Reorganization of the Mu Transpososome Active Sites during a Cooperative Transition between DNA Cleavage and Joining*

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Transposition of mobile genetic elements proceeds through a series of DNA phosphoryl transfer reactions, with multiple reaction steps catalyzed by the same set of active site residues. Mu transposase repeatedly utilizes the same active site DDE residues to cleave and join a single DNA strand at each transposon end to a new, distant DNA location (the target DNA). To better understand how DNA is manipulated within the Mu transposase-DNA complex during recombination, the impact of the DNA immediately adjacent to the Mu DNA ends (the flanking DNA) on the progress of transposition was investigated. We show that, in the absence of the MuB activator, the 3′-flanking strand can slow one or more steps between DNA cleavage and joining. The presence of this flanking DNA strand in just one active site slows the joining step in both active sites. Further evidence suggests that this slow step is due to a change in the affinity of the transpososome for the target DNA. Finally, we demonstrate that MuB activates transposition by stimulating the reaction step between cleavage and joining that is otherwise slowed by this flanking DNA strand. Based on these results, we propose that the 3′-flanking DNA strand must be removed from, or shifted within, both active sites after the cleavage step; this movement is coupled to a conformational change within the transpososome that properly positions the target DNA simultaneously within both active sites and thereby permits joining.

The successful relocation of mobile genetic elements, as with all DNA rearrangement processes, requires the precise spatial and temporal organization of DNA components within the active sites of large nucleoprotein complexes. This dynamic organization permits the proper series of DNA cleavage and joining reactions that comprise each DNA recombination pathway. Transposition and retroviral integration occur via one of two pathways that share common reaction steps (1, 2). Similar phosphoryl transfer reactions also take place during the early steps of VDJ recombination (3). Many transposases and integrases form a recombinase family related both by the three-dimensional structure of their catalytic domains and by a conserved set of acidic active site residues (4). Bacteriophage Mu transposase is a well studied member of this family.

Transposases and integrases promote recombination via either a replicative or cut-and-paste transposition pathway. These pathways share two common steps (see Fig. 1). First, one strand at each element end is hydrolyzed to yield a 3′OH at the terminus of the element sequence. This donor cleavage step separates this strand at each element end from the surrounding (or flanking) DNA. In the second common step, called DNA strand transfer or joining, each liberated 3′OH directly attacks closely spaced phosphodiester bonds in opposite strands of the DNA at the new location (the target DNA). Mu transposase promotes these two steps during replicative transposition to yield a transposition product in which each transposon end is covalently attached to both the flanking DNA and the target DNA. Cut-and-paste transposases catalyze one or more additional steps in which the non-transferred strand at each element end is cleaved, thus excising the transposon from the flanking DNA before joining.

The composition of the Mu nucleoprotein complex, or transpososome, in which these reaction steps take place is relatively well characterized (5). The stable Mu complex contains four identical transposase subunits (6–8). Each subunit contains several functional domains. Transposase subunits anchor both ends of the Mu genome within the transpososome (6, 9, 10) by specifically binding repeat sequences near the Mu DNA ends through an N-terminal domain (11, 12). Additional protein regions, within and adjacent to the catalytic core, have been implicated in nonspecific DNA binding (12–14) and are proposed to contribute amino acids to the active sites (8, 15–17). The catalytic core (14) contains the conserved DDE residues characteristic of the larger transposase/integrase family. These active site DDE residues are required specifically for the chemical steps of transposition (15, 18, 19) and coordinate the necessary catalytic divalent metal ions (20–22). The transposase catalytic core and described DNA-binding domains are the minimal regions required for transposition in vitro when the Mu DNA ends are contained within short DNA fragments (23).

The Mu transpososome promotes recombination through the repeated use of only two active sites, as defined by the required sets of DDE amino acids. Two of the four stably bound transposase subunits provide DDE residues to the active sites, with each active site containing DDE residues from a single subunit. Each active site catalyzes both donor cleavage and joining at the same Mu DNA end (24, 25). Repeated active site usage for multiple reaction steps is also observed for the Tn5 and Tn10 cut-and-paste transpososomes (26, 27). Within the Mu transpososome, the two “catalytic” subunits are arranged such that the subunit bound site specifically to one Mu DNA end donates DDE residues for recombination of the partner Mu DNA end (23–25, 28). This “criss-crossing” subunit geometry is also observed in the Tn5 transposase-DNA cocrystal (21, 27) and could be a conserved feature of transposase/integrase complexes.

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A second phage-encoded protein, MuB, can also contribute to the Mu transpososome. MuB interacts with the C-terminal domain of transposase (29, 30) and stimulates transposition in multiple ways. MuB activates assembly of the Mu transpososome, stimulates transposase to promote strand transfer, and guides the choice of target DNA (31–36). MuB possesses both nonspecific DNA binding and ATPase activities (31, 37); however, the specific DNA binding and protein conformational changes within the transpososome that are otherwise slow or inefficient in the absence of MuB remain to be discovered.

The Mu transpososome binds and manipulates both flank and target DNA during transposition. DNase I protection studies indicate that up to 20 nucleotides of flanking DNA are bound by transposase subunits within the transpososome (9). Before cleavage, the flanking DNA of the cleaved strand must lie within the active sites. The in-line mechanism of phosphoribosyltransferase dictates that, between the cleavage and joining reaction steps, an organizational change must occur within the two active sites such that the proximity of each 3′OH shifts from near the flanking DNA to near the target DNA. To gain insight into how the Mu transpososome organizes and manipulates these DNA regions during transposition, we studied the impact of the flanking DNA on the kinetics of transposition in the absence of MuB. We show that the presence of the flanking DNA strand that was attached to the 3′OH transposon DNA end before cleavage (the 3′-flanking strand) can specifically slow one or more reaction steps that occur after cleavage. This slow step is clearly observed in the presence of an inhibitor. In addition, the 3′-flanking strand at a single Mu DNA end can slow this step coordinately in both active sites. Furthermore, we provide evidence suggesting that initial binding of the target DNA is not altered by the presence of the 3′-flanking strand. Finally, we demonstrate that MuB enhances the rate of transposition by stimulating the step after cleavage that can otherwise be inhibited by the 3′-flanking strand. Based on these results, we suggest that release of the 3′-flanking strand from both active sites is required after cleavage for efficient and productive binding of the target DNA within the two active sites. A model is proposed for the conversion from cleavage to joining in which MuB allosterically stimulates a conformational change that accomplishes this DNA movement within the active sites.

**EXPERIMENTAL PROCEDURES**

**Protein and DNA**—MuA 77-663 was purified as described previously (25) and was used for all transposition reactions. Synthesis, purification, and 32P phosphorylation of the donor fragment component oligonucleotides and annealing of these oligonucleotides to create the donor fragments were as described previously (25). Donor fragments were made by annealing the following sets of oligonucleotides: long plus TB996 plus TB876 (phosphorylated at 5′OH transposon DNA strand at a single Mu DNA end can specifically slow this step coordinately in both active sites. Furthermore, we provide evidence suggesting that initial binding of the target DNA is not altered by the presence of the 3′-flanking strand. Finally, we demonstrate that MuB enhances the rate of transposition by stimulating the step after cleavage that can otherwise be inhibited by the 3′-flanking strand. Based on these results, we suggest that release of the 3′-flanking strand from both active sites is required after cleavage for efficient and productive binding of the target DNA within the two active sites. A model is proposed for the conversion from cleavage to joining in which MuB allosterically stimulates a conformational change that accomplishes this DNA movement within the active sites.

**RESULTS**

A Single 3′-Flanking DNA Strand within the Mu Transpososome Can Inhibit Transposition in the Absence of MuB—The impact of the flanking DNA on Mu transposition was assessed by following the progress of joined product accumulation in vitro. Time courses were initiated by the addition of Mu transposase monomers to a mixture containing MgCl2, a large supercoiled DNA to serve as the transposition target, and one of two different types of Mu DNA end fragment that functioned as the transposition donor (see Figs. 1B and 2A). In addition to the Mu DNA sequence, the “long” donor fragment contained flanking DNA that extended on both strands past the cleavage site. The “short” donor lacked most flanking DNA and was “pre-cleaved.” Activity was monitored by 32P labeling the donor fragment on the 5′ end of the bottom strand (Fig. 2A) and by following the joining of this strand to the target DNA (Fig. 1). These reactions lacked the MuB activator. MuB was included only in the experiments described in the final section, where its effect is described.

Analysis of the progress of joined product accumulation revealed that the long donor fragment was joined to the target DNA significantly more slowly than the short donor fragment. In reactions containing either donor type, agarose gel electrophoresis (Fig. 2B) of the joined DNA species present at early reaction time points showed that both donor fragments within a transpososome were joined to one target DNA molecule.
nearly simultaneously (Fig. 2B, DEST). Very few products (8–10%) in which a single donor fragment was joined to the target (SEST) were observed. Graphing the fraction of total donor fragments that were joined as a function of time indicated that joined products accumulated more slowly when the reaction contained the long donor fragment (Fig. 2C).

The long and short donor fragments differed from each other in two general ways, either (or both) of which could contribute
to the difference in progress curves. Unlike the short donor, the long donor fragment possessed both a longer segment of DNA flanking the cleavage site and an intact phosphodiester bond at the cleavage site. To test whether slower transposition was due to the intact cleavage site, the scissile phosphate was removed from the long donor fragment. This "precleaved" long donor analog was therefore identical to the original long donor except for the presence of a "nick" at the cleavage site. Time-course analysis indicated that this precleaved long donor was joined to the target at a rate nearly identical to that observed for the original long donor (Fig. 3A: compare filled circles to filled diamonds), suggesting that the phosphodiester bond at the cleavage site was not responsible for the slower transposition. These results are consistent with the notion that cleavage occurs quickly compared with the rate-determining step(s) (see also next section).

During transposition, the 3'OH of the cleaved Mu DNA end must shift proximity from the flanking DNA strand to the target DNA within an active site. This shift could be accomplished by removing only the 5'-phosphate on the 3'-flanking strand from the active site after cleavage (38) (see also "Discussion"). However, the observation that the "precleaved" long donor, which lacked this phosphate, exhibited the same transposition progress as the original long donor suggested that this phosphate had little impact. To verify this conclusion, transposition progress curves were compared using "precleaved" long donors that either contained or lacked this 5'-phosphate. This analysis indicated that the presence of the 5'-phosphate on the 3'-flanking strand did not appreciably change the transposition progress (Fig. 3B). Together, these results suggest that the slower accumulation of joined product in reactions containing the long donor is due to the presence of the flanking DNA.

To identify the flanking DNA feature responsible for slower transposition, time-course reactions were repeated using donor fragment analogs carrying different portions or configurations of the flanking DNA (Fig. 3A). Three were tested: 1) a donor fragment containing unbasepaired flanking strands, where the two strands were identical rather than complimentary (filled triangles), 2) a donor fragment carrying the 3'-flanking strand but only four nucleotides of the 5'-flanking strand (filled squares), and 3) a donor fragment including only the 5'-flanking strand (open triangles). Like the original long donor, transposition occurred slowly when donor analogs included the 3'-

Fig. 3. Transposition can be slowed by nucleotides of the 3'-flanking DNA strand. Time course reactions were as described in Fig. 2. Representative experiments are shown. The oligonucleotide composition of the donor fragments diagramed at the right derive from those depicted in Fig. 2A (see also "Experimental Procedures"); bottom line, cleaved strand; top line, uncleaved strand. A, presence of the 3'-flanking strand is sufficient to slow transposition. Only the first 250 min are shown; final time points for all curves cluster within 60–70% donor joined. B, slower transposition is independent of the presence of the 5'-phosphate of the 3'-flanking strand. Within the donor substrates diagrammed on the right, P depicts the addition of the 5'-phosphate to the 3'-flanking DNA strand; □ depicts the replacement of the 5'-phosphate with a 5'-hydroxyl group. The difference between △, ○, and ● is within the experimental error.
flanking strand, regardless of the nature of the 5'-flanking strand. In contrast, when the donor analog lacked the 3'-flanking DNA strand (but contained the 5'-flanking strand), joined product accumulated at a rate nearly identical to that of the original short donor fragment. Thus, some or all of the nucleotides of the 3'-flanking strand, but not the 5'-phosphate itself, slows one or more reaction steps during transposition. This inhibition is independent of both the basepairing between the two flanking strands and the covalent attachment of the 3'-flanking strand to the Mu DNA end.

Our previous studies show that, in Mu transpososomes containing one wild-type and one mutant (DDE') active site, the presence of an uncleaved Mu DNA end in the mutant active site inhibits joining in the wild-type active site (25). This study suggests that an event required for joining occurs coordinately in the two active sites and that an uncleaved Mu DNA end in just one active site antagonizes this event. To determine whether the 3'-flanking strand similarly slowed transposition in both active sites in wild-type transpososomes, complexes containing one short and one long donor fragment were analyzed. These mixed flank complexes were generated by including both types of donor fragment in the reaction mixture (mixed donor reactions) (25). This mixture contains not only the mixed flank complexes but also complexes with two long donor fragments and complexes with two short donor fragments. The activity of mixed flank complexes was assessed by 32P labeling either the long or short donor fragment and by then supplying this fragment in limiting quantity; thus, most radiolabeled complexes (~95%) will be the mixed variety (see Fig. 4A).

The transposition activity of the mixed donor reactions demonstrated that the presence of 3'-flanking DNA at just one Mu DNA end within a wild-type transpososome slowed the rate of transposition in both active sites. In reactions containing excess unlabeled long donor, the labeled short donor was joined to the target more slowly than when reactions included only the short donor fragment (Fig. 4B, compare circles to squares). As above, products in which only a single Mu DNA end was joined to the target were rarely observed (data not shown), indicating that joining of the two donor fragments within the transpososome occurred nearly synchronously. Comparison of the progress curve for these mixed donor reactions with the set of curves for the short donor alone indicated that these curves differed significantly (p = 1.6e-34, see Fig. 4). Likewise, when mixed donor reactions contained excess unlabeled short donor, the progress curve approached that observed in reactions containing only the long donor fragment. Although joined product accumulated more quickly in mixed donor reactions when the short donor was radiolabeled, this is likely due to the elevated activity of the contaminating complexes (see Fig. 4). Nonetheless, the progress curves for the mixed donor reactions suggest that the presence of a single 3'-flanking DNA strand within the transpososome can slow recombination of both Mu DNA ends.

An Inhibitor in the \( \phi X174 \) Target DNA Preparation Cooperates with the 3'-Flanking DNA to Slow Transposition—Al-
though the presence of the 3'-flanking strand within a transpososome inhibits transposition in the experiments described above, the rate of accumulation of joined products did not always differ between the short and long donor fragments. In the above reactions, we included δX174 RFI DNA as the target, which is the DNA used as target in numerous published Mu transposition in vitro experiments. We discovered that a contaminant present in the δX174 preparation specifically slowed transposition by transpososomes containing the 3'-flanking strand.

Several experimental observations support this hypothesis. First, joined products accumulated at a similar rate in reactions containing either the short or long donor fragment when the pUC19 plasmid (or pBR322, data not shown) was substituted for δX174 as the target DNA. Second, transposition into these other targets by complexes containing the 3'-flanking strand was slowed when the δX174 preparation was also included (Fig. 5A and data not shown). Third, gel filtration of the δX174 preparation partially removed the inhibitory contaminant (Fig. 5B). Together, these results suggest that an as yet unidentified inhibitor, present in multiple δX174 preparations (see “Experimental Procedures”), slowed transposition in a way that is specific to the presence of the 3'-flanking DNA strand.

We asked whether the combination of 3'-flanking DNA and inhibitor acted competitively with respect to target DNA binding to slow transposition. This analysis relies on two assumptions: 1) the inhibitor and the target DNA interact with the transpososome in a reversible manner and 2) binding of inhibitor and target to the complex is in rapid equilibrium with the unbound species. The assumption of reversible binding is supported by the observation that the total amount of joined product is similar in the inhibited and uninhibited cases. In addition, preincubation of target DNA with transpososomes under conditions that do not allow joining (e.g. in the absence of Mg2+) does not commit the transpososome to join Mu DNA ends to a particular target DNA (data not shown). Given these assumptions, if the inhibitor acts in combination with the 3'-flanking strand to alter initial target binding to transpososomes, increasing the concentration of target DNA is expected to speed the reaction progress. In contrast to this prediction, increasing the target DNA concentration from 9 to 156 nM pBR322 plasmid molecules while holding the inhibitor concentration constant did not overcome the effect of the inhibitor (Fig. 5C). These results suggest that the inhibitor does not change the affinity of the complex for initial binding of the target DNA, but instead slows transposition via an alternate mechanism.

Combination of the 3'-Flanking Strand and Inhibitor Specifically Slowed the Conversion of Cleaved Intermediate to Joined Product—The overall progress of transposition depends on the rates of individual reaction steps. At a minimum, these steps normally include complex assembly, donor cleavage, target DNA binding, and DNA end joining (Fig. 1A). To identify the transposition reaction steps(s) slowed by the 3'-flanking DNA strand in the presence of the inhibitor, the amounts of cleaved intermediate and joined product were quantified throughout a time course (Fig. 6A). Following both of these reaction species allowed us to fit rate constants to modeled mechanisms for the formation of the intermediate cleaved complex and the conversion of cleaved intermediate to joined product. The inhibitory effect of the 3'-flanking strand was then investigated by comparing the dependence of the rates of these steps on the presence or absence of the inhibitor.

This analysis revealed that the 3'-flanking strand specifically slowed the conversion of cleaved intermediate to joined product under the inhibited conditions. In reactions containing the inhibitor, the amount of cleaved long donor increased dur-

![Fig. 5](image-url)

**Fig. 5.** An inhibitor in the δX174 preparation slows transposition by complexes containing the 3'-flanking DNA. A, joined product progress curves for reactions containing the long donor fragment and pUC19 as a transposition target, δX174. A representative experiment is shown. The total joined products (into both pUC19 and δX174) are plotted; transposition into either pUC19 or δX174 is slowed equivalently in reactions containing δX174 (data not shown). The rate of transposition varied little with the different target DNA molecules in reactions containing the short donor fragment (data not shown). B, separation of an inhibitor from the δX174 DNA by gel filtration. Reactions contained the long donor fragment. C, incuiones contained 9.5 nM δX174 in addition to pBR322. Long, reactions contained the long donor fragment; short, reactions contained the short donor fragment.
Flanking DNA Slows Mu Transposition

A least squares fit of models to this data (see Supplementary Material) suggests that transpososomes containing the 3’-flanking strand converted the cleaved intermediate to the joined product at least 8 times (and perhaps as much as 88 times) more slowly under the inhibited conditions.

To resolve whether this effect was specific to the conversion from the cleaved to the joined state of the active site, we determined whether the 3’-flanking strand also slowed the generation of the cleaved intermediate. The total number of active complexes that have assembled and then cleaved the donor fragment at each time point is equal to the sum of the cleaved and joined reaction species at each time point, given that 1) assembled complexes are stable (25) and 2) donor cleavage is irreversible. Graphing the sum of cleaved and joined species (cl + j) at each time point revealed that generation of the intermediate cleaved complex was largely unaffected by either the 3’-flanking strand or the inhibitor; progress curves for this sum (cl + j) overlapped for reactions that contained the long or short donor fragment in the presence or absence of the inhibitor (Fig. 6C). This overlap supports the notion that transpososome assembly (or assembly and cleavage) occurred over a similar timescale regardless of the 3’-flanking strand or the inhibitor and that this process was rate-determining in the uninhibited reactions. Thus, the conversion of cleaved intermediate to joined product (cl→j conversion) was specifically slowed when both the 3’-flanking strand and the inhibitor were present.

Fig. 6. The 3’-flanking strand in conjunction with the inhibitor specifically slows the conversion of cleaved intermediate to joined product. A, separation of cleaved donor and joined product DNA species by denaturing PAGE. A representative experiment, including the long donor and φX174, is shown. B, progress curves for the cleaved donor and joined product. Representative experiments are shown. All reactions contained pBR322 as a target and the long donor fragment. Joined products include transposition into all types of target molecules. C, neither the 3’-flanking strand nor the inhibitor slowed the generation of the intermediate cleaved complex. The total amount of reacted donor (cleaved + joined) for each time point is graphed. Cl, cleaved donor; j, joined product.

course (Fig. 6B, right panel). A least squares fit of models to this data suggests that transpososomes containing the 3’-flanking strand converted the cleaved intermediate to the joined product at least 8 times (and perhaps as much as 88 times) more slowly under the inhibited conditions.

To resolve whether this effect was specific to the conversion from the cleaved to the joined state of the active site, we determined whether the 3’-flanking strand also slowed the generation of the cleaved intermediate. The total number of active complexes that have assembled and then cleaved the donor fragment at each time point is equal to the sum of the cleaved and joined reaction species at each time point, given that 1) assembled complexes are stable (25) and 2) donor cleavage is irreversible. Graphing the sum of cleaved and joined species (cl + j) at each time point revealed that generation of the intermediate cleaved complex was largely unaffected by either the 3’-flanking strand or the inhibitor; progress curves for this sum (cl + j) overlapped for reactions that contained the long or short donor fragment in the presence or absence of the inhibitor (Fig. 6C). This overlap supports the notion that transpososome assembly (or assembly and cleavage) occurred over a similar timescale regardless of the 3’-flanking strand or the inhibitor and that this process was rate-determining in the uninhibited reactions. Thus, the conversion of cleaved intermediate to joined product (cl→j conversion) was specifically slowed when both the 3’-flanking strand and the inhibitor were present.
MuB Stimulates the Conversion Step That Is Otherwise Inhibited by the 3'-Flanking Strand—The transpositional activator protein MuB stimulates both transpososome assembly and Mu DNA end joining. We wished to determine whether MuB activates transposition by stimulating the step during the cl→j conversion that was otherwise slowed by the 3'-flanking DNA strand and the inhibitor. To this end, the effects of MuB on the progress curves for both the cleaved and joined reaction species were investigated. These experiments were similar to those described above except that they also included MuB and ATP. ATP is an essential cofactor for MuB.

As expected, MuB stimulated a step prior to cleavage. Graphing the total amount of reacted donor DNA (i.e., the sum (cl + j)) as a function of time showed that ATP alone had little effect on the generation of cleaved complexes, whereas MuB increased this rate (Fig. 7A). Stimulation by MuB depended on protein-protein interaction between transposase and MuB; MuB failed to stimulate transposition when transposase lacked the C-terminal MuB-interaction domain (missing amino acids 606–663) (data not shown). MuB also stimulated transposition when reactions contained the short donor (data not shown). These results indicate that, under the conditions employed here, MuB stimulates complex assembly in a manner that is largely independent of the flanking DNA (and the inhibitor).

MuB also stimulated transposition by increasing the rate of conversion of the cleaved intermediate to the joined product. Comparison of progress curves obtained in the presence or absence of MuB indicated that MuB increased transposition overall to a rate faster than that seen in the uninhibited reactions (Fig. 7B, compare open diamonds to squares). Furthermore, MuB specifically increased the rate of conversion of cleaved intermediate to joined product (Fig. 7C). Stimulation by MuB required transposase-MuB interaction (data not shown). These data were fit to a kinetic model, as above, and gave estimated rate constants for the cl→j conversion of $2 \times 10^{-3}$ s$^{-1}$ and $5.7 \times 10^{-5}$ s$^{-1}$ for the MuB-stimulated and unstimulated reactions, respectively (see Supplementary Material). The rate constant for the MuB-stimulated cl→j conversion in these experiments is roughly equivalent to the rate constant for this step when both ϕX174 and ATP were omitted (either $1.6 \pm 0.8 \times 10^{-3}$ or $7 \pm 11 \times 10^{-3}$ s$^{-1}$, depending on the model used). These data reveal that MuB increased the rate of transposition in two ways: 1) by stimulating the generation of the intermediate cleaved complex, likely by promoting complex assembly and 2) by activating the cl→j conversion. MuB thus stimulates transpososomes to efficiently perform one or more steps during the cl→j conversion that is otherwise slowed by the presence of the 3'-flanking strand under the inhibitory conditions.

**DISCUSSION**

Reorganization of Flank and Target DNA upon Conversion from Cleavage to Joining—Within the Mu transpososome, a single active site, or two overlapping active sites that share a common set of catalytic DDE residues, promotes recombination at one Mu DNA end (24, 25). The repeated use of the same catalytic residues for the phosphotransfer steps of both cleavage and joining places restrictions on the organization of active site components during these two chemical steps. The Mu transpososome may proceed from cleavage to joining by moving donor and target DNA with respect to the catalytic DDE residues, by rearranging the location of specific catalytic residues and/or divalent metal ion cofactors within the active sites, or by a combination of these mechanisms. Transposase subunits within the transpososome bind the donor and target DNAs and promote transposition in the absence of the MuB activator protein in vitro. We have used this ability to dissect the mechanism of conversion between cleavage and joining and to delineate the roles of transposase subunits and MuB in this transition. In this work we show that, when MuB is absent, the strand attached to the transposon 3' end before cleavage (the 3'-flanking strand) can specifically slow a post-cleavage step. This slow cleavage to joining (cl→j) conversion step was observed when reactions also contained an inhibitor from the ϕX174 preparation (see “Experimental Procedures”). The specificity of this inhibition allowed us to probe how the flanking DNA is manipulated within the Mu transpososome. We demonstrated that the cl→j step was slowed by the 3'-flanking strand in a manner that was independent of: 1) the 5'-flanking strand, 2) basepairing between the two flanking DNA strands,
and 3) the 5'-phosphate of the 3'-flanking strand. Furthermore, we provide evidence that this cl–j conversion step occurs cooperatively between the two active sites, where the presence of the 3'-flanking strand in one active site can inhibit joining in both active sites. Finally, we show that MuB speeds this conversion step and thereby stimulates transposition.

These data extend previous work in which we proposed the existence of a cooperative transition within the transpososome in which the two active sites become ready for joining in concert (25). This early work is based on the activity of transpososomes containing mixtures of active and inactive transposase subunits, where mutations in the catalytic DDE motif render a subunit inactive. In the present work, evidence for a conformational change during the cl–j conversion has now been revealed for fully active complexes. This conformational step was not observed for wild-type transpososomes in the earlier experiments, which also included the inhibitor, because only the reaction end point was analyzed. Reduction of the cl–j conversion was likely more readily seen in the mixed DDE–/DDE+ transpososomes, because failure to cleave the Mu DNA end in the DDE+ active site would provide a strong block to this step (see below).

The slower cl–j conversion effected by the combination of 3'-flanking strand and inhibitor suggests that proper target DNA engagement within the transposase active sites occurs only after cleavage. Proper engagement must activate target DNA for joining and requires target DNA binding within the correct active site binding pocket. Here, we provide evidence that the combination of 3'-flanking DNA and the inhibitor does not compete with the target DNA for initial binding to transposase subunits. Although target DNA non-covalently interacts with transpososomes at various reaction stages in the presence of MuB (36), it is currently unclear whether the transposase subunits bind target DNA before cleavage. If target DNA binds transposase only after cleavage, the 3'-flanking strand and inhibitor may slow the cl–j conversion by preventing target binding by transposase subunits (see below). Alternatively, target DNA may bind a "preloading" site on transposase, where it is not yet engaged within the active sites. Target binding within this hypothetical preloading site must be "loose" (or metal ion-dependent), because Mu transpososomes preincubated with target DNA in the absence of MuB do not exhibit target commitment. In this case, the 3'-flanking strand and inhibitor may prevent the shift of target DNA from the preloading site into the proper binding pocket within the active sites.

**General Model for the cl–j Conversion for Replicative and Cut-and-paste Transpososomes**—Based on the results presented here, we propose two working models for the rearrangement of DNA components within a transpososome that occurs after cleavage of the Mu DNA ends and before the chemical step of joining these ends to a new target DNA (Fig. 8). Within each active site, joining a cleaved Mu DNA end to the target requires the relative movement of the 3’OH of the cleaved transposon end from within close proximity of the 5’-phosphate of the 3'-flanking strand (cleavage) to within close proximity of the scissile phosphate of the target DNA strand (joining). We propose that the 3'-flanking strand is removed from (Model 1) or repositioned within (Model 2) each active site after cleavage. Removal (or repositioning) of the 3'-flanking DNA from (or within) both active sites triggers a change in the transpososome that reorganizes active site components into a conformation that allows the joining chemistry. This reorganization allows proper "engaged" binding of the target DNA within the active sites and may rearrange catalytic amino acids and/or divalent metal ion(s) to activate phosphoryl transfer of the target DNA. In Model 1, this rearrangement permits a portion of the target DNA to contact the same binding pocket that previously held the 3'-flanking strand. In Model 2, the target DNA and 3'-flanking strand contact different binding surfaces in the active sites.

Models for the organization of DNA components during cleavage and joining have been previously proposed based on the observed stereospecificity of these steps for certain phosphorothioate substitutions of the non-bridging oxygens of the scissile phosphates (38). In one of these previous models, the 3'-flanking and target DNA strands interact with non-overlapping binding pockets in each active site (similar in this aspect to Model 2, Fig. 8). In this previous model, conversion from cleavage to joining can theoretically be accomplished by moving only the 5'-phosphate of the 3'-flanking strand. This early model thus predicts a stronger dependence of the cl–j conversion on the presence of the 5'-phosphate of the 3'-flanking strand than on the remaining nucleotides. In contrast to this prediction, we observe a much greater effect of the nucleotides of the 3'-flanking strand than the 5'-phosphate on the cl–j conversion. We are therefore attracted to models for the cl–j conversion in which the 3'-flanking strand moves substantially after cleavage from its original position within each active site.

A requirement for the removal of the 3'-flanking strand during this conversion could be easily explained if the 3'-flanking strand and a portion of the target DNA sequentially occupy the same binding pocket in an active site (Model 1). Reuse of the same active site binding pocket for these DNA segments has also been previously proposed (38). However, in this early model, reuse of the same active site for 3' end cleavage and joining is accomplished by moving the transposon DNA ends and by fixing the catalytic divalent metal ions and amino acids that activate the scissile phosphate. In the recent crystal structure of the related Tn5 transpososome, numerous protein-DNA contacts between transposase subunits and the transposon DNA ends are observed (21). Our model (Model 1) therefore suggests that the Mu transposon DNA ends are relatively fixed between the cleavage and joining states but that the catalytic divalent metal ions are reorganized around each scissile phosphate.

Whether target DNA binds to the same (Model 1) or a different surface (Model 2) as the 3'-flanking strand in the active sites, removal (or substantial movement) of the 3'-flanking strand from the active site is the most likely explanation for the possible action of the inhibitor. For example, the inhibitor could slow the cl–j conversion by binding the 3'-flanking strand and thereby impeding introduction of the target DNA into the active sites. If removal (or movement) of the 3'-flanking strand from the active site must occur before joining, then this action of the inhibitor would be exacerbated by “trapping” the 3'-flanking strand in one active site, as occurs when donor cleavage is blocked by active site mutations. Indeed, the presence of an uncleaved Mu DNA end in a mutant (DDE-') active site strongly blocks the cl–j conversion in the partner wild-type active site (25), whereas this conversion occurs much more efficiently when both active sites are wild-type. Another possibility is that the inhibitor may bind transpososomes when the 3'-flanking strand is in its "cleavage" binding pocket and thereby prevent the reorganization of active site components into a conformation that is competent for joining.

The models proposed here for the cl–j conversion promoted by the Mu replicative transpososome share similarity to models proposed for target DNA interaction by the Tn10 cut-and-paste transpososome. Like Mu, the Tn10 and Tn5 cut-and-paste transposases repeatedly utilize the same active site DDE residues to recombine each transposon DNA end with target DNA.
Mu and Tn10 transposition share phosphorothioate stereospecificity for the scissile phosphates of the common catalytic steps, transposon DNA 3' end cleavage and 3' end joining (38), suggesting a common arrangement of DNA components within the active sites for these two steps. Furthermore, Tn10 transpososomes stably interact with the target DNA (target commitment) only when both donor fragments lack the flanking DNA; stable target interaction is not observed when just one of the two paired donor fragments within the transpososome contains flanking DNA (39). These authors suggest that Tn10 transposase subunits stably bind target DNA within the active sites only after excision of the transposon DNA from the surrounding/flanking DNA. The work here extends this idea to include the Mu replicative transpososome and further suggests that transpososomes that repeatedly use the same active site for multiple phosphoryl transfer reactions may similarly link a conformational change in both active sites after cleavage to proper positioning of the target DNA within the active sites for joining.

Although these replicative and cut-and-paste transpososomes may similarly require a reorganization of flanking DNA and active site components after cleavage to properly position target DNA in the active sites, details for the cl–j conversion must differ for these two transposition processes. One potential difference between these two types of transpososomes may be the trigger for the conformational change. Cut-and-paste transposases, such as Tn10 and Tn5 (40, 41), also catalyze cleavage of the non-transferred strand at each transposon DNA end. This cleavage occurs via a two-step process in which the 3'-OH of the cleaved transferred strand at the transposon end directly attacks the non-transferred strand to generate a DNA hairpin intermediate. Subsequent hydrolysis of this hairpin directly precedes the end-joining step. Triggering the conformational change that allows productive target binding within the active sites may therefore require removal of the non-transferred strand of the hairpin from the active sites, instead of the 3'-flanking strand, which, as we suggest, occurs during Mu replicative transposition.

**MuB Stimulates Transposase to Permit the cl–j Conversion**—MuB exhibits nonspecific DNA binding activity and functions to “deliver” target DNA to transposase (42). MuB also allosterically activates Mu transposase, although the molecular mechanism by which MuB stimulates transposase is unknown. We show that MuB stimulates the step between cleav-
age and joining that can otherwise be slowed by the 3′-flanking DNA strand under the inhibited conditions. We further provide evidence suggesting that this slow step in the absence of MuB is not due to a defect in initial target DNA binding by the transposase subunits of the transpososome. Thus, MuB stimulates transposase to undergo the cl→j conversion not by promoting initial target binding, but through a distinct mechanism. We propose two possible modes, not mutually exclusive, by which MuB stimulates this conversion. First, MuB may bind the 3′-flanking DNA and thereby promote removal of the 3′-flanking strand from the active site after cleavage. (Alternatively, MuB could block binding of the inhibitor.) Second, MuB may allosterically activate MuA specifically during the cl→j conversion step to undergo the conformational shift, perhaps by stabilizing the joining-competent conformational state of MuA in the transpososome.

A role for MuB in binding the 3′ flank or promoting its movement within the transpososome subunits is attractive given the differences between replicative and cut-and-paste transposition. Activators of transposition such as MuB have not been generally found for cut-and-paste transposases that promote recombination of a transposon end through the repeated use of the same active site. (The cut-and-paste transposition system of Tn7 employs a MuB-like protein, although active site residues from two different protein subunits are required to recombine a single transposon DNA end (43, 44)). Cut-and-paste transposition severs the connection between both strands of the flanking DNA and the transposon end before the joining step. In contrast, the 3′-flanking strand still associates with the replicative transpososome after cleavage, through basepairing and covalent attachment of the non-transferred strand. Binding of the 3′-flanking strand by MuB could provide a mechanism by which MuB promotes transposition into a distant target DNA site rather than an adjacent flanking DNA site. It will be interesting to determine whether the DNA binding and ATPase activities of MuB are required to stimulate the conversion between cleavage and joining under conditions where the presence of the 3′-flanking strand slows this reaction step.

REFERENCES

1. Mizuuchi, K. (1992) Annu. Rev. Biochem. 61, 1011–1051
2. Kleckner, N., Chalmers, R. M., Kwon, D., Sakai, J., and Bolland, S. (1996) Curr. Top. Microbiol. Immunol. 204, 49–82
3. Fugmann, S. D., Lee, A. I., Shokett, P. E., Villey, I. J., and Schatz, D. G. (2000) Annu. Rev. Immunol. 18, 495–527
4. Rice, P. A., and Baker, T. A. (2001) Nat. Struct. Biol. 8, 302–307
5. Chaconas, G., Lavoie, B. D., and Watson, M. A. (1996) Curr. Biol. 6, 817–820
6. Lavoie, B. D., Chan, B. S., Allison, R. G., and Chaconas, G. (1991) EMBO J. 10, 3051–3059
7. Mizuuchi, M., Baker, T. A., and Mizuuchi, K. (1992) Cell 70, 303–311
8. Baker, T. A., Mizuuchi, M., Savilahti, H., and Mizuuchi, K. (1993) Cell 74, 723–733
9. Mizuuchi, M., Baker, T. A., and Mizuuchi, K. (1991) Proc. Natl. Acad. Sci. U. S. A. 88, 9931–9935
10. Zou, A. H., Leung, P. C., and Harshey, R. M. (1991) J. Biol. Chem. 266, 20476–20482
11. Craige, R., Mizuuchi, M., and Mizuuchi, K. (1984) Cell 35, 377–384
12. Nakayama, C., Teplow, D. B., and Harshey, R. M. (1987) Proc. Natl. Acad. Sci. U. S. A. 84, 1809–1813
13. Wu, Z., and Chaconas, G. (1995) EMBO J. 14, 3835–3843
14. Rice, P., and Mizuuchi, K. (1995) Cell 82, 209–220
15. Krementsova, E., Giffin, M. J., Pinus, D., and Baker, T. A. (1998) J. Biol. Chem. 273, 31358–31365
16. Namgoong, S. Y., Kim, K., Saxena, P., Yang, J. Y., Jayaram, M., Giedroc, D. P., and Harshey, R. M. (1998) J. Mol. Biol. 275, 221–232
17. Naigamwalla, D. Z., Coros, C. J., Wu, Z., and Chaconas, G. (1996) J. Mol. Biol. 262, 265–274
18. Baker, T. A., and Lao, L. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 6654–6658
19. Kim, K., Namgoong, S. Y., Jayaram, M., and Harshey, R. M. (1995) J. Biol. Chem. 270, 1472–1479
20. Wlodawer, A. (1999) Adv. Virus Res. 52, 335–350
21. Davies, D. R., Goryshin, I. Y., Reznikoff, W. S., and Raymert, I. (2000) Science 289, 77–85
22. Lovell, S., Goryshin, I. Y., Reznikoff, W. R., and Rayment, I. (2002) Nat. Struct. Biol. 9, 278–281
23. Aldaz, H., Schuster, E., and Baker, T. A. (1996) Cell 85, 257–269
24. Namgoong, S. Y., and Harshey, R. M. (1998) EMBO J. 17, 3775–3785
25. Williams, T. L., Jackson, E. L., Carritte, A., and Baker, T. A. (1999) Genes Dev. 13, 2725–2737
26. Bolland, S., and Kleckner, N. (1996) Cell 84, 223–233
27. Naumann, T. A., and Reznikoff, W. S. (2000) Proc. Natl. Acad. Sci. U. S. A. 97, 8944–8949
28. Savilahti, H., and Mizuuchi, K. (1996) Cell 85, 271–280
29. Wu, Z., and Chaconas, G. (1994) J. Biol. Chem. 269, 28829–28833
30. Levenken, I., Yamauchi, M., and Baker, T. A. (1997) Genes Dev. 11, 1561–1572
31. Maxwell, A., Craige, R., and Mizuuchi, K. (1987) Proc. Natl. Acad. Sci. U. S. A. 84, 699–703
32. Baker, T. A., Mizuuchi, M., and Mizuuchi, K. (1991) Cell 65, 1083–1013
33. Surette, M. G., Harkness, T., and Chaconas, G. (1991) J. Biol. Chem. 266, 3118–3124
34. Surette, M. G., and Chaconas, G. (1991) J. Biol. Chem. 266, 17306–17313
35. Mizuuchi, M., Baker, T. A., and Mizuuchi, K. (1995) Cell 83, 375–385
36. Naigamwalla, D. Z., and Kleckner, N. (1997) EMBO J. 16, 5277–5284
37. Chaconas, G., Gloor, G., and Miller, J. L. (1985) J. Biol. Chem. 260, 2662–2669
38. Kennedy, A. K., Hafid, D. B., and Mizuuchi, K. (2000) Cell 101, 293–305
39. Sakai, J., and Kleckner, N. (1997) Cell 89, 255–264
40. Kennedy, A. K., Gubhahakturka, A., Kleckner, N., and Hafid, D. B. (1998) Cell 95, 125–134
41. Elbashir, A., Goryshin, I. Y., and Reznikoff, W. S. (1999) J. Biol. Chem. 274, 37021–37029
42. Yamauchi, M., and Baker, T. A. (1998) EMBO J. 17, 5509–5518
43. Stellwagen, A. E., and Craig, N. L. (1998) Trends Biochem. Sci. 23, 486–490
44. Sarnovsky, R. J., May, E. W., and Craig, N. L. (1996) EMBO J. 15, 6348–6361
45. Savilahti, H., Rice, P. A., and Mizuuchi, K. (1995) EMBO J. 14, 4983–4980