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A domain sharing model for active site assembly within the Mu A tetramer during transposition: the enhancer may specify domain contributions

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The functional configuration of Mu transposase (A protein) is its tetrameric form. We present here a model for the organization of a functional Mu A tetramer. Within the tetramer, assembly of each of the two active sites for Mu end cleavage requires amino acid contributions from the central and C-terminal domains (domains II and III respectively) of at least two Mu A monomers in a *trans* configuration. The Mu enhancer is likely to function in this assembly process by specifying the two monomers that provide their C-terminal domains for strand cleavage. The Mu B protein is not required in this step. Each of the two active sites for the strand transfer reaction is also organized by domain sharing (but in the reverse mode) between Mu A monomers; i.e. a donor of domain II (also the recipient of domain III) during cleavage is a recipient of domain II (and the donor of domain III) during strand transfer. The function of the Mu B protein (which is required at the strand transfer step) and that of the enhancer element may be analogous in that their interactions with Mu A (domain III and domain Iα respectively) promote conformations of Mu A conducive to strand cleavage or strand transfer.

Key words: active site assembly/DNA transposition/enhancer/phage Mu/transposase

Introduction

Transposition of Mu occurs by a series of precisely regulated events wherein the left and right ends of Mu DNA (*att*L and *att*R) undergo cleavage, followed by joining to target DNA. Assembly of the active tetrameric configuration of the Mu transposase (A protein; Lavoie *et al.*, 1991) is a complex process requiring a negatively supercoiled DNA substrate, a set of six *att* subsites, a pair of internal enhancer sites, the *Escherichia coli* HU protein and divalent metal ions (reviewed in Mizuuchi, 1992; Lavoie and Chaconas, 1993; Wang and Harshey, 1994). Cleavage of Mu ends within this higher order DNA–protein assembly gives rise to the stable 'type I complex' (Figure 1). The Mu B protein then facilitates joining of the cleaved ends to target DNA to generate the strand-transferred 'type II complex' (Surette *et al.*, 1987).

The Mu A protein is organized into three principal domains (Figure 2; Nakayama *et al.*, 1987). Structure–function studies have mapped *att* and enhancer DNA binding activities to two separate regions in the N-terminal domain I (Nakayama *et al.*, 1987; Leung *et al.*, 1989). The central, proteolytically stable domain II is inferred to be the catalytic domain, since mutations that perturb or block various steps of catalysis map here (Leung and Harshey, 1991; Baker and Luo, 1994; Kim *et al.*, 1995). Potential active site residues include acidic amino acids that might be part of a motif analogous to the D-D-35-E found in several other transposases and retroviral integrases (Fayet *et al.*, 1990; Kulkosky *et al.*, 1992; Radstrom *et al.*, 1994). Domain II also shows non-specific (target?) DNA binding activity (Nakayama *et al.*, 1987). The catalytic region must extend into part of the C-terminal domain (IIIα), as determined by the activity of deletions that are localized here (Harshey and Cuneo, 1986; Bremer *et al.*, 1988; Betermier *et al.*, 1989; Leung and Harshey, 1991; Baker *et al.*, 1993). Domain IIIβ is required for interaction with the accessory transposition protein Mu B, which promotes strand transfer to target DNA (Harshey and Cuneo, 1986; Baker *et al.*, 1991; Leung and Harshey, 1991).

The transposase protein of the Mu-related phage D108 (D108 A) is nearly identical to the Mu A protein (see DuBow, 1987). The two proteins differ functionally only within the enhancer binding domain Iα (Gill *et al.*, 1981; Toussaint *et al.*, 1983; Harshey *et al.*, 1985; Mizuuchi *et al.*, 1986; Leung *et al.*, 1989). This domain consists of the first ~80 N-terminal residues of the two transposases. When provided with the cognate enhancer sequence *in cis* with respect to the *att* sites, Mu A and D108 A can functionally substitute for each other (Toussaint *et al.*, 1983; J.-Y.Yang and R.M.Harshey, unpublished data).

In this study we addressed the role of the enhancer element and of the Mu B protein in promoting the strand breakage and strand transfer reactions respectively. We wished to know how, within the transposase tetramer, individual Mu A monomers contribute to the chemistry of transposition. We tested combinations of wild-type Mu A, D108 A and their variants containing deletions of specific domains or alterations of specific amino acid residues in complementation experiments *in vitro* using suitably designed DNA substrates. Our results are consistent with a model in which the active site for one strand cleavage or one strand transfer event is assembled by primary contributions from at least two separate transposase monomers within the protein tetramer. The enhancer directs building of the strand cleavage pockets by specifying the role of two Mu A monomers within the tetramer. The role of Mu B protein in the strand transfer reaction is less clear. It may actively facilitate the reaction by functioning as an allosteric effector of the strand transfer pockets or it may play a role in presenting the target phosphodiester to a pre-assembled active site in the proper orientation for strand transfer. The two possibilities are not mutually exclusive.

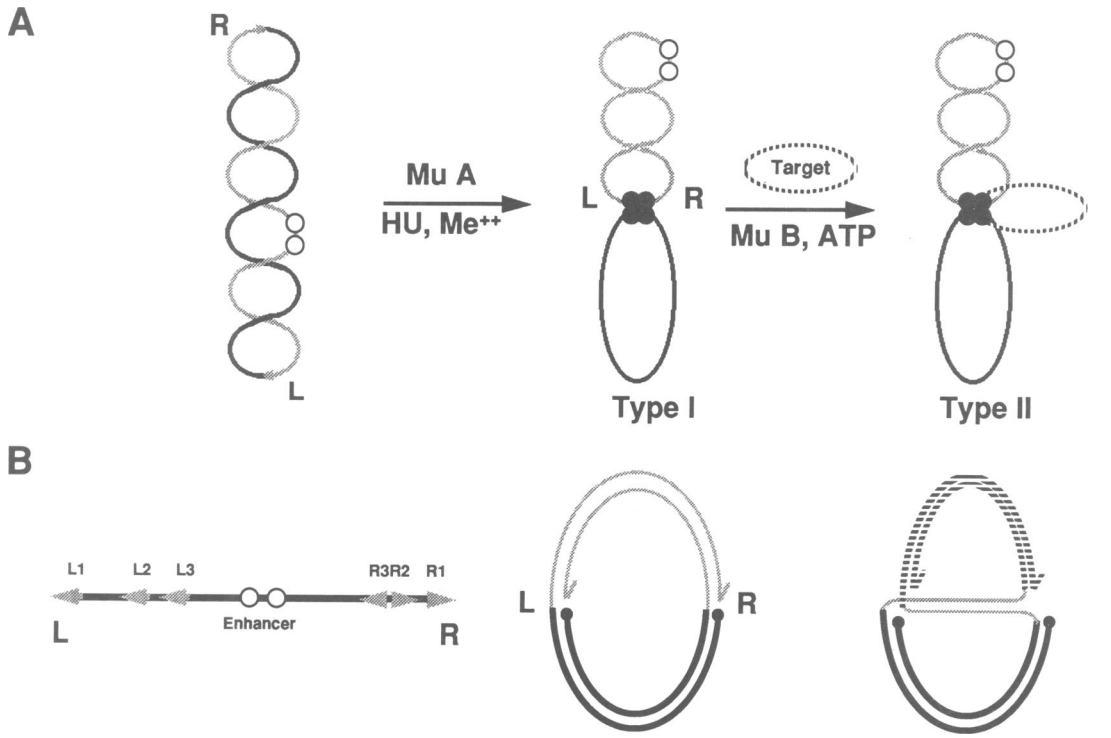

Fig. 1. Mu end cleavage and strand transfer. (**A**) Mu ends (L and R) on a negatively supercoiled plasmid (left) are brought together (synapsed) by Mu A protein [in the presence of HU protein and divalent metal ions (Me^{2+})] and cleaved (one single strand nick at each end) to generate a stable nucleoprotein complex (type I; Mu DNA is supercoiled, non-Mu or vector DNA is relaxed) wherein Mu A has tetramerized. This process requires the presence of the Mu enhancer (shown as a pair of open circles in the Mu genome), normally *in cis* with respect to the Mu ends. Cleaved Mu ends in the type I complex are joined or strand transferred to target DNA, in a reaction requiring Mu B protein and ATP (type II nucleoprotein complex). Not shown in this scheme is the intermediate complex (type 0, prior to type I) which can be trapped in the presence of Ca^{2+}, wherein Mu A has tetramerized but the Mu ends are not cleaved (Mizuuchi *et al.*, 1992). (**B**) The Mu A binding sites (three at each end, L1–L3 and R1–R3) and their relative orientations are indicated by arrowheads. The configurations of the cleaved and strand transferred ends are diagrammed below the type I and type II complexes. 5′ Phosphate groups are denoted by circular knobs and 3′ hydroxyl groups are represented by split arrowheads. See text for references and other details.

Results

Mixed tetramers containing Mu A and Mu A(E392A) can mediate strand cleavage at Mu ends

Recent studies have implicated Glu392 within Mu A as a potential catalytic residue (Baker and Luo, 1994; Kim *et al.*, 1995). For example, Mu A(E392A) can assemble the Mu A tetramer and mediate synapsis of Mu ends, but cannot support strand cleavage or strand transfer under normal reaction conditions in the presence of Mg^{2+} (Kim *et al.*, 1995). It has been suggested that Glu392 may either be an active site residue or contribute significantly to organization of the reaction pocket.

We found that Mu A(E392A) can be incorporated into cleavage-competent mixed tetramers with wild-type Mu A (K.Kim and R.M.Harshey, unpublished). When Mu A(E392A) was mixed with a suboptimal amount of Mu A, there was distinct stimulation of the formation of the type I complex containing cleaved Mu ends. However, in reactions in which Mu A(E392A) was mixed with non-limiting amounts of Mu A, the yield of type I complex was inhibited. The presence of Mu A(E392A) in the type I complex could be demonstrated when the reaction included radioactively labeled protein (containing [^{35}S]-methionine). A subset of the mixed tetramers into which the mutant version of Mu A has been incorporated is therefore capable of strand cleavage. Furthermore, yields of the type I complexes (in which both Mu ends were nicked) at different ratios of the two proteins would be consistent with occupancy, within the active tetramer, of two specific positions by the wild-type protein. The experimental data summarized above are not displayed here, since the results of analogous mixing experiments have recently been published by Baker *et al.* (1994). Based on the yields of the different types of strand cleavage and strand transfer products from the mixed protein reactions, they have proposed a model for the normal wild-type reaction. According to their model, a pair of Mu A monomers cooperate to perform the two strand cleavage events and then a pair of monomers cooperate to perform the two strand transfers. Our analyses of the strand cleavage and strand transfer reactions using combinations of Mu A and Mu A variants (see below) lead to a different model for DNA transposition.

Only two monomers of Mu A need to interact with the enhancer to assemble the functional tetramer

As pointed out earlier, the only functional distinction between the Mu A and D108 A proteins is in their individual binding specificities for the respective enhancer elements. Enhancer recognition is mediated by the protein domain Iα (see Figure 2). Does every Mu A subunit within the tetrameric protein assembly have to harbor the enhancer binding domain for it be proficient in strand

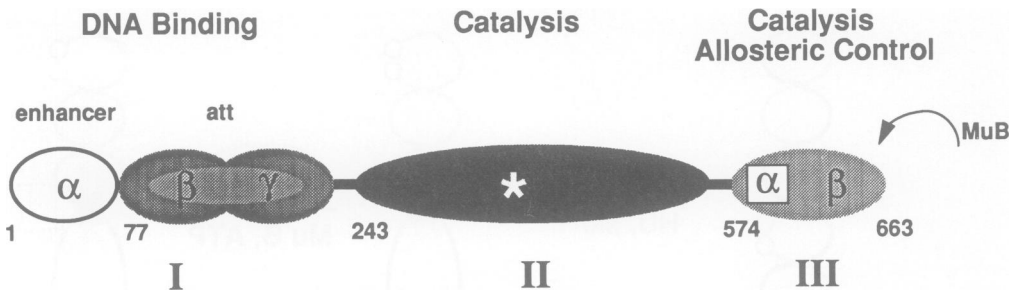

Fig. 2. Domain structure of Mu A protein. On the basis of limited proteolysis, three domains were assigned to the Mu A protein. Amino acid numbers corresponding to the N-terminus of each major domain are shown beneath the structure. The N-terminal domain I contains subdomains α, β and γ, which encode site-specific DNA binding activity. Domain Iα binds to Mu enhancer and Iβγ binds to Mu *att* sites. Glu392 (*) is one of several potential catalytic residues that map to domain II, alterations of which affect both cleavage and strand transfer. The proximal third of domain III (α) is also required for catalysis, while the distal two thirds of domain III (β) are responsible for interactions with the Mu B protein. See text for references and other details.

cleavage? We addressed this question by exploiting the differential enhancer specificities of Mu A and D108 A, together with the cleavage competence of mixed tetramers containing Mu A and Mu A(E392A) monomers (see previous section).

The results of *in vitro* complementation analyses with four pairwise transposase combinations are displayed in Figure 3. They are also summarized for clarity and quick reference in Table I (along with salient data not shown in Figure 3). The substrate used in Figure 3A and B contained the Mu enhancer element. Mu A(E392A), D108 A or Mu A(Δ1–62) (lacking three quarters of the enhancer binding domain) were individually inactive in mediating strand cleavage within this substrate and failed to generate the type I complex (lanes 1 and 7, Figure 3A and B). This result demonstrates that the reaction cannot proceed when Glu392 is intact but enhancer recognition is not possible or when enhancer recognition is normal but Glu392 is lacking. Contributions of the two components were then assessed by using the appropriate pairs of proteins in the reaction. When Mu A(E392A) was mixed with either D108A (Figure 3A) or with Mu A(Δ1–62) (Figure 3B), the mixed protein pairs yielded the type I complex (lanes 2–6, Figure 3A and B). No type I complex was obtained upon mixing D108 A with Mu A(Δ1–62), further substantiating the indispensability of enhancer recognition for cleavage of Mu ends (data not shown). However, the complexes produced by the protein pairs were unable to convert the type I to a type II complex via strand transfer to target DNA when Mu B protein and ATP were present in the reaction (data not shown; see also results in Figure 7). Hence, we infer that these type I complexes do not harbor the active sites required for strand transfer.

The simplest interpretation of the above results is that the active sites required for the two strand cleavage events at the ends of Mu derive the Glu392 residues from transposase monomers that are not obligated to interact with the enhancer element. This conclusion is corroborated by the results shown in Figure 3C and D. Here the substrate contained the D108 enhancer rather than the Mu enhancer. Now Mu A or Mu A(Δ1–62) could be paired with D108 A(E392A) to yield the type I complex (lanes 2–6, Figure 3C and D). In contrast, each of the three proteins by itself failed to yield the strand cleavage product (lanes 1 and 7, Figure 3C and D). Furthermore, a Mu A variant in which the N-terminal 30 residues were replaced by the corresponding segment from D108 A and which had lost the ability to recognize the Mu or the D108 enhancer [Mu A(D30M)] could be paired with Mu A(E392A) to produce strand cleavage only when the enhancer was Mu derived and with D108 A(E392A) only when the enhancer was D108 derived (data not shown).

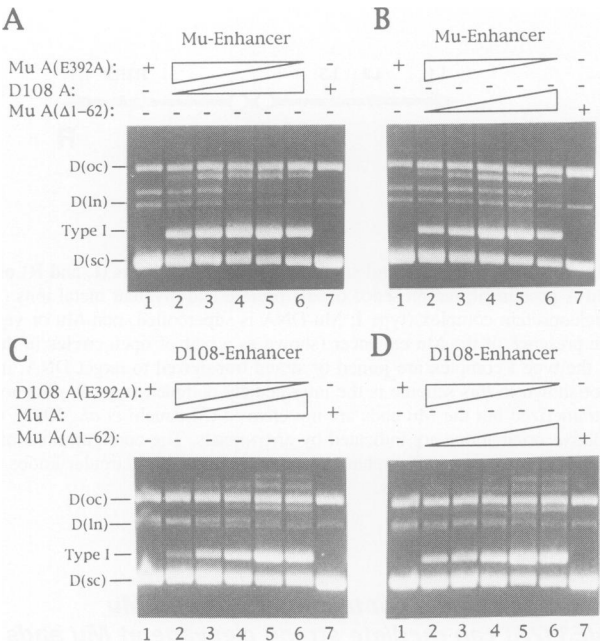

Fig. 3. Complementation between Mu A protein mutant pairs defective in catalysis and in enhancer recognition. (**A** and **B**) Mu A(E392A) was mixed with either D108A (A) or Mu A(Δ1–62) (B) and assayed for type I formation (cleavage) on mini-Mu plasmid pRA170 (containing the Mu enhancer). Lanes 1 and 7 are controls with only one of the proteins added as indicated. The molar ratios of Mu A(E392A) to its partner protein in lanes 2–6 were: 3:1, 2:1, 2:2, 1:2 and 1:3. The final Mu A amount was ~4 pmol in all lanes. Supercoiled (sc), open circular (oc) and cleaved type I forms of the donor (D) mini-Mu plasmid are indicated. A small amount of the linear form present in the donor plasmid is marked ln. The bands not labeled in the figure have not been characterized. (**C** and **D**) Reactions as in (A and B) except that D108 A(E392A) was mixed with either Mu A (C) or Mu A(Δ1–62) (D) and assayed for type I formation on mini-Mu plasmid pJY170 (containing the D108 enhancer). The uncleaved type 0 complexes formed with Mu A(E392A) alone did not resolve from unreacted (sc) plasmid substrate, due to the larger size of the pRA170 (A and B) and pJY170 (C and D) plasmids.

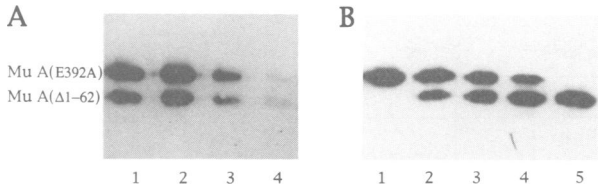

Fig. 4. Stoichiometry of Mu A(E392A) and Mu A(Δ1–62) within the type I complex. (**A**) Type I complexes formed on a Mu enhancer-containing plasmid upon incubation with Mu A(E392A) and Mu A(Δ1–62) were challenged with heparin (Materials and methods) and isolated following agarose gel electrophoresis. The type I-associated proteins were fractionated in SDS–polyacrylamide, transferred to nylon membrane and probed with Mu A antibodies. Lanes 1–4 correspond to type I reactions containing Mu A(E392A) and Mu A(Δ1–62) in the following protein ratios: 1:1, 1:3, 1:5 and 1:10. (**B**) Mu A(E392A) and Mu A(Δ1–62) alone (lanes 1 and 5) or a mixture of the two proteins in the ratios 3:1, 2:2 or 1:3 (lanes 2–4) were fractionated in SDS–polyacrylamide and probed with Mu A antibodies as in (A).

Stoichiometry of Mu A(E392A) and Mu A(Δ1–62) within the type I complex

To assess the protein composition of the type I complex formed by Mu A(E392A) and Mu A(Δ1–62), reactions were set up using the two proteins in the molar ratios 1:1, 1:3, 1:5 and 1:10 (lanes 1–4 respectively, Figure 4A). At protein ratios of 1:3, 1:5 and 1:10 the molar ratio of Mu A (E392A) to Mu A(Δ1–62) within the isolated type I complexes was ~2:2 (lanes 2–4, Figure 4A), although a slightly higher abundance of Mu A(E392A) was detected at input ratios of 1:3 and 1:5. At equimolar amounts of the two proteins there was a clear enrichment of Mu A(E392A) over Mu A(Δ1–62) (~1.7: 1) within the type I complex (lane 1, Figure 4A). These estimates were based on quantitation of Western blots of standard mixtures of Mu A(E392A) and Mu A(Δ1–62) in the molar ratios 1:0, 3:1, 1:1, 1:3 and 0:1 respectively (lanes 1–5, Figure 4B). Baker *et al.* (1994) have shown that in the type I complex formed by Mu A and Mu A(E392Q) tetramers, a significant fraction of the DNA molecules are cleaved at only one of the two Mu ends. Hence the higher abundance of Mu A(E392A) over Mu A(Δ1–62) in the type I complexes observed here (lanes 1–3, Figure 4A) can be accounted for by the fraction of singly cleaved complexes containing Mu A(E392A) and Mu A(Δ1–62) in the ratio 3:1. We interpret the protein stoichiometry [the virtual absence of the 3 Mu A(Δ1–62):1 Mu A(E392A) tetramer in the type I complex] to mean that the Mu A monomers bound to the *att* subsites can be fixed into a reactive tetrameric complex if and only if at least two of the monomers are capable of enhancer recognition. An alternative explanation is that a Mu A tetramer assembled following occupancy of the *att* subsites acquires chemical competence only if two of the monomers can interact with the enhancer. We suspect that the positions of these two monomers are likely to be fixed within the tetramer (reflecting the relative functional orientation of the enhancer and the *att* sites; see Discussion). An alternative explanation for our results is that a tetramer containing three monomers of Mu A(Δ1–62) and one monomer of Mu A(E392A) can mediate strand nicking, but the resultant complex is unstable and is not recovered as part of the type I complex population analyzed here. However, the failure to observe an increase in the nicked circular substrate fraction even when the protein mixture was skewed greatly towards Mu A(Δ1–62) discounts this possibility.

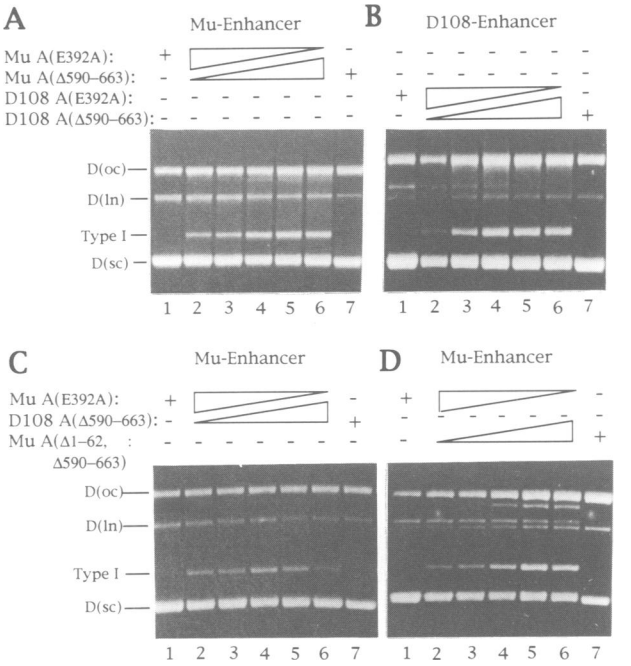

Fig. 5. Complementation between A protein mutants defective in catalysis and those lacking domain IIIα. (**A** and **B**) Mixtures of Mu A(E392A) with Mu A(Δ590–663) (A) and D108 A(E392A) with D108 A(Δ590–663) (B) were assayed for type I formation (strand cleavage) on mini-Mu plasmid pRA170 (Mu enhancer) or pJY170 (D108 enhancer). Lanes 1 and 7 are control reactions containing only one of the proteins. Reactions represented by lanes 2–6 contained the indicated proteins in the molar ratios: 3:1, 2:1, 2:2, 1:2 and 1:3. The final Mu A amount was ~4 pmol in all lanes. (**C** and **D**) Mixtures of Mu A(E392A) with either D108 A(Δ590–663) or Mu A(Δ1–62, Δ590–663) were assayed for type I complex formation on plasmid pRA170. Lane descriptions are as in (A and B). Designations of type I complex and DNA species are as in Figure 3.

Transposase mutants lacking domain IIIα can be complemented in trans during strand cleavage

Domain IIIα (see Figure 2; residues 574–603) of Mu A is essential for its catalytic function, as inferred from deletion analyses; removal of C-terminal residues beyond 603 does not affect transposition. In order to test whether this domain can be effectively provided *in trans* to a Mu A monomer lacking it, we assayed Mu A(Δ590–663) (which lacks a functional IIIα domain) in combination with Mu A(E392A) for strand cleavage activity. Although neither protein by itself was functional in Mu end cleavage (lanes 1 and 7, Figure 5A), cleavage ability was partially restored in the mixture (lanes 2–6, Figure 5A). Mu A(Δ574–663), a mutant missing the entire domain IIIα, behaved similar to Mu A(Δ590–663) (data not shown). Analogous results were obtained when the experiment was repeated with D108 A(Δ590–663) and D108 A(E392A) in the presence of the D108 enhancer (Figure 5B). Consistent with the results from the experiments of Figure 3, no complementation could be detected with the Mu A variant pair when the enhancer element was D108 derived; similarly, the D108 A variant pair failed to complement in the presence of the Mu enhancer element (data not shown).

The results from Figure 5 (also summarized in Table I,

Table I. Summary of complementation between pairs of Mu A mutants

Transposase or its variants	Strand Cleavage			Strand Transfer		
	Mu-Enh. ○–○	D108-Enh. ●–●	Ref.	Supercoiled (mini-Mu)	Pre-cleaved (attR)	Ref.
Mu A(E392A) + D108 A	+		Fig.3A	-		
Mu A(E392A) + Mu A(Δ1–62)	+	-	Fig.3B	-		
Mu A(E392A) + Mu A(D30M)	+	-		-		
D108 A(E392A) + Mu A		+	Fig.3C	N.D.		
D108 A(E392A) + Mu A(Δ1–62)	-	+	Fig.3D	N.D.		
D108 A(E392A) + Mu A(D30M)	-	+		N.D.		
Mu A(Δ1–62) + D108 A	-			N.D.		
Mu A(E392A) + Mu A(Δ590–663)	+	-	Fig.5A	-	+	Fig.7
Mu A(E392A) + Mu A(Δ574–663)	+	-		-	+	
Mu A(E392A) + D108 A(Δ590–663)	+	-	Fig.5C	N.D.	N.D.	
Mu A(E392A) + Mu A(Δ1–62,Δ590–663)	+	-	Fig.5D	N.D.	N.D.	
D108 A(E392A) + D108 A(Δ590–663)	-	+	Fig.5B	N.D.	N.D.	
D108 A(E392A) + Mu A(Δ590–663)	-	+		N.D.	N.D.	
D108 A(E392A) + Mu A(Δ1–62,Δ590–663)	-	+		N.D.	N.D.	
Mu A(Δ590–663) + D108 A	-		Fig.6A	N.D.		
Mu A(Δ590–663) + Mu A(Δ1–62)	-	-		N.D.		
Mu A(Δ590–663) + Mu A(Δ1–62,E392A)	-	-		N.D.	+	
D108 A(Δ590–663) + Mu A		-				
D108 A(Δ590–663) + Mu A(Δ1–62)	-	-		N.D.		
D108 A(Δ590–663) + Mu A(Δ1–62,E392A)	-	-		N.D.	N.D.	

The results obtained with various pairwise combinations of wild-type and mutant A proteins are summarized. Mu A (D30M) contains the first 30 N-terminal residues of D108 A and is unable to bind either Mu or D108 enhancer. Where appropriate, the figure in the text depicting the experimental outcome is referred to. The Table also includes results for which the data are not shown in figure form. A blank box indicates that one of the A proteins in the pair is by itself active on the substrate DNA. Reactions not done are denoted by N.D. Enhancer binding domains Iα of the transposase (circles) are drawn to match the open or filled dumb-bells representing Mu or D108 enhancers. The active and inactive forms of domain II are indicated by ✓ and × respectively. Domain IIIα is symbolized by a square. Deletions of these domains are schematically represented.
The following rule, consistent with the proposed model for active site assembly, will make it easier to appreciate the large set of results compiled here. Domain Iα matched with its cognate enhancer (○ with ○–○ and ● with ●–●) and domain IIIα (□) *in cis* together with domain II (✓) *in trans* will be positive in cleavage of a supercoiled donor substrate. Domain IIIα (□) together with domain II (✓) *in trans* is sufficient for strand transfer in a pre-cleaved substrate.

along with data not shown) together with those shown in Figure 3 demonstrate that the Mu A protomer contributing Glu392 (present within domain II) to strand cleavage need harbor neither domain IIIα nor the enhancer binding domain. This surmise was verified by reacting a substrate containing the Mu enhancer with Mu A(E392A) mixed with either D108 A(Δ590–603) or with Mu A(Δ1–62, Δ590–663). Both reactions led to formation of the type I complex (lanes 2–6, Figure 5C and D). We have verified by Western blot assays that in these reactions both protein partners were present within the type I complexes (results not shown). When the enhancer was derived from D108, the combinations of D108 A(E392A) with a Mu A variant lacking an intact domain IIIα or lacking the enhancer binding domain as well as domain IIIα yielded the type I complex (data not shown; see Table I).

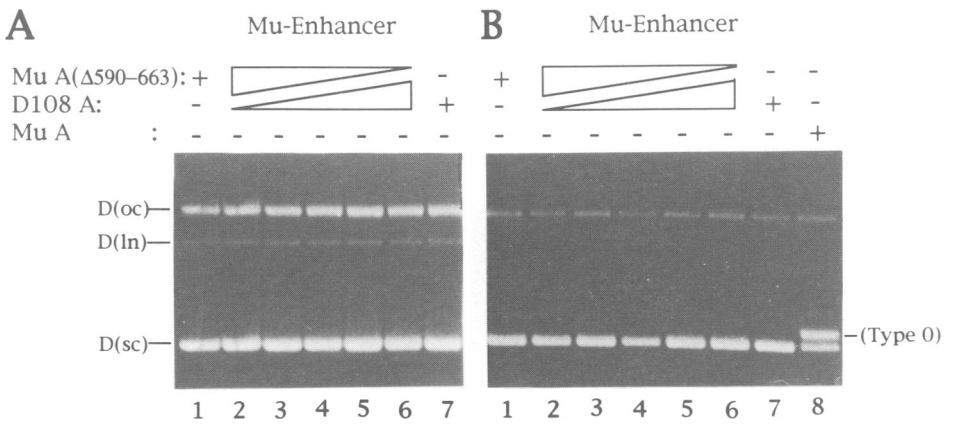

Fig. 6. Complementation between A protein mutants defective in enhancer recognition with those lacking domain IIIα. (**A**) Mixtures of Mu A(Δ590–663) and D108 A were assayed for type I complex formation on plasmid pRA170. Lanes 1 and 7 are controls with only one of the proteins added as indicated. Lanes 2–6 are reactions that contained Mu A(Δ590–663) and D108A in the ratios: 3:1, 2:1, 2:2, 1:2 and 1:3. The final Mu A amount was ~4 pmol in all lanes. (**B**) Reactions were done with plasmid pMK21 (Mu enhancer) in the presence of Ca^{2+} to assay for type 0 complex formation (assembled tetramers, uncleaved Mu ends). The type 0 complex formed on pMK21 migrates slightly above the supercoiled plasmid band (Materials and methods; Kim *et al.*, 1995). Lanes 1–7 contained the protein mixtures as in (A); lane 8 is a type 0 reaction with wild-type Mu A. Symbols are as in Figure 3.

Mu A subunits that interact with the enhancer preparatory to strand cleavage must have a functional domain IIIα in cis

Thus far, results with pairwise combinations assembled from Mu A (or D108 A) and appropriate Mu A variants (or D108 A variants) reveal the following facets of the Mu A tetramer in the context of the strand cleavage reaction: (i) the monomer donating Glu392 need not interact with the enhancer nor does it provide domain IIIα to the reaction (Figures 3 and 5); (ii) formation of the type I complex requires that at least two Mu A monomers be able to recognize the enhancer (Figures 3 and 4). It is known that domain IIIα is required for assembly of the Mu A tetramer (Baker *et al.*, 1993; J.-Y.Yang and R.M.Harshey, unpublished data). However, the ability of a Mu A variant lacking this domain to form mixed tetramers with wild-type Mu A (Baker *et al.*, 1993) or to complement a Mu A variant defective in catalysis (Figure 5A and B) implies that not all four Mu A monomers need to harbor domain IIIα for successful tetramerization. What is the functional configuration of domain IIIα with respect to the enhancer binding domain during strand cleavage? Results from Figure 5 show that IIIα *in cis* with respect to the enhancer binding domain (and *trans* with respect to domain II) is active. Can IIIα function when placed *in trans* to the enhancer binding domain? To test this, a mixture of D108A and Mu A(Δ590–603) was reacted with a substrate containing the Mu enhancer. No type I complex was obtained from this combination (lanes 2–6, Figure 6A). Furthermore, this combination also failed to produce the uncleaved type 0 complex (in which a Mu A tetramer is organized, but no strand cleavage occurs) (lanes 2–6, Figure 6B).

We conclude that only the *cis* and not the *trans* arrangement of domain IIIα and the enhancer binding domain will support assembly of the catalytically active Mu A tetramer.

Pattern of catalytic complementation by Mu A mutants during strand transfer to target DNA in the presence of Mu B

During a normal transposition event, cleavage of Mu ends is followed by joining of the cleaved ends to target DNA (strand transfer), a reaction promoted by the Mu B protein (see Figure 1). When a pairwise transposase combination that can yield the type I complex [for example Mu A(E392A) and D108A; see Figure 3A] was reacted with a donor DNA containing the Mu enhancer and pUC19 target DNA in the presence of Mu B and ATP, no strand transfer products were obtained (data not shown; see Table I). The same result was obtained when Mu A(E392A) was paired with Mu A(Δ590–663) (Figure 7A). Strand cleavage could be observed (formation of the type I complex), but no strand transfer products could be detected (lanes 4–6, Figure 7A). However, when a substrate containing pre-formed 3′ hydroxyl groups at the normal Mu cleavage sites was incubated with Mu A(E392A) and Mu A(Δ590–663) in the presence of Mu B, ATP and target DNA, strand transfer was detected (lanes 4–6, Figure 7B). It is known that strand transfer from a pre-cleaved donor is independent of enhancer function (Mizuuchi and Mizuuchi, 1989). Consistent with this mechanism, Mu A(E392A, Δ1–62) could also complement Mu A(Δ590–663) in the strand transfer reaction (data not shown; see Table I).

A simple explanation that accommodates this set of results is that the tetramer derived from the complementing monomers can assemble only one good pair of active sites. If these are used for the cleavage step, the lack of the second pair of active sites precludes the strand transfer step. Since the strand breakage and strand transfer reactions are thought to follow similar chemistry (in-line nucleophilic attack by a hydroxide ion or the 3′ hydroxyl group from DNA on scissile phosphodiester bonds; see Mizuuchi, 1992), the active sites mediating them are likely to be similar. It is appealing then to imagine that the four active sites required for one round of transposition (two for cleavage and two for strand transfer) may be assembled by a two-step swapping of the same domain pairs (domain II and domain IIIα) among the Mu A monomers constituting the active tetramer. In such a case, provided the donor substrate had been pre-cleaved, the active sites for strand transfer may be derived from a mixture of Mu A(E392A) (which can supply domain IIIα) and Mu A(Δ590–663) (which can supply domain II).

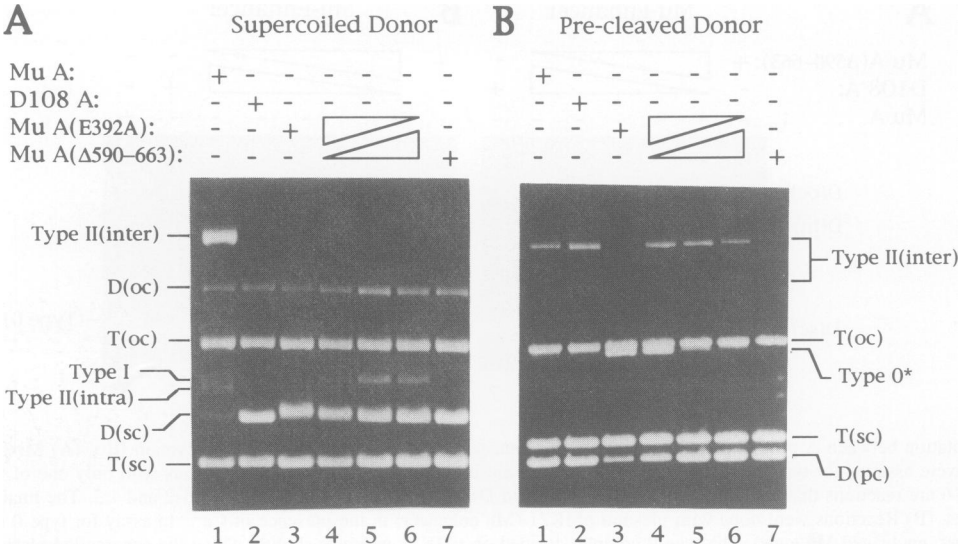

Fig. 7. Complementation between Mu A mutants during strand transfer in the presence of Mu B. (**A**) Mixtures of Mu A(E392A) and Mu A(Δ590–663) were assayed for cleavage and strand transfer in the presence of target DNA (pUC19), Mu B and ATP. Plasmid pRA170 served as the donor. Lane 1, wild-type Mu A control; lane 2, D108 A control; lanes 3 and 7, Mu A(E392A) and Mu A(Δ590–663) controls; lanes 4–6, indicated proteins in the molar ratios 3:1, 2:2 and 1:3. Reactions were fractionated without SDS treatment. (**B**) Assays done as in (A) except that the donor DNA was pre-cleaved at the right end of Mu. The target plasmid is indicated by T and the pre-cleaved donor plasmid by (pc). The intramolecular and intermolecular strand transfer products are labeled Type II(intra) and Type II(inter) respectively. Type 0* is the Mu A(E392A) complex assembled (but not strand transferred) on pre-cleaved donor.

Validity of the domain swap model

The domain swap model states that the IIIα domains derived from the two Mu A monomers (pair 1) interacting with the enhancer element and the domain II regions derived from the other two monomers (pair 2) cooperate to assemble the active sites for strand cleavage. Conversely, the IIIα domains from pair 2 and the domain II regions from pair 1 associate to assemble the active sites for strand transfer. The experiments shown in Figure 8 test the validity of this model using a substrate containing the Mu enhancer. Assembly of the strand cleavage and strand transfer pockets was assayed simultaneously under reaction conditions that stimulate intramolecular strand transfer in the absence of Mu B and ATP (see Materials and methods). Figure 8A and B represent electrophoretic fractionation of the same reactions with or without SDS treatment respectively. In the absence of protein dissociation, the type I complex migrates between the supercoiled and linear forms of the substrate plasmid and the strand transfer products (type II complex) are clustered just below the type I band (Figure 8A). Upon protein dissociation prior to electrophoresis, the type I complex is indirectly represented by an increase in the open circular form of the substrate and the strand transfer products are positioned as a series of bands below the open circlular form and a band below the supercoiled form of the substrate plasmid (Figure 8B). Based on a set of Mu A titrations, two suboptimal amounts of Mu A were chosen to produce a low level of type I and type II complexes (lanes 1 and 8 and 2 and 9, Figure 8A and B). When the reaction was spiked with two concentrations of D108 A, stimulation of type I and type II formation was observed (compare lanes 3 and 5 with lane 1 and lanes 4 and 6 with lane 2, Figure 8A and B). The results are consistent with the domain swap model. Cleavage is accomplished by using domain IIIα from two Mu A monomers (pair 1) and domain II from

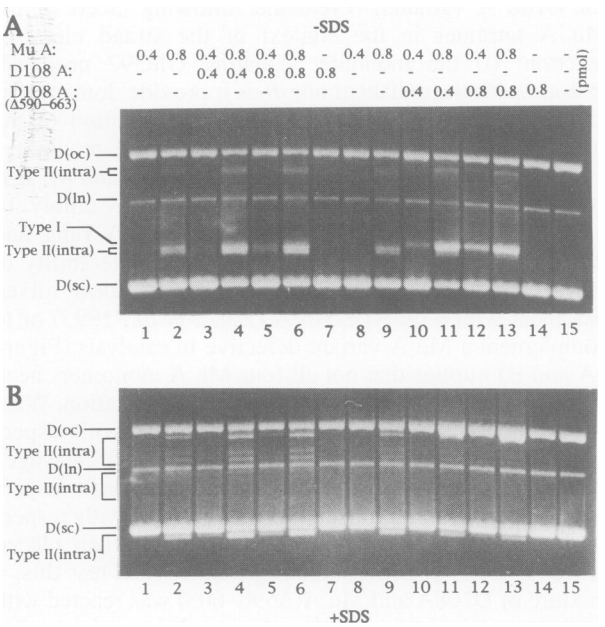

Fig. 8. Strand cleavage and strand transfer potential derived from pairing Mu A with D108 A or D108 A(Δ590–663). (**A**) Cleavage and intramolecular strand transfer were assayed using pRA170 as the donor substrate. The amounts (in pmol) of the Mu A proteins or the deletion variant present in the reactions are shown above each lane. The reactions were fractionated without SDS treatment. (**B**) Reactions carried out as in (A) were treated with SDS prior to electrophoresis. Designations of type I and II complexes and DNA species are as in Figure 7.

two D108 A monomers; strand transfer follows by using IIIα from D108A and domain II from Mu A. A strikingly distinct, and revealing, result was obtained when the spiking was done with D108 A(Δ590–663). There was indeed stimulation of type I complex formation (compare

lanes 10 and 12 with lane 8 and lanes 11 and 13 with lane 9, Figure 8A and B). This is predicted by the model, since D108 A and D108 A(Δ590–663) should be equivalent in their ability to contribute domain II. In contrast, strand transfer was inhibited in reactions containing D108 A(Δ590–663) (compare lanes 10 and 12 with lane 8 and lanes 11 and 13 with lane 9, Figure 8A and B). This is also predicted by the model. Unlike D108 A, the deletion variant cannot provide the domain IIIα component of the strand transfer pockets. Furthermore, consistent with the model, the catalytic stimulation by equivalent amounts of D108 A (cleavage plus strand transfer; type I plus type II) and D108 A(Δ590–663) (cleavage alone; type I) was roughly equal.

Discussion

Analyses of strand cleavage and strand transfer reactions using pairwise protein combinations derived from wild-type Mu A and Mu A variants defective in enhancer recognition, Mu A oligomerization or the chemical steps of transposition lead to a simple model for active site assembly within the Mu A tetramer. The model is based on the following assumptions: (i) the effects of deleting domain Iα from Mu A are directly related to the loss of normal enhancer interactions; (ii) the lack of activity of the Mu A variant Mu A(E392A) implies a role for Glu392 in catalysis. Since the transposition reaction is a complex reaction involving several levels of DNA–protein and protein–protein interactions, there is some concern that alterations within the transposase may cause unsuspected perturbations that could then affect the reaction in ways that cannot be rigorously controlled. We have attempted to minimize this risk by applying our experimental tests to two different enhancers and their cognate protein domains and to multiple pairwise protein combinations that include two wild-type transposases and their altered forms. We present here the simplest possible model based on the most straightforward assumptions, in the hope that it will stimulate vigorous attempts to falsify it.

One key inference from our experiments is that protein domains contributed by more than one transposase monomer are utilized in organizing a catalytic pocket in which a strand cleavage or a strand joining event occurs. They strongly suggest a domain sharing mechanism for building the cleavage and joining pockets. The modular architecture of the active site provides insights into the potential roles of the enhancer element and the Mu B protein in specific steps of the transposition pathway. In our study we have not addressed the role, if any, of subdomains Iβ and Iγ of Mu A in strand cleavage or strand transfer. The Iβ–Iγ domain is essential for *att* binding and no mutations that affect catalytic functions without affecting DNA binding have been mapped to this region.

The shared domain model for the transposase active site

The principal conclusion from this study is that a monomer of Mu A does not harbor a functional active site, which is built by sharing of specific domains by more than one transposase monomer (Figure 9). For example, the catalytic pocket for a strand cleavage event requires cooperativity among the enhancer binding domain (Iα), the central catalytic domain containing Glu392 (domain II) and part of the C-terminal domain (IIIα). Domains Iα and IIIα are donated by one monomer, while domain II is derived from a second monomer. Furthermore, in order to organize a pair of strand cleavage pockets (required for cutting the two ends of Mu), at least two of the Mu A monomers within the tetramer must contain domain Iα. Similarly, the organization of each of the two strand transfer pockets requires the sharing of domain II from one monomer and domain IIIα from a second monomer.

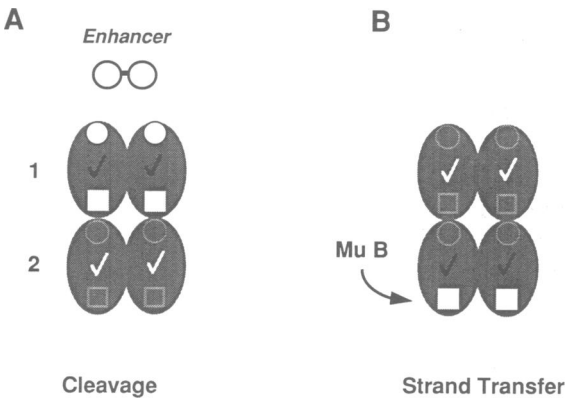

Fig. 9. A domain sharing model for assembly of strand cleavage and joining pockets within the Mu A tetramer. Each monomer of Mu A is represented as an oval within which the enhancer binding domain Iα is depicted by a circle (○). The C-terminal subdomain IIIα (required for tetramer and active site assembly) and the central domain II (harboring key catalytic residues) are symbolized by □ and ✓ respectively. The Mu A domains active in organizing the pair of active sites in the strand cleavage step (A) and the strand transfer step (B) are open; those that are silent at each of these two steps are filled. (**A**) The enhancer, symbolized by the dumb-bell, interacts with domain Iα (○) from pair 1, specifying it as donors of domain IIIα (□) in organizing the cleavage pockets. The active sites for Mu end cleavage are built by contributions from domain IIIα (□) of the top two Mu A monomers (pair 1) and from domain II (✓) of the bottom two monomers (pair 2). (**B**) The strand transfer pockets are built by a role reversal between pairs 1 and 2. Pair 2 is now the donor of domain IIIα and pair 1 is the donor of domain II. The Mu B protein, which interacts with domain IIIβ (not shown), may act as an allosteric effector of the strand transfer pocket. The representation of the Mu A monomers is only schematic. We do not know the positioning of the monomers with respect to the *att* cleavage sites. We also do not know the spatial and functional relationship of a monomer within pair 1 with respect to each of the two monomers of pair 2. In reality, the strand cleavage and strand transfer pockets are likely to be tightly associated.

Two simple models can be proposed to account for the shared active site configuration within the Mu A tetramer. In one model we may imagine that two monomers cooperate to build the two active sites for strand cleavage, while the other two monomers interact to build the two active sites for strand transfer. In this model, a dimer of Mu A is sufficient for chemical competence, and interactions with the other two monomers within the tetramer are purely structural or allosteric. In a second model, catalytic contributions from all four monomers are necessary for assembling the two strand cleavage pockets or the two strand transfer pockets. Here the pair of strand cleavage active sites is derived by domain swapping between two pairs of Mu A monomers; the pair of strand transfer active sites is derived by a reverse domain swap between the same monomer pairs. As discussed below, the latter model is more consistent with our results.

Role of the enhancer element and domain Iα of Mu A in transposition

The function of the Mu enhancer has remained enigmatic and elusive until now. It is required in steps leading up to strand cutting, but is not directly involved in cleavage (Leung and Harshey, 1989; Mizuuchi and Mizuuchi, 1989; Surette and Chaconas, 1992). Its interactions with domain Iα of the transposase appear to be transient; attempts to trap these interactions during the progression of the transposition pathway have not been successful. It is likely that enhancer recognition may be mediated by Mu A associated with the accessory *att* subsites L2 and L3 in *att*L and R3 in *att*R during assembly of the transposition complex (see Figure 1B; Allison and Chaconas, 1992). Our results offer a plausible functional role for the enhancer during transposition. The enhancer probably specifies which two Mu A monomers will provide the IIIα domains for the cleavage reaction. This determination may be achieved spatially by fixing the relative location of these monomers within the tetramer with respect to the scissile phosphodiesters. Alternatively, interaction of domain Iα with the enhancer may be a prerequisite for some conformational change in domain IIIα to permit its recruitment into active site assembly. The latter scheme presupposes some mode of communication between the N- and C-terminal regions of Mu A. Structural studies on Mu A might shed light on this issue.

We wish to make it clear that the domain sharing model does not imply that residues directly participating in the chemical steps of transposition are shared between domains II and IIIα. While this is likely, it is possible that domain IIIα is essential for the correct spatial orientation of catalytic residues located entirely within domain II. A somewhat related issue is whether the contribution of domain IIIα to tetramer assembly is separable from its contribution to active site organization. In the absence of domain IIIα, Mu A cannot assemble a tetramer, and a Mu A variant altered within this domain that can uncouple tetramer assembly from active site assembly has not been identified. Nevertheless, formation of strand cleavage-competent mixtures between Mu A and Mu A(Δ575–663) (Baker *et al.*, 1993) and between Mu A and D108 A(Δ590–663) (Figure 8; this study) and the formation of strand transfer-competent mixtures from Mu A(E392A) and Mu A(Δ590–663) (Figure 7; this study) shows that the transposase tetramer, but not a full complement of the active sites, can be derived from less than four (probably two) equivalents of the IIIα domain. The inability of a type I complex assembled with Mu A and D108 A(Δ590–663) to carry out strand transfer (Figure 8; this study) can be attributed to the missing IIIα domains within it. It should also be emphasized that, while the implied mode of action of domain Iα in our model is allosteric, none of the results rule out a direct role for this domain in catalysis, at least in the strand cleavage step. However, the dispensability of domain Iα in the strand transfer step and, under special reaction conditions, in the cleavage step (for example in the presence of dimethylsulfoxide) argues against its direct participation in catalysis.

The functional assignment of Mu A monomers from our results do not agree with the conclusions of Baker *et al.* (1993) regarding the 'division of labor' within the Mu A tetramer. Their results were based upon the incorporation of Mu A(Δ575–663), a cleavage-incompetent domain IIIα deletion mutant of Mu A, into mixed type I tetramers with the wild-type protein. The presence of the two protein partners in a 2:2 molar ratio within the type I complex led Baker *et al.* to propose the following scheme. Strand cleavage was effected by the two wild-type monomers within the mixed tetramer, hence, in the normal reaction only two monomers contribute to the strand cutting step. No strand transfer products were obtained with the mixed tetramer, even when the donor substrate contained pre-cleaved Mu ends, hence, all four wild-type monomers are essential for this step of transposition. Baker *et al.* (1994) have modified this inference to account for their recent observation that a mixture of Mu A(E392Q) and wild-type Mu A can mediate strand transfer on pre-cleaved Mu ends. In the revised model, two monomers are principally responsible for cleavage and two different monomers are responsible for strand transfer. In the light of our observations, we would reinterpret the Baker *et al.* results as follows. Contributions from domain II of Mu A(Δ575–663) and domain IIIα of Mu A or domain II of Mu A and domain IIIα of Mu A(E392Q) can mediate strand breakage. However, although domain II from Mu A is available to the first pair, no strand transfer pockets can be built for want of domain IIIα within Mu A(Δ575–663). In the second pair, although domain IIIα from Mu A is available, no strand transfer pockets can be built for want of a functional domain II within Mu A(E392Q). In contrast to the Baker *et al.* 'two first–then four' or 'two first–then two' models, our model proposes domain contributions from all four transposase monomers in each of the two steps of transposition. The strand cleavage and strand transfer potential of mixtures of Mu A with D108 A or D108 A(Δ590–663) supports our model (Figure 8).

Enhancers (*cis* acting sequences that markedly stimulate a reaction) have been characterized in at least two other site-specific recombination systems (Hin and Gin; Kahmann *et al.*, 1985; Johnson and Simon, 1985). In the Hin system, evidence has been obtained for simultaneous interaction of the enhancer binding protein Fis with the enhancer on the one hand and the recombinase bound to the recombination sites on the other to promote assembly of the synaptic complex (Heichman and Johnson, 1990). It is possible that the recombination and transposition systems may have commonalities in the mode of enhancer action. While the former encodes the recognition specificities for two distinct and distant DNA sequences in two separate proteins, the latter encodes them in separate domains of a single polypeptide.

The organization of the enhancer site within the Mu transposon has interesting regulatory implications for the physiology of Mu bacteriophage (see Harshey, 1988; Berg and Howe, 1989). Transcription of the transposase (Mu A protein) and the accessory protein Mu B is controlled by the action of the Mu repressor on a set of operator sites. The enhancer element is located within the operator region and forms a subset of the operator sites (Leung *et al.*, 1989; Mizuuchi and Mizuuchi, 1989; Surette *et al.*, 1989). In addition, domain Iα of Mu A shows strong amino acid homology to the DNA binding domain of the Mu repressor (Harshey *et al.*, 1985; Mizuuchi *et al.*, 1986; Vogel *et al.*, 1991). Thus, by binding to the operator,

the Mu repressor not only prevents expression of the transposition proteins but also blocks activity of the transposase by effectively sequestering the enhancer.

Role of Mu B in the strand transfer reaction

According to the model presented here, assembly of the strand cleavage active sites specified by the enhancer–Mu A interaction automatically fixes the configuration of the strand transfer active sites. How does the Mu B protein function in the joining of the cleaved Mu ends to the target DNA? It might act (i) as a passive carrier of the target DNA to the strand transfer pocket or (ii) as an active allosteric effector of Mu A. Although the target transporting or positioning role of Mu B is consistent with all of our results, several observations suggest that the potential allosteric activator function of Mu B may be more pertinent to strand transfer (Baker *et al.*, 1991; Surette *et al.*, 1991). Thus interaction of Mu B with domain IIIβ of Mu A may be a necessary event, for example, to induce the active configuration of IIIα within the strand transfer pocket. In this sense, the action of the enhancer element during the strand cleavage step and of Mu B during the strand transfer step may indeed be functionally analogous.

General implications of the shared active site in strand breakage and joining in DNA

The mechanism of Mu transposition shares many common chemical features with transposition of other transposable elements, as well as integration of retroviruses into their host genomes (see Mizuuchi, 1992). Most of these elements share a conserved catalytic triad motif, D-D-35-E (Fayet *et al.*, 1990; Kulkosky *et al.*, 1992; Radstrom *et al.*, 1994). The Glu392 residue in domain II of Mu A may be functionally analogous to one of the acidic residues of the retroviral D-D-E motif (Baker and Lou, 1994; Kim *et al.*, 1995). Pairwise mixing experiments with Mu A(E392A) and other catalytically defective mutants in domain II [Mu A(D269V) and Mu A(G348D)] suggest that these residues (Asp269, Gly348 and Glu392) must be present *in cis* on the same monomer and cannot be shared between monomers for catalytic competence (K.Kim and R.M.Harshey, unpublished data). A similar situation obtains with the D-D-E residues of the retroviral integrases (Engelman *et al.*, 1993; van Gent *et al.*, 1993). The architecture of the retroviral integrases reveals an N-terminal domain with a zinc finger-like motif (required for catalysis), the main catalytic domain harboring D-D-E and a C-terminal DNA binding domain. A catalytically inactive integrase variant mutated within the D-D-E triad, but containing wild-type N- and C-terminal domains (in the *cis* configuration), can be functionally complemented by providing the D-D-E domain *in trans*. These results are highly suggestive of a mechanism of active site assembly similar to that of Mu A.

The organization of a shared active site for strand breakage and reunion was first demonstrated in the case of the yeast site-specific recombinase Flp (Chen *et al.*, 1992). Here, during the strand cleavage step, a monomer of Flp bound to its recognition target orients an adjacent phosphodiester for nucleophilic attack by the active site tyrosine derived *in trans* from a second Flp monomer. Strand breakage results in the formation of a 3′-*O*-phosphotyrosyl diester and a 5′ hydroxyl group. In the strand joining reaction, the phosphotyrosyl bond is oriented by the Flp monomer bound adjacent to it for attack by the 5′ hydroxyl derived *in trans* from the cleaved partner DNA molecule (Lee and Jayaram, 1993). In the Mu transposition reaction, the shared active sites arranged by one set of domain swaps orient the phosphodiesters at the ends of Mu and the hydroxide ions (water-derived nucleophiles) in line with each other during the strand breakage step. Following cleavage, the active sites derived by a second set of domain swaps orient the target phosphodiesters and the 3′ hydroxyl groups (cleavage-generated nucleophiles) for strand joining.

The partial active site configuration within the monomer and the assembly of a full active site at the interface of monomers are highly desirable features for enzymes carrying out phosphoryl transfer reactions within or between DNA molecules. The shared active site can be a safeguard against the onset of partial reactions prior tc assembly of the full reaction complex. The concurrent assembly of a pair of active sites by reciprocal sharing of residues between monomers is an elegant device for coordinating two phosphoryl transfers simultaneously. It is no accident therefore that recombinases, transposases and integrases have incorporated this architectural design into their active sites.

Materials and methods

Plasmids

Plasmids pRA170 (Leung *et al.*, 1989) and pMK21 (Kim *et al.*, 1995) carrying the left and right ends of Mu and the internal Mu enhancer were used as donors in transposition assays. pRA170 was manipulated to replace the Mu enhancer with the D108 enhancer, yielding pJY170 (details to be described elsewhere). The plasmid engineered to be cut at the Mu right end by *Hin*dIII digestion (pMS358) was a gift from M.Surette and G.Chaconas (University of Western Ontario) and is described in Namgoong *et al.* (1994). Target DNA was a dimeric form of pUC19.

Plasmid pET158, which has the wild-type Mu A gene cloned into the T7 expression vector pET11-a (Novagen), is described in Kim *et al.* (1995). Mutant Mu A proteins were expressed from this vector after exchanging appropriate restriction fragments with pET158.

Transposase variants

Mu A(E392A) has been described (Kim *et al.*, 1995). Mu A(Δ590–663) carries 589 residues from the N-terminus and is thus partially deleted in domain IIIα. A Mu A hybrid D108 A carrying the enhancer binding domain of D108 A was constructed by exchanging the relevant *Pst*I fragments between the D108 and Mu A genes (Harshey *et al.*, 1985; DuBow, 1987). This protein has been referred to as D108 A for convenience, since Mu A and D108 A are almost identical beyond the first 80 residues (Gill *et al.*, 1981). Mu A mutations were moved into D108 A by exchange of appropriate restriction fragments between the two genes.

Protein purification

The wild-type Mu A protein and the variant proteins were expressed from pET158 as described by Kim *et al.* (1995). These proteins were judged to be >95% pure from Coomassie brilliant blue staining of SDS–polyacrylamide gels in which they were fractionated. The concentration of Mu A or Mu A variants was derived from the extinction coefficient $\varepsilon_{1\ mg/ml} = 1.83$ (Kuo *et al.*, 1991).

Purification of HU and Mu B proteins has been described earlier (Leung and Harshey, 1991).

In vitro assays for Mu DNA cleavage and strand transfer

Standard assays for pre-cleavage assembly of the Mu A tetramer on Mu ends (type 0 complex), strand cleavage (type I complex) and strand transfer to target DNA in the presence of Mu B and ATP (type II

complex) were done as described by Mizuuchi *et al.* (1992) and Surette *et al.* (1987). Intramolecular strand transfer assays were done in the presence of 25% glycerol as described by Maxwell *et al.* (1987).

Conditions for observing a distinct type 0 complex band upon agarose gel electrophoresis were as described in Kim *et al.* (1995). Briefly, the size of the mini-Mu plasmid is important for separation of the type 0 complex from unreacted supercoiled substrate, as is the addition of 10 μg/ml heparin prior to electrophoresis, to remove loosely bound Mu A protein.

Determination of stoichiometry of Mu A mutants in mixed tetramers

Type I reactions were treated with 500 μg/ml heparin to remove all loosely bound Mu A protein and electrophoresed in 0.9% low gelling agarose. Type I complexes were visualized under brief UV illumination after staining the gel with ethidium bromide. The excised gel slices containing the complex were melted at 90°C in Laemmli sample buffer (Laemmli, 1970) and proteins were fractionated on a 10% SDS–polyacrylamide gel. The gel was Western blotted and developed with anti-Mu A polyclonal antibody using the ECL kit (Amersham). Protein standards were run in parallel and the bands quantitated as described below for DNA.

Quantitation of type I complexes

Negative films were scanned with a BioRad video densitometer and DNA bands were quantitated using software supplied with the system.

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