A unique right end–enhancer complex precedes synopsis of Mu ends: the enhancer is sequestered within the transpososome throughout transposition

Shailja Pathania, Makkuni Jayaram and Rasika M.Harshey¹

Section of Molecular Genetics and Microbiology and Institute of Cellular and Molecular Biology, University of Texas at Austin, Austin, TX 78712, USA

¹Corresponding author
e-mail: rasika@uts.cc.utexas.edu

Assembly of the Mu transpososome is dependent on interactions of transposase subunits with the left (L) and right (R) ends of Mu and an enhancer (E). We have followed the order and dynamics of association of these sites within a series of transpososomes prior to and during formation of a three-site complex (LER), engagement of Mu ends by the transposase active site (type 0 complex), cleavage of the ends (type I complex) and their transfer to target DNA (type II complex). LER appears to be preceded by a two-site complex (ER) where E and R are interwrapped twice, as in the mature transpososome. At each stage thereafter, the overall topology of five DNA supercoils is retained: two between E and R, one between E and L and two between L and R. However, L–R interactions within LER appear to be flexible. Unexpectedly, the enhancer was seen to persist within the transpososome through cleavage and strand transfer of Mu ends to target DNA.

Keywords: attL/attR/*Escherichia coli*/Mu/transpososome

Introduction

High-order nucleoprotein complexes direct the execution of various cellular DNA processes such as replication, transcription, repair, recombination and segregation. Several of these transactions involve the recruitment of distant or noncontiguous DNA sites (Pathania *et al.*, 2002; and references therein). Their characterization has relied upon lower resolution genetic, biochemical and physical methods, such as deletion analysis, footprinting experiments or electron microscopy. Results thus obtained are subject to the caveat that the assembled structures may not represent their native states because of the genetic manipulations imposed on them or the invasive nature of the procedures employed to probe them. We have recently described a more benign ‘difference topology’ assay that revealed the organization of a three-site DNA synapse involved in phage Mu transposition (Pathania *et al.*, 2002).

Assembly of the Mu transposition complex (the ‘transpososome’) requires the correct orientation of the two ends, attL (L) and attR (R), and an enhancer DNA element (E) (Figure 1A and B) in a negatively supercoiled substrate, as well as MuA and MuB proteins, *Escherichia coli* HU protein and divalent cations (Chaconas and

Harshey, 2002). The MuA transposase protein has two distinct DNA binding specificities, one for the att sites and the other for the enhancer. On the 37 kb Mu genome the enhancer is proximal to attL, located ~1 kb from this end (Morgan *et al.*, 2002). Originally mapped as the operator region to which the Mu repressor binds to regulate lysogeny/lysis, the enhancer plays a dual role in phage Mu physiology, being also required for the assembly of the transpososome (Figure 1B; Chaconas and Harshey, 2002). Maturation of the transpososome proceeds from a metastable LER complex, via the more stable type 0 complex in which MuA has assumed its active tetrameric configuration, to the highly stable type I complex in which strand nicking has occurred at L and R. Strand transfer of the cleaved Mu ends to target DNA results in the most stable type II complex. Even after strand cleavage within the type I complex the Mu DNA remains supercoiled, anchored by the MuA tetramer, while the non-Mu DNA is relaxed. MuB plays a stimulatory role throughout transposition and is involved in capturing target DNA for strand transfer (Chaconas and Harshey, 2002).

In order to map the DNA path within the Mu synapse by difference topology, the transposition complex was first assembled in the presence of a DNA substrate and the requisite protein components (Pathania *et al.*, 2002). Subsequently, a Cre-mediated exchange at two strategically placed loxP sites was used to trap the DNA crossings between a given Mu site and the other two. These crossings were counted as knot nodes or catenane nodes in the recombination products. From a series of coupled assembly and recombination assays, we deduced that the type 0 complex sequesters five negative supercoils by the combined L–E, R–E and L–R interactions. Earlier biochemical analyses had concluded that the enhancer–MuA association is transient, and is relevant only to the pre-type 0 state (Mizuuchi *et al.*, 1992; Watson and Chaconas, 1996). However, the difference topology analysis suggested that the enhancer remains associated with the type 0 complex and contributes three DNA crossings by plectonemic wraps with L and R (Figure 1B; Pathania *et al.*, 2002).

The results from the present study provide snapshots of events prior to LER formation and follow the fate of the enhancer through to completion of transposition. We show that the enhancer interacts uniquely with R, even in the absence of L and HU, and this interaction is topologically the same as that seen in a synapse containing all three sites. The association between E and R to establish the E–R topology appears to be an early event that is completed prior to the entry of L into the synaptic complex. Our data indicate that the enhancer remains associated with the transpososome even in the type I and type II complexes. We find that the topology of the L–R interaction is not as stable in LER as it is in type 0 and type I complexes.

Fig. 1. (A) The *attL* and *attR* ends of Mu can each be divided into three subsites: L1, L2 and L3 and R1, R2 and R3. In the Mu genome, *attL* and *attR* are in a head to head orientation. The enhancer, located between *attL* and *attR*, overlaps the operators O1–O3. (B) Nucleoprotein assemblies during transposition. Interaction between MuA and *attL* (L), *attR* (R) and enhancer (E) sequences triggers transpososome assembly in the presence of the *Escherichia coli* protein HU and divalent metal ions. Assembly proceeds sequentially from the earliest identified complex, LER, to type 0 where the transposase has assumed its tetrameric configuration but the ends are not cleaved, to type I wherein the ends are cleaved and finally to type II in which Mu ends are joined to target DNA, a reaction promoted by MuB protein in the presence of ATP (Chaconas and Harshey, 2002). The enhancer has thus far shown to be a part of the LER (Watson and Chaconas, 1996) and type 0 complexes (Pathania *et al.*, 2002). Although the tetrameric core of MuA (which footprints on L1, R1 and R2) is drawn to represent catalytically active complexes, all six *att* binding sites are occupied by MuA in these complexes (Chaconas and Harshey, 2002).

Results

The logic of difference topology assays

A detailed rationale for the difference topology analysis employed in this study can be found in Pathania *et al.* (2002). Briefly, each assay utilizes a pair of matched plasmid substrates that are similar in their arrangement of Mu sites and the location of *loxP* sites. They differ in the relative *loxP* orientations: direct in one case and inverted in the other. After assembling the Mu synapse, each plasmid is subjected to Cre recombination and the product topology analyzed. Imagine that the Mu synapse contains l interdomainal DNA crossings, n being odd. As explained previously (Pathania *et al.*, 2002), Cre inversion reaction from this synapse will display these crossings as a knot product with n nodes. The product of the corresponding Cre deletion reaction will be a catenane with $l + 1$ links. Now consider a Mu synapse with $l - 1$ (even) DNA crossings. In this case, the deletion catenane and inversion knot formed by Cre will contain $l - 1$ and l nodes respectively. The single additional catenane or knot node observed in each case is extraneous to the synapse and is introduced only to align the *loxP* sites in the correct geometry for the Cre reaction.

Two-site complexes preceding LER

Among the intermediates in transpososome assembly characterized to date, the three-site LER complex is the earliest. It appears to be quite unstable during conventional analytical procedures and could be detected initially only after glutaraldehyde cross-linking (Watson and Chaconas, 1996). On the other hand, using Cre recombination as a

reporter, the input substrate could be efficiently trapped as LER (Pathania *et al.*, 2002). Apparently, a majority of the ‘unstable’ LER molecules do not dissociate, at least not until after the Cre reaction has been completed. We reasoned that this assay had a good chance of revealing potential two-site complexes that precede LER, but could have escaped detection previously due to their transience.

A series of plasmid substrates were constructed to probe pairwise interactions among the three Mu sites (L, E and R) (Figure 2A). The control plasmids pSPIn and pSPDir contain these sites in their normal relative orientations (Pathania *et al.*, 2002). In addition, they harbor two *loxP* sites in inverted (In) or direct (Dir) orientation: one placed adjacent to *attL* and the other adjacent to *attR*. The distance between the center of each *loxP* and its neighboring *att* site was less than 200 bp (Pathania *et al.*, 2002). This short spacing was intended to minimize the random trapping of supercoils during Cre recombination from the transpososome–Cre hybrid synapse. Three derivatives were constructed from pSPIn and pSPDir by deleting the enhancer, *attL* or *attR* (indicated by ΔE , ΔL or ΔR respectively). In pSP ΔE (R), the *loxP* sites were positioned close to and on either side of *attR* (symbolized by placing R in parentheses) to separate it from the *attL* DNA domain. Potential DNA crossings between *attL* and *attR* could thus be recovered as catenane or knot nodes in the Cre recombination products. In pSP ΔL and pSP ΔR , designed to reveal E–R and E–L crossings respectively, the *attL* and *attR* sequences were deleted, while the *loxP* sites remained at their original locations. Deletion of an *att* site increases the distance from the proximal *loxP* site to the remaining *att* site by several hundred base pairs. Potential

Fig. 2. Interaction between pairwise combinations of *attL* (L), enhancer (E) and *attR* (R) sites. (A) Schematic maps of plasmids used to assess the binary interactions are shown. In one set of plasmids, the *loxP* sites (small rectangles) were in an inverted orientation [denoted by the suffix 'In' in (B)]. In a corresponding second set, the *loxP* sites were in a direct orientation [denoted by the suffix 'Dir' in (C)]. The results of Cre recombination performed on a subset of these substrates are shown in (B) and (C). The parentheses around R indicate that it was flanked by *loxP* sites. Reactions were deproteinized prior to agarose gel electrophoresis. Supercoiled, open circular and linear forms of the substrates are indicated as 'sc', 'oc' and 'l' respectively. D2 is the larger deletion circle resulting from Cre recombination. The supercoiled inversion and deletion products of MuA- and HU-assisted Cre recombination are labeled Kn(sc) and Ca(sc) respectively. The three- and five-noded knots in lanes 3 and 7 (B) or the 2-Cats in lanes 7 and 10 (C) were the result of some DNA nicking during the reaction.

non-specific trapping of supercoils in the topology assays was therefore a concern; hence, we derivatized pSPΔL to pSPΔL(R) such that the *loxP* sites flanking *attR* had the requisite short spacings so as not to contaminate E-R topology by random DNA crossings. A similar derivative was not constructed with pSPΔR, since we noted in preliminary experiments that pSPΔR did not give topologically complex products in MuA and HU-assisted Cre reactions (see, for example, Figure 2B).

In experiments that examine the topology of the LER complex using native *attL* and *attR* sites, the catalytically inactive MuA variant, MuA(E392A), was employed to

prevent the cleavage of Mu ends. Either MuA or MuA(E392A) was suitable for the assembly of LER complex on *attL* and *attR* sites containing point mutations that prevent their cleavage. However, both proteins were used with non-cleavable substrates, since the steady-state yield of the complex was significantly higher for MuA(E392A) than MuA.

Only two-site interactions between E and R add to the topology of Cre recombination products

A subset of the reporter plasmids shown in Figure 2A was subjected to Cre recombination with or without

Fig. 3. Topology of the ER complex. (A–C) Products of Cre recombination formed on the indicated In or Dir substrates after incubation of MuA or MuA(E392A) in the presence or absence of HU were examined by agarose gel electrophoresis after DNase I nicking. Lane M in each panel is a Cre alone reaction performed at high pH in order to generate marker knot or catenane ladders (see Materials and methods). All symbols are as in Figure 2. (D) Diagram illustrating how supercoil branch migration outside the ER synapse in the absence of HU results in the entrapment of additional random interdomainal nodes in pSP but not pSP(R).

pre-incubation in the presence of MuA or MuA(E392A) and HU [MuA(E392A) was used when the substrate was wild-type in order to prevent cleavage at Mu ends]. In the Cre alone reaction, nearly all of the recombinants from the inversion and deletion substrates were unknotted or unlinked for all plasmids (Figure 2B and C, lanes 2, 5, 9 and 12). The inversion products could not be distinguished from the corresponding substrates because of their identical electrophoretic mobilities. However, by appropriate restriction enzyme digestion, the extent of recombination in these plasmids was estimated to be in the range of 50–60% of the input substrate (data not shown). Of the unlinked deletion circles D1 and D2 resulting from Cre recombination, the smaller D1 circle had migrated off the gel. Cre reactions performed after pre-incubating the substrates with MuA or MuA(E392A) and HU gave supercoiled knots or catenanes [Kn(Sc) and Ca(Sc) respectively] with the parent pSP plasmids (Figure 2B and C, lane 3) and with pSPΔL(R) (Figure 2B and C, lane 10). The pSPΔE(R) and pSPΔR plasmids did not yield Kn(Sc) or Ca(Sc) products (Figure 2B and C, lanes 6 and 13). However, when the enhancer DNA was supplied *in*

trans as a linear fragment, topologically complex recombinants were formed from pSPΔE(R) (Figure 2B and C, compare lanes 6 and 7; Pathania *et al.*, 2002).

The sum of the inversion and deletion results from plasmids containing binary combinations of Mu sites show that it is only the E–R interactions that result in DNA crossings that are transmitted to the Cre recombination products. Neither E–L interactions nor L–R interactions in the absence of the enhancer lead to such DNA crossings. The paired inversion/deletion assays can detect even a single stable node between the DNA domains harboring the two sites. The inversion reaction will not generate a knotted product in this case. This is because a minimum of three crossings is required to form the simplest knot. However, because of the addition of a second node to properly orient the *loxP* sites in the deletion reaction, the product will be a two-noded catenane. Hence, an inversion unknot and a deletion 2-cat will indicate one crossing between the two DNA domains marked by the *loxP* sites. Interactions between sites that do not produce a stable crossing between their respective DNA domains will not be revealed by the topological assay.

We noticed that knotted or catenated recombinants were formed (even though at slightly reduced levels) following the action of Cre on pSPΔL(R) incubated with MuA in the absence of HU (data not shown). Since HU binds specifically to the L end (Lavoie and Chaconas, 1993), it is probably required only for the specific recruitment of *attL* into the transpososome. The present data suggest that productive interaction between E and R can proceed in the absence of L (and even of HU; Figure 3) and is perhaps a prerequisite for enlisting the MuA and HU bound L into the high-order assembly process.

Dissecting the topology of the ER complex

Is the ER complex suggested by the results in Figure 2 relevant to the normal transposition pathway? If it is, one might expect the topology of E–R interactions to remain constant whether or not they are established in the three-site plasmid pSP(R) or in the two-site plasmid pSPΔL(R). Reactions analogous to those depicted in Figure 2 were therefore analyzed by DNase I nicking followed by electrophoresis. In this set of assays and all subsequent assays, the topology of a relevant product was inferred from marker knot and catenane ladders generated by Cre recombination reactions carried out at high pH (see Materials and methods; Kilbride *et al.* 1999). These ladders are displayed in Figure 3, but are omitted from subsequent figures for simplicity. The authenticity of the three-noded knot or the four-noded catenane has previously been verified by electron microscopy (Pathania *et al.*, 2002).

For all plasmids, the major inversion products from the unassisted Cre reactions were open circles co-migrating with the parental plasmids. The corresponding deletion products were primarily the free circles D1 (which have migrated off the gels) and D2 (Figure 3C, lane 7). In the MuA plus HU-assisted Cre reaction, pSPΔL(R)In gave the trefoil knot as the prominent product (Figure 3A, lane 3). A similar reaction with pSPΔL(R)Dir yielded, in addition to the unlinked circles, 2-Cat as the major product and smaller amounts of 4-Cat (Figure 3A, lane 6). From the 2-Cat and trefoil combination of products, we conclude that the ER complex contains two crossings between these sites. This number agrees with our previous estimate of E–R crossings in the LER complex (Pathania *et al.*, 2002) and with the results from pSP(R) shown in Figure 3B.

In pSP(R), which contains L, R and E, the *loxP* sites were inserted on either side of *attR* to report, via Cre recombination, on the number of crossings it makes with *attL* and the enhancer. The expected number from previous work is four (Pathania *et al.*, 2002), two between L and R and two between E and R. Consistent with this expectation, the complete reaction with MuA(E392A) plus HU followed by Cre gave, in addition to the simple recombinants, the five-noded knot from pSP(R)In (Figure 3B, lane 3) and the four-noded catenane from pSP(R)Dir (Figure 3B, lane 7) as the strongest products. In the absence of HU, the corresponding products were the trefoil knot and the 2-Cat (Figure 3B, lanes 4 and 8), indicating the loss of two nodes. This result agrees with the absence of *attL* from the synapse (and of the two crossings it makes with *attR*) due to the lack of HU. However, the formation of a two-noded complex between *attR* and the enhancer appears to proceed without HU.

The MuA-mediated interwrapping of the enhancer and *attR* is expected to restrict the freedom of movement of the DNA regions harboring these sites. Entry of *attL* into this complex with assistance from MuA and HU and completion of the ‘three branched’ architecture of the synapse (Figure 3D, MuA plus HU; Pathania *et al.*, 2002) would impose a barrier to diffusion of supercoils (by slithering, for example) across these branches. When HU is absent, the DNA outside the ER branch, including *attL*, should be free from this constraint. When the flanking *loxP* sites are in close proximity to *attR*, as in pSP(R) substrates (Figure 3B), the mobility of supercoiled branches containing *attL* would not influence the product topology (Figure 3D, left, MuA alone). However, if one *loxP* site is fixed in the ER branch while the other is mobile (as the *loxP* sites would be in pSPIn and pSPDir; Figure 2A) recombination between the two is predicted to trap a distribution of knot nodes (for inversion) and catenane nodes (for deletion) (Figure 3D, right, MuA alone). Accordingly, a ladder of recombinant bands was observed in Cre reactions of pSPIn and pSPDir following incubation with MuA but no HU (Figure 3C, lanes 3 and 7). When HU was also present during pre-incubation, recombination suppressed the distribution of products, specifically enriching the three-noded knot and the four-noded catenane (Figure 3C, lanes 4 and 8). The product topology is consistent with the total of three crossings that the enhancer makes with *attL* and *attR*.

In summary, the combined outcomes from the data in Figures 2 and 3 suggest that the assembly of the transpososome is initiated by *attR* and enhancer interactions that can become topologically fixed without contributions from *attL* or HU. This topology is the same as the final ER topology observed in LER (Pathania *et al.*, 2002). Even when bound by MuA, *attL* appears to be freely mobile at this stage. Occupancy by HU restricts *attL* mobility and guides it to the ER complex, where it becomes assimilated into the final synapse by establishing one DNA crossing with the enhancer and two with *attR* (Pathania *et al.*, 2002).

Enhancer persists within the transpososome in type I and type II complexes

Our previous analyses suggested that the enhancer remains associated with L and R, crossing them a total of three times, in both the LER and type 0 (Figure 1; Pathania *et al.*, 2002). This result was not expected for type 0, since other unrelated experiments had suggested that the enhancer was released concomitant with type 0 assembly (Mizuuchi *et al.*, 1992; Watson and Chaconas, 1996). We therefore wished to test the status of the enhancer following the cleavage of Mu ends (type I complex) and their transfer to a target DNA (type II complex).

The enhancer topology in type I was probed using pSPIn and pSPDir (Figure 2A) in which the *loxP* sites marked the boundaries of the *attL*–*attR* domain and the enhancer domain. Roughly 50% of each substrate was assembled into the type I complex by MuA and HU in the absence of Cre (Figure 4A and B, lane 2). The Cre reactions on the free plasmid or following type I assembly were analyzed after deproteinization with or without DNase I nicking. Because Mu ends are nicked by the transposase, DNase I treatment is redundant for the knot products (Figure 4A,

Fig. 4. Association of the enhancer with type I and type II complexes. Cre recombination was performed after formation of type I (A and B) or type II (C) complexes on the indicated substrates. I and II refer to type I and type II products. Ca, catenane; hn, half nicked; T, target; Do, mini-Mu donor; D², donor dimer. Unlabeled bands above II in lanes 2–5 are most likely strand transfer products arising from the donor dimer. All other symbols are as in Figure 2.

lanes 3 and 4). Nicking is relevant for the catenane products since the cleaved Mu ends would be present on one of the two circles and the other would be supercoiled (Figure 4B, lanes 3 and 4). Note that the MuA tetramer in type I sequesters the supercoils in the Mu portion of the cleaved plasmid and strand exchange during Cre recombination permanently traps them. The faint bands migrating below the supercoiled substrate in lane 3 were due to supercoiled recombinants [Kn(sc)] formed from traces of uncleaved complexes (LER or type 0). The relevant

outcome is that Cre recombination from the type I complex gave almost all trefoils by inversion (Figure 4A, lanes 3 and 4) and four-noded catenanes by deletion (Figure 4B, lane 4). The corresponding products from the control Cre reaction were unknotted and unlinked (Figure 4A and B, lane 5). Thus, E crosses the L and R ends three times in the type I complex as it does in the LER and type 0 complexes (Pathania *et al.*, 2002). Topological characterization of products from Cre reactions coupled to the type II complex is complicated

because the target DNA is covalently linked to the cleaved Mu ends. Preparation of a reference knot or catenane ladder that carries this appendage, against which a recombinant band can be compared, is not straightforward. To simplify the analysis, we carried out the type II-Cre reaction only with the deletion substrate (pSPDir) and tested by restriction digestion whether the product circles were catenated or not.

The type II reaction mixture contained, in addition to the donor plasmid pSPDir, MuA and HU, MuB protein, ATP and the target plasmid. With an ~2-fold molar excess of the target (labeled 'T' in Figure 4C) over the donor (labeled 'Do' in Figure 4C), the supercoiled form of the latter was nearly quantitatively converted to type II and migrated, prior to deproteinization, as a slow mobility band (Figure 4C, lane 2). Upon SDS treatment, it resolved into a ladder of topoisomers (Figure 4C, lane 3). Recall that the supercoils in the Mu segment of the plasmid are barricaded by the MuA tetramer even after strand cleavage, even though the non-Mu part is relaxed. Once the theta shaped strand transfer product has been formed, the supercoils cannot be dissipated even after protein dissociation. Following Cre recombination, but without SDS treatment, the type II product ran as a doublet (Figure 4C, lane 4), ~60% being the faster species consisting of the recombination product (data not shown). In the SDS treated case, there was one prominent band (labeled '*') migrating just above the type II ladder that could be attributed to the Cre reaction product (Figure 4C, compare lanes 3 and 5). The unlinked small deletion circle D1 (containing the enhancer) could not be detected, suggesting that it was topologically linked to the larger circle [containing the strand transfer product; labeled 'II(D2)']. It was readily seen in a reaction in which pSPDir was treated with Cre alone (Figure 4C, labeled 'D1(oc)' in lane 8). Treatment of the type II-Cre reaction with *EcoRV*, which cuts within the smaller circle, released it as the linear D1 species (Figure 4C, lane 7). We conclude that the enhancer is not released from the transpososome even after strand transfer, even though we do not know its degree of linkage with the *att* sites.

In summary, data presented in Figure 4 show that the enhancer remains associated with the Mu ends during the chemical steps of strand cleavage and strand transfer and even after they have been completed.

L and R crossings in LER and type I complexes

In our previous study, we solved the complete topology of the type 0 complex organized by the cleavage incompetent MuA(E392A) and Mg^{2+} by determining the number of nodes that each Mu site makes with the other two: two R-E, one L-E and two L-R crossings (Pathania *et al.*, 2002). We have verified this estimate by assembling the type 0 complex differently, using wild-type MuA in the presence of Ca^{2+} (data not shown). The combined results from earlier work (Pathania *et al.*, 2002) and this study (Figures 3C, 4A and B) revealed the sum of the L-E and R-E crossings in the LER and type I complexes to be also three. We have now completed the topological analysis of the transposition pathway by examining the status of L-R crossings in the LER and type I complexes. The experimental strategy here was to perform Cre recombination after assembling the required complexes from the

pSPΔE(R)In and pSPΔE(R)Dir pair of plasmids (*loxP* sites flanking *attR*; Figure 2A) by supplying the enhancer *in trans* (Pathania *et al.*, 2002). Since the enhancer is unlinked, the knot-catenane configurations would provide exclusively the L-R topology. The results were further confirmed by using plasmid substrates containing the enhancer *in cis*.

Complete LER topology. The LER complexes (with enhancer *in trans*) were assembled on substrate variants containing point mutations at the cleavage sites in *attL* and *attR* (Watson and Chaconas, 1996; Pathania *et al.*, 2002) using MuA or MuA(E392A) in the presence of Mg^{2+} or MuA in the presence of Ca^{2+} . Since there is no simple method to directly visualize the LER complex, the extent of its formation was indirectly assayed by assessing the supercoiled knots or catenanes generated by Cre recombination. The results with the inversion substrate pSP*ΔE(R)In (the asterisk denotes that the Mu ends are non-cleavable) are shown in Figure 5A. The yield of supercoiled knots by MuA/ Mg^{2+} (Figure 5A, lane 3) was much lower than that with MuA(E392A)/ Mg^{2+} (Figure 5A, lane 2) or MuA/ Ca^{2+} (Figure 5A, lane 4). The same was also true of supercoiled catenanes obtained with the deletion plasmid pSP*ΔE(R)Dir (data not shown). When the pSP*ΔE(R)In reactions were analyzed after nicking, the trefoil knot was detected as the most prominent product from the LER/Cre reaction (Figure 5B, lanes 2–4). The corresponding product for pSP*ΔE(R)Dir was the two-noded catenane (Figure 5C, lanes 2–4). Therefore, the number of L-R crossings in the LER complex must be two. As was suspected from the levels of supercoiled Cre recombination products in Figure 5A, the MuA/ Mg^{2+} reactions resulted in the least amounts of the trefoil knot (Figure 5B, lane 3) and the two-noded catenane (Figure 5C, lane 3). Reactions carried out with pSP*ΔE(L)In and pSP*ΔE(L)Dir plasmids (*loxP* sites flanking *attL*) also revealed the same two-crossing L-R topology (data not shown).

To verify the enhancer *in trans* results, the LER/Cre reactions were repeated with the enhancer *in cis* using the following matched plasmid pairs: pSP*(R)In and pSP*(R)Dir for one reaction set and pSP*(L)In and pSP*(L)Dir for the other. In the nicked LER/Cre reactions from the pSP*(R) plasmids, the five-noded knot (Figure 5D, lanes 2 and 4) and the four-noded catenane (Figure 5E, lanes 2 and 4) were primarily enriched. Considerably smaller amounts of three- and seven-noded knots from inversion and two- and six-noded catenanes from deletion were also observed. Reactions with MuA/ Mg^{2+} also gave the five-noded knot and the four-noded catenane (Figure 5D and E, lanes 3) but in much poorer yields. Thus, four of the LER nodes can be trapped by Cre recombination (five noded-knot/four-noded catenane) when *attR* is flanked by *loxP* sites. This outcome is in agreement with two L-R crossings (inferred from the enhancer *in trans* assays) plus two additional E-R crossings (contributed by the enhancer *in cis*). For the pSP*(L) plasmids, the principal LER/Cre reaction products were the three-noded knot (Figure 5F, lanes 2–4) and the four-noded catenane (Figure 5G, lanes 2–4). Therefore, three synaptic crossings from LER

Fig. 5. LER topology. (A) Generation of Kn products from enhancerless substrates carrying point mutations at the cleavage sites. The enhancer was supplied *in trans* under the indicated reaction conditions. (B and C) Kn or Ca products formed on either pSP*ΔE(R)In or pSP*ΔE(R)Dir substrates were nicked with DNase I and examined by agarose gel electrophoresis. (D–G) Topology of LER formed on substrates with enhancer present *in cis*. Kn or Ca products formed on In and Dir versions of pSP*(L) and pSP*(R) substrates were nicked with DNase I and analyzed as in (C). All other symbols are as in Figure 2.

(three-noded knot/four-noded catenane) are included in the Cre reaction product when *attL* is flanked by *loxP* sites. They correspond to two L–R crossings and one E–R crossing. The sum of the results from Figure 5 demonstrates that the LER complex has the same five-noded DNA topology that was previously assigned to the type 0 complex.

The low levels of the knotted or catenated recombination products formed when the LER complex was assembled by MuA/Mg²⁺, rather than by MuAE392A/Mg²⁺ or MuA/Ca²⁺ is most likely due to unstable L–R crossings. In the presence of Mg²⁺ ions, the MuA active site is capable of acquiring its cleavage configuration. However, because of the mutant nucleotides at the cleavage position in the substrate plasmids, it may go through futile cycles of engaging and disengaging the Mu ends. At the same time, Cre attempts to recombine the *loxP* sites, one of which is located proximal to a Mu cleavage site. Potential steric conflicts arising from these DNA–protein dynamics could force the LER synapse to disassemble during strand exchange by Cre. The different behavior of wild-type MuA versus MuA(E392A) under Mg²⁺ conditions could be due to the observed differences

between these proteins in their intersubunit co-operativity (Jiang *et al.*, 1999).

Complete topology of the type I complex. In the first set of assays, the type I complex was assembled in pSPΔE(R) and pSPΔE(L) plasmids containing native *attL* and *attR* using MuA and Mg²⁺ with the enhancer supplied *in trans* (Figure 6A–D, lane 2). The subsequent Cre reactions uniquely enriched the trefoil knot and the two-noded catenane from the pSPΔE(R)In and pSPΔE(R)Dir plasmids respectively (Figure 6A and B, lane 3). The same results were obtained with the pSPΔE(L)In and pSPΔE(L)Dir plasmids as well (Figure 6C and D, lanes 3). No DNase I treatment was performed here because the products were already nicked by the action of MuA at the two ends. Thus, in the type I complex, *attL* and *attR* intertwining traps two DNA nodes. Similar assays were then performed with the enhancer present *in cis*. Now the pSP(R)In and pSP(R)Dir plasmids gave primarily the five-noded knot and the four-noded catenane as the type I/Cre recombination products (Figure 6E and F, lane 3). The corresponding products from pSP(L)In and pSP(L)Dir plasmids were the three-noded knot and the four-noded

Fig. 6. Topology of type I complexes when enhancer was provided *in trans* (A–D) or present *in cis* (E–H). The Kn and Ca products of Cre recombination in lanes 3 were not treated with DNase I since they were already nicked by MuA. All other symbols are as in Figure 2.

catenane (Figure 6G and H, lane 3). These results indicate that the Cre reaction traps four and three DNA crossings from the type I complex when the *loxP* sites border *attR* and *attL* respectively. They are consistent with two L–R crossings plus two E–R crossings in the first case and two L–R crossings plus one E–L crossing in the second. In other words, the type I complex sequesters five nodes in all (two L–R, two E–R and one E–L), as do the type 0 and LER complexes (Figure 7).

Discussion

Transposition of phage Mu requires the juxtaposition, cleavage and transfer of the *att* sites in a complex reaction promoted by the enhancer DNA and the transposase with assistance from accessory proteins and divalent cations. Distinct high-order protein–DNA assemblies are obligatory intermediates in the pathway leading to the acquisition of chemical competence for strand cutting and joining to target DNA. Topological characterization of these complexes suggests a defined order in the interaction of the enhancer with the two *att* sites (*attR* precedes *attL*) and demonstrates the persistence of the enhancer within the transpososome during strand cleavage and transfer. Furthermore, the DNA crossing pattern established by

the three sites upon synapsis is retained at least until the strand cleavage step.

Architecture of the transposition synapse

Data from the present study, in conjunction with our previous results (Pathania *et al.*, 2002), reveal two DNA crossings between the enhancer and *attR*, one between the enhancer and *attL* and two between *attL* and *attR* in the LER, type 0 and type I complexes. However, we noted that Cre recombination (used to entrap the synaptic nodes in the difference topology assays) tends to dissociate the LER complex assembled by MuA/Mg²⁺ (but not MuA/Ca²⁺ or MuA(E392A)/Mg²⁺) when the *loxP* sites border *attL* or *attR*. If L–R interactions during the early stages of transpososome assembly are still fluid, steric conflicts between synapsis of the *loxP* sites by Cre and attempts by a potentially active transposase active site to engage the Mu ends may disrupt LER. This finding is consistent with the previously reported instability of the LER complex (Watson and Chaconas, 1996) and with the more recent finding that the L1 footprint within LER is weaker than that within type 0 (Kobryn *et al.*, 2002). The conversion of LER to the type 0 transpososome is accompanied by tetramerization of MuA, as well as DNA melting around the Mu ends (Wang *et al.*, 1996; Kobryn *et al.*, 2002). Since LER cannot progress to type 0 when *attL* and *attR*

Fig. 7. A model for the ordered pathway of interaction of three distant sites during Mu transposition. The MuA-mediated interaction between E and R traps two supercoils and nucleates the assembly of the transpososome. L is recruited into this complex next, and contributes one more crossing with E. The two L–R crossings within LER are rather fluid (indicated by the interrupted lines), but become stable in type 0 (Figure 1). The five-noded topology is maintained in type I as well. The enhancer remains associated with the transpososome throughout transposition.

carry mutations that prevent cleavage, it has been suggested that Mu ends are not engaged in the transposase active sites prior to type 0 formation (Watson and Chaconas, 1996).

ER, an early intermediate in the LER pathway

The only stable two-site interactions reported by difference topology are those between the enhancer and *attR*. The E–R interactions occur in complete absence of L and are independent of the HU protein. Even in the presence of all three sites, upon omission of HU, only E–R interactions are topologically identified. In the absence of HU, however, the ER complex cannot capture L (Figure 3C). HU is known to bind and bend the DNA between the L1 and L2 subsites of *attL* (Figure 1A; Lavoie and Chaconas, 1993) and to deliver L1 LER (Kobryn *et al.*, 2002). HU can also bend DNA at the enhancer (Chaconas and Harshey, 2002). It is possible that a specific HU-induced conformation at one or both of these sites is required for entry of L into the transposition synapse.

According to our data, a stable E–L crossing is not formed even when HU is present and L–R crossings are not established unless the enhancer is present *in cis* or provided *in trans*. Earlier glutaraldehyde cross-linking experiments had indicated the existence of LR complexes in both the presence and absence of the enhancer (Watson and Chaconas, 1996). Potentially, an LR complex may be formed as an intermediate during the dissociation of the LER complex. Our data argue against functional L–R association in the absence of the enhancer. Note, however, that at least one stable node has to be formed between the two sites for detection by the topological assay. In contrast, glutaraldehyde cross-linking may trap transitory

interactions between these sites. In reactions using oligonucleotide substrates performed under different experimental conditions, L–R interactions were not observed unless enhancer was included in the reaction (Mizuuchi and Mizuuchi, 2001).

We suggest that ER is a critical early intermediate that nucleates the initiation of transpososome assembly and is a pre-requisite for the HU-assisted capturing of the L end to form LER. The functional dominance of the interactions between O1 and R1 (within the enhancer and *attR* respectively) revealed by hybrid enhancers and transposases encoding different enhancer specificities (Jiang *et al.*, 1999) are consistent with this proposal.

The first productive interaction of the enhancer with the right end, despite its proximity to the left end of the Mu genome, has regulatory implications. This effective shortening of the distance between *attL* and *attR* may facilitate the synapsis of these sites and trigger concomitant conformational changes in the transposase for its chemical competence. The E–R interactions could also serve as a ‘topological filter’ (Gellert and Nash, 1987) to ensure that the subsequent L–R interactions lead to a functional synapse. This site selectivity would be relevant during active transposition that generates multiple Mu ends within a host genome.

The enhancer is an integral part of the transpososome from start to finish

We have demonstrated that the three-noded enhancer–*att* site topology persists in the LER, type 0 and type I complexes. The enhancer also remains associated with the *att* sites even after strand transfer has been completed (in the type II complex), although the precise linkage of this

association has not been determined. While the enhancer is essential for conversion of LER to type 0, it is not essential for the chemical steps of strand cleavage and strand transfer (Surette and Chaconas, 1992). Perhaps the sequestration of the enhancer within the synapse prevents the Mu repressor from binding to it (see below), thus signaling a commitment to transposition. Alternatively, the enhancer may influence some as yet unknown aspect of strand transfer. When excess MuA is removed from the type I complex to leave only the tetrameric core, a procedure that probably strips the enhancer from the complex, strand transfer fails to occur unless MuB protein is added (Wu and Chaconas, 1997). Furthermore, functional similarities between MuB–target DNA complexes during strand transfer and the enhancer during MuA tetramer assembly have been proposed (Mizuuchi *et al.*, 1995). A potential role for the enhancer during the transition from transposition to Mu DNA replication is also not unlikely (Nakai *et al.*, 2001; Chaconas and Harshey, 2002).

Action of enhancers in different systems: a unifying theme?

Enhancers of DNA transposition have so far been discovered only in Mu and the related phage D108. The enhancers span the phage operator sites and are critical in lysis/lysogeny decisions. The repressors and transposases share extensive homology at their N-terminal domains and consequently have overlapping binding specificities. Thus, depending on which of the two proteins is bound, the enhancer/operator can bring about two mutually opposing outcomes: turning off phage gene expression or turning on transposition. The enhancer binding region of MuA is at least partly responsible for keeping the MuA monomer catalytically silent. Certain deletions removing this region from the N-terminus of MuA confer enhancer independence on transposition (Yang *et al.*, 1995). As shown by the present analysis, the sequential interaction of the enhancer and the *att* sites with concomitant entrapment of specific DNA crossings involving all three sites is critical in the stepwise maturation of the transpososome.

The Mu enhancer resembles enhancers or accessory DNA sites studied in site-specific recombination systems (Wasserman and Cozzarelli, 1985; Kanaar *et al.*, 1990; Heichman *et al.*, 1991; Stark *et al.*, 1992; Grindley, 1994; Colloms *et al.*, 1997) in that all of these DNA elements facilitate the organization of a unique stereospecific synapse that promotes DNA cleavage. In a unifying view, the enhancer is a DNA chaperone that channels the transposase or recombinase along a specific assembly pathway to yield a 'properly folded' high-order reaction complex (Yang *et al.*, 1995). Whether this analogy may be extended to enhancers of transcription systems (Schleif, 1992; Tjian and Maniatis, 1994; Tahirov *et al.*, 2002) remains to be seen. Currently, little is known regarding the topology of transcription complexes or the dynamics of enhancer interactions with the basal transcription apparatus. Whether the enhancer remains associated with the basal complex during promoter clearance is also not clear. Answers to these questions may clarify whether transposition/recombination enhancers and transcription enhancers share a unifying theme in their mechanism of action.

Materials and methods

Plasmids

pSPIn(Δ L) and pSPDir(Δ L) are deletion variants of the pSPIn and pSPDir plasmids, described in Pathania *et al.* (2002), and were constructed by deleting the *attL* containing *Bgl*III–*Xba*I fragment. pSPIn(Δ R) and pSPDir(Δ R) are the *attR* deletion variants of pSPIn and pSPDir. These were constructed by first deleting the *Aat*II–*Sal*I fragment containing *attR* from pMK21 (Kim *et al.*, 1995). *loxP* sites (Kilbride *et al.*, 1999) were introduced into the resulting plasmid, pMK21(Δ R), at *Nsi*I and *Pst*I sites in two orientations to obtain pSPIn(Δ R) and pSPDir(Δ R). pSPIn(Δ E) and pSPDir(Δ E) were constructed by deleting the enhancer fragment flanked by *Xho*I sites in pSPDir. The 1332 bp deleted fragment was substituted by a 1524 bp *Xho*I fragment from pEK26 (Grainge *et al.*, 2000). This substitution resulted in the enhancerless plasmid being larger than its parent plasmid. An *Ssp*I site in the substituted fragment was used to introduce a blunt-ended *Nsi*I fragment from pSPDir, carrying the *loxP* site, to give pSPIn(Δ E) and pSPDir(Δ E). Construction of pSP(L)In, pSP(L)Dir, pSP(R)In, pSP(R)Dir and their enhancerless variants pSP(L, Δ E)In, pSP(L, Δ E)Dir, pSP(R, Δ E)In and pSP(R, Δ E)Dir is described in Pathania *et al.* (2002). In pSP(R)In there were 85 and 120 bp between R1 and *loxP* and R3 and *loxP* respectively. In pSP(R)Dir, these same distances were 100 and 120 bp. In pSP(L)In there were 139 bp and 105 bp between L3 and *loxP* and L1 and *loxP*, respectively. In pSP(L)Dir these same distances were 139 bp and 149 bp.

Proteins

MuA, MuA(E392A) and *E. coli* HU proteins were purified as described (Yang *et al.*, 1995). Cre protein was also obtained as described (Grainge *et al.*, 2000). IHF was a generous gift from Steve Goodman (University of Southern California, CA).

Mu transposition complexes and Cre recombination reactions

LER, type 0, type I and type II complexes were assembled with donor mini-Mu plasmids (30 μ g/ml) at pH 7.6 (20 mM HEPES–KOH) in presence of 10 mM MgCl₂, 140 mM NaCl, 10 μ g/ml *E. coli* HU protein and 30 μ g/ml MuA or MuA(E392A). Reactions were incubated for 20 min at 30°C. MuB and ATP were present for the type II reactions at 10 μ g/ml and 2 mM respectively. pUC19 was used as target DNA (40 μ g/ml) for type II reactions. In some assays, the enhancer (O1–O2) was supplied as a DNA fragment *in trans* in 50-fold molar excess of the plasmid substrate. These reactions included the *E. coli* IHF protein at a molar ratio of enhancer to IHF of 1:1.6 (Surette and Chaconas, 1992). IHF binds between the O1 and O2 sites and is absolutely required (most likely for DNA bending) on linear enhancer substrates or on circular substrates when the superhelical density is low.

Cre was added to the transposition complexes and the reactions were further incubated for 30 min at 30°C. Cre recombination was stopped by adding SDS (final concentration 0.1%) and heat inactivating the protein at 75°C for 10 min. Samples were then processed as described by Grainge *et al.* (2000).

To obtain marker knot and catenane ladders Cre recombination was carried out at pH 9.0 (Kilbride *et al.*, 1999).

Acknowledgements

This work was funded by the National Institutes of Health (GM33247 to R.M.H. and GM35654 to M.J.) and in part by the Robert Welch Foundation (F-1351 to R.M.H. and F-1274 to M.J.).

References

- Chaconas, G. and Harshey, R.M. (2002) Transposition of phage Mu DNA. In Craig, N., Lambowitz, A., Gellert, M. and Craigie, R. (eds), *Mobile DNA II*. ASM Press, Washington, DC, pp. 384–402.
- Colloms, S.D., Bath, J. and Sherratt, D.J. (1997) Topological selectivity in Xer site-specific recombination. *Cell*, **88**, 855–864.
- Gellert, M. and Nash, H. (1987) Communication between segments of DNA during site-specific recombination. *Nature*, **325**, 401–404.
- Grainge, I., Buck, D. and Jayaram, M. (2000) Geometry of site alignment during int family recombination: antiparallel synapsis by the Flp recombinase. *J. Mol. Biol.*, **298**, 749–764.
- Grindley, N.D.F. (1994) Resolvase-mediated site-specific recombination.

- In Eckstein, F. and Lilley, D.M.J. (eds), *Nucleic Acids and Molecular Biology*. Springer-Verlag, Berlin, Germany, pp. 236–267.
- Heichman, K.A., Moskowitz, I.P. and Johnson, R.C. (1991) Configuration of DNA strands and mechanism of strand exchange in the Hin invertasome as revealed by analysis of recombinant knots. *Genes Dev.*, **5**, 1622–1634.
- Jiang, H., Yang, J.Y. and Harshey, R.M. (1999) Criss-crossed interactions between the enhancer and the *att* sites of phage Mu during DNA transposition. *EMBO J.*, **18**, 3845–3855.
- Kanaar, R., Klippel, A., Shekhtman, E., Dungan, J.M., Kahmann, R. and Cozzarelli, N.R. (1990) Processive recombination by the phage Mu Gin system: implications for the mechanisms of DNA strand exchange, DNA site alignment and enhancer action. *Cell*, **62**, 353–366.
- Kilbride, E., Boocock, M.R. and Stark, W.M. (1999) Topological selectivity of a hybrid site-specific recombination system with elements from Tn3 res/resolvase and bacteriophage P1 loxP/Cre. *J. Mol. Biol.*, **289**, 1219–1230.
- Kim, K., Namgoong, S.Y., Jayaram, M. and Harshey, R.M. (1995) Step-arrest mutants of phage Mu transposase. Implications in DNA-protein assembly, Mu end cleavage, and strand transfer. *J. Biol. Chem.*, **270**, 1472–1479.
- Kobryn, K., Watson, M.A., Allison, R.G. and Chaconas, G. (2002) The Mu three-site synapse: a strained assembly platform in which delivery of the L1 transposase binding site triggers catalytic commitment. *Mol. Cell*, **10**, 659–669.
- Lavoie, B.D. and Chaconas, G. (1993) Site-specific HU binding in the Mu transpososome: conversion of a sequence-independent DNA-binding protein into a chemical nuclease. *Genes Dev.*, **7**, 2510–2519.
- Mizuuchi, M. and Mizuuchi, K. (2001) Conformational isomerization in phage Mu transpososome assembly: effects of the transpositional enhancer and of MuB. *EMBO J.*, **20**, 6927–6935.
- Mizuuchi, M., Baker, T.A. and Mizuuchi, K. (1992) Assembly of the active form of the transposase–Mu DNA complex: a critical control point in Mu transposition. *Cell*, **70**, 303–311.
- Mizuuchi, M., Baker, T.A. and Mizuuchi, K. (1995) Assembly of phage Mu transpososomes: cooperative transitions assisted by protein and DNA scaffolds. *Cell*, **83**, 375–385.
- Morgan, G.J., Hatfull, G.F., Casjens, S. and Hendrix, R.W. (2002) Bacteriophage Mu genome sequence: analysis and comparison with Mu-like prophages in *Haemophilus*, *Neisseria* and *Deinococcus*. *J. Mol. Biol.*, **317**, 337–359.
- Nakai, H., Doseeva, V. and Jones, J.M. (2001) Handoff from recombinase to replisome: insights from transposition. *Proc. Natl Acad. Sci. USA*, **98**, 8247–8254.
- Pathania, S., Jayaram, M. and Harshey, R.M. (2002) Path of DNA within the Mu transpososome. Transposase interactions bridging two Mu ends and the enhancer trap five DNA supercoils. *Cell*, **109**, 425–436.
- Schleif, R. (1992) DNA looping. *Annu. Rev. Biochem.*, **61**, 199–223.
- Stark, W.M., Boocock, M.R. and Sherratt, D.J. (1992) Catalysis by site-specific recombinases. *Trends Genet.*, **8**, 432–439.
- Surette, M.G. and Chaconas, G. (1992) The Mu transpositional enhancer can function *in trans*: requirement of the enhancer for synapsis but not strand cleavage. *Cell*, **68**, 1101–1108.
- Tahirov, T.H. *et al.* (2002) Mechanism of c-Myb-C/EBP β cooperation from separated sites on a promoter. *Cell*, **108**, 57–70.
- Tjian, R. and Maniatis, T. (1994) Transcriptional activation: a complex puzzle with few easy pieces. *Cell*, **77**, 5–8.
- Wang, Z., Namgoong, S.Y., Zhang, X. and Harshey, R.M. (1996) Kinetic and structural probing of the precleavage synaptic complex (type 0) formed during phage Mu transposition. Action of metal ions and reagents specific to single-stranded DNA. *J. Biol. Chem.*, **271**, 9619–9626.
- Wasserman, S.A. and Cozzarelli, N.R. (1985) Determination of the stereostructure of the product of Tn3 resolvase by a general method. *Proc. Natl Acad. Sci. USA*, **82**, 1079–1083.
- Watson, M.A. and Chaconas, G. (1996) Three-site synapsis during Mu DNA transposition: a critical intermediate preceding engagement of the active site. *Cell*, **85**, 435–445.
- Wu, Z. and Chaconas, G. (1997) The Mu transposase tetramer is inactive in unassisted strand transfer: an auto-allosteric effect of Mu A promotes the reaction in the absence of Mu B. *J. Mol. Biol.*, **267**, 132–141.
- Yang, J.Y., Jayaram, M. and Harshey, R.M. (1995) Enhancer-independent variants of phage Mu transposase: enhancer-specific stimulation of catalytic activity by a partner transposase. *Genes Dev.*, **9**, 2545–2555.

Received March 3, 2003; revised May 22, 2003;
accepted May 23, 2003