

Criss-crossed interactions between the enhancer and the att sites of phage Mu during DNA transposition

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A bipartite enhancer sequence (composed of the O1 and O2 operator sites) is essential for assembly of the functional tetramer of phage Mu transposase (MuA) on supercoiled DNA substrates. A three-site interaction (LER) between the left (L) and right (R) ends of Mu (att sites) and the enhancer (E) precedes tetramer assembly. We have dissected the role of the enhancer in tetramer assembly by using two transposase proteins that have a common att site specificity, but are distinct in their enhancer specificity. The activity of these proteins on substrates containing hybrid enhancers reveals a 'criss-crossed' pattern of interaction between att and enhancer sites. The left operator, O1, of the enhancer interacts specifically with the transposase subunit at the R1 site (within the right att sequence) that is responsible for cleaving the left end of Mu. The right operator, O2, shows a preferential interaction with the transposase subunit at the L1 site (within the left att sequence) that is responsible for cleaving the right end of Mu.

Keywords: DNA transposition/enhancer/MuA transposase/phage Mu/transpososome assembly

Introduction

Fundamental biological reactions such as replication, transcription and recombination are carried out by architecturally elaborate and geometrically precise multiprotein catalytic assemblies. The complexity of these reactions arises, in large part, from the double-helical geometry of the DNA substrate/template, and the limits to its flexibility and dynamics imposed by its relatively large size, intrinsic elastic properties, topological attributes such as negative superhelicity, association with other macromolecules including RNAs and proteins, and packaging into higher order structures such as nucleosomes. For a number of these systems, DNA sequences have been identified that normally act *in cis*, often in an orientation- and distance-independent manner, to facilitate the reaction or modulate its efficiency (see Yang *et al.*, 1995a; Hertel *et al.*, 1997; Zhang *et al.*, 1997). These sequences have been generally classified as 'enhancers'.

The mechanisms underlying enhancer function in the different systems may have common as well as varied

themes. In site-specific recombination systems, for example, enhancers (or accessory DNA sites) play a central role, via DNA-protein interactions, in the precise alignment of the recombining sites within a geometrically defined synaptic structure (Heichman and Johnson, 1990; Kanaar *et al.*, 1990; Stark *et al.*, 1992; Grindley, 1994; Colloms *et al.*, 1997). The enhancer element required for the transposition of phage Mu genome is functionally complex, and is involved in two distinct regulatory pathways. The Mu enhancer participates directly, though transiently, in the assembly of the Mu-end synaptic structure harboring the tetrameric configuration of the transposase (MuA protein). It is only within this high order protein-DNA complex that the strand cleavage and joining reactions of transposition can occur (see Mizuuchi, 1992; Lavoie and Chaconas, 1995; Chaconas *et al.*, 1996). The enhancer also regulates transposition at the level of gene expression. It encompasses the operator sites at which the Mu lysogenic repressor binds to shut-off transcription of the genes encoding Mu transposition proteins (see Goosen and Van de Putte, 1987).

The Mu enhancer was originally discovered as a *cis*-acting element required for the cleavage reaction of transposition (Leung *et al.*, 1989; Mizuuchi and Mizuuchi, 1989), in a distance-independent orientation-specific manner (Leung *et al.*, 1989; Surette *et al.*, 1989). Subsequent studies showed that the enhancer can be active *in trans* when supplied at a 50-fold molar excess on an unlinked short linear DNA molecule in the presence of the *Escherichia coli* protein IHF (Surette and Chaconas, 1992). An unstable three-site interaction between the enhancer and the left and right ends of Mu (attL: L1, L2, L3 sites; attR: R1, R2, R3 sites) was trapped using glutaraldehyde cross-linking (the LER complex; see Figure 1; Watson and Chaconas, 1996). The *E.coli* HU protein is also required for LER formation. On negatively supercoiled DNA, which is the normal substrate for transposition, LER progresses into a stably assembled type 0 complex, with concomitant tetramerization of the MuA protein (Chaconas *et al.*, 1996). The tetramer footprints on only three of the six att sites: L1, R1 and R2, but not on L2, L3 and R3. The enhancer is not associated with the type 0 complex, and is not required for the transposase-mediated cleavage of Mu ends (type I complex formation) induced by Mg²⁺ or Mn²⁺ ions, or for the joining of the cleaved ends to target DNA (promoted by MuB protein and ATP). The strand-transferred product, still associated with the transposition proteins, is called the type II complex (Figure 1).

The MuA protein exists as a monomer in solution, and contains two separate DNA-binding regions, I α and I $\beta\gamma$, in its N-terminal domain I (Chaconas *et al.*, 1996). The I α sequence is specific to the enhancer, the I $\beta\gamma$ sequence is specific to the att DNA (Figure 2A). The central domain

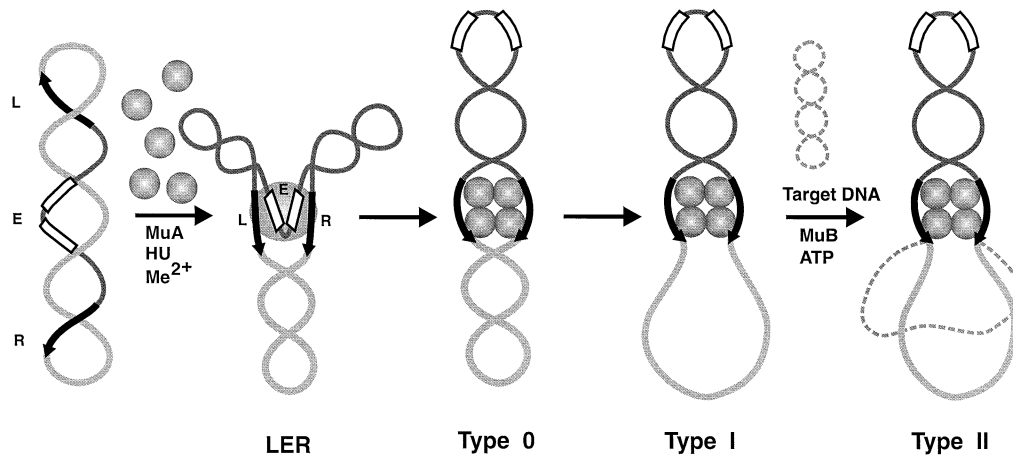


Fig. 1. Nucleoprotein complexes in Mu transposition. Monomeric MuA protein binds to the two Mu ends L and R (each composed of three att sites), and interacts with an enhancer element E on a negatively supercoiled plasmid, to promote rapid formation of the LER complex in the presence of divalent metal ions and *E. coli* HU protein. Ca²⁺ ions support conversion of LER to type 0, in which MuA has tetramerized and the enhancer is no longer associated with the ends. Mg²⁺ or Mn²⁺ ions also support formation of type 0, promoting cleavage of the synapsed Mu ends to produce the type I complex. MuB protein modulates the activity of MuA at each stage of the reaction, and captures target DNA in the presence of ATP to generate the type II strand transfer complex (Chaconas *et al.*, 1996).

II of MuA, which includes a DDE motif (presumed to coordinate Mg²⁺), is the main catalytic domain, while the C-terminal domain III is required for both tetramer assembly and interaction with the MuB protein. Recent studies have shown that only two subunits within the MuA tetramer, those associated with the attL1 and attR1 sites, provide their DDE residues towards the strand cleavage/transfer reactions (Figure 2B; Namgoong and Harshey, 1998). The DDE contribution occurs *in trans*, as revealed by directed protein placements in negatively supercoiled substrates (Namgoong and Harshey, 1998) or in linear attR substrates under altered reaction conditions (Aldaz *et al.*, 1996; Savilahti and Mizuuchi, 1996). Taken together, the outcomes from the various experimental strategies conform to the interpretation that it is the L1-bound monomer that orients the nucleophile (water or the hydroxide ion) for cleavage at R1, and vice versa.

We have analyzed the bipartite interactions of a transposase monomer with the enhancer and att sequences that are responsible for its incorporation into an active tetramer. To do so, we exploited similarities and differences between the transposition systems of Mu and the Mu-related phage D108 (see DuBow, 1987). The MuA and D108A proteins are highly specific for their cognate enhancers (Toussaint *et al.*, 1983; Yang *et al.*, 1995a,b) by virtue of their non-homologous I α subdomains (Harshey *et al.*, 1985; Mizuuchi *et al.*, 1986; Leung *et al.*, 1989; Clubb *et al.*, 1994; Yang, 1995). Otherwise the two proteins are essentially identical, and can bind to and cleave either Mu or D108 ends under reaction conditions that bypass the enhancer. For our experiments, we have used a MuA derivative in which the native I α subdomain has been replaced by the equivalent peptide region from D108A (Yang *et al.*, 1995b). For the purposes of this report, we refer to this chimeric protein (which can transpose D108 but not Mu) as D108A. By assaying how enhancers of Mu-D108 hybrid specificity respond to mixtures of MuA and D108A proteins or their variants, we have mapped the interactions between elements within the enhancer sequence and the L1 and R1 att sites.

Results

Rationale for the construction of substrates with hybrid enhancers

Before its function in transposition was discovered, the enhancer was recognized as the operator whose interaction with the Mu/D108 repressor controlled divergent transcription of the immunity and transposition/replication functions of the two phages *in vivo* (Goosen and Van de Putte, 1987; Levin and DuBow, 1989). Three sites for repressor binding (O1–O3) were identified in Mu, and two in D108 (O1–O2). MuA was shown to bind the Mu operator sites *in vitro* (Craigie *et al.*, 1984), two of which (O1–O2) were sufficient for enhancer function (Surette *et al.*, 1989). The *E. coli* protein integration host factor (IHF) was demonstrated to bind the enhancer region between the O1 and O2 sites on both phage genomes (Krause and Higgins, 1986; Kukulj and DuBow, 1992). IHF was shown to be required for Mu enhancer function *in vitro* only when the superhelical density of the plasmid was low (Surette and Chaconas, 1989). While a single IHF-binding consensus was identified in the Mu enhancer (nt 947–959), two potential IHF sites in opposite orientations (sites 1 and 2; nt 906–894 and 911–923, respectively) were identified in the D108 enhancer (Kukulj and DuBow, 1992; see Figure 3).

The design of the hybrid enhancer substrates was based on the available footprinting patterns, derived using neocarzinostatin (NCS) protection, for the association of Mu and D108 transposases with their respective enhancers (data summarized in Figure 3; Yang, 1995). While the MuA NCS footprints were similar to the DNase I footprints obtained with the Mu repressor (Goosen and Van de Putte, 1987), the D108A footprints revealed a third O3 protection region not reported with the D108 repressor (Levin and DuBow, 1989; Kukulj and DuBow, 1992). In designing hybrid Mu-D108 enhancers, we utilized the similarly oriented IHF-binding sequences between O1 and O2 as the points of exchange (Figure 3). Although there are two potential IHF-binding sequences (sites 1 and 2) in D108,

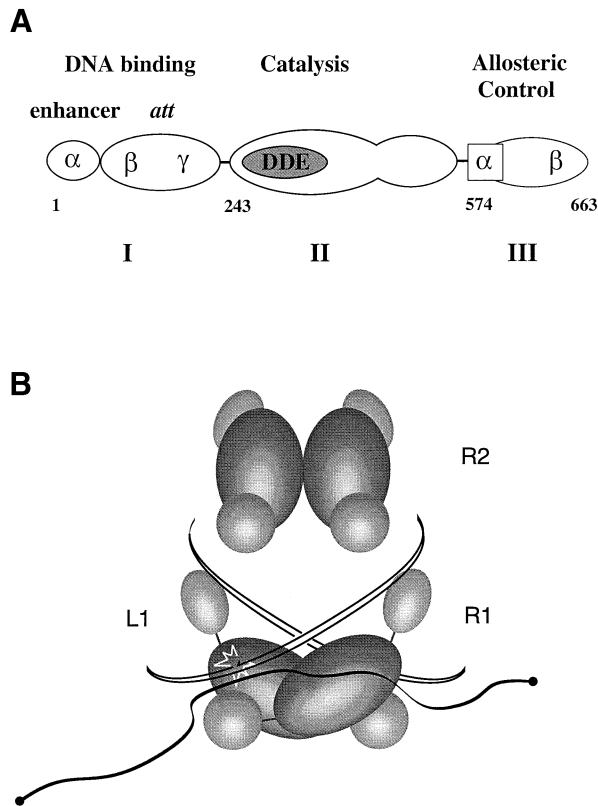


Fig. 2. Organization of MuA and contribution of DDE domains in transposition. (A) On the basis of limited proteolysis, three domains (I–III) were assigned to MuA protein (Nakayama *et al.*, 1987), whose general functions are indicated. Amino acid numbers corresponding to the amino terminus of each major domain are shown beneath the structure. (B) A model for the arrangement of the DDE-contributing MuA subunits during transposition from supercoiled Mu DNA is depicted. Within the transposase tetramer, the two subunits bound through domains I to attL1 and attR1 donate domains II (containing DDE) *in trans* towards the cleavage (broken DNA chain), and subsequent strand transfer of the right and left Mu ends, respectively. The association of target DNA (identified by filled circles at each end) with the subunits at L1 and R1 is hypothetical. Specific structural/catalytic functions have not yet been assigned to the two other subunits in the tetramer. See text for details.

the relative orientation of site 1 is opposite to that of the Mu IHF site. The site 2 sequence has a better match to the IHF consensus, and includes a flanking AT-rich element that enhances IHF binding to DNA (Hales *et al.*, 1994). The DNA exchange strategy ensures that the relative positioning of the operators with respect to the ‘hybrid IHF site’ is the same as that with respect to the native IHF sites in the phage genomes. The two- or three-site substrates (O1–O2 or O1–O2–O3, respectively) used in this study are schematically diagrammed in Figure 3. For simplicity, the origin of the enhancer site is designated by the first letter of each phage. For example, MM refers to O1–O2 being derived from Mu, MD to O1 from Mu and O2 from D108 etc. The plasmid substrates containing all three enhancer sites derived either from Mu (MMM; pZW140) or from D108 (DDD; pJY140D) have been described previously [Wang *et al.* (1996) and Yang *et al.* (1995a), respectively].

Enhancer and att site interactions in Mu transposition

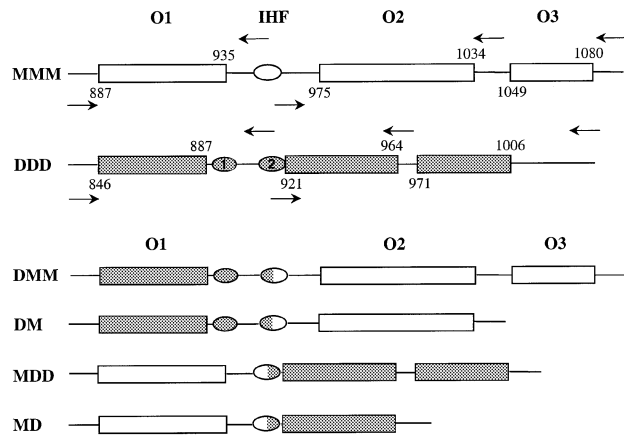


Fig. 3. Transposase footprints on enhancer DNA and strategy for constructing hybrid enhancer substrates. Neocarzinostatin protection experiments (not shown) revealed three footprints (O1–O3) of each transposase on its respective enhancer (the numbers refer to the nucleotide positions from the left end of each phage genome; see Mizuuchi *et al.*, 1986; Priess *et al.*, 1987). MuA footprints extended from nt 887–935 (O1), 975–1034 (O2) and 1049–1080 (O3), while D108A footprints extended from nt 846–887 (O1), 921–964 (O2) and 971–1006 (O3). IHF-binding consensus sequences are indicated as ovals. They are found from nt 947–959 in Mu, and 906–894 (site 1) and 911–923 (site 2) in D108. Site 2 is opposite in orientation to site 1. Oligonucleotide primers at the positions indicated (arrows) were used for PCR amplification of either O1, O2 or O2–O3, followed by ligation to create O1/O2 or O1/O2–O3 hybrid substrates. See text for details.

Reactivity of plasmid substrates containing the Mu-D108 hybrid enhancers

The efficiency and transposase-specificity of the native and hybrid substrates in type I assays is shown in Figure 4. The non-hybrid O1–O2 and O1–O2–O3 substrates (lanes 1–8) were reactive only with their respective cognate transposases (absence of type I in lanes 2, 4, 5 and 7). A small (~5%) but reproducible enhancement in the formation of the type I cleaved complex was noted with the three site substrates (DDD and MMM) relative to the two-site substrates (DD and MM) (Figure 4, compare lane 3 with 1 and lane 8 with 6). Results with the hybrid substrates were as follows. When the O1 site was derived from D108 (DM and DMM; lanes 9–12), D108A was active (lanes 9 and 11), while MuA was inactive (lanes 10 and 12). When the substrates contained the Mu O1 site (MD and MDD; lanes 13–16), MuA reacted efficiently to yield type I (lanes 14 and 16). D108A was also active on the MD substrate (lane 13); in contrast, with the MDD substrate, the activity of D108A dropped sharply (lane 15).

The following conclusions may be derived from the results in Figure 4. First, the O1 enhancer site is an important determinant of the compatibility between a DNA substrate and a given transposase. An exception to the O1-transposase specificity rule is the MD substrate. It responds to MuA and D108A equally well in type I production (lanes 13 and 14). The reason that MD is active with D108A may perhaps be due to a less stringent enhancer specificity of D108A than MuA. Note that the addition of the D108 O3 to MD to make it MDD results in strong discrimination against D108A, but does not alter the reactivity to MuA. This result would be consistent with the titration of D108A protein away from M at the

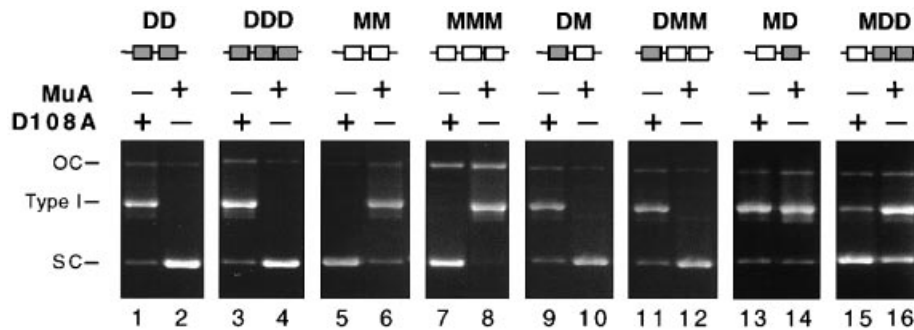


Fig. 4. Activity of MuA and D108A on two- and three-site wild-type and hybrid enhancer substrates. Plasmids substrates were incubated with 0.4 μ g of MuA or D108A proteins and assayed for type I complex formation as described in the Materials and methods. The supercoiled (SC) and open circular (OC) forms of the plasmids are indicated.

O1 site by the additional D at the O3 site [molar ratio of DNA (which includes six att sites and either two or three enhancer sites) to A protein is 1:14 in all lanes]. The resulting decrease in the effective concentration of D108A coupled with its weaker affinity for M might be sufficient to make it function less well on MDD compared with MD. A second conclusion from the results in Figure 4 is that although the O1–O2 sequences constitute the minimal enhancer, the participation of O3 in the reaction is non-trivial. O3 not only plays a role in promoting optimal reaction (the DDD and MMM substrates are better substrates for type I formation than the DD and MM substrates, respectively), but may also contribute to enhance transposase selectivity by O1, at least when the latter is derived from Mu (MDD shows strong discrimination against D108A compared with MD).

Reactions of the DM hybrid enhancer substrates with mixtures of wild-type and mutant transposases

Previous studies have shown that cleavage of the left and right Mu ends by the MuA tetramer requires the presence of the catalytic DDE triad on the monomers stationed at the R1 and L1 sites, respectively (Figure 2B; Namgoong and Harshey, 1998). What role, if any, does the enhancer play in positioning the transposase subunits at these two sites? To address this issue, we tested a substrate containing a hybrid enhancer (DM) against pairwise mixtures containing the wild-type transposase specific to one enhancer and a mutant transposase specific to the other enhancer (Figure 5).

The catalytically inactive DDE⁻ variant used in these studies carries an alanine substitution at Glu392 (Kim *et al.*, 1995). The results of pairing wild-type D108A with MuA(E392A) or MuA with D108A(E392A) on the DM substrate are shown in Figure 5A. As expected from the data in Figure 4, only D108A was active by itself on this substrate, generating either the cleaved type I complex with the wild-type protein (Figure 5A, lane b), or the uncleaved type 0 complex with D108A(E392A) (lane g). MuA or MuA(E392A) were not active (Figure 5A, lanes a and c). However, in reactions containing D108A and MuA(E392A) in the molar ratios indicated (Figure 5A, lanes d–f), formation of the type I complex as well as the nicked or open circular form of the substrate (OC) was observed. (The increase in OC probably results from disintegration of the type I complex.) There was very little

accumulation of the uncleaved type 0 complex in the mixed reactions. Under these reaction conditions 0.1 μ g of D108A by itself yielded a barely detectable type I reaction (data not shown). Thus MuA(E392A), together with limiting amounts of D108A, was able to stimulate the assembly of cleavage-competent tetramers on the DM substrate. Type I complexes were also generated by mixtures of MuA and D108A(E392A) at different molar ratios (Figure 5A, lanes h–j), even though each protein by itself was unable to cleave this substrate (lanes a and g). With this protein combination, there was no accumulation of the nicked circular form of the substrate. However, detectable amounts of the uncleaved type 0 form were produced at all protein ratios.

It is known that type I complexes cleaved only at the left end are unstable, and a significant fraction is dissociated into the free DNA form when assayed by gel electrophoresis. They also show poor strand transfer activity when provided with a target DNA (Namgoong and Harshey, 1998). Complexes cleaved at the right end alone are more stable, and yield small amounts of single-ended strand transfer products. When strand-transfer assays were performed in the presence of MuB protein, ATP and target DNA, efficient double-ended strand transfer was obtained with wild-type D108A, no strand transfer was detected with the D108A/MuA(E392A) mixture (equivalent to lane e in Figure 5A), and weak single-ended strand transfer was obtained with the D108A(E392A)/MuA mixture (equivalent to lane i in Figure 5A) (data not shown). Based on these observations, it seemed plausible that cleavage had occurred at the left end alone of Mu in the d–f reactions in Figure 5A, and at the right end alone in the h–j reactions (see below). When assays similar to those in Figure 5A were repeated with the DMM substrate, qualitatively similar results were obtained (results not shown).

Identification of the cleaved ends in the DM substrate

To determine whether one or both ends were cleaved in the reactions described above, DNA from both intact type I complexes as well as from the relaxed species (OC) was analyzed by digestion with suitable restriction enzymes followed by labeling the cut ends with terminal transferase (see Materials and methods). As shown in Figure 5B, *Bam*HI–*Xba*I double digestion followed by 3' end labeling would yield two fragments, LC1 and LC2,

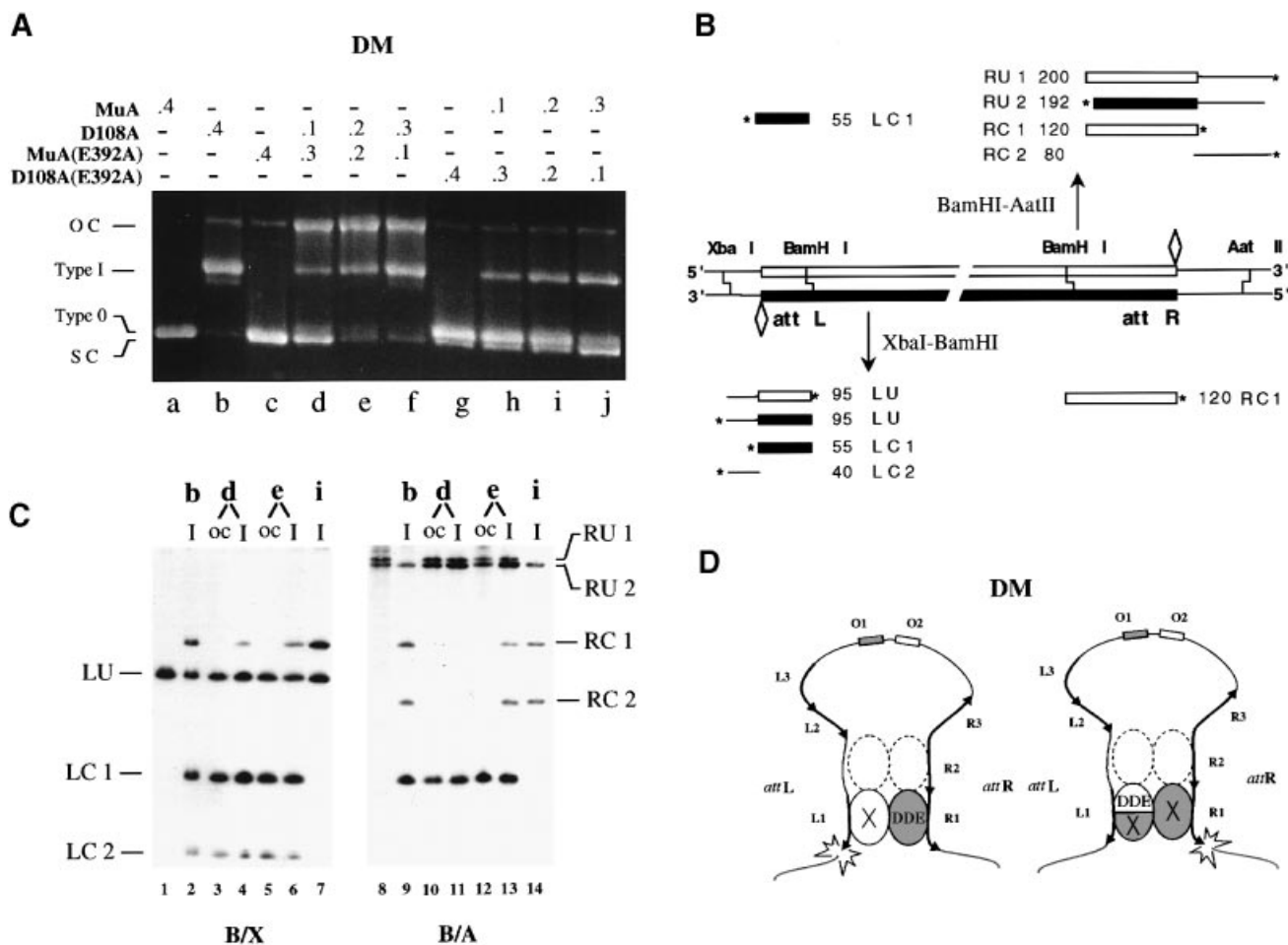


Fig. 5. Reaction of MuA, D108A, and their variants with the DM hybrid enhancer substrate. (A) The DM substrate was incubated with mixtures of the amounts indicated (in micrograms) of wild-type MuA or D108A proteins and their DDE⁻ variants, and assayed for type I or type 0 complex formation. Symbols as in Figure 4. (B) Since cleavage at Mu ends yield free 3' hydroxyl groups, a 3' end labeling strategy was used to assay left or right end cleavages in the type I complex. The top and bottom strands of the Mu genome are represented by the unfilled and filled bars, respectively. The diamonds indicate the strand cleavage positions. Double digestion with *Bam*HI-*Xba*I or with *Bam*HI-*Aat*II, followed by 3' end labeling (indicated by the asterisk) would give rise to the radioactive products indicated. They can be revealed by electrophoresis in denaturing polyacrylamide [see (C)]. Uncleaved attL generates an LU doublet consisting of a 95 nt fragment from the bottom strand, which has the same length as the fragment from the top strand; uncleaved attR generates RU1 from the top strand, which differs in length by 8 nt from the corresponding bottom strand fragment RU2. The products specific to left and right end cleavages are denoted by LC and RC, respectively. (C) The reactions corresponding to b, d, e and i of (A), were analyzed by the strategy outlined in B. Lanes 1 and 8 represent the substrate DNA that was not treated with MuA or D108A proteins. B/X and B/A stand for *Bam*HI-*Xba*I and *Bam*HI-*Aat*II restriction digestions. The symbols OC and I correspond to the isolated OC product and the type I complex, respectively. (D) Deduced position of transposase subunits at L1 and R1. DDE = DDE⁺ subunit; X = DDE⁻ subunit. Gray oval = D108A or its variant; white oval = MuA or its variant. The occupancy of an att site by either of the two transposases is represented by the half white/half gray oval. The two subunits in the tetramer whose enhancer specificity could not be addressed in these experiments are drawn with broken lines.

diagnostic of left end cleavage. The same reaction will also produce the RC1 fragment diagnostic of right end cleavage, and the coincident LU doublet. One component of the LU doublet is derived from molecules that are uncleaved by transposase on the bottom strand at the left end; the second LU component is contributed by all molecules, cleaved or uncleaved by MuA (since the transposase cleaves only one strand at each end). Similarly, in the *Bam*HI-*Aat*II-treated reactions, the RC1 and RC2 fragments would indicate right end cleavage, and the LC1 fragment would indicate left end cleavage. The RU1 band would result from molecules in which no right end cleavage has occurred on the top strand, while RU2 arises from the complementary bottom strand, independent of transposase-mediated cleavage.

The results of the cleavage analysis are shown in

Figure 5C. No cleavage at the Mu ends was detected in the control DNA not reacted with transposase (lanes 1 and 8). In the type I complexes generated with D108A alone (Figure 5A, lane b), cleavage was observed at both the left and right ends (Figure 5C, LC1, LC2 and RC1 in lane 2; RC1, RC2 and LC1 in lane 9). As was suspected from the assumption that the nicked circles (OC) result from the breakdown of the type I complex cleaved only at the left end, the reaction in lane d of Figure 5A [1:3 ratio of D108A: MuA(E392A)] showed nearly exclusive left end cleavage in the OC product (Figure 5C, lanes 3 and 10), and in the type I (I) product (Figure 5C, lanes 4 and 11). The trace of RC1 seen in lane 4, and of RC1 and RC2 in lane 11 can be accounted for by the fraction of the homotetramer of D108A (or a tetramer containing D108A on the L1 and R1 sites) assembled on the substrate.

Similarly, in the reaction with the 1:1 protein mixture (Figure 5A, lane e), the OC product contained only left end cleavage (Figure 5C, lanes 5 and 12), whereas the type I product contained, in addition, a small amount of right end cleavage (Figure 5C, lanes 6 and 13). The increase in the proportion of right end cleavage in lane 13 over lane 11 is consistent with the expected increase in the D108A homotetramer population (or in the occupancy of D108A on the L1 and R1 sites within the assembled tetramer) in the e reaction over the d reaction in Figure 5A. The type I complex generated by the MuA/D108A(E392A) mixture (Figure 5A, lane i; 1:1 molar ratio of proteins) showed exclusive right end cleavage (Figure 5C, lanes 7 and 14). Cleavage analyses with the DMM substrate and the binary protein mixtures gave the same results as the DM substrate (data not shown).

The differences in the intensities of the cleavage fragments in Figure 5C (and also in Figure 6B) need clarification. They result from differences in the efficiencies of the labeling reaction at the various 3' ends (see Figure 5B and Materials and methods). The ratio between the intensities of a particular cleavage fragment and the corresponding uncleaved fragment in a given reaction would be a valid estimate of the extent of transposase-mediated cleavage. By this criterion, the left and right end cleavages are equivalent for the control reaction containing wild-type transposase (Figure 5C, lanes 2 and 9 of reaction b). The ratio of LC1 plus LC2 to LU in Figure 5C, lane 2, roughly matched that of RC1 plus RC2 to RU1 plus RU2 in lane 9. Note that MuA cleavage at the left and right ends depletes one of the two LU fragments and the RU1 fragment, respectively, by splitting LU into LC1 plus LC2 and RU1 into RC1 plus RC2. As a result, in reactions containing left end cleavage products, the intensity of the LU band was reduced relative to that in the control reaction (Figure 5C, compare lanes 2–6 with lane 1). For the same reason, in reactions containing right end cleavage, the relative abundance of RU1 (RU1:RU2) was reduced with respect to the control reaction (Figure 5C, compare lanes 9, 13 and 14 with lane 8) or to reactions containing only left end cleavage (Figure 5C, compare lanes 9, 13 and 14 with lanes 10 and 12).

The conclusions from the data in Figure 5A and C are summarized in Figure 5D. Recall that according to the *trans* rule for DDE donation in MuA active site assembly, cleavage of the left and right ends requires a wild-type (for DDE) transposase monomer to be placed at R1 and L1, respectively. Furthermore, a cleavage competent monomer positioned at L1 or R1 is chemically active even when the other three monomers are mutant in DDE (Namgoong and Harshey, 1998). The cleavage patterns obtained with the DM substrate using a mixture of D108A and MuA(E392A) place the D108A monomer at the R1 site, and exclude the placement of MuA(E392A) at this site. The absence of significant amounts of the type 0 (uncleaved) complex in the d–f reactions in Figure 5A, and the preponderance of left end cleavage in reaction e (Figure 5C, lane 6) are consistent with D108A being present at R1 in virtually all of the complexes, and MuA(E392A) being present at L1 in a large majority of the complexes (Figure 5D, left). Similarly, the absence of left end cleavage in a reaction containing a mixture of MuA and D108A(E392A) (i in Figure 5A; see lane 7 of

Figure 5C) attests to the absence of MuA at R1. However, the detectable accumulation of the type 0 complex in the reactions h–j in Figure 5A (approximately 1:1 of type 0 to type I in i) indicates that among complexes containing D108A(E392A) at R1, the L1 site may be occupied by either MuA or D108A(E392A) with roughly equal probability (Figure 5D, right).

Taken together, our results ascertain that the distribution of the transposase subunits at the Mu ends is determined by their specificities for the enhancer elements. The O1 site of the enhancer sequence specifies the occupancy of the transposase monomer at R1. The O2 site, on the other hand, promotes the placement of its cognate transposase at L1. The O2–L1 rule is less rigid than the O1–R1 rule. This is exemplified by the fact that the plasmid containing the DM enhancer is a good substrate for wild-type D108A, but refractory to wild-type MuA. Thus, even wild-type D108A can break the O2–L1 rule. However, the O2–L1 preference is revealed in reactions containing both transposases, MuA and D108A, only one of which harbors the O2 recognition. Our data suggest that transposase occupancy of L1 is influenced by whether R1 is filled by wild-type D108A or D108A(E392A). In the former case, it is MuA(E392A) rather than D108A that is most often positioned at L1; in the latter case, either MuA or D108A(E392A) has equal chances of occupying L1. These findings may reflect subtle differences between the wild-type and mutant proteins in their affinities for the enhancer and/or att sequences, or in their intersubunit cooperativity.

An MDD hybrid substrate confirms the geometric correspondence between the enhancer and att sites

If the specific att to enhancer interactions derived above are valid, then reversing the order of the O1–O2 elements (from DM to MD) should reverse the relative distribution of the transposase subunits (Figure 6). The MD substrate is not desirable for testing this hypothesis, since MuA and D108A are strongly reactive towards it (see Figure 4). On the other hand, the MDD substrate is suitable, since MuA acts on it efficiently, while the background reaction due to D108A is acceptably low (Figure 6A, compare lanes a and b).

The experiments were performed as in Figure 5, using the same pairs of wild-type and mutant protein combinations. There is remarkable concordance between the results in Figures 6 and 5. The reactions with MuA(E392A) and D108A (Figure 6A, lanes d–f) yielded type I, along with roughly equivalent levels of type 0 at 1:1 molar ratio of the proteins (lane e). The isolated type I complex from reaction e contained only right end cleavage (Figure 6B, RC1 in lane 3; RC1 and RC2 in lane 10); in contrast, the type I complex from the MuA reaction (Figure 6A, lane a) contained both left and right end cleavages (Figure 6B, LC1, RC1 and RC2 in lane 2; LC1, RC1 and RC2 in lane 9). The MuA/D108A(E392A) reactions (Figure 6A, lanes h–j) gave rise to nicked circles and type I, with no detectable trapping of type 0 at an equimolar protein ratio (lane i). While the nicked circles were cleaved exclusively at the left end (Figure 6B, LC1 and LC2 in lane 4; LC1 in lane 11), the type I contained some right end cleavages as well (Figure 6B, presence of RC1 in lane 5, and of

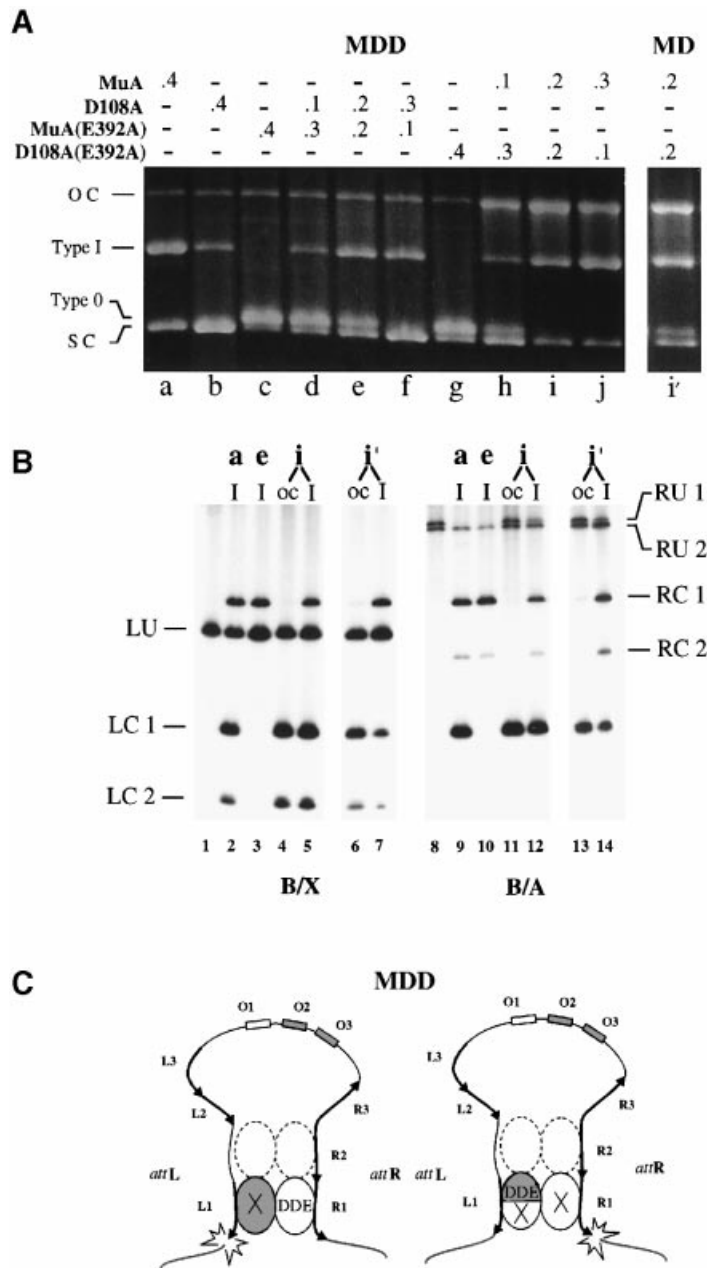


Fig. 6. Reactions of the MDD (or MD) hybrid enhancer substrates with MuA, D108A and their variants. (**A**, **B** and **C**) as in Figure 5A, C and D, respectively, except with the MDD (or MD) substrates.

RC1 and RC2 in lane 12). Since there were roughly twice as many nicked circles as type I in the i reaction, we estimate that the fraction of right end cleavages must be <10% of the total cleavages. Control DNA not reacted with transposase was not cleaved at either end (lanes 1 and 8).

As summarized in Figure 6C, the outcome of the reactions with MuA plus D108A(E392A) on the MDD substrate places MuA at the R1 site and the D108A mutant at the L1 site (arrangement at left). The strong cleavage bias against the left end in the MuA(E392A)/D108A reactions, together with the formation of detectable amounts of type 0, positions MuA(E392A) at R1, and either D108A or Mu(E392A) at L1 (arrangement at right). Thus, the hybrid enhancers DM and MDD reveal the strong correspondence between transposase recognition of

the left operator element of the enhancer (O1) and its association with the right att site (R1) during the assembly of the transposition complex. They also reveal a similar, though more relaxed, association between the right operator element (O2) and the left att site (L1). Note that the D108A transposase can assemble a functional tetramer on the MDD substrate, as revealed by the small amount of type I formation by D108A (Figure 6A, lane b), and by the more abundant formation of type 0 by D108A(E392A). Yet, in the presence of MuA at one-third its own amount (Figure 6A, lane h), the D108 protein fails to occupy the R1 site. This is revealed by the large reduction of type 0, and the concomitant formation of nicked circles cleaved only at the left end, and type I cleaved predominantly at the left end. The rarity of right end cleavage even when half the protein fraction is MuA (Figure 6A, lane i;

Figure 6B, the sum of lanes 4 and 5 or 11 and 12 indicates that MuA does not effectively compete with its D108 counterpart for the L1 site when the right operator site (O2) is derived from D108. This effect is unlikely to be mediated by the O3 operator present on MDD. Even in an MD reaction equivalent to that of *i* with MDD [Figure 6A, lane *i*', 1:1 ratio of MuA to D108A(E392A)], the extent of right end cleavage relative to left end cleavage was quite small (Figure 6B, sum of lanes 6 and 7 or 13 and 14). This is despite the fact that, in the absence of the competing transposase, MD is a strong substrate for either MuA or D108A (see Figure 4, lanes 13 and 14). Consistent with the cross-reactivity of MD, a trace amount of type 0 was detectable in the *i*' reaction (Figure 6A), indicating the occupancy of both L1 and R1 by D108A(E392A).

Finally, we note that even on natural enhancer substrates (MMM and DDD) mixtures of either MuA and D108A(E392A) or D108A and MuA(E392A) reveal a distribution of the transposase proteins according to the rules derived above with the hybrid enhancer substrates (our unpublished data).

Discussion

Enhancer-specified transposase arrangement on Mu att sites

During the assembly of the Mu transpososome complex, there is a 'crosswise' communication between the operator sites of the enhancer and the R1 and L1 att sites at which strand cleavages occur. Interaction of a transposase monomer with the left operator O1 is a prerequisite for its occupancy of the R1 site in the functional tetramer. Similarly, recognition of the right operator O2 by a transposase monomer results in its preferential placement at the L1 site. However, unlike the strict O1–R1 connection, L1 occupancy by a transposase monomer is not proscribed by a noncognate O2 sequence.

In order to derive the above enhancer/att site assignments, we have relied on the known catalytic contributions made by the MuA subunits located at the R1 and L1 sites (Namgoong and Harshey, 1998). Since the functions of the other two MuA subunits in the strand cleavage reaction have not yet been deciphered, our current strategy cannot reveal the role, if any, played by the enhancer in their positioning within the transposase tetramer. There is circumstantial evidence, though, that their placement may also be directed by the enhancer. First, maximal efficiency of tetramer assembly requires that all four MuA subunits contain the enhancer recognition region (Mizuuchi *et al.*, 1995). Secondly, the low basal activity of enhancer-independent deletion derivatives of MuA is stimulated by catalytically inactive variants harboring an intact enhancer-binding domain (Yang *et al.*, 1995a). Thirdly, mutational analyses have suggested enhancer interactions with all the six att sites (Allison and Chaconas, 1992; Lavoie and Chaconas, 1995). The latter experiments combined single mutant att sites with partially deleted O1 or O2 elements in assays for type I formation. A positive interaction was inferred if the activity of the att or enhancer mutants alone was no worse than a combination of the two. Accordingly, it was concluded that O1–R2, O2–R3 and O1–L3 interactions are functionally important (Allison and Chaconas,

1992). We wish to emphasize that there is no conflict between these results which examined enhancer interactions with the R2, R3 and L3 sites, and our present study which has examined enhancer interactions with the R1 and L1 sites. The mutational approach of Lavoie and Chaconas (1995) has also indicated O1–R1 and O2–L1 as the interacting partners (cited as unpublished data). Since the O1 and O2 regions protected by MuA/D108A are large (see Figure 3), interaction between a given operator site and more than one *att* site is quite plausible.

Functional dominance of O1–R1 interaction

The primary determinant of the reactivity of a hybrid enhancer substrate is the O1–R1 recognition mediated by the two DNA binding regions of a single transposase monomer. Even in the instance where this rule is apparently broken (the MD substrate that can be acted on by MuA or by D108A; Figure 4), a binary mixture of the wild-type and mutant forms of the two transposases reveals a highly preferred association of MuA at the R1 site (Figure 6B). The dominance of the long-range interaction between R1 and the L1-proximal O1 might facilitate synapsis of the right and left ends of Mu, which are separated by ~37 kb on the Mu genome. The sum of our results is consistent with a scheme in which the bipartite transposase interactions at O1–R1 and O2–L1 are central steps in establishing the correct synaptic configuration of the att sites, and the associated transposase tetramer. These interactions likely serve as geometric and/or topological filters (Gellert and Nash, 1987) to foster the functionally relevant associations among the individual transposase subunits during their maturation into the active tetrameric form.

Let us assume that the assembly of the transposition complex proceeds from an initial state consisting of an ensemble of substrate molecules containing all possible arrangements of MuA on the six att sites and the three operator sites. This assumption may not be strictly correct, since *in vitro* experiments indicate that MuA has a higher affinity for the att sites relative to the operator sites (Craigie *et al.*, 1984; Leung *et al.*, 1989). Nevertheless, it is clear that the multiplicity of DNA sites and their protein partners can potentially give rise to a large number of abortive inter-protein/inter-site associations. The enhancer may, in principle, facilitate the formation of the reactive MuA tetramer by stabilizing the correctly configured intermediates along the assembly pathway. The O1–R1/O2–L1 rules signify at least a subset of the requirements for the forward progression of the LER complex towards the type 0 complex. The analogy of this process to folding pathways in proteins has prompted Yang *et al.* (1995a) to characterize the enhancer as a 'DNA chaperone'.

Topology of the LER complex

The topology and geometry of the synaptic structures for the Gin and Hin invertases, the Tn3 and $\gamma\delta$ resolvases, and the Integrase family recombinase XerC/XerD have been surmised from the number and sign of recombination-specific crossings retained by the reaction products (Kanaar *et al.*, 1990; Heichman *et al.*, 1991; Stark *et al.*, 1992; Grindley, 1994; Colloms *et al.*, 1997; Murley and Grindley, 1998). Since the MuA cleavage reaction is hydrolytic (leading to nicked products), standard topological methods

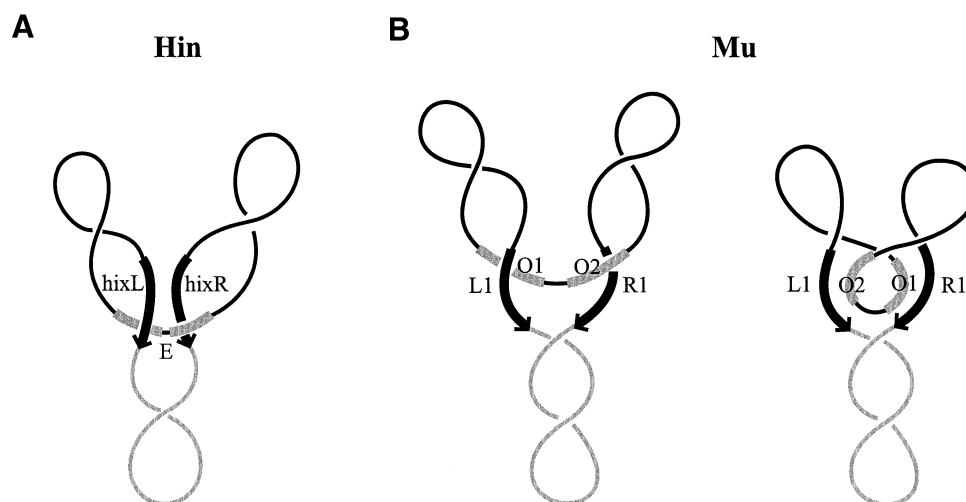


Fig. 7. Comparison of a possible synaptic arrangement of the Mu LER with the synaptic arrangement inferred for the Hin site-specific recombination system. The recombination target sites and their relative orientations in both systems are indicated by the arrows. In the Hin system (**A**), a three site alignment between the recombining sites (hixL and hixR) and the bipartite enhancer (E) is proposed to occur at the base of a plectonemic branch as shown. For the Mu system (**B**), a similar arrangement would lead to an O1–L1/O2–R1 interaction [(B), left]. The observed O1–R1 and O2–L2 interactions within the MuA mediated LER synapse can be accommodated by a solenoidal wrap of the enhancer DNA segment [(B), right], perhaps stabilized by the bivalent DNA binding properties of MuA. The DNA crossing that brings about O1–R1 and O2–L1 proximity may be facilitated by high negative superhelicity of the substrate. Or, at low superhelical densities, the same result may be achieved by the sharp DNA turn induced by IHF binding between O1 and O2 (see text).

cannot be employed to derive the topology of the transpososome. Nevertheless, the precise positioning and relative orientation of the att sites on the Mu genome, the essential requirement of negative supercoiling in the substrate, and the selective interactions between the att sites and enhancer (as implied in the O1–R1/O2–L1 rule) suggest that the transposition reaction is preceded by the assembly of a synaptic structure of defined topology.

The topology of the Mu system is expected to be most similar to that of the Hin/Gin systems (Figure 7A), given their common requirement for negative supercoiling and the inverted orientation of the reactive DNA sites (Craigie and Mizuuchi, 1986; Heichman and Johnson, 1990). However, the path to the two synaptic assemblies appears not to be the same. A three-site alignment (attL, attR and enhancer) at the base of a plectonemic branch would be most readily accommodated by an O1–L1/O2–R1 interaction (Figure 7B, left) as proposed for the Hin system (Figure 7A). However, interactions observed are the opposite: O1–R1 and O2–L1. A plausible model that would accept the criss-crossed operator–att site juxtaposition incorporates a solenoidal wrap in the LER complex (as in Figure 7B, right). Negative supercoiling in the substrate would favor a left-handed wrap that may be stabilized by MuA–enhancer interaction. Alternatively, the sharp DNA turn (Rice *et al.*, 1996) caused by IHF binding between O1 and O2 could facilitate this wrap. The absolute requirement for IHF in transposition at low substrate superhelicity (Surette and Chaconas, 1989) and its dispensability at high superhelicity would be consistent with this architectural role for IHF. Although the topology of the type 0 complex would be fixed by the LER complex, the att–enhancer interactions are disrupted prior to type 0 formation (Surette and Chaconas, 1992).

Coordination and control of strand breakage and joining reactions by enhancers

Enhancers control the rate and directionality of site-specific recombination reactions by promoting the assembly of

topologically correct, recombination competent structures only when the targets sites are arranged in a defined orientation in negatively supercoiled substrates. Mutations in the recombinase or the enhancer-binding protein that confer enhancer independence on a given reaction have been characterized (Haffter and Bickle, 1988; Klippel *et al.*, 1988; Deufel *et al.*, 1997; Arnold *et al.*, 1999). Consistent with the expected regulatory role of enhancers, the enhancer-independent reaction is released from the constraints of substrate supercoiling, synapse topology and target site orientation that the normal reaction is subject to (Klippel *et al.*, 1993; Crisona *et al.*, 1994; Arnold *et al.*, 1999). In addition to their structural role, enhancers can also modulate the activity of the recombinase by promoting specific protein–protein interactions. For example, the enhancer-bound Fis dimers induce conformational changes in the Hin recombinase dimers, orienting the active site residues for concerted cleavage in both the recombination partners (Merickel *et al.*, 1998). Similarly, the bipartite association of the catalytically inert MuA monomer with the enhancer and an att site promotes tetramerization by initiating its association with additional MuA monomers, concomitantly unlocking the catalytic potential for coordinated strand cleavages at the Mu ends. At least part of the negative controls operating on the monomer to keep it catalytically silent must be contributed by the enhancer binding region of MuA, since its deletion results in partial enhancer independence (Yang *et al.*, 1995b). Artificial reaction conditions [that include the addition of 15–25% dimethylsulfoxide (DMSO); Mizuuchi and Mizuuchi, 1989] can obviate the enhancer requirement, while simultaneously eliminating the need for substrate circularity or supercoiling. Thus, the properties of the Mu transposition enhancer parallel those of the enhancers characterized in site-specific recombination systems. In addition, the Mu enhancer plays a second critical role in regulating transposition by providing the binding target

for the lysogenic repressor that shuts off transposase expression (Goosen and Van de Putte, 1987).

Materials and methods

Construction of transposition substrates

Plasmids pJMM and pJDD were obtained by deleting the O3 enhancer site from pZW140 (Wang *et al.*, 1996) and pJY140D (Yang *et al.*, 1995a), respectively. This entailed creating an *EcoRI* site (by PCR mutagenesis; Innis *et al.*, 1990) at 1038 bp on the Mu genome and 969 bp on the D108 genome, followed by the deletion of an *EcoRI* fragment harboring the O3 site. Plasmids pJMD and pJMDD were constructed as follows. The DNA encompassing the O1 site of Mu and the left half of the IHF site on the Mu genome (from pZW140) was amplified by PCR (see Figure 3). Similarly, amplified DNA fragments encompassing the right half of the IHF site2 on the D108 genome (Figure 3) and either O2 or O2–O3 of D108 (from pJY140D) were prepared. The Mu DNA fragment was ligated in separate reactions with either the O2 fragment or the O2–O3 fragment from D108. Plasmids pJDM and pJDM were generated by an analogous procedure, except that O1 was derived from D108, and O2 or O2–O3 from Mu. In both sets of hybrids, the IHF sites were exchanged between 953 and 954 bp on the Mu genome, and between 919 and 920 bp on the D108 genome. All recombinant joints were verified by DNA sequencing. Plasmid maps can be obtained from the authors upon request.

Wild-type and variant transposase proteins

MuA, D108A, MuA(E392A) and D108A(E392A) have been described previously (Yang *et al.*, 1995b). They were purified to near homogeneity by previously published procedures (Kuo *et al.*, 1991).

In vitro assays for Mu DNA cleavage and strand transfer

Standard assays for strand cleavage (type I complex) and strand transfer to target DNA in the presence of MuB and ATP (type II complex) were done as described by Surette *et al.* (1987), except that 10 mM HEPES–KOH (pH 7.6) and 140 mM KCl were substituted for 25 mM Tris–HCl and 140 mM NaCl, respectively. Besides the amounts indicated of transposase proteins (0.1–0.4 µg), type I reactions contained 1 µg of donor DNA, and 0.2 µg of *E.coli* HU protein in 20 µl reaction volumes. Reaction mixtures were incubated at 30°C for 20 min, and analyzed by agarose gel electrophoresis.

Determination of Mu end cleavage

DNA bands were excised from ethidium bromide-stained agarose gels, purified using QIA Gel Extraction Kit (Qiagen), and digested with either *Bam*HI plus *Xba*I or with *Bam*HI plus *Aat*II. The DNA fragments were denatured by boiling for 3 min, quickly cooled to room temperature, and labeled with [α -³²P]cordycepin phosphate using terminal nucleotidyl transferase at 37°C for 1 h. Labeled products were fractionated over a Sephadex G-50 column to remove unincorporated label, electrophoresed on 6% denaturing polyacrylamide gels, and detected by autoradiography.

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