resuspended in sufficient HEDG (25 mM Hepes pH 7.3 (at 250 mM), 0.1 mM EDTA, 1 mM DTT, 10% glycerol) plus 100 mM KCl until the conductivity was equal to HEDG plus 300 mM KCl. For MuA1-527 and MuA1-490 the KCl was replaced in all buffers with NaCl.

The diluted protein was loaded onto a 8 ml bed volume P-11 phosphocellulose (Whatman, Maidstone UK) column preequilibrated in HEDG plus 300 mM KCl. The protein was eluted off the column with a step gradient of HEDG plus 500 mM KCl on a Pharmacia (Upsala Sweden) FPLC system. Peak fractions as assayed by SDS-PAGE were pooled and diluted with HEDG plus 100 mM KCl until the conductivity of the solution was equal to that of HEDG plus 300 mM KCl. The reduced conductivity solution was reloaded onto the same column (freshly stripped with HEDG plus 1M KCl and equilibrated with HEDG plus 300 mM KCl) and eluted using a linear gradient from HEDG plus 300 mM KCl to HEDG plus 1 M KCl. For MuA1-527 and MuA1-490 the initial step gradient was omitted. Peak fractions of MuA1-562 as assayed by SDS-PAGE were pooled and dialized against MuA dilution buffer (300 mM NaCl, 25 mM Hepes pH 7.3 at 250 mM, 0.1 mM EDTA, 10% glycerol, 1 mM DTT) plus 10% glycerol (final glycerol concentration

was 20%) overnight. The dialysate was aliquoted, frozen in liquid nitrogen and stored at -80 degrees until used.

Subsequent to the phosphocellulose column, peak fractions of MuA1-527 as assayed by SDS–PAGE were pooled and diluted with HEDG plus 100 mM NaCl until its conductivity equaled that of HEDG plus 300 mM NaCl. The protein was then loaded onto a Pharmacia Mono S HR 5/5 FPLC column preequilibrated with HEDG plus 300 mM NaCl. MuA1-527 was eluted off the Mono S column using a linear gradient of HEDG plus 300 mM NaCl to HEDG plus 1 M NaCl. Peak fractions as assayed by SDS-PAGE were pooled and loaded directly onto a small (2 ml bed volume) hydroxyapatite (Bio-Rad) column that had been preequilibratedwith HDG (HEDG less the EDTA) plus 300 mM NaCl. The protein was eluted off the hydroxyapatite column using a linear gradient of HDG plus 300 mM NaCl to HDG plus 300 mM NaCl plus 0.5 mM potassium phosphate (pH 7.4). Peak fractions as assayed by SDS-PAGE were pooled, dialyzed against MuA dilution buffer plus 10% glycerol overnight, then briefly dry dialyzed against PEG-8000 (Sigma). The resulting dialysate was frozen in liquid nitrogen and stored at -80 degrees until use.

Subsequent to the phosphocellulose column, peak fractions of MuA1-490 as assayed by SDS-PAGE were pooled and diluted with HEDG plus 100 mM NaCl until the conductivity of the solution equaled that of HEDG plus 300 mM NaCl. The protein was then loaded onto a Pharmacia Smart System Mono-S column (bed volume 0.1 ml) preequilibrated with HEDG plus 300 mM NaCl. MuA1-490 was eluted off the Mono-S column using a linear gradient of HEDG plus 300 mM NaCl to HEDG plus 1 M NaCl. Peak fractions as assayed by SDS–PAGE were pooled and frozen in liquid nitrogen and stored at -80 degrees until use.

A high absorptivity contaminant of approximately 130 kDa apparent molecular weight copurified both with MuA1-527 and MuA1-490. Because of this contaminant the final protein concentrations were determined by quantitation of the Coomassie Brilliant Blue R-250 stained bands displayed in

Figure 4. Final protein concentrations were determined by loading 250 ng, 1000 ng, 1750 ng, 2500 ng, and 3250 ng WT MuA and approximately 500 ng of MuA1-574, MuA1-562, MuA1-527 and MuA1-490 onto an 8% SDS-PAGE. Subsequent to electrophoresis, the gel was stained with Coomassie Brilliant Blue R-250, transilluminated with UV light and photographed with a Hitachi KP-M1U CCD camera (Hitachi Denshi Ltd., Japan). The photograph's file was saved in TIFF format and the bands quantified using ImageQuant v4.1 software (Molecular Dynamics, Sunnyvale CA) with a grey scale intensity range of 0-256 (reproduced in Figure 4). The concentrations of the purified proteins were determined by comparison to a standard curve generated by the WT MuA loaded into lanes 1-5. The final concentrations of the purified proteins were: MuA1-562 (0.03 μg/μl), MuA1-527 (0.04 μg/μl) and MuA1-490 (0.017 μg/μl).

Sequence Specific DNA Binding

Reaction mixtures contained in a final reaction volume of 10 μl: 25 mM Tris-HCl (pH 8 at room temperature), 10 mM $MgCl_2$, 1 mM DTT, 25 μg/ml BSA, 1% Triton X-100, 100 mM NaCl, 5.5 μg/ml pKN2 digested with restriction enzyme Hae II, and 100 ng of one of the following proteins: WT MuA, MuA1-574, MuA1-562 or MuA1-527. The reaction was incubated on ice for 10 minutes to allow sequence specific binding to occur. 2 μl of 10% Ficoll was then added and the samples were loaded onto a prerun 5% acrylamide/bis-acrylamide (29:1) native gel containing 1xTAB buffer (40 mM Tris acetate at pH 7.8, 5 mM sodium acetate and 1 mM EDTA). The gel was electrophoresed at 70 V for 3 hours with a running buffer of 1xTAB plus 0.1% Triton X-100 at 4 degrees. Subsequent to electrophoresis the gel was stained with ethidium bromide to visualize the DNA products. A photograph of the stained gel is presented in Figure 5.

Mixed Tetramer Formation with Wild Type MuA

Reaction mixtures contained in a final reaction volume of 25 μl: 25 mM Tris-HCl, 10 mM $MgCl_2$, 1 mM DTT, 100 μg/ml BSA, 15% glycerol, 100 μM NaCl, 2mM ATP, 10 μg/ml donor DNA (pSG1), 10 μg/ml target DNA (ΦX174, RF I), 40 ng HU protein, and 100 ng MuB protein. In

addition some reactions contained 0, 50, 100, 200, or 400 ng of WT MuA protein and either 100 ng of MuA1-574, MuA1-562, MuA1-527 or MuA1-490 (see Figure 6). In reactions containing MuA1-490, ATP, MuB and target DNA were omitted. The reaction components were mixed together on ice and then incubated at 30 degrees for one hour. Reactions containing MuA1-490 were incubated for 1.5 hours at 30 degrees. 0.2 volumes of stop mix (0.1% bromophenol blue, 2.5% SDS, 50 mM EDTA and 25% glycerol) was added to stop the reaction. Aliquots were then loaded onto an 0.8% HGT agarose gel and electrophoresed for 2 hours at 70 V in 1xTAB buffer with constant buffer recirculation. Subsequent to electrophoresis the agarose gels were stained with ethidium bromide to visualize the DNA products. A negative image using a grey scale intensity of 0 to 256 produced by photography with a charge coupled device camera is displayed in Figure 6.

Cleaved Donor Complex Formation

Reaction mixtures contained in a final reaction volume of 25 µl: 25 mM Tris-HCl, 10 mM $MgCl_2$, 1 mM DTT, 100 µg/ml BSA, 15% glycerol, 100 mM NaCl, 10 µg/ml donor DNA (pSG1), and 40 ng HU protein. Some of the reactions contained 100 ng of the catalytic point mutant MuA D269N/E392Q and/or 100ng of MuA1-574, MuA1-562, MuA1-527 or MuA1-490 (see Figure 7). The reaction components were mixed together on ice and then incubated at 30 degrees for one hour. 0.2 volumes of stop mix without SDS or bromophenol blue (50 mM EDTA and 25% glycerol) was added to stop the reaction. Aliquots were then loaded onto an 0.8% HGT agarose gel and electrophoresed for 2 hours at 70 V in 1xTAB buffer supplemented with 1mM DTT as a reducing agent with constant buffer recirculation. Both the agarose gel and the buffer also contained 80 µg/ml BSA as carrier protein and 10 µg/ml heparin as an inhibitor of DNA binding in order to dissociate any MuA not stably bound to the DNA. Aliquots of the appropriate reactions were reserved for use as load marker for the subsequent immunoblot. Subsequent to electrophoresis the agarose gel was stained with ethidium bromide to visualize the DNA products.

The cleaved donor complexes were then excised from the gel with a clean razor blade for use in an immunoblot.

Immunoblot of Cleaved Donor Complexes

Bands containing cleaved donor complexes from the above assay were excised from the ethidium bromide stained agarose gel with a clean razor blade. The gel slices were melted by boiling and 0.2 volumes of 5x protein sample buffer were added directly to the molten slices. One half the sample was then loaded directly onto a 8% SDS-PAGE. Samples of load reactions from the cleaved donor complex formation assay were diluted 7 fold with 1x protein sample buffer prior to electrophoresis. 10 ng of MuA D269N/E392Q, MuA1-574, MuA1-562, MuA1-527 and MuA1-490 were also loaded onto the gel as molecular weght markers. Electrophoresis was carried out at 140 V for 0.9 hours in Tris-glycine running buffer at room temperature. After electrophoresis the gel was blotted onto nitrocellulose paper in a semi-dry blotting apparatus for 0.7 hours at 15 V in Tris-glycine buffer plus 20% methanol. The polyacrylamide gel was then stained with Coomassie Brilliant Blue R-250 to insure complete protein transfer. Subsequent to transfer the nitrocellulose blot was blocked in TBST (10 mM Tris-HCl pH 7.5 at room temperature, 150 mM NaCl, 0.1% Tween 20) with 5% Carnation Non-Fat Dry Milk for 10 minutes with constant agitation at room temperature. Primary antibody (polyclonal rabbit anti-MuA) was added and agitation continued for 6 hours. The blot was then washed three times for 5 minutes with TBST and secondary antibody (goat anti-rabbit) was added and incubated for one hour. Residual secondary antibody was removed by three more five minute washes in TBST and the gel was then blotted dry. The immunoblot was then developed using ECL chemiluminescent western blotting reagent (Amersham Life Sciences, Arlington, IL) according to the manufacturer's protocol. Multiple exposures on Kodak (Rochester, NY) X-OMAT AR film were made to enable observation of weak signals (see Figure 8 panels A and B).

Phosphorylation and Annealing of Oligonucleotides

To assay whether mixed tetramers containing carboxyl terminal truncation proteins and the catalytic point mutant MuA D269N/E392Q can carry out strand transfer, oligonucleotides containing the R1 and R2 end binding sites, and an exposed 3' hydroxyl group at the appropriate terminal adenosine (termed precleaved) were synthesized, radiolabeled and annealed to one another. Deprotected oligonucleotides TB 225 and TB 226 were synthesized at the MIT Biopolymers Lab. The sequence of radiolabeled (*) TB 226 annealed to TB 225 is:

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*5'CGCTTT CGCGTTTTTCGTGAAACGCTTT CGCGTTTTTCGTGCGCCGCTT CA3'
 3'GCGAAAGCGCAAAAAGCACTTT GCGAAAGCGCAAAAAGCACGCGGCGAAGTGA5'
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Phosphorylation of the oligonucleotides was performed in a reaction volume of 50 μl containing 70 mM Tris-HCl (pH 7.6 at 25 degrees), 10 mM $MgCl_2$, 5 mM DTT, 20 U T4 Polynucleotide Kinase, 50 μCuries [γ-^{32}P]-ATP and 60 pMol oligonucleotide TB 226. The reaction mixture was incubated at 37 degrees for one hour and then 25 μl chloroform and 25 μl Tris-buffered phenol were added. The mixture was vortexed and centrifuged at 15,000 g to separate the aqueous and organic phases. The aqueous phase was pipetted off and NaCl added to a final concentration of 100mM, and 60 pMoles oligonucleotide TB 225 was added to it. The oligos were heated to 100 degrees for 2 minutes and cooled to room temperature in a heat block to allow for annealing over a period of one and a half hours. The mixture was then loaded onto a ProbeQuant G-50 Micro Column (Pharmacia Biotech, Piscataway, NJ) and the annealed double stranded oligonucleotiides were eluted from the column according to the manufacturer's protocol. The radioactive oligonucleotides were stored at 4 degrees.

Strand Transfer Complex Formation using Precleaved Oligonucleotides

Reaction mixtures contained in a final reaction volume of 25 μl: 25 mM Tris-HCl, 10 mM $MgCl_2$, 1 mM DTT, 100 μg/ml BSA, 15% glycerol, 100 mM NaCl, 0.1% Triton X-100, 10 μg/ml target DNA (ΦX174 RF I), 12% DMSO, 2 mM ATP, 100 ng MuB, 100 ng of either WT MuA, MuA1-574, MuA1-527, MuA1-562, MuA1-490 and/or the catalytic mutant MuA D269N/E392Q (see

Figure 9), and 1 pMol annealed radioactive oligonucleotide (TAB-225 annealed to TAB-226*). The reaction components were added together on ice, and then incubated at 30 degrees for one and a half hours. 0.2 volumes 5x protein sample buffer was then added to stop the reaction, and the samples were loaded onto a 0.8% HGT agarose gel and electrophoresed in 1xTAB buffer at 70 V for two hours with constant buffer recirculation. The reaction containing WT MuA was diluted 20 fold in 1x protein sample buffer prior to loading in order to allow its signal to be in the same range as the other reactions. After electrophoresis the gel was vacuum dried onto filter paper and exposed overnight to a Molecular Dynamics (Sunnyvale, CA) PhosphorImager screen. The screen was developed on a Molecular Dynamics PhosphoImager 445SI using a grey scale intensity range of 0-100. A reproduction of the exposure is displayed in Figure 9.

RESULTS

The crystal structure of the core domain of the Phage Mu transposase, which resolved amino acids 258-560, revealed the presence of two subdomains (see Figure 3 for a ribbon model of the core domain). Domain IIA (amino acids 243-490) contained the acidic DDE catalytic triad. Domain IIB (amino acids 490-574) displayed a large positively exposed patch of unknown function (Rice and Mizuuchi, 1994). In light of these data, experiments were undertaken to provide a more complete characterization of the function of domain IIB. A series of truncation mutants deleting either part or all of domain IIB were constructed and purified. As with all truncated proteins, there exists the possibility that the expressed protein is misfolded. In order to address whether at least a portion of the purified truncation proteins were properly folded, a qualitative assay for retention of sequence specific binding to Mu end binding sites was performed. Previously reported carboxyl terminal truncations deleting part of domain IIB had been shown to be unable to form tetramers or display any transposition chemistry by themselves *in vitro*, but partial activity was observed when mixed with full length MuA (Baker *et al.*, 1993). The purified truncation proteins were assayed for their ability to perform cleavage or strand transfer reactions in the presence of WT MuA. The truncation proteins were also assayed for their ability to perform donor cleavage in the presence of a full length, but catalytically incompetent, mutant form of MuA, MuA D269N/E392Q. This mutant protein has two of its three carboxylates comprising the DDE catalytic motif altered to their respective amide derivatives. MuA D269N/E392Q is able to assemble into a Mu transpososome but is completely deficient in catalysis. Cleaved donor complexes thus formed were immunoblotted to probe for their protein content. Ability to perform strand transfer after bypassing the requirement for donor cleavage was assayed in the presence of MuA D269N/E392Q.

Recombinant Protein Expression and Purification

The truncation proteins displayed essentially similar biochemical characteristics as wild type MuA during purification. However, the amount of ammonium sulfate neccessary to precipitate the

proteins increased with decreasing protein size. MuA1-562 precipitated at a final ammonium sulfate concentration of 30%, MuA1-527 required 35% and MuA1-490, 40%. All three truncation proteins adsorbed well onto the initial phosphocellulose column, under the purification conditions, as viewed by the absence of expressed protein in the flow through fraction. There was a decreasing affinity for the phospocellulose matrix which correlated with protein size. The smaller the protein, the lower the concentration of salt neccessary to fully elute it off the phosphocellulose column (data not shown). Rice *et al.* (Rice and Mizuuchi, 1994) reported domain IIB to contain a large positively charged exposed patch. Deletion of this patch could explain the reduced afinity of the smaller proteins to the negatively charged phosphate moeities of the phosphocellulose.

MuA1-490 displayed reduced affinity for the Pharmacia Mono-S (cation exchange) column when compared to MuA1-527. A significant portion of MuA1-490 eluted off the column in the flow through fraction, whereas MuA1-527 adhered well with little elution into the flow through. In addition, MuA1-490 readily eluted off the Mono-S column in the earliest fractions, whereas MuA1-527 eluted further along the linear salt gradient. Figure 4 shows each of the purified proteins on a SDS-PAGE gel. The volume of protein sample loaded was adjusted to achieve 100ng of protein in the sbsequent assays.

Sequence Specific DNA Binding

Upon purification of truncated proteins, it is important to determine whether they retain a native conformation. One way of determining this is to assay for functions that are known to be localized in domains of the protein that have been retained. Sequence specific Mu end binding has been localized to domain IB (residues 77-243) of MuA (Nakayama et al., 1987; Kim and Harshey, 1995) which is separated from domain IIB by 247 amino acids. Retention of this sequence specific DNA binding activity thus serves as a probe for a native fold (of at least domain IB) in the truncation mutants.

MuA1-574, MuA1-562, and MuA1-527 were assayed for their ability to bind sequence specifically to Mu end DNA. MuA1-490 was not tested. Plasmid pKN2 was purified and digested with restriction enzyme Hae II. Plasmid pKN2 contains an Eco RI fragment encoding 220 bp of the right end of the Mu genome (Adzuma and Mizuuchi, 1988). This region contains the three MuA right end binding sites R1, R2, and R3. By digesting this plasmid with HaeII, three fragments are produced of different mobility and composition (see Figure 4). The highest and lowest mobility fragments contain only vector DNA and no Mu end binding sites. The fragment of intermediate mobility contains the R1, R2, and R3 end binding sites, as well as vector sequence, and serves as the target for sequence specific DNA binding. The presence of the two fragments comprised only of vector sequence provides, on a molar ratio, a very large excess of nonspecific DNA sequence and serves as an internal control for sequence specific versus nonspecific DNA binding. This internal control eliminated the requirement for additional carrier DNA in the assay. WT MuA protein completely bound the fragment containing end binding sites and also caused a smear of the highest molecular weight fragment containing only vector DNA. Under the same conditions, MuA1-574, MuA1-562 and MuA1-527 bound only to the fragment containing the end binding site sequences (see Figure 5) This suggested that sequence specific binding was retained, at least to some degree, by the truncated proteins indicating that at minimum domain IB was properly folded.

Mixed Tetramer Formation with Wild Type MuA

Carboxyl terminal truncations of Mu transposase to amino acid 574 had previously been shown not to have any activity when assayed in a standard *in vitro* transposition assay. But when MuA1-574 was mixed with WT MuA and assayed, the truncated protein was incorporated into a mixed tetramer, and the resulting complex had partial activity in a standard transposition assay (Baker *et al.*, 1993). In order to assay whether MuA1-562, MuA1-527 and MuA1-490 retained some activity *in vitro*, they were mixed together with WT MuA and assayed *in vitro.*

Three noticeable results emerged from these mixing experiments. The first is that, as expected, the truncated proteins did not show any transposition activity by themselves. The donor and target DNA assayed in the absence of any protein resembled the DNA products in the presence of MuA1-574, MuA1-562 or MuA1-527 (see Figure 6, panel A, lanes 1, 6, 11 and 16). MuA1-490 was assayed for mixed tetramer formation in the absence of target DNA and the results resembled donor DNA assayed without protein (see Figure 6, panel B lanes 1 and 6).

The second observable effect of the deletion proteins was that formation of strand transfer complexes was inhibited by their presence when compared to a parallel reaction with WT MuA alone. In Figure 6, panel A, the intermolecular strand transfer products are the set of topoisomers at the top of the gel while in panel B (in which the assay was done in the absence of target DNA) the different intramolecular strand transfer products have either increased mobility and run just ahead of the supercoiled donor DNA or have intermediate mobility and migrate between the supercoiled and open circle forms of the donor DNA. At concentrations of WT MuA which would normally support strand transfer complex formation (Figure 6, panel A, lane 3, and panel B, lane 2), the addition of truncated proteins caused a diminution or elimination of appearance of strand transfer products (Figure 6, panel A, lanes 8, 13, and 18, and panel B lane 4). Third, there was a concominant accumulation of cleaved donor products that, in these agarose gels, is visualized by an increase in the open circle form of the donor DNA (this is most marked in Figure 6, panel A, lanes 8, 13, and 18 and panel B, lane 4). As the ratio of WT MuA to truncation proteins was increased from 1:2 to 4:1 (from left to right in Figure 6, panel A), the reaction products tended to resemble those produced by the reaction with WT MuA alone. This implied that the carboxyl terminal truncation proteins, missing either all or part of domain IIB, were augmenting the formation of cleaved donor products while also inhibiting the formation of strand transfer products in the presence of low levels of WT MuA.

Cleaved Donor Complex Formation

When mixed with WT MuA, the carboxyl terminal truncations in the above standard *in vitro* transposition assay accumulated cleaved donor products, while individually they were enzymatically inactive. This accumulation of cleaved donor products could be explained by two alternate hypothesis. One, the truncation proteins could be inhibiting WT MuA catalyzed strand transfer activity without affecting donor DNA cleavage. Alternatively the truncation proteins could themselves be displaying partial enzymatic activity. In order to determine which protein was responsible for the observed catalysis, an alternate full length form of MuA was utilized in a mixing experiment with the truncation proteins. Mixed tetramers were assembled using the carboxyl terminal truncated forms of MuA in conjunction with MuA D269N/E392Q. MuA D269N/E392Q is a catalytic mutant of MuA that is competent for stable synaptic complex formation, but deficient in all transposition catalysis (Baker *et al.*,1994). MuA D269N/E392Q has substitutions that replace two acidic amino acids, glutamate 269 and aspartate 392, with glutamine and asparagine respectively. These two residues are the first aspartate and the glutamate of the DDE catalytic triad motif that is conserved among many members of the transposase/integrase superfamily (Fayet *et al.*, 1990; Rowland and Dyke, 1990; Kulkosky *et al.*, 1992; Radstrom *et al.*, 1994; Baker and Luo, 1994). Any cleaved donor complexes formed by this assay would have to be a direct result of catalysis by the truncation proteins, because the point mutant cannot carry out catalysis due to a nonfunctional active site.

Cleaved donor complexes were formed by incubation of each of the carboxyl terminal truncation proteins (MuA1-574, MuA1-562, MuA1-527 and MuA1-490) with and without MuA D269N/E392Q under standard *in vitro* transposition conditions. After incubations an aliquot of those reactions containing both MuA D269N/E392Q and either MuA1-574, MuA1-562, MuA1-527, or MuA1-490 were saved for use as the "load" fractions in the later immunoblot. The remaining reactions were split. Stop buffer containing SDS was added to one half of each reaction. Stop buffer lacking SDS was added to the other half. All the reactions were then loaded

onto the same heparin/BSA agarose gel and electrophoresed. These electrophoresis conditions were chosen for several reasons. Firstly, by loading the samples in the presence or absence of SDS, resolution of both the protein/DNA complexes (-SDS) and also the DNA products remaining after removal of any associated protein (+SDS), was possible. Both cleaved donor complexes and donor cleavage products will be resolved concominantly. Heparin in both the gel and the electrophoresis buffer acts as a nonspecific competitor for DNA binding. By adding this competitor, only those proteins that are in a stable protein/DNA complex will remain bound to the DNA. Transpososomes are stable under these conditions, but simple MuA end binding is not. The results of the assay are displayed in the ethidium bromide stained gel in Figure 7.

As expected from the previous assays, none of the proteins, neither the catalytic mutant nor any of the carboxyl terminal truncation proteins, displayed any activity by themselves (see Figure 7, lanes 1-5 and 11-15) when compared to donor DNA incubated in the absence of any form of MuA (see Figure 7, lanes 6 and 16). In all of the reactions that contained a mixture of a carboxyl terminal truncation and the catalytic mutant, a product was formed that had altered mobility in the absence of SDS. In the presence of SDS the product migrated as open circle DNA. This change in mobility implied that the band was comprised of a stable protein/DNA complex. This intermediate mobility complex is most likely a cleaved donor complex. In cleaved donor complexes MuA restrains the supercoils in the Mu portion of the mini-Mu donor plasmid. The non-Mu portion of the mini-Mu donor plasmid relaxes its superhelicity due to donor cleavage. Cleaved donor complexes display reduced electrophoretic mobility, and if disassembled by SDS, the cleaved donor DNA is free to completely relax all its supercoils and migrate as open circle DNA (Surette *et al.*, 1987). The product of intermediate mobility disappeared upon addition of SDS, with a concominant increase in the amount of DNA migrating in the open circle form. This strongly suggests that the product was a cleaved donor complex (see Figure 7, lanes 17-20 and then 7-10). The novel products in Figure 7, lanes 17-20 were excised from the gel with a clean razor blade for analysis of their protein content by immunoblotting.

Immunoblot of Cleaved Donor Complexes

The cleaved donor complexes from the previous assay were recovered and subject to immunobloting to probe the protein contents of the protein/DNA complex. Aliquots of the cleaved donor complex formation assay reactions that had been saved as "load" fractions were diluted 7 fold with 1x protein sample buffer and loaded onto the same acrylamide gel as the recovered cleaved donor complexes. By doing this, the immunoblot would provide a "before and after purification" shot of the protein components. Both the proteins initially present in the cleaved donor complex formation reactions, and the protein components present in the stable protein/DNA complex excised from the gel would be represented together on the same immunoblot. A reproduction of the results are displayed in Figure 8.

An initial exposure of the immunoblot did not show any recovered proteins in the cleaved donor complexes. (Figure 8 panel A). Upon longer exposure, the protein/DNA complexes excised from the previous assay indicated the presence of both forms of the Mu transposase. Figure 8, panel B shows a longer exposure of the immunoblot and even though the exposure did not reproduce well, the presence of both forms of MuA is readily apparent. It is crucial to note that the carboxyl terminal truncation proteins by themselves fail to form cleaved donor complexes (as noted by the absence of intermediate mobility product in Figure 7, lanes 12-15). The double point mutant MuA D269N/E392Q does assemble into stable synaptic complexes by itself. The stable synaptic complex migrates only slightly slower than the supercoiled donor DNA (just visible in Figure 7, lane 11, and absent in lane 1 (+SDS)), and is well resolved from the cleaved donor complexes. Unlike the full length catalytic mutant, none of the truncation proteins displayed any ability to form stable tetramers as evident by the absence of any stable synaptic complex formation. When mixed with the catalytic mutant however, stable synaptic complexes were observed (Figure 7 lanes 17-20) as the bands with just slightly lower mobility than the supercoiled donor. Because neither the truncation mutants nor the catalytic mutant can independently carry out any transposition

chemistry, and because both proteins were recovered from the excised cleaved donor complexes, the results suggest that the carboxyl terminal truncation proteins can all form mixed tetramers with MuA D269N/E392Q and that these mixed tetramers can proceed to the stage of cleaved donor complex formation.

Strand Transfer Complex Formation using Precleaved Oligonucleotides

It has previously been shown that relaxed buffer conditions ease the topological and enhancer requirements of the donor DNA in a Mu transposition reaction. WT MuA has been shown to assemble into functional tetramers on short oligonucleotide substrates and carry out transposition chemistry in the presence of DMSO and Triton X-100 (Savilahti *et al.*,1995). Single stranded oligonucleotides were synthesized and radiolabeled on their 5' ends. The were annealed to one another forming a double stranded oligonucleotide that has a two base 5' overhang, an exposed 3' hydroxyl moiety on a terminal adenosine and the Rl and R2 binding sites (see Materials and Methods for oligonucleotide sequence). This substrate is competent to support tetramer formation followed by strand transfer using WT MuA (Aldaz *et al.*,1996). The ability of the carboxyl terminal truncation proteins to carry out strand transfer on precleaved (having a preexexposed 3' hydroxyl on the terminal adenosine bypassing the need for donor DNA cleavage) oligonucleotide substrates was assayed in the presence of the catalytic point mutant under these relaxed buffer conditions.

As expected, reactions containing only WT MuA were able to carry out strand transfer (see Figure 9, lane 2). MuA1-574, MuA1-562, MuA1-527, MuA1-490 and MuA D269N/E392Q assayed independently were unable to carry out any detectable strand transfer (see Figure 9, lanes 3-7). However, mixtures of each of the truncation proteins in conjunction with the catalytic mutant were able to carry out strand transfer (see Figure 9, lanes 8-11). It is important to note that even under these relaxed buffer conditions, the carboxyl terminal truncations which have some or all of domain IIB deleted do not carry out any transposition chemistry. The catalytic mutant,which does

not have the catalytic residues neccessary to carry out any transposition chemistry, also fails to proceed with any catalysis. However, when mixed together these inactive proteins were each able to complement the defect of the other. The catalytic point mutant allows the truncation mutant to assemble into a mixed tetramer, and the carboxyl terminal truncation mutants are able to then donate their active site residues to perform the strand transfer reactions.

DISCUSSION

In this work a series of carboxyl terminal truncations of the Phage Mu transposase of were constructed and purified. The series consisted of three proteins that contained a partial or complete deletion of domain IIB. All three truncation proteins displayed similar *in vitro* biochemical characteristics. As expected, the truncation proteins did not display any catalytic activity when assayed individually. MuA1-574, MuA1-562 and MuA1-527 retained sequence specific binding to Mu right end binding sites indicating that at minimum domain IB, in which Mu end binding function has been localized (Nakayama *et al.*,1987), retained a functional native fold. MuA1-490 was not assayed for Mu end binding ability. As expected, none were able to assemble into a stable transpososome as viewed by their inability to form stable synaptic complexes. However, as with a previously described partial carboxyl terminal truncation of domain IIIA (MuA1-574, Baker *et al.*, 1993), they all were able to interact with WT MuA and display partial function. As had been seen with MuA1-574, in the presence of WT MuA (at WT MuA concentrations too low to otherwise support transposition), an accumulation of cleaved donor product and an inhibition of strand transfer was observed. Their ability to interact with full length forms of MuA to form mixed tetramers also strongly suggested that the truncated proteins retained a native fold.

The truncation proteins were assayed for their ability to carry out donor cleavage in the presence of the catalytic mutant MuA D269N/E392Q. This full length catalytic mutant retains the ability to assemble into a transpososome, but is unable to perform any transposition biochemistry because two carboxylates, crucial for catalysis, have been mutated to their amide derivatives (Baker and Luo, 1994). The two residues form two thirds of the DDE motif that has been implicated as an active site triad in a variety of transposases and integrases (Kulkosky *et al.*, 1992; Engelman and Craigie, 1992; Baker and Luo, 1994; Krementsova *et al.*, 1996; Kim *et al.*, 1995). None of the truncation mutants were able to form stable synaptic complexes. Formation of a transpososome is a required step prior to catalysis. The absence of catalytic activity by the truncation mutants could

thus be due to two possible defects. One, residues crucial for catalysis might reside in domain IIB, and in their absence catalysis is not supported. The other possibility is that the neccessary catalytic determinants are present in the truncation proteins, but key multimerization determinants neccessary for tetramer formation are deleted. Since tetramer formation is a prerequisite function prior to catalysis, even if all the determinants neccessary for catalysis were present in the truncation proteins, they would never get the opportunity to utilize them due to their inability to first form a tetramer. While neither the truncation proteins nor the catalytic mutant can independently carry out donor cleavage, in the presence of one another, donor cleavage was possible. This result indicates that the two types of mutants can complement each other's defects. The catalytic mutant is able to provide a scaffold on which the truncation protein can assemble. Once assembled into a stable complex, the truncation proteins are able to donate their active site residues and carry out the endonucleolytic cleavage of the donor DNA that the catalytic mutant cannot. To insure that mixed tetramers of the truncation proteins and the catalytic mutant indeed contained both forms of the protein, the cleaved donor complexes were excised from a native gel and their protein constituents analyzed by immunobloting. The immunoblot clearly showed that both forms of the protein were present within the excised cleaved donor complexes. This suggested that the two mutant forms of MuA were together forming stable mixed tetramers, and that these mixed tetramers were functional for donor cleavage.

MuA has been shown to assemble into a catalytically active transpososome on oligonucleotides containing the R1 and R2 end binding sites under relaxed reaction conditions (Savilahti *et al.*, 1995). Oligonucleotides containing the appropriate exposed 3' hydroxyl moieties provide a substrate for strand transfer without requiring that donor cleavage occur (Savilahti *et al.*, 1995; Aldaz *et al.*, 1996). The truncation proteins, independently and in concert with the catalytic mutant, were assayed for their ability to catalyze strand transfer. As expected, all of the carboxyl terminal truncation proteins, irrespective of whether some or all of domain IIB was deleted, failed to show any activity for strand transfer. The catalytic mutant assayed alone was also unable to perform

strand transfer. In conjunction with the catalytic mutant, however, all of the truncation proteins demonstrated strand transfer activity. This strongly suggests that the defects of the two types of proteins can be complemented by one another to form partially active mixed tetramers. The catalytic mutant can provide an assembly scaffold for the truncation proteins, and the truncation proteins, once assembled, can donate their catalytic residues for use in strand transfer. It should also be noted that in addition to displaying an assembly defect, the truncation proteins are not able to catalyze both donor cleavage and strand transfer in the same reaction. This implies that there is a second defect, in addition to the assembly defect, in the truncation proteins that the full length proteins cannot complement.

In analyzing the results of the sequence specific DNA binding assay it is important to realize the inherent limitations of this type of assay. Firstly it is a qualitative activity assay. Even if a titration of the proteins to the DNA were performed, a binding constant distilled from the data could be inaccurate. This is because, as with the purification of any novel protein, the fraction of misfolded and unfolded protein in the sample remains an unknown. The amount of this unknown fraction would bias any binding constant calculation. Calculating a specific activity for end binding could be done, and if the truncation proteins showed a lower specific activity than WT MuA, it would be indicative that a fraction of the purified protein was inactive. If that were the case, then the concentration of each protein added to subsequent assays would have to be adjusted for specific activity. Nonetheless, as a qualitative assay, it does show that at least a fraction of the purified proteins retains native binding activity. The significance of assaying Mu end binding is that that activity has been functionally mapped to a different domain (domain I, Nakayama *et al.,* 1987) than the one being deleted (domain IIB). Retention of Mu end binding by the truncation proteins is thus an indication of a native-like fold.

The logical next step would be to assay for sequence nonspecific DNA binding, as domain IIB has been implicated in that function due to the presence of a large positively exposed patch (Rice and

Mizuuchi, 1995). The results of a simple nonspecific DNA binding assay, even if titrated to determine binding constants, would have to be viewed with several caveats. Once again the fraction of properly folded protein is an unknown that would have to be taken into account. This could be done by determining the specific activity of sequence specific DNA binding in the truncation proteins with respect to WT MuA. Even if nonspecific DNA binding were greatly reduced in the truncation proteins, it would be bold to claim that the nonspecific DNA binding activity was exclusively localized in the deleted portion for the following reason. Nonspecific DNA binding activity was mapped to domain II by isolating the products of partial proteolytic digestion (Nakayama *et al.,* 1987), and was assayed in the absence of domain I, which displays sequence specific end binding. In the absence of a putative nonspecific DNA binding domain, the Mu end binding domain (domain IB) could bind DNA nonspecifically. This binding would most likely occur with a lower affinity than binding to the Mu end, but binding activity of this type could nonetheless mask nonspecific DNA binding activity present in domain IIB. Because all of the truncation proteins in this study contain domain IB, localizing the site of nonspecific DNA binding activity could prove non-trivial.

The mixed tetramer formation assay with WT MuA and the truncation proteins yielded informative results. At WT MuA concentrations too low to otherwise support transposition, addition of any of the truncated proteins stimulated cleaved donor product formation. At WT MuA concentrations that can normally support transposition, addition of any of the truncated proteins inhibited strand transfer product formation. This indicated that the truncated proteins, while inactive when assayed by themselves, could alter the activity of full length MuA. This mirrored the results of T. A. Baker who showed that MuA1-574 assembled into mixed tetramers with WT MuA and displayed partial activity (Baker *et al.,* 1993). The accumulation of cleaved donor products implied that the truncation proteins were participating in donor cleavage with WT MuA. The presence of both truncation proteins and WT MuA in mixed tetramers capable of carrying out donor cleavage, has been shown by Baker (Baker *et al.,* 1993) for MuA1-574 and by Pincus (D. Pincus unpublished

observations) for MuA1-562 and MuA1-527 by probing the protein content of isolated cleaved donor complexes immunologically.

One question the above experiment does not address is whether it is the truncation protein or the WT MuA which is donating it's catalytic residues for the partial catalytic activities observed. To address this issue raised by the above experiment, a catalytically inactive, but full length form of MuA (MuA D269N/E392Q) was assayed in the presence of the truncation mutants. Neither the catalytic mutant nor the truncation mutants display any transposition chemistry when assayed individually. Together, however, they are able to form stable cleaved donor complexes. This restoration of function implies that these two forms of MuA can complement each other's defects. A mechanism based on the stimulation of assembly of the full length catalytic mutant by the truncation mutants without incorporation of the truncation mutants into a mixed tetramer would predict an absence of cleaved donor product as well as incorporation into stable tetramers of only one form of the proteins. This is because no matter how much catalytic mutant assembles into transpososomes, the resulting transpososomes would still lack the neccessary carboxylate residues to carry out transposition chemistry and would be stalled in stable synaptic complexes. Analysis of the protein content of the cleaved donor complexes formed in the presence of both the truncation proteins and the catalytic mutant revealed the physical presence of both proteins. This strongly suggests that the two forms of the transposase are coassembling into mixed tetramers and complementing each other's defects with the full length mutant acting as an assembly scaffold for the truncation mutants. Once assembled into a mixed tetramer, the truncation proteins could then donate their acidic residues to the active site to carry out donor DNA cleavage.

One of the noticeable results that is difficult to reconcile, is that the mixed tetramers formed with either the catalytic mutant or WT MuA, in concert with the truncation proteins, do not regain complete activity. If the only defect in the truncation mutants was an assembly defect, then once incorporated into a mixed tetramer they should have been able to complete both donor cleavage and

strand transfer during the same reaction. Strand transfer activity, however, was only observed when donor cleavage was bypassed by providing a precleaved DNA substrate. This is indicative of a second type of defect in the truncation mutants in addition to purely a block in assembly. Wu and Chaconas have described a cryptic nuclease activity, as well as nonspecific DNA binding activity in a peptide derived from domain IIIA containing residues 575-600 (Wu and Chaconas, 1995). Carboxyl terminal truncations of D108A, which is the transposase of the Mu like phage D108 that shares an almost identical sequence to MuA in all parts of the protein except the enhancer binding site situated in domain IA, (Lowe J. and Baker, T. A., unpublished data) indicate that the minimum size of D108A neccessary for all stages of transposition is amino acids 1-598, which corresponds to residues 1-597 in MuA. Deletion of four more amino acids to residue 594 (the equivalent to amino acid 593 in MuA) yields a protein that can assemble into a tetramer and carry out strand transfer, but is deficient in donor cleavage. In light of this data it is intriguing to hypothesize that the amino terminal end of domain IIIA is taking part in donor cleavage.

The truncation proteins purified and assayed in this work all have similar *in vitro* biochemical characteristics. A complete deletion of domain IIB (MuA1-490) is still able to incorporate into mixed tetramers together with a full length MuA and carry out partial transposition chemistry. Thus, at least some of the determinants neccessary for tetramer assembly must lie amino terminal to domain IIB. However, for self assembly into tetramers to occur, domain IIIA must be present (Baker *et al.*, 1993). This division of the determinants neccessary for assembly is reminiscent of the split determinants neccessary for the multimerization of the integrase from the mouse tumor virus Avian Sarcoma Virus (Andrake and Skalka, 1995). Even when all of domain IIB is deleted, partial catalytic activity is obtainable in the presence of full length MuA. Thus the active site catalytic triad must still be available to be donated to the active site. The truncation proteins in the presence of full length MuA, however, will not catalyze both donor cleavage and strand transfer in the same reaction. Because the Mu end binding domain is localized in domain IB, that there is a large positively charged exposed patch visible in the crystal structure (Rice and Mizuuchi , 1995),

and that a peptide containing residues 575-600 displays nucleolytic ability, it makes it attractive to consider that the function of the large positively charged patch is to bind either sequence nonspecifically to the target DNA, or to the donor DNA-target DNA junction.

Purification and analysis of the carboxyl terminal truncations of the Mu transposase has suggested several things. As expected, none of the truncation proteins displayed any activity independently. In concert with full length MuA they assembled into mixed tetramers and displayed partial activity. This indicated that some, but not all, of the determinants neccessary for tetramerization reside amino terminal to residue 490. The partial activity displayed suggests that the truncation proteins can donate their active site residues to perform catalysis. It also suggests that there is an additional function missing in the truncation proteins besides an assembly defect. The presence of a large positively charged patch (Rice and Mizuuchi, 1995) and possible DNA binding and endonucleolytic activity (Wu and Chaconas, 1995), suggest that domain IIB and domain IIIA possess determinants neccessary for nonspecific DNA binding (either target DNA binding, or donor DNA/target DNA junction binding) and residues crucial for donor cleavage.

FIGURES

FIGURE 1. Three Types of Transposition

Panel A. Diagram of the "cut and paste" mode of transposition. The donor DNA is cleaved on both strands to produce a blunt ended product. The 3' hydroxyl ends of the element are then used as the nucleophiles in the subsequent strand transfer event. A several base pair gap is introduced in the target DNA. The gaps are repaired by host DNA repair enzymes. This mode of transposition does not require a replication fork to pass through the transposon and thus is also termed non-replicative transposition. An example of a transposable element which displays this type mechanism of transposition is Tn7.

Panel B. Diagram of replicative transposition. The donor DNA is cleaved only once on each strand exposing a 3' hydroxyl at each end of the element. The 3' hydroxyl ends of the element are used as the nucleophiles in the subsequent strand transfer event. A several base pair gap is introduced in the target DNA. The three-way junction can be resolved either by the construction of a replication fork with concominant replication through the element to produce a cointegrate or by enzymatic cleavage followed by DNA repair. Because the cointegrate has two copies of the element, this mode is termed replicative transposition. The quintessential representative of this type of transposition is Phage Mu.

Panel C. Diagram of retroviral integration and retrotransposition by LTR-retrotransposons. The RNA genome is reverse transcribed into cDNA. The cDNA is cut once on each strand two nucleotides from the ends of the element, and the terminal dinucleotide dissociates from the element revealing a 3' hydroxyl at each end. This reaction is termed 3' end processing. The exposed 3' hydroxyl ends are used as the nucleophiles in the subsequent integration step. The several base pair gap is repaired by host DNA repair enzymes. HIV-1 displays this type of mechanism.

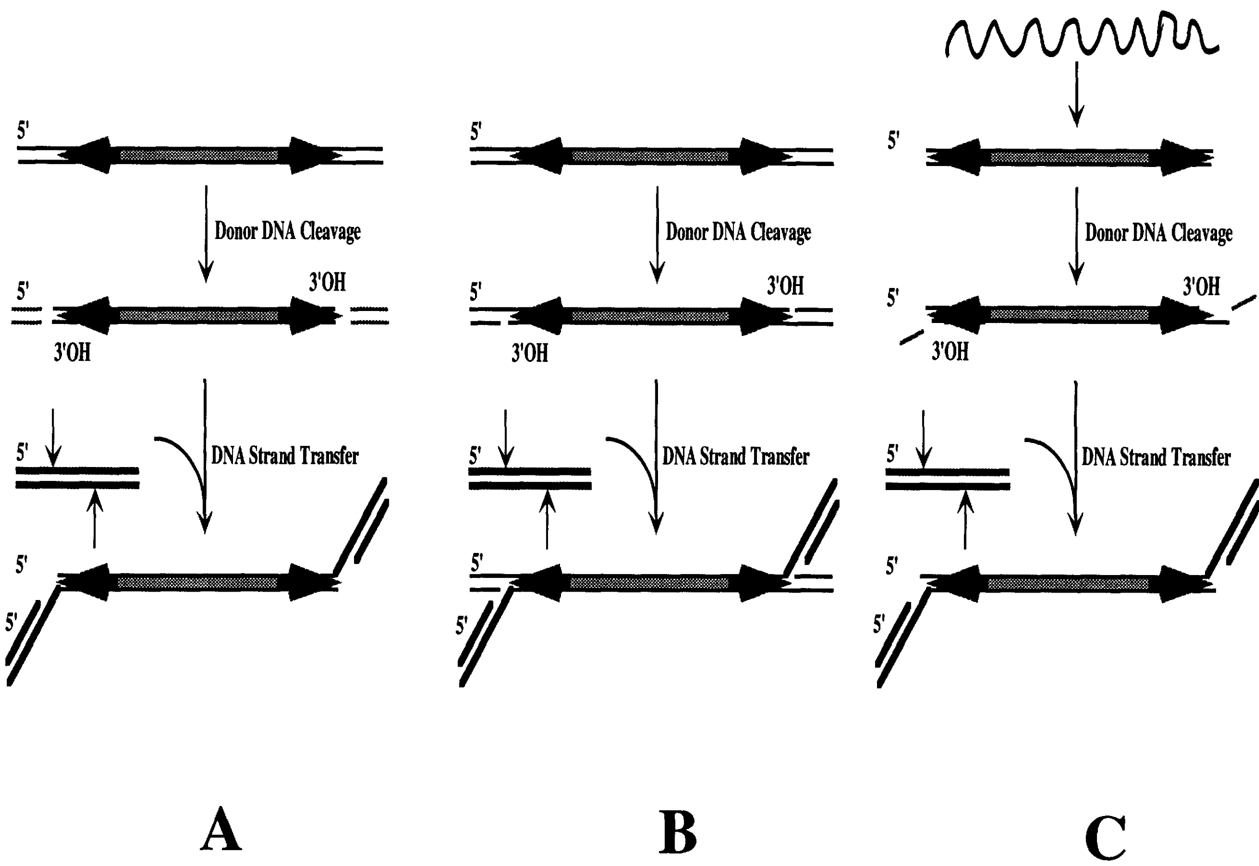

5'
Donor DNA Cleavage
3'OH
DNA Strand Transfer
A
B
C

FIGURE 2. Domain Structure of MuA

MuA is a 75 kDa protein comprised of three main domains. Various functions have been ascribed to each of the domains or subdomains. Domain IA binds to the Internal Activating Sequence, a cis-acting transpositional enhancing element. Domain IB binds sequence specifically to the Mu end binding sites. Domain IIA contains the conserved catalytic DDE motif. Domains IB, IIA and IIB are all required for tetramer formation. Nonspecific DNA binding could be due to domain IIB because of a large exposed positively charged patch. The extreme end of the protein, domain IIIB is involved in interaction with the transpositional enhancer protein MuB and interacts with the chaperone ClpX which is involved in the disassembly of the Strand Transfer Complex.

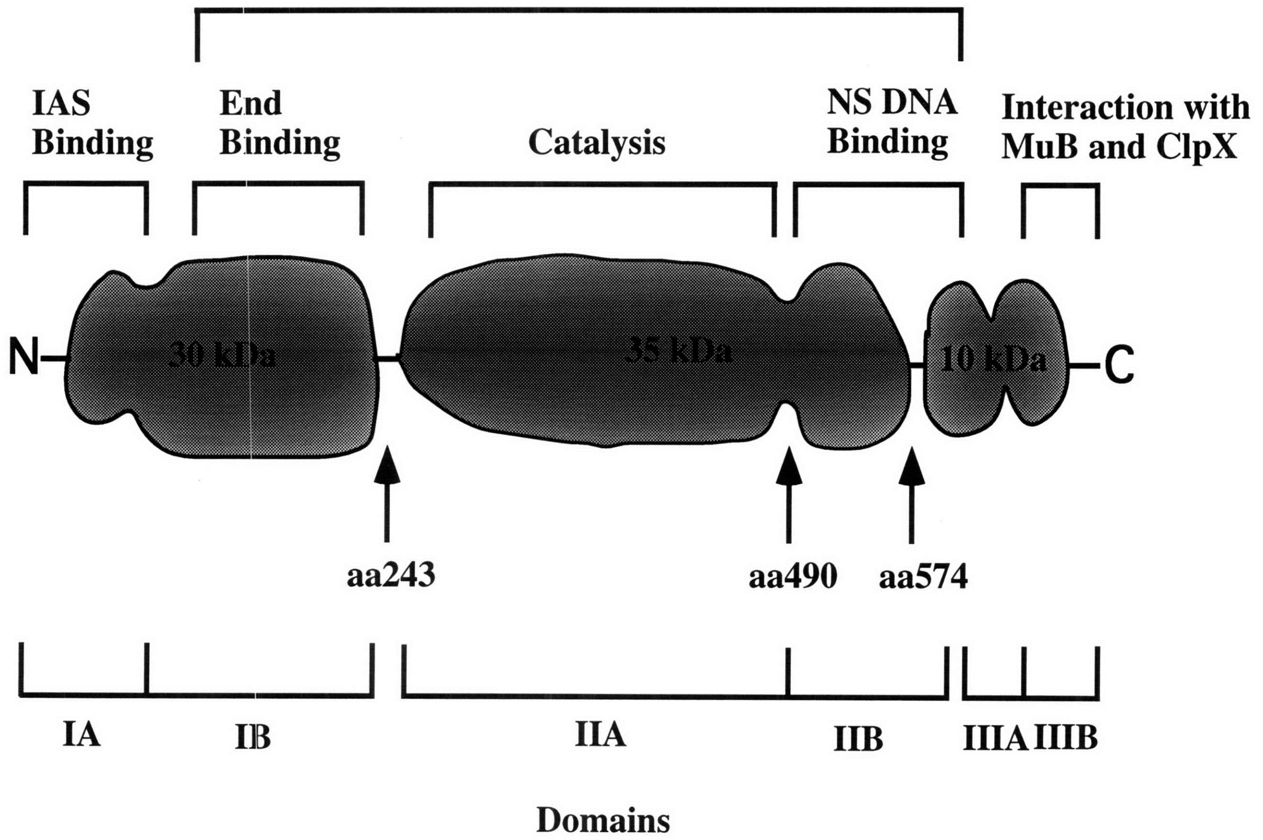

Tetramer Formation
IAS Binding
End Binding
Catalysis
NS DNA Binding
Interaction with MuB and ClpX
N
30 kDa
35 kDa
10 kDa
C
aa243
aa490
aa574
IA
IB
IIA
IIB
IIIA
IIIB
Domains

FIGURE 3. Ribbon Model of the Core Domain

Ribbon model of the core domain of MuA highlighting its two subdomains. This figure was generated by accessing file 1BCO, which contains the coordinates generated by Rice (Rice and Mizuuchi, 1995) by X-ray crystallography of the core domain of MuA, from the Brookhaven Protein Data Bank and visualising the file with the RasMol Molecular Renderer v2.5.1, copyright October 1984 Roger Sayle, Glaxo Research & Development (Greenford UK)

Domain II-A **Domain II-B**

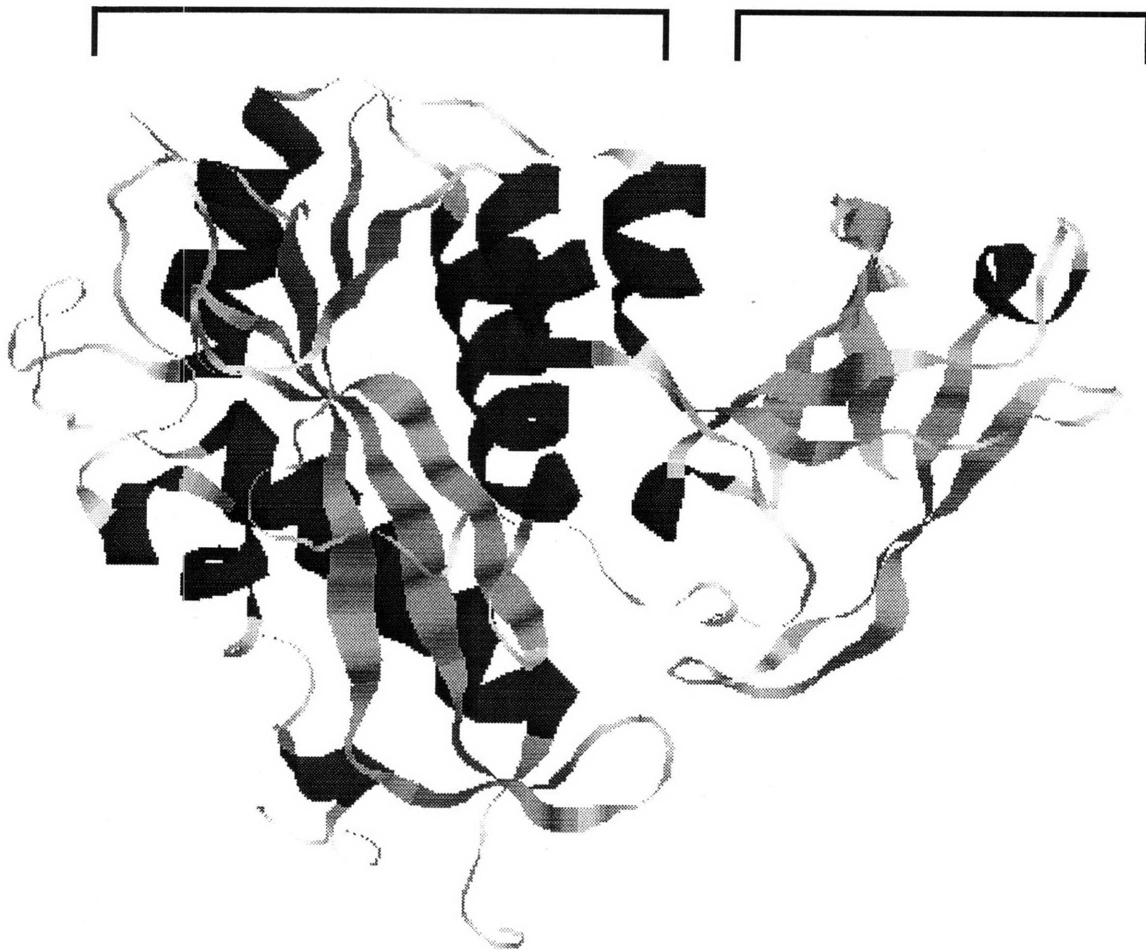
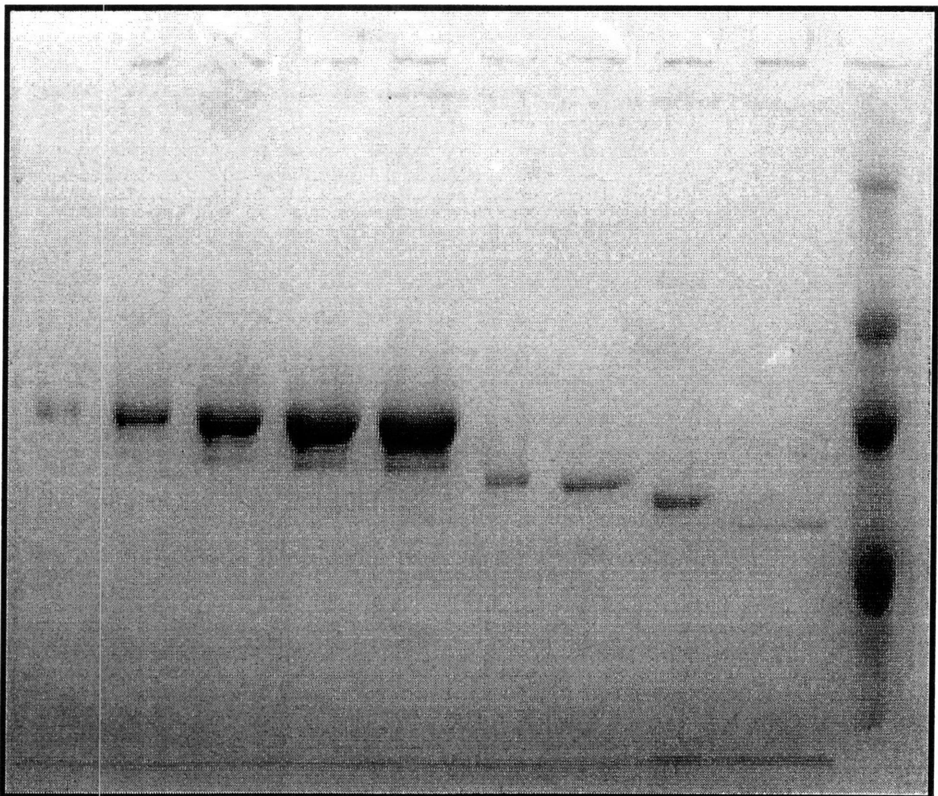


FIGURE 4. Purified Carboxyl Terminal Truncations

Lanes 1-5 contain 250 ng, 1000 ng, 1750 ng, 2500 ng, and 3250 ng WT MuA. Lanes 6-9 contain 390 ng MuA1-574, 460 ng MuA1-562, 560 ng MuA1-527 and 500 ng MuA1-490 respectively. Protein concentrations were determined by densitometry quantitation of each band as related to a standard curve drawn from the known concentration of the loaded WT MuA using Molecular Dynamics (Sunnyvale CA) ImageQuaNT v4.1 software with a grey scale intensity range of 0-256.



217 Kd

111Kd

71Kd

44Kd

WT 574 562 527 490 M

FIGURE 5. Sequence Specific DNA Binding

Lanes 1-5 contain 100 ng MuA1-527, MuA1-562, MuA1-574, WT MuA and no protein respectively. All lanes contain 55 ng pKN2 digested with restriction enzyme HaeII. The high and low mobility fragments contain only vector derived DNA. The fragment of intermediate mobility contains a 220 bp sequence containing the Mu right end binding sites. MuA-bound DNA displays slightly lower mobility than the fragment containing the right end binding sites.

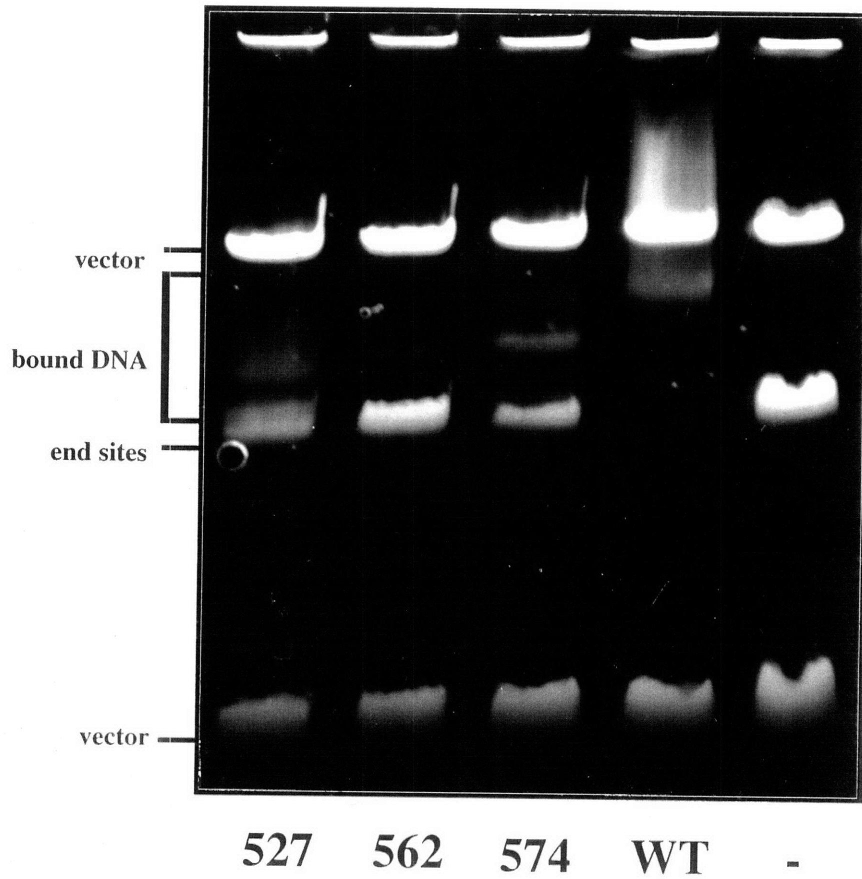
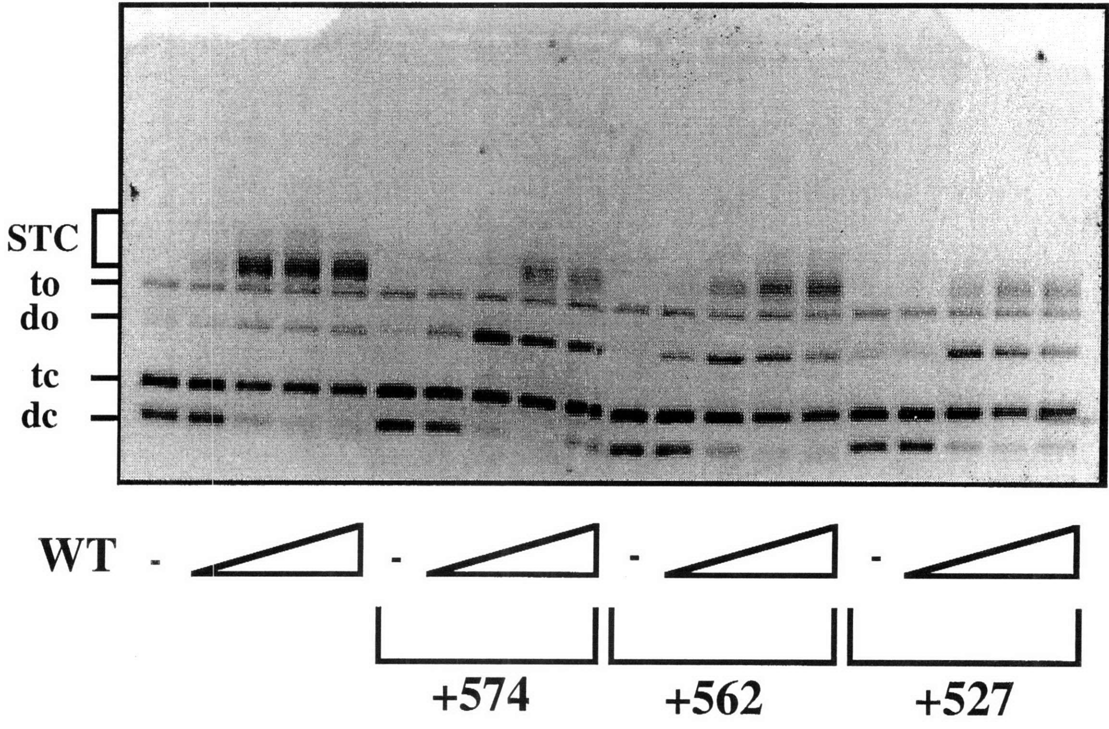

vector
bound DNA
end sites
vector
527
562
574
WT
-

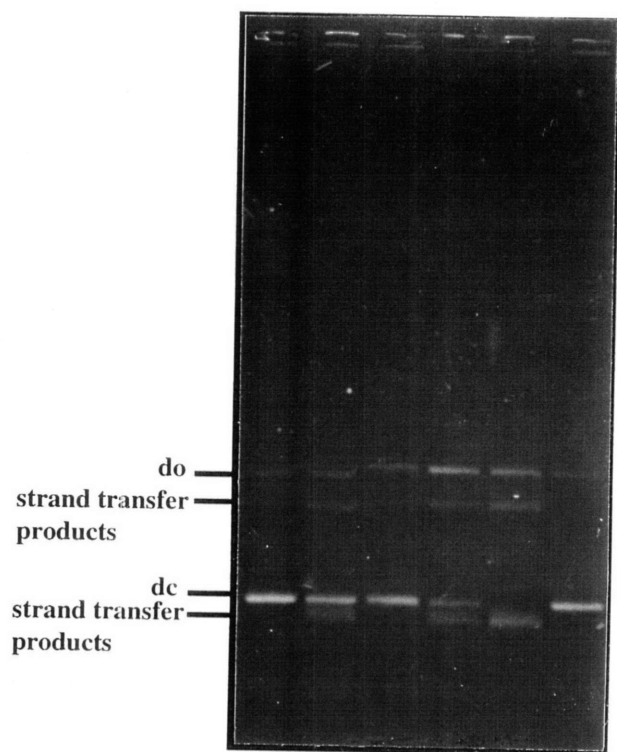
FIGURE 6. Mixed Tetramer Formation with WT MuA

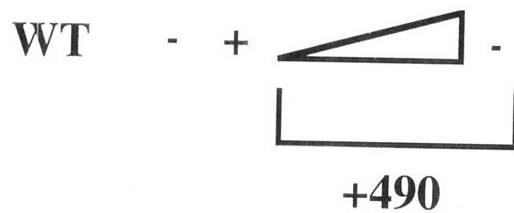
Panel A. Ethidium bromide stained DNA products of transposition reactions containing mixtures of WT MuA, MuA1-574, MuA1-562 and MuA1-527. Concentrations of WT MuA present are: lanes 2, 7, 12, 17, 50 ng; lanes 3, 8, 13, 18, 100 ng; lanes 4, 9, 14, 19, 200 ng; lanes 5, 10, 15, 20, 400 ng. Concentrations of carboxyl terminal truncation proteins as indicated are all 100 ng. Lane 1 contains no form of MuA. Cleaved donor DNA accumulates and has the same mobility as open circle donor DNA (do). Intermolecular strand transfer products run as the set of topoisomers with lower mobility than open circle target DNA (to). Supercoiled pSG1 donor DNA (dc) and supercoiled ΦX174 RF I target DNA (tc) have the greatest mobility and run at the bottom of the gel. A negative image using a grey scale intensity of 0 to 256 produced by photography with a CCD camera is displayed.

Panel B. Ethidium bromide stained DNA products of transposition reactions containing mixtures of WT MuA and MuA1-490. Concentrations of WT MuA present are: lanes 2 and 4, 100 ng, lane 3, 33 ng, lane 5, 300 ng. Concentrations of carboxyl terminal truncation protein MuA1-490 in the lanes indicated are 100 ng. Lane 1 contains no form of MuA. This assay was done in the absence of target DNA. Intramolecular strand transfer products run slightly faster than the supercoiled donor DNA (dc) as well as the set of topoisomers of medium mobility. Cleaved donor DNA accumulates and has the same mobility as open circle donor DNA (do).



A

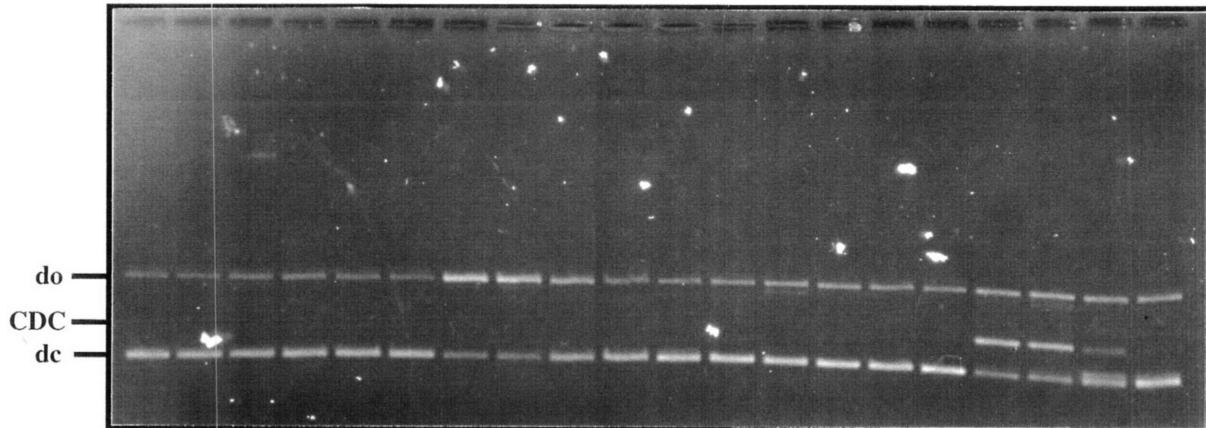




B

FIGURE 7. Cleaved Donor Complex Formation

Cleaved donor complexes were formed with MuA1-574, MuA1-562, MuA1-527 and MuA1-490 in the presence of MuA D269N/E392Q. The left and right halves of the heparin/BSA HGT agarose gel were loaded in the presence and absence of SDS respectively. Cleaved donor complexes are visible as the medium mobility product in lanes 17-20. Stable synaptic complexes formed with MuA D269N/E392Q are visible in lanes 11, 17, 18, 19 and 20 and have only slightly less mobility than the fastest migrating band (supercoiled pSG1 donor DNA, dc). The cleaved donor complexes shift in mobility in the presence of SDS and migrate as the lowest mobility band (open circle pSG1 donor DNA, do). Cleaved donor complexes from lanes 17-20 were excised from the gel for protein content analysis by immunoblotting.



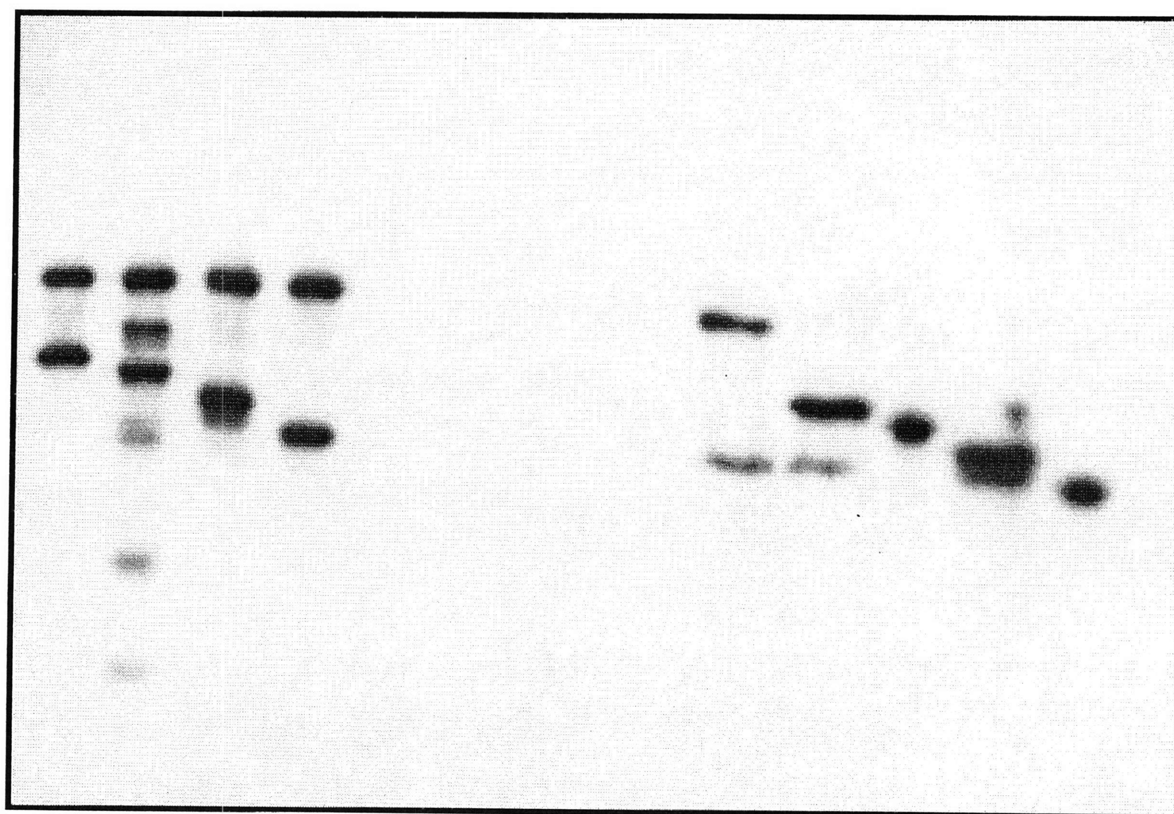
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
DE/NQ	+	-	-	-	-	-	+	+	+	+	+	-	-	-	-	-	+	+	+	+
574	-	+	-	-	-	-	+	-	-	-	-	+	-	-	-	-	+	-	-	-
562	-	-	+	-	-	-	-	+	-	-	-	-	+	-	-	-	-	+	-	-
527	-	-	-	+	-	-	-	-	+	-	-	-	-	+	-	-	-	-	+	-
490	-	-	-	-	+	-	-	-	-	+	-	-	-	-	+	-	-	-	-	+

+SDS (lanes 1–10) **-SDS** (lanes 11–20)

FIGURE 8. Immunoblot of Cleaved Donor Complexes

Panel A. Cleaved donor complexes from Figure 7, lanes 17-20 were excised from the gel, loaded onto a 8% SDS-PAGE gel, electrophoresed and immunoblotted. Lanes 9-13 contain 10 ng MuA D269N/E392Q, MuA1-574, MuA1-562, MuA1-527, and MuA1-490 respectively as molecular weight markers. A seven fold dilution of an aliquot of the samples loaded onto lanes 17-20 of the cleaved donor complex formation gel were loaded onto lanes 1-4 as "load" markers. Lanes 5-8 contain the excised cleaved donor complexes from lanes 17-20 respectively.

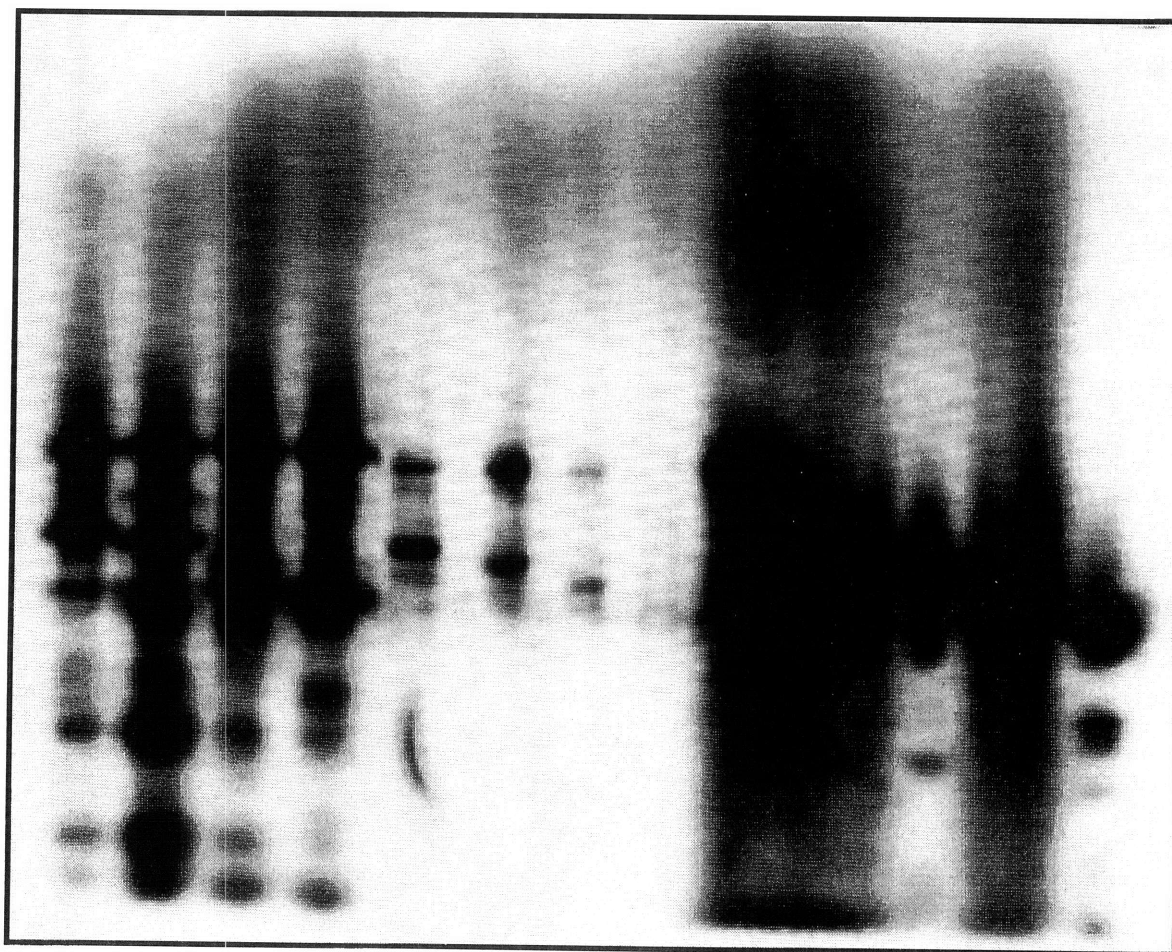
Panel B. An extended exposure of the same gel used to make panel A. The dual protein components of the cleaved donor complexes are visible in lanes 5-8.



A

+ D269N/E392Q

574 562 527 490 574 562 527 490 - 574 562 527 490

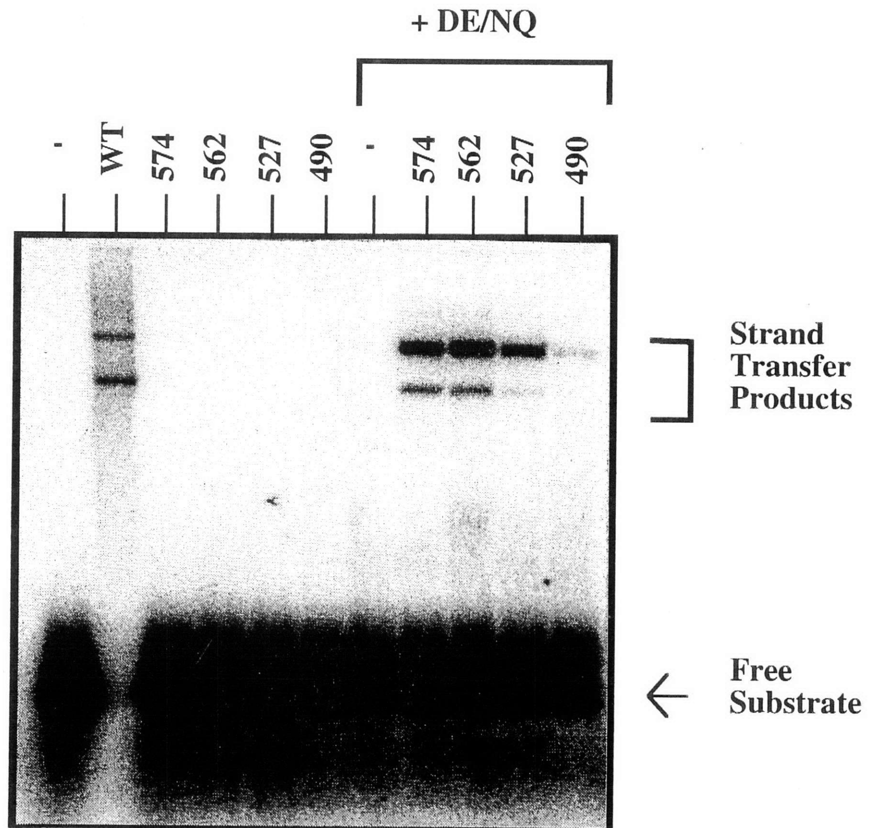


Loads CDC Markers

B

FIGURE 9. Strand Transfer Complex Formation Using Precleaved Oligonucleotides

DNA products of strand transfer reactions containing mixtures of MuA D269N/E392Q and either MuA1-574, MuA1-562, MuA1-527, or MuA1-490. Protein and DNA concentrations are as in Materials and Methods. The lane containing WT MuA alone was diluted 20 fold in order to reduce its signal to the range of the other reactions.


+ DE/NQ
-
WT
574
562
527
490
-
574
562
527
490
Strand
Transfer
Products
Free
Substrate

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