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The same two monomers within a MuA tetramer provide the DDE domains for the strand cleavage and strand transfer steps of transposition

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The chemistry of Mu transposition is executed within a tetrameric form of the Mu transposase (MuA protein). A triad of DDE (Asp, Asp35Glu motif) residues in the central domain of MuA (DDE domain) is essential for both the strand cleavage and strand transfer steps of transposition. Previous studies had suggested that complete Mu transposition requires all four subunits in the MuA tetramer to carry an active DDE domain. Using a mixture of MuA proteins with either wild-type or altered *att*-DNA binding specificities, we have now designed specific arrangements of MuA subunits carrying the DDE domain. From analysis of the abilities of oriented tetramers to carry out DNA cleavage and strand transfer from supercoiled DNA, a new picture of the disposition of DNA and protein partners during transposition has emerged. For DNA cleavage, two subunits of MuA located at *att*L1 and *att*R1 (sites that undergo cleavage) provide DDE residues *in trans*. The same two subunits contribute DDE residues for strand transfer, also *in trans*. Thus, only two active DDE$^+$ monomers within the tetramer carry out complete Mu transposition. We also show that when the *att*R1–R2 arrangement used on supercoiled substrates is tested for cleavage on linear substrates, alternative chemically competent DNA–protein associations are produced, wherein the functional DDE subunits are positioned at R2 rather than at R1.

Keywords: active site assembly/altered DNA-binding specificity/DDE motif/DNA transposition/Mu transposase

Introduction

Phage Mu transposase (MuA protein) in its tetrameric form promotes DNA cleavage and joining reactions of transposition (Figure 1; reviewed in Mizuuchi, 1992; Lavoie and Chaconas, 1995). While MuA can bind six *att* sites (L1–L3 at the left or *att*L end, and R1–R3 at the right or *att*R end) and a bipartite enhancer element (O1–O2) on Mu DNA, the MuA tetramer footprints on only three of the *att* sites (L1, R1 and R2; Figure 1A). Strand cleavage occurs at two specific phosphodiester bonds (adjacent to L1 and R1) on opposite DNA strands. The resulting 3′-OH groups are joined via transesterification to two phosphodiesters placed 5 bp apart on the two strands of a target DNA.

Several nucleoprotein complexes have been identified throughout the process of Mu transposition (reviewed in Chaconas *et al*., 1996). The first is the short-lived LER complex, wherein the left and right *att* ends interact with the enhancer (Watson and Chaconas, 1996). This complex converts to a stable type0 complex (in the presence of Ca^{2+}), the formation of which is the rate-determining step of the overall cleavage reaction (Wang *et al*., 1996). MuA assumes its active tetrameric configuration in this complex. Addition of Mg^{2+} to type0 promotes cleavage of Mu ends, giving rise to the typeI complex. Capture of target DNA can occur at several points along the reaction pathway, assisted by MuB protein and ATP (Naigamwalla and Chaconas, 1997), eventually giving rise to the strand-transferred typeII complex.

The MuA monomer (its normal form in solution; Kuo *et al*., 1991) is a complex protein with a modular organization (Nakayama *et al*., 1987). A variety of functions have been mapped to the different domains (Figure 1B). The N-terminal domain I contains the recognition motifs for two types of DNA sites; the enhancer and *att* sites (Leung *et al*., 1989; Clubb *et al*., 1994; Kim and Harshey, 1995; Clubb *et al*., 1997; Schumacher *et al*., 1997). Domain Iα binds the enhancer sites, while domain Iβγ binds *att* sites. The central domain II contains a triad of DDE residues present within IIα (the N-proximal subdomain), that are essential for the strand-cleavage and strand-transfer steps of transposition (Baker and Luo, 1994; Kim *et al*., 1995; Rice and Mizuuchi, 1995). (We shall henceforth refer to domain IIα as the DDE domain.) The corresponding residues in the integrase protein of ASV have been shown to co-ordinate metal ions (Bujacz *et al*., 1996, 1997). Subdomain IIβ has a large positive charge potential (Rice and Mizuuchi, 1995) and has been implicated in metal-assisted assembly of the MuA tetramer and in intramolecular DNA strand transfer (Namgoong *et al*., 1998a). The C-terminal domain (domain III) is also required for assembly (and probably the chemical competence) of the MuA tetramer, and for interactions with the accessory transposition factor, the MuB protein (see references cited in Yang *et al*., 1996). The two subdomains responsible for these two functions have been named IIIα and IIIβ, respectively. A 26-residue peptide in IIIα has been shown to possess non-specific DNA-binding and nuclease activity (Wu and Chaconas, 1995).

Previous studies have implicated all four MuA subunits in contributing DDE domains for the two cleavage and two strand-transfer reactions associated with complete Mu-transposition. For example, analyses of transposition products obtained using mixtures of wild-type and mutant (in DDE residues) MuA monomers on supercoiled Mu substrates were interpreted to suggest that each DDE$^+$ subunit promoted one cleavage or one joining event (Baker *et al*., 1994). Yang *et al*. (1995) observed that when a

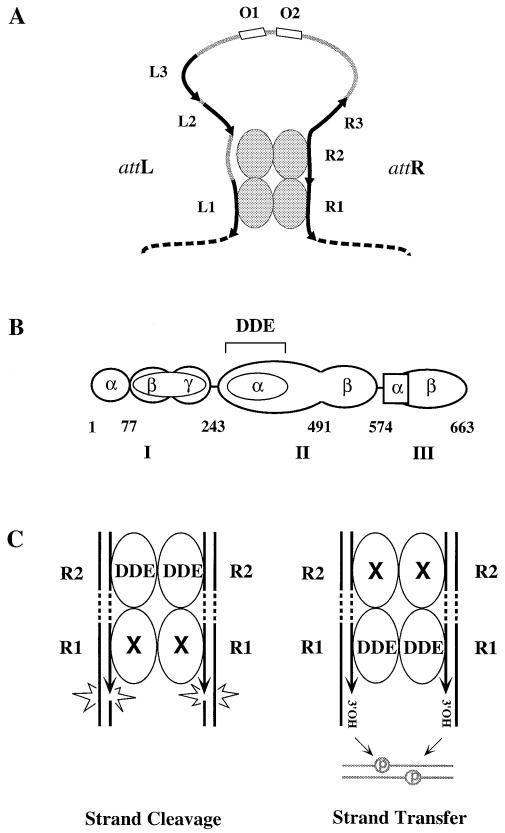

Fig. 1. (**A**) Orientation of *att*L(L1–L3), *att*R(R1–R3), and enhancer (O1–O2) sites on Mu DNA. The MuA tetramer footprints on only three *att* sites (L1, R1, R2). Non-Mu DNA is indicated by broken lines. (**B**) Domainal organization of MuA. On the basis of limited proteolysis, three domains (I–III) were assigned to MuA protein. Amino acid numbers corresponding to the amino terminus of each major subdomain (designated α, β or γ) are shown beneath the structure. DDE residues in domain IIα are required for catalysis. (**C**) Position of DDE domains during cleavage and strand transfer derived from complementation experiments performed using linear R1–R2 substrates (Yang *et al*., 1996). DDE^- subunits are indicated by X, and DDE^+ subunits by DDE. The subunits were loaded separately on R1 or R2 'subsites' that were brought together by complementary base-pairing (dotted lines). The 3′-OH groups (larger arrowheads) generated upon cleavage are shown attacking two phosphodiester bonds (P) within target DNA during strand transfer. See text for details.

tetramer is assembled from two MuA variants, one lacking a functional DDE domain and another lacking a functional assembly domain IIIα (which also harbors the non-specific nuclease activity; Wu and Chaconas, 1995), either strand cleavage within a substrate or strand transfer of a pre-cleaved substrate, but not strand cleavage plus strand transfer, could be performed. A model accomodating these results invoked that the MuA active site for strand cleavage or strand transfer is built by reciprocal sharing of structural/catalytic residues between the DDE and IIIα domains from separate MuA monomers (Yang *et al*., 1995). The donors of DDE (and recipients of IIIα) in the strand cleavage step act as donors of IIIα (and recipients of DDE) in the strand-transfer step. Thus, all four subunits were proposed to contribute DDE domains for complete transposition. The validity of these conclusions was tested under artificial reaction conditions (in the presence of 15% DMSO and 10% glycerol) that permit cleavage and strand transfer with linear R1–R2 substrates. These conditions bypass the normal requirement for a negatively supercoiled substrate, the enhancer element, as well as the *Escherichia coli* HU protein (Mizuuchi and Mizuuchi, 1989). These experiments showed that MuA monomers contributing DDE to the cleavage reaction occupy R2 (Yang *et al*., 1996), while those contributing DDE to the strand-transfer reaction occupy R1 (Aldaz *et al*., 1996; Yang *et al*., 1996; Figure 1C). Assuming that under these artificial conditions an R1–R2/R1–R2 synapse mimics a normal *att*L/*att*R synapse, the combined results were consistent with four DDE subunits promoting complete transposition. In related experimental protocols, the monomers contributing DDE were shown to act *in trans*, i.e. mediate cleavage and strand transfer in the partner *att* site rather than the *att* site with which they were associated (Aldaz *et al*., 1996; Savilahti and Mizuuchi, 1996).

In order to decipher protein–DNA partnerships on supercoiled DNA under normal reaction conditions, we have isolated a MuA variant with an altered *att* DNA binding specificity, whose properties are described in detail elsewhere (Namgoong *et al*., 1998b). By directing this variant to specific *att* sites on supercoiled DNA we have mapped the MuA monomers that contribute DDE domains for transposition. In contrast to previous conclusions (Yang *et al*., 1996), our results show that the same two DDE domains are responsible for the cleavage of Mu ends as well as their strand transfer. Furthermore, the DDE contribution occurs *in trans* on supercoiled substrates, as previously observed with linear substrates.

Results

The altered *att* site specificity mutant of MuA used in this work carries an Arg→Val substitution at residue 146 in the *att* DNA binding domain, Iβ (see Figure 1B). Compared with wild-type MuA, MuA(R146V) has a 60-fold lower affinity for a wild-type *att*R2 site ($K_d = 3.3\times10^{-8}$ M and 1.9×10^{-6} M for wild-type MuA and MuA(R146V), respectively; Namgoong *et al*., 1998b). *In vitro* selection for re-association of MuA(R146V) to mutagenized *att*R2 DNA resulted in isolation of an altered *att*R2 site carrying multiple changes in a 5 bp (S2) region overlapping the rightmost essential G residue (see Zou *et al*., 1991, and Materials and methods). MuA(R146V) binds this altered DNA with a K_d of 2.4×10^{-7} M, and shows equivalent affinities for all other *att* sites carrying the S2 alteration (Namgoong *et al*., 1998b). Wild-type MuA also binds the altered *att* S2 sites with affinities comparable with those of MuA(R146V), which are lower than those displayed by wild-type MuA for wild-type *att* sites (Namgoong *et al*., 1998b).

We have exploited the ability of MuA(R146V) to strongly discriminate between wild-type and *att* S2 sites to determine which MuA subunits within the transpososome (assembled on a supercoiled DNA substrate) contribute DDE domains for the chemical steps of transposition. We have focused on the MuA subunits placed on the 'core' *att* sites L1, R1 and R2. Footprinting analyses of the MuA tetramer assembled on negatively supercoiled DNA have shown that all three sites are occupied by MuA (Kuo *et al*., 1991; Lavoie *et al*., 1991; Mizuuchi *et al*., 1991) (see Figure 1A); no MuA-specific footprint has been obtained at any of the other *att* sites. Our assays were

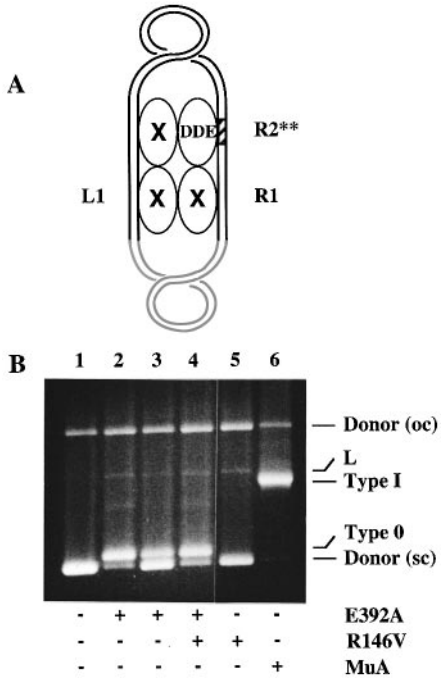

Fig. 2. DDE$^+$ subunit at *att*R2 does not promote Mu end cleavage. (**A**) Arrangement of mutant (hatched + double asterisk) and wild-type *att* sites on supercoiled pR2**. MuA (ovals) X = DDE$^-$ subunit; DDE = DDE$^+$ subunit. (**B**) Complementation between MuA(E392A) and MuA(R146V) for DNA cleavage (typeI formation) was monitored when suboptimal amounts (0.2 μg) of MuA(E392A) (lane 3) were mixed with equal amounts of MuA(R146V) (lanes 4). Lanes 1, 2, 5 and 6 are controls with no protein, MuA(E392A), MuA(R146V) and wild-type MuA (0.4 μg each), respectively. The positions of supercoiled (sc), open circular (oc) and linear (L) forms of the donor plasmid are indicated, as are those of type0 and typeI complexes.

based on the ability of two MuA variants, one DDE$^+$ and the other DDE$^-$, to form mixed tetramers that are functional in transposition. In each major set of experiments, a MuA tetramer was assembled in which a single DDE$^+$ monomer was directed to occupy L1, R1 or R2.

MuA subunit at attR2 does not provide DDE for DNA cleavage in a supercoiled substrate but does so in linear R1–R2 substrates

According to the current positional map (derived from transposition assays conducted on a pair of linear *att*R substrates), the DDE domains for cleavage are probably provided by MuA subunits at the R2 position (Figure 1C; Yang *et al*., 1996). To test this conclusion, we monitored Mu-end cleavage by placing a monomer of DDE$^+$ MuA(R146V) at the R2** site, and DDE$^-$ MuA(E392A) at all other sites on the supercoiled donor plasmid, pR2** (Figure 2A; ** indicates the presence of the S2 mutation at a given site. Note that although the plasmids used in this study contain all six *att* sites, for clarity, only those on which the tetramer footprints are indicated.) In all reactions containing a MuA(R146V) variant plus a second protein, the substrate was pre-incubated with the R146V-containing protein prior to the addition of its partner protein. In this way, we could ensure that the altered sites were selectively blocked from binding to the protein without the R146V substitution. The activity of MuA or its variants on pR2** is shown in Figure 2B. Wild-type MuA, which can bind to R2** as well as the other sites, converted essentially all of pR2** into the cleaved typeI complex under these assay conditions (lane 6). Similarly, the catalytically inactive variant MuA(E392A) yielded nearly 90% of the uncleaved type0 complex from this substrate (lane 2). MuA(R146V), which can bind R2** but not the other sites, was inactive on this substrate (lane 5). At a fixed, suboptimal amount of MuA(E392A) (corresponding to that in lane 3), the addition of an equivalent amount of MuA(R146V) (the sum of the two quantities of protein added up to the amount of MuA present in lane 6) caused efficient stimulation in type0 formation (nearly 90% conversion of the donor substrate) (lane 4). However, no typeI formation was detectable. These results demonstrate that MuA(R146V) placed at R2 cannot support cleavage at either the L1 or the R1 end. Western blot analysis with a truncated but functional DDE variant of MuA(R146V) was used to confirm that MuA(R146V) was incorporated into the type0 complex in the mixed reaction, exhibiting the expected stoichiometry of 1:3.00 ± 0.75 in favor of MuA(E392A) (see Materials and methods).

In an attempt to resolve the apparent contradiction between these results and those obtained earlier using 'subsites' R1 and R2, brought together by complementary base-pairing (Yang *et al*., 1996), we repeated the cleavage assay with a linear R1–R2** substrate, labeled at the 3′-end (Figure 3). Note that this substrate has the same configuration as the R1–R2** region of the *att*R DNA of pR2** (Figure 2A). While MuA cleaved this substrate readily (Figure 3B, lane 2), neither MuA(E392A) (lane 3) nor MuA(R146V) (lane 4) was capable of cleavage. These results agree with the cleavage results on pR2** obtained with these proteins (see Figure 2B). However, an equimolar mixture of MuA(E392A) and MuA(R146V) yielded strand cleavage (Figure 3B, lane 5). The level of cleavage was ~40% of that seen in the MuA reaction. This is in clear contradiction of the result of the mixed-protein reaction with pR2** (Figure 2B, lane 4). The reciprocal experiment with linear R1**–R2 did not give meaningful results due to the inherently low cleavage of the R1** site (as explained for pR1** in Figure 5; data not shown). Nevertheless, the lack of cleavage in R1–R2** with MuA(R146V) alone certifies that the cleavage obtained with the protein mixture could not have resulted from fortuitous association of MuA(R146V) with R1. Thus, in the unnatural situation of the linear substrate, the DDE domain of a MuA monomer bound to R2 was functional in the cleavage of the R1 site.

Based on the contrasting cleavage results obtained with the negatively supercoiled plasmid and the analogous linear substrates, we conclude that there is more than one type of MuA–MuA interaction that can yield a functional cleavage pocket (Figure 3A). Under artificial reaction conditions, and on artificial substrates assembled from linear DNA segments, an R2-bound MuA can act as the DDE donor in strand cleavage. However, within the structural restrictions of the native substrate, this mode of active site assembly is forbidden. As shown below, in the context of a supercoiled substrate, only R1 or L1 bound MuA can provide a DDE function for the cleavage reaction.

MuA subunits positioned at L1 and R1 contribute DDE domains for cleavage in trans

To test whether MuA placed at L1 could serve as a DDE donor in strand cleavage, the activity of MuA or its

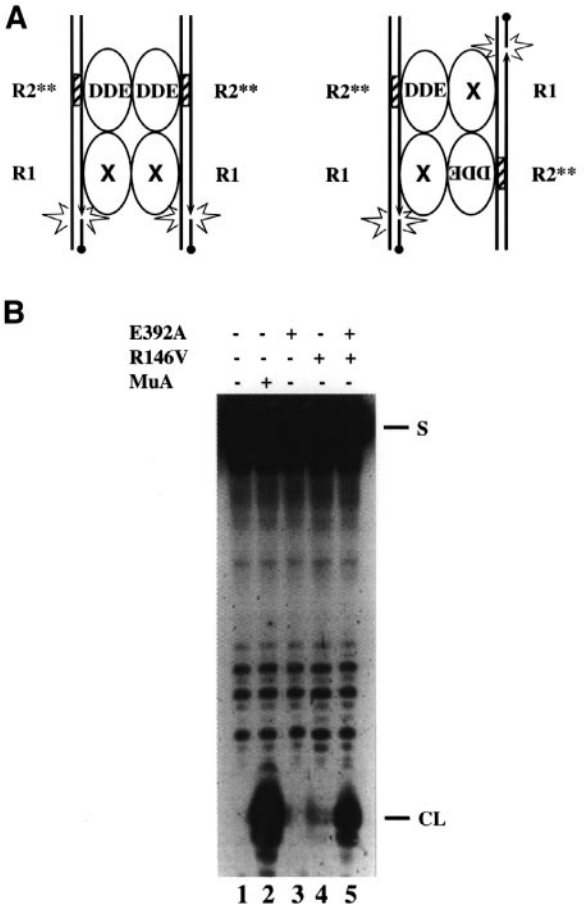

Fig. 3. DDE$^+$ subunit at *att*R2 promotes Mu end cleavage on a linear substrate. (**A**) Two possible arrangements of MuA on R1–R2 substrates synapsed in the parallel (left) or antiparallel (right) alignment. Other alignments (e.g. parallel but staggered) are also possible (not indicated). The labeled 3′-end is indicated by a filled circle. Arrowhead indicates 3′-OH. All other symbols as in Figure 2A. (**B**) 0.2 pmol of the substrate (S) (obtained by hybridizing two deoxyoligonucleotides that span the R1 and R2** sequences, and labeling the strand that undergoes cleavage with [α-^{32}P]cordycepin phosphate at the 3′ end) was incubated with 0.4 μg each of wild-type MuA, MuA(E392A), MuA(R146V), or a complementing mixture of MuA(E392A) and MuA(R146V) under DMSO reaction conditions (see Materials and methods). Reaction products were electrophoresed on a 12% denaturing acrylamide gel, and detected by autoradiography. The labeled product of strand breakage (CL) is 11 nucleotides in length.

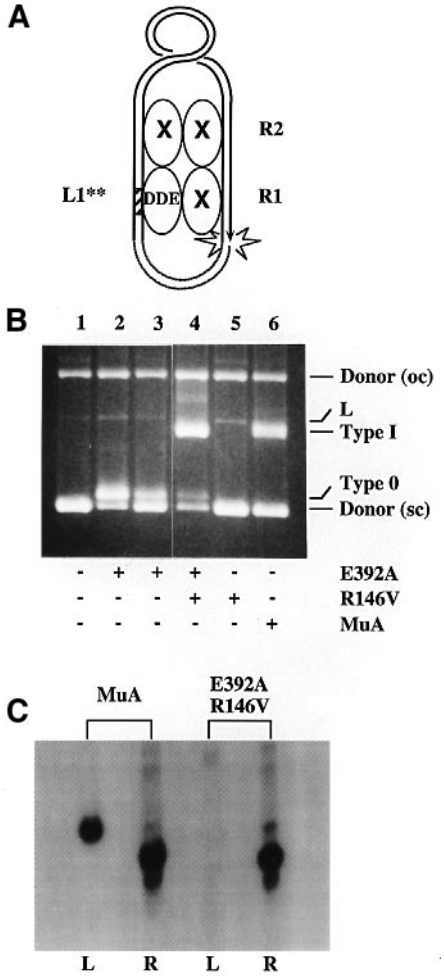

Fig. 4. DDE$^+$ subunit at *att*L1 promotes right end cleavage. (**A**) Arrangement of MuA subunits on pL1**. Symbols as in Figures 2 and 3A. (**B**) Complementation between MuA(E392A) and MuA(R146V) for DNA cleavage (typeI formation). Reactions were as in Figure 2B. (**C**) Determination of Mu end cleavage. TypeI complexes formed in lanes 6 (MuA) and 4 (E392A + R146V) in (B) were hybridized with radiolabeled primers designed to detect either left- or right-end cleavage (see Materials and methods). Chain-extension products were analyzed on a 6% denaturing polyacrylamide gel. L and R indicate left- and right-end-specific primer extension products, respectively. The radiolabeled R primer had a slightly higher specific activity in this experiment, accounting for the higher intensity of the primer extension product.

variants on pL1** (Figure 4A) was tested as shown in Figure 4B. Wild-type MuA (lane 6) yielded the cleaved typeI complex, and MuA(E392A) (the DDE$^-$ mutant) yielded the uncleaved type0 complex (lane 2), similar to the results obtained with pR2** (Figure 2B). TypeI yield in lane 6 and type0 yield in lane 2 were ~40% and 70% of the input substrate, respectively. These values are lower than those observed under similar reaction conditions for the pR2** substrate (see Figure 2B, lanes 6 and 2, respectively). This variation may reflect either differences in the binding affinities of MuA and MuA(E392A) for L1** and R2** sites, or positional effects of the altered sites. Consistent with its inability to bind normal *att* sites, MuA(R146V) yielded neither the type0 nor the typeI complex with pL1** (Figure 4B, lane 5). In a reaction in which pL1** was treated with an equimolar mixture of MuA(R146V) and MuA(E392A), efficient strand cleavage (typeI formation) was observed (lane 4). The amount of MuA(E392A) in the mixture was the same as in lane 3, and was sufficient to yield ~30–40% type0 complex by itself. The sum of the two protein amounts equalled the quantity of MuA present in lane 6.

From the above experiment, we conclude that a DDE$^+$ MuA monomer placed at L1, with DDE$^-$ partners placed at all other sites, can mediate strand cleavage. (The question of single- versus double-end cleavage is addressed below.) Western blot analysis of the cleaved complex (performed as described above for pR2**) showed a stoichiometry of 1:2.8 ± 0.23 in favor of MuA(E392A) (see Materials and methods), confirming that only a single DDE$^+$ monomer was present in the mixed complex.

Since there was only one good DDE donor in the MuA(R146V)/MuA(E392A) tetramer, we suspected that the typeI complex was produced under this condition by

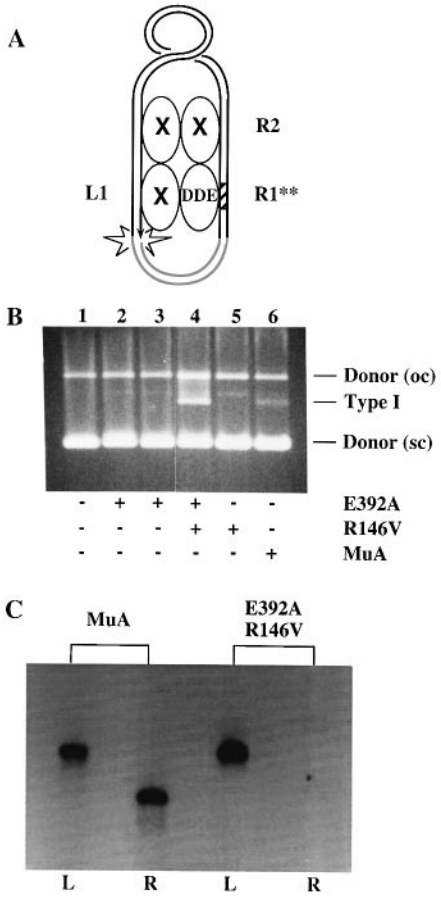

Fig. 5. DDE$^+$ subunit at *att*R1 promotes left end cleavage. (**A**) Arrangement of MuA subunits on pR1**. Symbols as in Figure 4A. (**B**) Complementation between MuA(E392A) and MuA(R146V) for DNA cleavage (typeI formation). Reactions were as in Figure 2B. (**C**) Determination of Mu end cleavage by primer extension. TypeI complexes formed in lanes 6 and 4 in (B) were analyzed as described in Figure 4C.

single-end cleavage. In order to determine which Mu end underwent cleavage in this arrangement, the typeI band was gel isolated and subjected to primer extension analysis using primers designed to monitor left (L) or right (R) end cleavage (see Materials and methods) (Figure 4C). While products indicative of both end cleavages were identified in the complex generated with wild-type MuA on pL1** (lanes L and R), only right-end cleavage was observed in the complex formed by a mixture of MuA(E392A) and MuA(R146V) (see the absence of product band in lane L). We conclude that the DDE domain from a MuA subunit placed on L1 is responsible for cleavage at the right end of Mu (R1).

We then introduced the S2 mutation at the R1 site in plasmid pR1** (Figure 5A). Type1 formation by wild-type MuA (Figure 5B, lane 6) and type0 formation by MuA(E392A) (Figure 5B, lane 2) from pR1** was extremely weak, although the binding affinity of wild-type MuA for R1** was similar to that determined for L1** or R2** sites (Namgoong *et al*., 1998b). Thus, the R1** site has a strong positional effect, causing an intrinsic drop in the reactivity of the plasmid. However, when an equimolar mixture (at the same molar ratio of plasmid to protein as in Figure 4B, lane 4) of MuA(E392A) and MuA(R146V) was reacted with pR1**, low but easily detectable levels of typeI were produced (Figure 5B, lane 4). Primer extension analysis of the typeI complex formed with the protein mixture, as well as that formed with wild-type MuA on pR1** (lane 6), are shown in Figure 5C. While both ends were cleaved in the reaction containing wild-type MuA, only left Mu ends were cleaved in the mixed protein reaction (Figure 5C, lanes L and R). Thus, the DDE domain from MuA located at R1 is responsible for cleavage at the left end of Mu (L1).

Results with plasmid substrates containing the S2 mutation at the accessory sites L2, L3 or R3 were similar to those obtained with pR2** (data not shown). In each case, stimulation of type0 formation was observed when MuA(R146V) was mixed with suboptimal concentrations of MuA(E392A) (see Figure 2B, lane 3 versus 4). No typeI was detected in any of these experiments. While these observations do not track the fourth (non-footprinting) monomer to any particular accessory *att* site (L2, L3 or R3), they demonstrate that cleavage of Mu ends is not promoted by MuA subunits from any site except L1 and R1 (Figures 4 and 5).

The arrangement of DDE domains is the same during DNA-cleavage and strand transfer

An important question is whether the cleavage-competent configurations, derived from the above experiments (Figures 4 and 5), were also capable of strand transfer.

Experiments shown in Figure 6 test strand transfer from plasmid pL1** when a single functional DDE subunit was placed on L1** (Figure 6A). Strand transfer reactions (Figure 6B) were similar to the cleavage reactions described under Figures 4 and 5, except they included target DNA, MuB protein and ATP. SDS was added to the reactions prior to electrophoresis, in order to dissociate DNA–protein complexes. Therefore, the typeI complex would be detected as the open circular plasmid in this assay [Donor(oc)]. While wild-type MuA generated double-ended strand transfer products that migrated as a series of distinct bands (reflecting the distribution of topoisomers retained in the Mu sequence of the donor plasmid) just above the open circular form of the target DNA (DEP; lane 6), a mixture of MuA(E392A) and MuA(R146V) generated products in which only one end was transferred to the target DNA (lane 4). The single-ended product (SEP) migrated distinctly above DEP (compare lanes 4 and 6; single-end integrations do not retain donor supercoils). Transfer of one end was expected, since only the right end was cleaved when the lone DDE donor was bound to the L1 site (Figure 4). PCR analysis confirmed that SEP contained junctions in which the right end was joined to the target DNA; similar analysis failed to reveal left-end joints in SEP (data not shown). Thus, the DDE domain from the MuA subunit at L1 is responsible not only for DNA cleavage at R1, but also for the subsequent transfer of the cleaved R1 end to the target DNA.

Note that the conversion of the cleaved complex into strand transfer products was less efficient in the reaction with the complementing MuA variant mixture relative to the reaction with MuA. This is evident when one compares the ratio of SEP to Donor(oc) in lane 4 with the DEP to Donor(oc) ratio in lane 6. Longer reaction times (>1 h) were needed to chase more of the cleaved DNA into SEP

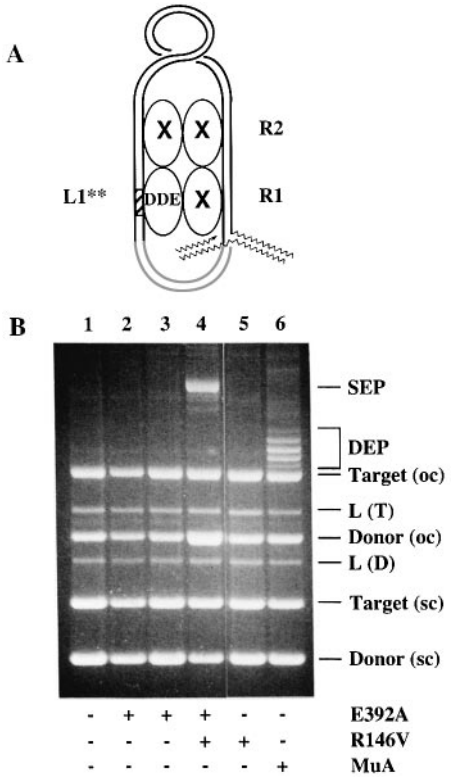

Fig. 6. DDE$^+$ subunit at *att*L1 promotes right-end cleavage and strand transfer. (**A**) Arrangement of MuA subunits on pL1**. Symbols as in Figure 4A; strand-transferred target DNA is indicated by wavy lines. (Note that the target DNA in this experiment is actually circular.) (**B**) Complementation between MuA(E392A) and MuA(R146V) for strand transfer. Reactions were as in Figure 4B, except that target DNA, MuB protein and ATP were included, and SDS was added prior to electrophoresis. Position of open circular (oc), supercoiled (sc) and linear (L) forms of the target (T) plasmid, as well as double-end (DEP) and single-end (SEP) strand-transfer products as indicated.

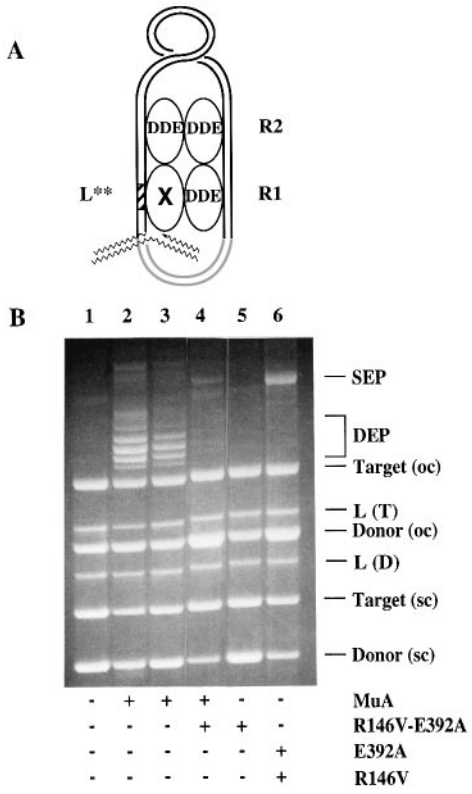

Fig. 7. Left-end cleavage and strand transfer. (**A**) Arrangement of MuA subunits on pL1**. Symbols as in Figure 6A. (**B**) Complementation between MuA(R146V, E392A) and wild-type MuA for strand transfer. Reactions were as in Figure 6B.

(data not shown). We do not believe that the placement of three MuA(E392A) subunits within the tetramer *per se* is responsible for the drop in the strand-transfer efficiency. For example, results with left-end strand transfer (see Figure 7) show that even when three wild-type monomers were present in the tetramer, single-ended strand transfer was inefficient. We believe that the asymmetry imposed on the typeI complex as a result of uncoordinated cleavages may impede strand transfer.

Assaying strand transfer of the left end from an arrangement containing MuA(R146V) at R1** and MuA(E392A) at all other sites using pR1** was not efficient, due to the inherent sluggish reactivity of the pR1** substrate. Given the low yield of typeI complex from this substrate (Figure 5B, lane 4) and the suboptimal conversion of singly cleaved typeI into strand-transfer products (Figure 6), the probability of obtaining detectable levels of SEP was low. Southern blots were needed to detect single-ended strand-transfer products in this reaction (data not shown). We therefore arranged the left-end cleavage configuration on the more efficient substrate pL1**, by placing the double variant MuA(R146V, E392A) at L1** and wild-type MuA at other sites (Figure 7A). This was accomplished (Figure 7B) by adding a fixed amount of MuA(R146V, E392A) sufficient to virtually saturate the L1** site in pL1**, followed by a suboptimal amount (equivalent to that in lane 3) of MuA (lane 4). As expected from the results shown in Figure 5, cleavage occurred at the left end, and was signified by an increase in quantity of the open circular form of pL1** [lane 4, Donor (oc)]. This result was confirmed by primer extension (results not shown). Low but detectable amounts of SEP were also formed in this reaction. The fraction of the cleaved complex that underwent strand transfer to give SEP was significantly smaller than that converted to SEP in the right-end reaction. This is illustrated by the reaction of pL1** in the presence of MuA(E392A) and MuA(R146V) that induces right-end cleavage (lane 6; analogous to the reaction shown in Figure 6B, lane 4). We have observed that typeI complexes cleaved only at the left end are unstable compared with those cleaved only at the right end (data not shown). This instablity may account for the very low frequency of their maturation into strand-transfer products. The small amount of double-ended strand transfers seen in the MuA–MuA(E392A, R146V) reaction (Figure 7B, lane 4) can be explained as resulting from a small fraction of complexes containing all four wild-type MuA monomers.

To show that two DDE$^+$ subunits at L1 and R1 can carry out efficient double-end cleavage and strand transfer, a pL1**R1** substrate would be desirable. However, the pR1** substrate by itself is poorly active (Figure 5), and introduction of more than one altered site results in significant reduction of transposition efficiency in all combinations of doubly mutant substrates tested (data not shown). An indirect experiment was therefore carried out to test if DDE subunits in the tetramer other than those

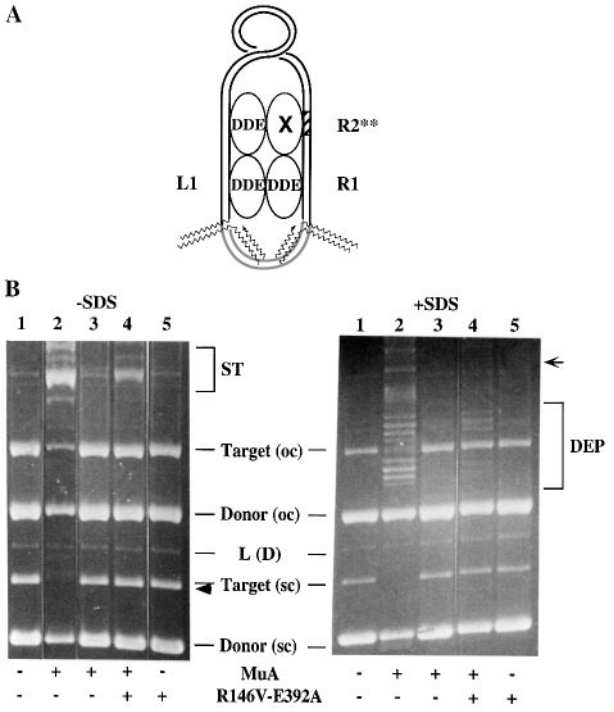

Fig. 8. DDE⁻ subunit at *att*R2 does not interfere with double-end strand transfer. (**A**) Arrangement of MuA subunits on pR2**. Symbols as in Figure 6A. (**B**) Complementation between MuA(R146V, E392A) and wild-type MuA for strand transfer, when suboptimal amounts (0.1 μg) of MuA (lane 3) were mixed with 0.3 μg of MuA(R146V, E392A) (lane 4). Lanes 1, 2 and 5 are controls with no protein, MuA and MuA(R146V, E392A) (0.4 μg each), respectively. Duplicate reactions were electrophoresed either without (–) or with (+) SDS. Arrows point to expected positions for migration of cleaved typeI (–SDS lanes) or SEP (+SDS lanes).

at L1 and R1 were normally utilized for strand transfer. If this were the case, a DDE⁻ subunit positioned at R2 (Figure 8A) would be expected to interfere with double-end strand transfer, and generate single-end strand transfer when paired with DDE+ subunits at all other sites. The results are shown in Figure 8B. (The reactions were electrophoresed either without or with SDS.) When pR2** was first incubated with a fixed amount of MuA(R146V, E392A) sufficient to saturate the R2** site, followed by a suboptimal amount (equivalent to that in lane 3) of MuA, a marked stimulation of strand transfer products was observed (lane 4; ST in –SDS and DEP in +SDS lanes). In contrast to the results from single-end cleavage/strand-transfer experiments shown in Figures 6 and 7 (lanes 4), there was no accumulation of cleaved products in this reaction (typeI complex in –SDS and Donor (oc) in +SDS), suggesting that all the cleaved products formed were converted into strand transfer products. Significantly, all the strand-transfer products were DEP (lane 4, +SDS; DEP and SEP cannot be distinguished under –SDS conditions), and no SEP products were detected. These results are consistent with those shown in Figures 2–7, and support the conclusion that the DDE domain of the R2 subunit does not directly participate in either the cleavage or the strand-transfer steps of transposition. Similar results were obtained when MuA(R146V, E392A) was placed at L2**, L3** or R3** (data not shown).

In summary (Figure 9), our results demonstrate that, in native negatively supercoiled substrates, MuA monomers

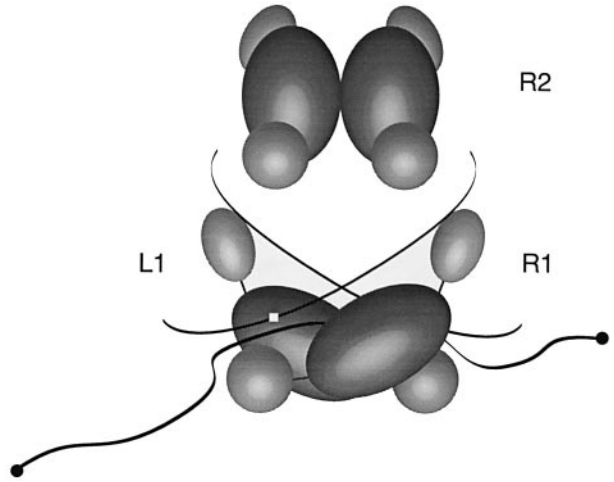

Fig. 9. A model depicting arrangement of DDE+ MuA subunits during transposition from supercoiled Mu DNA. Two DDE+ subunits (only major three domains indicated; see Figure 1B), bound through domain I to *att*L1 and *att*R1 and acting *in trans*, cleave and strand transfer (white square) Mu DNA ends within the MuA tetramer. The association of target DNA (tagged by filled circles) with the subunits at L1 and R1 is purely hypothetical. Specific structural/catalytic functions have not yet been assigned to the two other subunits in the tetramer.

positioned at L1 and R1 contribute their DDE domains for Mu DNA cleavage in the *trans* configuration. The same monomers then provide the DDE domains for the subsequent strand-transfer reaction (also *in trans*). We find that complexes cleaved only at one end are inefficient in strand transfer relative to those cleaved at both ends. In addition, type I complexes cut singly at the right end are more successful in strand transfer than those cut singly at the left end.

Discussion

Previous attempts to understand the catalytic contributions of individual MuA monomers within the functional tetramer had to rely on simplified linear substrates, an artificial R1–R2 to R1–R2 synapse presumed to mimic the normal *att*R–*att*L synapse, and altered reaction conditions (Aldaz *et al*., 1996; Savilahti and Mizuuchi, 1996; Yang *et al*., 1996). The combined results extrapolated to the native transposition system suggested that the DDE domains for strand cleavage as well as strand transfer are provided *in trans*, that is, left-end cleavage/transfer depends on the DDE domain of a MuA subunit bound to the right end, and vice versa (Aldaz *et al*., 1996; Savilahti and Mizuuchi, 1996). They also suggested that the strand transfer of the left end requires the DDE domain of MuA to be located at the R1 site and the IIIα domain of MuA located at the R2 site (Aldaz *et al*., 1996; Yang *et al*., 1996). A further implication was that the strand cleavage reaction utilizes the DDE domains not from the MuA monomers adjacent to the break points (R1 and L1), but from those positioned distal to them (Yang *et al*., 1996). Or, DDE contributions from two separate monomers are required for the cleavage and transfer of a single Mu-end. It should be clarified that the published experiments of Aldaz *et al*. (1996) addressed only the issue of DDE contributions during strand transfer of pre-cleaved ends, but not during strand cleavage.

A study of position-dependent catalytic roles of MuA monomers in native, negatively supercoiled substrates under standard reaction conditions, was made possible by the isolation of a variant of MuA, MuA(R146V), with altered *att* DNA specificity. MuA(R146V) binds poorly to each of the six binding sites within *att*L and *att*R, but will bind with significantly higher affinity to an altered site (Namgoong *et al*., 1998b). Analyses of the cleavage and strand-transfer reactions in oriented tetramers arranged on plasmids containing the altered site at specific positions (L1**, R1** or R2**) provides a new picture of the DDE contribution during the cleavage and joining steps of transposition. Our studies focus on these three sites because three of the four MuA subunits in the tetramer map to them. Experiments with plasmids containing the altered site at the three accessory positions (L2**, L3** or R3**) showed a stimulation of assembly when MuA(R146V) was mixed with suboptimal amounts of the DDE^- variant MuA(E392A). Western blot analysis confirmed that MuA(R146V) was incorporated into the complex in these experiments (data not shown). These results are consistent with genetic studies, where mutation of any one of these accessory sites still permitted tetramer formation (Lavoie *et al*., 1991). Although our experiments do not clarify whether the odd fourth monomer in the MuA tetramer is derived from a specific *att* site, neither this monomer, nor the one that footprints on the R2 site, contributes active DDE residues to the steps of cleavage and strand transfer. It needs to be emphasized that, since the structure of the *att* DNA–MuA complex is unknown, positioning of a MuA monomer within it can only be defined in terms of its cognizance for the normal binding sequence or the altered binding sequence. Since only a single binding site was altered in a given experiment, only one MuA variant was expected to be incorporated into the assembled mixed tetramer. Analysis of the protein composition within the tetramer provides supportive evidence for this assumption. It is extremely unlikely that the directed monomer of MuA rearranges during tetramer assembly. The outcomes from the multiple experiments utilizing single but separate altered sites cannot easily be accommodated by a common, internally consistent interpretation if the variant protein were to alter its initial site-association. Furthermore, the interpretation of our results tacitly assumes that neither the altered attachment site nor its cognate MuA variant has any effect upon the mechanism of strand cleavage and target joining reactions.

Three significant findings have resulted from the current analyses (summarized in Figure 9). In a supercoiled substrate, the DDE domains for strand cleavage are donated by MuA monomers located at L1 and R1. This contribution occurs *in trans*, from L1-associated MuA for cleavage of R1, and R1-associated MuA for cleavage of L1. The DDE domain utilized in end cleavage is also utilized in transferring that end to a target DNA. In a linear substrate however, the DDE domain from an R2-associated MuA is functional in the cleavage of an R1-end.

Two DDE^+ subunits within the MuA tetramer catalyze both cleavage and strand transfer of Mu DNA ends

A number of previous experiments, based on the ability of a DDE mutant of MuA to catalytically complement a IIIα domain mutant, suggested that the tetramer assembled from the mutant pair is capable of assembling either the active site for strand cleavage or the active site for strand transfer, but not both active sites simultaneously (Yang *et al*., 1995). The reciprocal domain-sharing model (Yang *et al*., 1995) accommodates this observation by the following proposals: (i) MuA active sites are built by sharing of DDE and IIIα domains from separate MuA monomers; (ii) the donor of the DDE domain during cleavage is different from the donor of the domain during strand transfer; and (iii) the IIIα domain functioning during strand cleavage is not the same as that functioning during strand transfer. *In vitro* assays performed on linear *att*R1–R2 or *att*R1–R2 reconstituted from R1 and R2 subsites were consistent with these proposals (Yang *et al*., 1996). However, results with supercoiled substrates (Figures 2–8) contradict proposal (ii); the cleavage event promoted by a MuA tetramer containing a single DDE donor can be channelled into strand transfer by the same complex. Note that there is an important difference between the present study and earlier complementation assays on which the domain-sharing model was founded. In the experiments reported here, the two MuA partners contain an intact IIIα domain whereas in the Yang *et al*. (1995) experiments, this domain was deleted from one partner. Thus, it is possible that the IIIα domain, and not the DDE domains, may be provided by separate MuA monomers for cleavage and transfer reactions [proposal (iii) of the Yang *et al*. model]. Alternatively, the same active site may carry out cleavage and target joining, but the transition from one mode to the other may require participation of monomers that do not directly contribute to the chemical steps. These issues are currently being tackled using the altered-specificity variant of MuA.

The conclusion that two DDE^+ subunits are sufficient for complete transposition is inconsistent with that of Baker *et al*. (1994). Using a supercoiled substrate and mixtures of MuA and MuA(E392Q) (both contain an intact IIIα domain), Baker *et al*. concluded that four DDE^+ subunits are required for complete transposition. They found that mixed tetramers generated products stalled at cleavage and products with only one end joined to target DNA. Increasing wild-type MuA in the mixture reduced the accumulation of cleaved products and promoted double-end strand transfer. To fit these results into our current model, we have to assume that the single-ended products were derived from complexes containing wild-type MuA at either L1 or R1. The increase in double-ended products with increasing MuA may be accommodated by the simultaneous occupancy of L1 and R1 by MuA. However, it is difficult to reconcile the Baker *et al*. observation that, even in the single-end strand-transfer complexes, the ratio of MuA to MuA(E392Q) was 2:2. In experiments patterned after those of Baker *et al*. (1994), Bolland and Kleckner (1996) have concluded that the same DDE domain is utilized during DNA-cleavage and strand-joining steps of Tn10 transposition. Our current results agree with a similar model for Mu transposition.

The distinct non-equivalence in the cleavage-transfer reactions at the left and right ends of Mu as seen in our assays merits comment. While the cleavage-transfer at R1 with the DDE donor placed at L1** was a fairly strong

reaction (Figure 6), the reaction at L1 with the DDE donor placed at R1** was much weaker (see Figure 7 also). This observation is most easily explained by an intrinsic positional effect of the altered site (Figure 5), and the inherent instability of the typeI nucleoprotein complex containing single left-end cleavages. Results showing production of only DEP when the subunit at R2 is DDE$^-$ (Figure 8) lend support to the idea that DDE$^+$ subunits at L1 and R1 carry out efficient strand transfer only when both ends are cleaved. The non-equivalence in the stability of singly cleaved substrates was also observed in the experiments of Surette *et al*. (1991), where substrates carrying a mutation at either the left or the right end underwent cleavage at the wild-type end in the presence of MuA and MuB proteins; complexes cleaved at the left end were far more unstable than those cleaved at the right end. We have extended the observations of Surette *et al*. (1991) to show that in these mutant substrates, strand transfer is observed only in complexes cleaved at the right end, and not in those cleaved at the left end, consistent with the behavior of singly cleaved substrates reported here (unpublished results).

Influence of DNA topology on the configuration of functional DNA–protein associations

One striking finding from this study (which is consistent with the results of Yang *et al*., 1996) is the distinct modes of DDE contribution by MuA during strand cleavage in topologically distinct substrates. This may be a consequence of shared active sites in MuA, which might permit more than one catalytic configuration within a given oligomeric state of the protein. In the context of the tetramer, a given MuA monomer could potentially share its DDE domain with the IIIα domain from any one of the other three monomers. The structural constraints imposed on the native transpososome by DNA topology and by multiple DNA–DNA, DNA–protein and protein–protein interactions may eliminate all but one unique mode of active-site assembly during the cleavage step or the strand transfer step. When these constraints are at least partially removed by altering substrate topology, the protein composition and solvent conditions, normally disallowed modes of DNA–DNA and protein–DNA associations may become manifest. The cleavage of an R1-end in a linear substrate by donation of DDE from an R2-bound MuA (Yang *et al*., 1996; this study) can be explained if an R1 to R2 association that mimics the R1 to L1 association during a normal reaction is possible in this artificial reaction.

It is appropriate to compare the action of MuA on linear substrates with the action of the Flp site-specific recombinase on analogous artificial substrates. Like MuA, the Flp protein binds to DNA as a monomer and establishes a dimer or a tetramer only in the DNA-bound state. Flp utilizes shared active sites to mediate strand breakage and strand transfer between two DNA molecules (Chen *et al*., 1992). When the normal Flp target site is split into two half-sites (a left and a right half-site), the enzyme loses its left-to-right orientation, and brings together two left or two right half-sites in a complex competent in strand breakage and exchange (Serre *et al*., 1992). The proposed R2 to R1 synapse in reactions containing linear R1–R2 or R1, R2 subsites (Figure 3; Yang *et al*., 1996) represents a very similar situation. Furthermore, Flp can build two active sites by sharing catalytic residues among three monomers or from four monomers (Qian and Cox, 1995; Lee *et al*., 1996, 1997). Selectivity in the assembly and orientation of active sites as dictated by the topology of the substrate or the stacking freedom of DNA arms may be widespread among recombinases and transposases that act via shared active sites or require specific inter-subunit interactions to attain catalytic competence. In this context, it is relevant to note that two of the Integrase family recombinases, λInt and P1Cre, pose a paradox as to whether they utilize the catalytic tyrosine *in cis* or *in trans* to mediate strand breakage. While a number of reaction conditions reveal *cis* DNA-cleavage by Int (Nunes-Duby *et al*., 1994), at least one set of assays is suggestive of *trans* cleavage (Han *et al*., 1994). Similarly, while the crystal structure of a cleaved DNA–Cre complex reveals DNA cleavage *in cis* (Guo *et al*., 1997), reactions in solution are consistent with *trans* DNA cleavage (Shaikh and Sadowski, 1997).

We wish to emphasize that besides differences in topology, the arrangement of Mu *att* sites is also different in supercoiled and linear substrates (*att*L/*att*R versus R1–R2/R1–R2), respectively. In fact, only *att*R/*att*R configurations are transposition competent on linear substrates, whether or not the Mu ends are pre-cleaved; *att*L/*att*R or *att*L/*att*L configurations are not (Craigie and Mizuuchi 1987; Namgoong *et al*., 1994). Thus, results with linear substrates, while providing important mechanistic information, may not faithfully represent the catalytic configuration of the transposition complex on native supercoiled substrates.

Concluding thoughts

Site-specific recombinases that mediate strand exchange between two double-helical DNA partners generally utilize a tetramer as the functional entity. Members of the integrase and resolvase/invertase families follow this paradigm (Craig, 1988; Landy, 1993; Sadowski, 1993). Whereas a site-specific recombinase tetramer mediates four strand breakage-joining reactions via transesterification, the transposase tetramer (MuA in this study) mediates only two strand breakage-joining reactions via hydrolysis followed by transesterification. Thus, in principle, two active sites would be sufficient for the transposition reaction. The results obtained in this study are most easily accommodated by two DDE domains of MuA monomers (those bound at the L1 and R1 sites) being responsible for the chemical steps of transposition. However, the active transposase unit is still a tetramer, the other two monomers probably contributing either chemically, structurally or allosterically to the catalytic competence of the tetramer. Biochemical evidence in the case of the Flp recombinase, and structural evidence in the case of the Cre recombinase, strongly suggest that all four enzyme monomers participate at each stage of the two-step reaction that is completed via two rounds of pairwise single-strand exchanges (Guo *et al*., 1997; Lee *et al*., 1997). Two of the four Cre monomers are catalytically self-sufficient to execute the cleavage and exchange of one pair of strands, but can do so only in the context of the tetramer. The Flp protein provides a more striking example of catalytic cooperation, the assembly of two active sites (for breaking

and exchanging one pair of strands) requiring catalytic contributions from all four Flp monomers. While our studies on Mu transposition have revealed how two MuA subunits provide the DDE domains for the reaction, the role of the other two subunits in organizing the functional tetramer remains to be explored. Since MuA exists in solution as a monomer, binds to DNA as a monomer, but promotes transposition only within the tetramer, the system can, in principle, postpone strand breakage until the fully functional DNA–protein complex has been assembled by sharing catalytic/structural residues from more than one protein subunit. Furthermore, the action of DDE *in trans* can serve to co-ordinate cleavages at the left and right ends of the Mu DNA. Our finding that a cleaved single end is only poorly strand transferred to the target DNA suggests the operation of an additional checkpoint that prevents a rare uncoordinated cleavage event from giving rise to a dead-end transposition product.

Materials and methods

DNA substrates and proteins

Plasmid pMK21 (Kim *et al*., 1995) was used for replacement of individual *att* sites with the S2 mutation, using PCR mutagenesis. The sequence of one strand of the altered sites (change from wild-type indicated in bold) is as follows: L1, 5′-TGTATTGATTCACTTGA-A**CCCCC**AAAAAAA; R1, 5′-TGAAGCGGCGCACGAAAA**CCCC-C**AAAGCGT; R2, 5′-GAAAGCGTTTCACGATAA**CCCCC**AAAA-CTT; R3, 5′-ACATCTGTTTCATTTGAA**CCCCC**AAAGCTA; L2, 5′-TAGTCGTTAATCAATGAA**CCCCC**AAAGATA; L3, 5′-GCTTTGT-TTCATTGAAA**CCCCC**AAAAACA. The resultant plasmids (indicated in the text by the sites altered) are as follows: pMK51 (pR1**); pMK52 (pR2**); pMK53 (pL1**); pMK54 (pL2**); pMK55 (pL3**); and pMK63 (pR3**). Target DNA used in strand transfer assays was a dimeric form of pUC19.

The linear *att*R subtrate with the S2 mutation at R2 was assembled by annealing the following two deoxyoligonucleotides: 5′-GATCACT-CATTGAAGCGGCGCACGAAAAACGCGAAAGCGTTTCACGATA-A**CCCCC**AAAACTT (top strand); and 5′-AAGTTTT**GGGGG**TT-ATCGTGAAACGCTTTCGCGTTTTTCGTGCGCCGCTTCAATGAG-TGATC (bottom strand). The underlined sequences are the flanking residues outside the Mu *att* site. The bold sequences indicate the change from wild-type.

The construction of MuA(R146V) is described elsewhere (Namgoong *et al*., 1998b). This mutation was moved into MuA(E392A) and MuA(Δ1-62) by an appropriate exchange of restriction fragments. Wild-type and mutant forms of MuA, as well as MuB and HU proteins were purified as described in Yang *et al*. (1995).

Mu DNA cleavage and strand transfer

TypeI cleavage reactions were carried out in 20 μl reaction mixtures containing 25 mM Tris–HCl (pH 7.5), 130 mM NaCl, 10 mM $MgCl_2$, 2 μg of donor supercoiled DNA, 0.4 μg of MuA or its variants (unless otherwise indicated) and 0.2 μg HU, for 20 min at 30°C (Surette *et al*., 1987). When using mixtures of complementing proteins, the donor DNA was pre-incubated in buffer with MuA(R146V) for 5 min, before addition of other components. Reactions were electrophoresed on 1% agarose gels, and the DNA bands visualized by ethidium bromide staining and UV illumination.

TypeII strand transfer reactions were similar to typeI except that 2 μg of target DNA, 0.2 μg of MuB protein, and 2 mM ATP were included. SDS (0.1% final concentration) was added to the reactions before electrophoresis.

DMSO assay conditions for cleavage of linear *att*R substrates were as described by Yang *et al*., 1996.

Primer extension analysis

The typeI complex band was excised from ethidium bromide-stained agarose gels, immersed in 400 μl of TAE buffer [40 mM Tris–acetate, 1 mM EDTA (pH 8)] in a Spectro dialysis membrane (MW cutoff 6000–8000), and electroeluted for 30 min at 60 V. DNA was precipitated with 100% ethanol after addition of 0.3 M (final) NaOAc, washed with 80% ethanol and re-dissolved in 15 μl of water. The DNA was denatured by addition of 4 μl of 1 M NaOH and 1 μl of 10 mM EDTA at 37°C for 10 min, re-precipitated as described above and re-suspended in 5 μl of water. Primer extension was carried out by first annealing [γ-^{32}P]ATP-labeled primers (LT and RB; see Wang *et al*., 1996) to the isolated DNA at 65°C for 2 min in Sequenase reaction buffer (supplied by the USB). Annealing mixtures were cooled slowly to room temperature, and the primers extended by incubation for 2 min at room temperature followed by 5 min at 37°C, after addition of 2 μl each of 1 mM dNTP, 100 mM DTT and Sequenase (diluted 8-fold). Reactions were terminated by addition of 8 μl of 'Sequenase Stop' solution, and electrophoresed on 6% denaturing polyacrylamide gels.

Determination of stoichiometry of MuA mutants in mixed tetramers

Type0 and typeI reactions identical to those described in Figures 2 and 4 (lanes 4) were carried out, with the exception that MuA(Δ1-62, R146V), a truncated variant of MuA(R146V), was used. This variant is functional for cleavage when incorporated into mixed tetramers with MuA(E392A) (Yang *et al*., 1995). The complexes were treated with heparin to remove all loosely bound MuA, and subjected to Western blot analysis as described by Yang *et al*. (1995). Protein amounts from three separate experiments were quantified, and the ratio of MuA(Δ1-62, R146V) to MuA(E392A) was estimated by normalizing the values of the former to one.

Quantification

DNA and protein band intensities were quantified using a Bio-Rad video densitometer (GS-700).

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