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The AAA+ ClpX machine unfolds a keystone subunit to remodel the Mu transpososome

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A hyperstable complex of the tetrameric MuA transposase with recombined DNA must be remodeled to allow subsequent DNA replication. ClpX, a AAA+ enzyme, fulfills this function by unfolding one transpososome subunit. Which MuA subunit is extracted, and how complex destabilization relates to establishment of the correct directionality (left to right) of Mu replication, is not known. Here, using altered-specificity MuA proteins/DNA sites, we demonstrate that transpososome destabilization requires preferential ClpX unfolding of either the catalytic-left or catalytic-right subunits, which make extensive intersubunit contacts in the tetramer. In contrast, ClpX recognizes the other two subunits in the tetramer much less efficiently, and their extraction does not substantially destabilize the complex. Thus, ClpX targets the most stable structural components of the complex. Left-end biased Mu replication is not, however, determined by ClpX's intrinsic subunit preference. The specific targeting of a stabilizing "keystone subunit" within a complex for unfolding is an attractive general mechanism for remodeling by AAA+ enzymes.

AAA+ ATPase | ClpX unfoldase | ClpXP protease | integrase | transposase

Chaperone-mediated remodeling of stable nucleoprotein complexes is a ubiquitous reaction that drives many physiological processes, including DNA replication, recombination, and pathological events that involve viral integration. The austere genomic economy imposed on viruses makes them particularly dependent on using host chaperones for essential remodeling reactions, allowing these nucleoprotein complexes to fulfill diverse functions during the viral life cycle. One such viral complex, the Mu transpososome, is an extremely stable multimer that catalyzes phage Mu transposition and consists of four MuA subunits and Mu DNA (1). After insertion of the Mu genome into the host chromosome, the transpososome (in a form known as the strand transfer complex, or STC1) is inhibitory to the DNA-replication machinery, and thus blocks completion of a cycle of replicative transposition (2, 3). This complex is destabilized in the cell by ClpX, a host-encoded AAA+ protein-unfolding machine, but is resistant to dissociation by heat or denaturants *in vitro* (4, 5).

ClpX remodels the STC1 into a fragile complex known as the STC2. This remodeling event is essential for Mu replication (6). ClpP, the peptidase that interacts with ClpX to form the ClpXP protease, is not necessary for transpososome remodeling, indicating that unfolding of MuA from the transpososome and not MuA degradation is the essential activity (2). Although the stable transpososome contains a MuA tetramer, *in vitro* experiments indicate that ClpX can remodel this complex by unfolding only one subunit (7). The resulting STC2 complex is fragile and dissociates upon gel electrophoresis or high-salt challenge, providing a convenient assay for remodeling. By dissecting the geometry of this remodeling reaction, we can probe transpososome architecture and define the local structural elements of the multimeric substrate that must be remodeled by ClpX to destabilize the complex.

The Mu transpososome is asymmetric (Fig. 1A). The DNA sites bound by MuA in the assembled transpososome (L1, R1, L2 and R2) are ~24 bp, with the exception of L2, which is a

half-site (8). There is also a ~80 bp segment of DNA between L1 and L2, which is unique to the left side of the transpososome and is looped out when L1 and L2 are bound by MuA (9, 10). In contrast, the R1 and R2 sites are contiguous on the right DNA end. Although the DNA sites share a consensus sequence, they differ in their affinity for MuA; R2 binds with the tightest affinity, whereas L2 forms the weakest contacts and is not stably bound by MuA in the final assembled complex (10, 11). These sites also differ in their contribution to transpososome function and architecture. MuA subunits bound to R1 and L1 catalyze the DNA cleavage and joining reactions needed for transposition, whereas subunits bound to R2 and L2 are structurally important but do not contribute directly to catalysis of recombination (12–15).

Footprinting studies revealed that ClpX-mediated remodeling causes changes in DNA accessibility, which are most pronounced on the left side of the complex, particularly within the left-end loop (7). It is unclear whether these changes reflect ClpX extraction of a MuA subunit primarily from the L1 or the L2 DNA site. Alternatively, extraction of a subunit on the right side of the complex might lead to the DNA-footprinting changes on the left, as a consequence of the interwoven nature of the transpososome. A goal of this work is to distinguish between these possibilities. One potential implication of finding that ClpX preferentially extracts a MuA subunit from the left end of the Mu DNA is that this subunit bias may influence downstream steps in the transition between recombination and initiation of DNA replication. Both with phage Mu and an remodeling-replication system *in vitro*, it has been shown that Mu replication preferentially initiates at the left end (16, 17). The mechanistic basis of this initiation-end bias is, however, unknown.

We sought to determine which subunit within the transpososome is unfolded by ClpX using an experimental set-up developed to target MuA subunits to specific DNA sites in the complex (18). This experimental design allowed us to dissect the different roles played by MuA subunits in the remodeling reaction, including which subunits contribute the bulk of the stability to the tetramer and must be unfolded by ClpX (the L1 and R1 subunits), as well as which subunits serve a more accessory role and do not substantially destabilize of the tetramer when removed (the L2 and R2 subunits).

Results

ClpX Disassembles Transpososome Containing Altered-Specificity MuA Variants. To probe ClpX-mediated transpososome disassembly, we adapted a system to target MuA subunits with altered DNA-binding specificity (MuA^{R146V}) to sites specific for this variant (18). We inserted the altered-specificity DNA sequence

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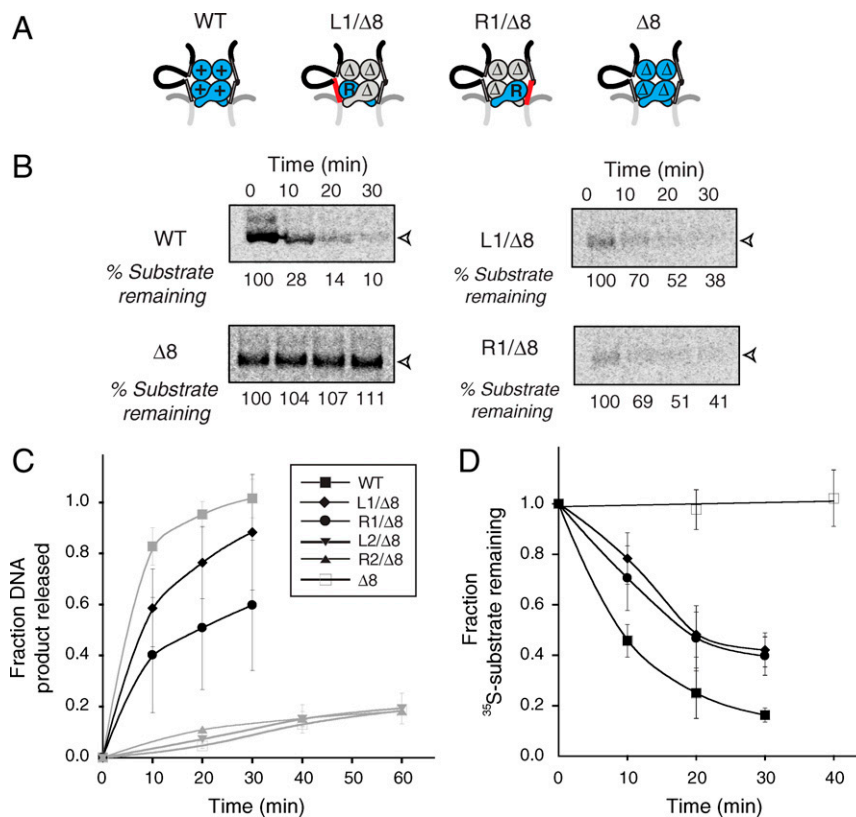


Fig. 3. ClpX disassembly of transposomes with tagged MuA subunits at L1 or R1. **A.** ^{35}S -MuA^{R146V} was targeted to either L1 or R1. ^{35}S -MuA^{R146V} was targeted to either L1 or R1 and remaining positions were filled with unlabeled MuA^{Δ8}. Control complexes were assembled with ^{35}S -MuA or ^{35}S -MuA^{Δ8}. **B.** Labeled subunit release assays for the targeted complexes and control complexes described in **A** were monitored by ^{35}S -subunit disappearance (phosphor storage detection). *Open Arrows* indicate assembled complexes and the radioactivity in each sample is shown under each lane as a percentage of the 0 min sample. **C.** Global disassembly of the same complexes as monitored by DNA-product appearance. Each reaction was performed in at least triplicate. Error bars represent one standard deviation from the mean. *Gray Lines* are the superimposed data from Fig. 2C. Assembly of transposomes on miniMu plasmid containing the MuA^{R146V}-specific site at R1 was ~3-fold less efficient than assembly on plasmids containing the same MuA^{R146V}-specific sequence substitutions at R2, L2 and L1. The poor efficiency made quantification of the global disassembly rate difficult, leading to a large standard deviation on the average rate. However, the global disassembly rate of this variant was within error of that of the L1 variant, and in combination with data showing identical rates of release of ^{35}S -MuA^{R146V} from both R1 and L1 (**D**), we conclude that removal of a MuA subunit from either of these sites results in disassembly of the complex. **D.** Labeled subunit release assay of complexes with ^{35}S -MuA^{R146V} targeted to either L1 or R1 and remaining positions filled with unlabeled MuA^{Δ8} (L1/Δ8 and R1/Δ8, respectively) as well as control ^{35}S -MuA or ^{35}S -MuA^{Δ8} complexes, monitored as described in **B**. Legend is the same as for **C**. Each reaction was performed in at least triplicate. Error bars represent one standard deviation from the mean.

fact, during remodeling of the complexes with the full-length subunit at either L1 or R1, there was general concordance between the global disassembly assay and release of the ^{35}S subunit from the targeted position. Thus, these experiments indicate that ClpX recognition and unfolding of full-length MuA subunits at the L1 and R1 sites results in destabilization of the STC1. The same preference for the L1 and R1 subunits during disassembly was observed when reactions were performed with ClpXP rather than ClpX alone (Fig. S2). Because the experimental design involves assembling transposomes with only a single subunit carrying a functional ClpX-recognition signal, these experiments support the conclusion drawn from earlier studies (7) that ClpX action on a single subunit in the tetramer is sufficient to destabilize the entire complex. The slower rate of disassembly of complexes with a single full-length MuA^{R146V} subunit and three MuA^{Δ8} subunits compared with wild-type complexes indicates that there is some recognition role for the C-terminal MuA tags at other DNA sites in remodeling (see below).

STCs Containing Two Mu DNA Right Ends Are Disassembled Efficiently.

We sought to confirm by a different approach that MuA subunits at R1 and L1 are functionally equivalent for ClpX disassembly. One prediction of this model is that a transposome assembled on two right ends (with an R1-R1 combination at the catalytic

sites) would be disassembled as efficiently as a wild-type transposome by ClpX. STCs efficiently assemble on two MuA DNA right ends and these symmetrical complexes have been widely used to characterize the transposome biochemically and structurally (15, 20). Therefore, to approach the question of recognition determinants within the transposome, we replaced the L1 and L2 binding sites with an R1 and an R2 site in the same mini-Mu plasmid, so that the wild-type and double right-end STC1s would be the same except for these binding-site substitutions. STC1s assembled efficiently on this plasmid (Fig. 4). Previous studies showed that ClpXP disassembles the wild-type STC1 with an apparent K_M of ~1.0 μM and an apparent V_{max} of ~3.1/min (21). We found that ClpXP disassembled the double right-end transposome with an apparent K_M of ~1.1 μM and an apparent V_{max} of ~3.5/min (Fig. 4). Because the kinetic parameters for remodeling the double right-end complex were essentially the same as for the wild-type complex, we conclude that no special features of the left end of the Mu DNA contribute to recognition of the complex. Furthermore, as the experiments presented above reveal that ClpX targets one of the catalytic subunits (R1- or L1-bound) to destabilize the complex, these data indicated that an R1-R1 combination at the catalytic sites is recognized as efficiently as an L1-R1 combination by ClpX in the context of a transposome.

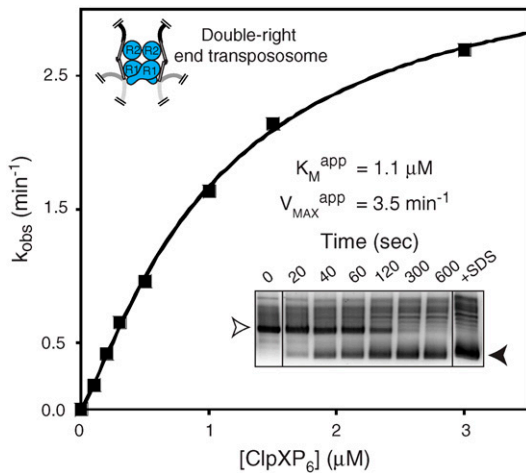


Fig. 4. Transpososomes containing two right sides are recognized by ClpXP with the same efficiency as wild-type transpososomes. ClpXP-mediated disassembly curves for transposomes assembled on a miniMu plasmid containing two right ends ($K_M^{app} = 1.1 \pm 0.1 \text{ mM}$; $V_{max}^{app} = 3.5 \pm 0.2 \text{ min}^{-1}$). The rate of disassembly was determined by increasing enzyme concentration as described (21). Data were fit to a modified Hill equation [reaction rate = $(V_{max}^{app}) / (1 + (K_M^{app} / [\text{ClpXP}])^n)$], where n is the Hill coefficient ($n \sim 1.0$ for this reaction). Inset shows appearance of the DNA product upon addition of enzyme. Open Arrow indicates the position of the assembled complex; black arrow indicates the disassembly product used for rate quantification. The same affinity (K_M^{app}) of ClpX₆ for this double right-end complex is observed in the absence of ClpP, however the rate of the reaction for each concentration of enzyme, as well as the V_{max} , are lower, as was observed in previous studies (22).

Discussion

ClpX as a Machine for Disassembly of Macromolecular Complexes.

Many ClpX substrates are multimeric proteins or proteins that participate in multisubunit complexes. However, the way in which ClpX selects and unfolds subunits to achieve remodeling of these complexes is only beginning to be understood. Because the transpososome is a relatively well characterized complex, it is an ideal multimeric ClpX substrate to use to parse the molecular interactions required to mediate remodeling. Multiple studies have established that ClpX destabilizes the transpososome by unfolding only a limited number of subunits from the complex (4, 7, 22), but it remained unclear which subunit(s) must be unfolded to achieve remodeling. We find that ClpX remodels the transpososome by unfolding of either of these subunits results in generally similar disassembly rates and extents, although inefficient complex assembly when the altered-specificity mutant was positioned at the R1 site made quantification somewhat difficult. However, MuA positioned at R1 and L1 was released from complexes at the same rate as monitored by the ³⁵S-subunit assay, global disassembly rates for these two complexes were within experimental error, and the transposomes assembled on two right DNA ends were disassembled with the same kinetic parameters as the wild-type complexes. Thus, we conclude that there is no significant difference between ClpX recognition of the R1- vs. L1-bound MuA subunit. In contrast, ClpX unfolds subunits bound to the L2 and R2 sites more slowly than those bound to L1 and R1. Furthermore, unfolding of the L2- and R2-bound subunits does not promote complex disassembly, as global disassembly occurred at the same rate and extent as observed with a control MuA variant that is not recognized by ClpX. These results suggest that L2- and R2-bound subunits are not required for the stability of the assembled transpososome. ClpX's preference for unfolding the catalytic L1/R1 subunits indicates that the enzyme targets the most stable elements of the transpososome to destabilize the complex. The

observation that unfolding the L1/R1 subunits is most destabilizing provides further evidence that the L1/R1 and L2/R2 pairs make different intersubunit contacts within the complex.

ClpX unfolds one subunit or occasionally two subunits from the STC1 (7). We propose that ClpX stochastically recognizes and unfolds a single MuA subunit bound at either L1 or R1. Once this subunit is unfolded, the remodeled complex would typically not be subject to further unfolding (Fig. 5). The end result of remodeling, therefore, is a mixture of two principal complexes that make up the majority of the STC2 population—one fragile complex created by unfolding the L1-bound subunit, and another created by unfolding the R1-bound subunit (Fig. 5).

Previous biochemical analyses using DNase protection and heparin competition show that of the four subunits of the transpososome, only those at the L1, R1, and R2 DNA sites are stably bound; these studies thus suggested that these three subunits were necessary to maintain transpososome stability (10, 11). However, these experiments could not determine whether the L2 subunit contributed to stability of the transpososome via protein-protein contacts not observable by DNA-protection assays. Our finding that ClpX mediated unfolding of a MuA subunit bound to either L2 or R2 does not result in any substantial appearance of DNA disassembly products demonstrates that these two subunits do not make contacts essential for the stability of the assembled transpososome. Rather, our results demonstrate that the presence of an intact L1/R1 subunit pair is necessary to maintain the stability of the complex. The stability provided by the L1/R1 MuA interaction pair may mimic transposition complexes such as the Tn5 transpososome, which is a stable dimer both during and after the transposition reaction (23). Additionally, our results are supported by cryo electron-microscopy data, which reveal extensive protein-protein contacts unique to the two catalytic subunits in a double right-end Mu complex (20). The network of contacts between the L1 and R1 MuA subunits therefore appears to be the major factor responsible for the exceptional thermodynamic stability of the transpososome.

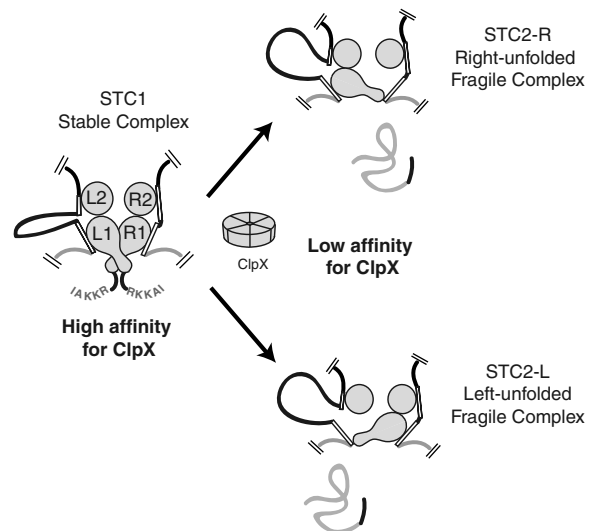


Fig. 5. Model for destabilization of the STC by ClpX. The assembled STC1 presents the interwoven L1-R1 subunits to ClpX for high-affinity binding. In the assembled state, the left-end loop is severely bent. ClpX can select either the L1 or R1 subunit to destabilize the complex, resulting in a heterogeneous STC2 mix containing complexes that were destabilized on the right side (STC2-R) and complexes that were destabilized on the left (STC2-L); in both types of complexes, constraints on the left-end loop are relaxed. The interwoven structure of the STC1, initially responsible for high-affinity presentation of the substrate to ClpX, is lost upon destabilization, and the STC2 is released from the enzyme.

By using altered binding specificity to target MuA subunits to different DNA sites in the transpososome, we were able to pinpoint which subunits ClpX unfolds to remodel the transpososome and to determine how different subunits contribute to complex stability. Conceptually, one could use targeted ClpX unfolding to dissect the role of subunits within a wide variety of complexes by tagging specific subunits with appropriate recognition tags. Indeed, such selective unfolding of subunits from a complex has been previously applied to analyze the contribution of the L2 subunit to the stability and function of the ribosome (24). Our study provides proof-of-principle for this method using a natural oligomeric substrate, which has evolved to allow disassembly by ClpX-mediated unfolding of its most stable elements.

Although ClpX destabilizes complexes containing a MuA recognition tag at either L1 or R1, the rate of disassembly is only about half 50% that observed with complexes composed of all wild-type subunits. Thus, although ClpX unfolding of one tagged subunit bound at L1 or R1 is sufficient for destabilization, other tagged subunits in the complex appear to play a role in optimal recognition or destabilization of the complex. This stimulatory role of tags on “secondary” MuA subunits probably involves creating a higher affinity interaction between ClpX and the transpososome via tethering interactions with the N-domains of the ClpX hexamer, as MuA tags are known to contribute to recognition in this fashion (21). For example, the C-terminal MuA tags in both subunits of the L1/R1 pair could bind ClpX. Once one of these subunits was removed from the transpososome by unfolding, the remaining subunit would not be interwoven with its partner and therefore would be recognized poorly by ClpX (21). A mechanism of this type would make the probability of a second unfolding event by ClpX extremely low (Fig. 5). The tethering activity provided by the tags uniquely within the STC1 may thus be a mechanism to limit the unfolding by ClpX to one catalytic MuA subunit, preserving the subunit composition of the STC2 required to ultimately recruit the DNA-replication machinery.

Although our results show that ClpX has preference for the L1 and R1 subunits of the transpososome compared to L2 and R2, our experiments were performed using complexes with only one MuA tag, which removes all tag-dependent secondary tethering interactions. The preference for the catalytic subunits may, in fact, be more pronounced in a wild-type complex in which tethering contacts by all four tags could target a high affinity interaction between ClpX and the L1 or R1 subunits.

ClpX Preference for MuA Subunits Does Not Signal Left-End Initiation of DNA Replication. Our results indicate that ClpX has no intrinsic preference for left versus right catalytic subunits during remodeling. However, previous footprinting studies showed that ClpX-mediated remodeling of the STC1 is accompanied by large DNA conformational changes on the left side of the transpososome (7). Most of the footprinting changes observed occur within the left-end loop and the L2 site, whereas minimal changes were detected within the actual L1 and R1 sites (7). The unique left-end loop is not bound by MuA and is thought to be in a severely bent configuration when both L1 and L2 are bound by MuA in the STC1. It is conceivable that the same interwoven structure that is the major source of stability for the transpososome is also responsible for restraining the conformation of the left-end loop. Upon destabilization of the complex by ClpX, either by unfolding the L1 or R1 subunit, releasing the constraints on the loop could result in the large conformational change detectable by footprinting (7) (Fig. 5). This change in the loop may bias changes observed by footprinting in the remodeled transpososome to the left end of the complex. Indeed, if ClpX can remove MuA subunits bound either to L1 or R1 with equal probability, as we observe, then the interactions at these sites would remain in half of the STC2 population, explaining why minimal changes in footprinting are observed at these sites.

If ClpX does not discriminate between the left and right catalytic subunits during the conversion from STC1 to STC2, at what point is the preference for left-end initiation of replication established? Unlike most genomes, phage Mu does not have a replication origin. Instead, it uses the forked DNA structures generated during recombination as the nucleation sites for replication-fork assembly in a reaction that mimics replication restart (25). Why the DNA fork at the left end is preferentially used for initiation is not understood. Studies from Nakai and colleagues have shown that a multicomponent (partially purified) fraction, known as MRF α , is responsible for the conversion of the STC1 to a replication-competent complex known as the prereplisome (25). Although ClpX is one of the factors in MRF α required for the transition from STC1 to STC2, there are other incompletely characterized factors (known as faction MRF α DF) that completely disassemble MuA from the STC2, creating the prereplisome scaffold for the assembly of the replication machinery (26). The large conformational change in left-end DNA caused by ClpX-mediated remodeling of the STC1 may provide a signal for MRF α DF to disassemble the loosely bound MuA subunits in the STC2, allowing the replication machinery to initiate replication on the left side of the Mu genome. Other structural asymmetries between the left and right side of the transpososome may also play a role in the left end replication preference, such as the presence of the DNA-binding site for HU, a DNA-bending protein, as well as the *pac* DNA packaging site within the left-end loop of the transpososome (27).

The transition from the stable to the fragile transpososome occurs concomitantly with disruption of the stably interwoven subunits in the transpososome. Destabilization of complexes by unfolding of the most stable local structural elements by ClpX and other AAA+ unfoldases may be a general mechanism for remodeling other multimeric substrates. For example, Dps is a dodecamer that protects DNA by forming extremely stable biocrystals upon entry into stationary phase (28). Upon exit into exponential phase, ClpX may target only those Dps subunits that are critical for biocrystal stability, allowing destabilization of the complex using minimal energy. Further discovery of remodeling substrates may help reveal such commonalities in the mechanisms of complex destabilization by ClpX and other unfoldases.

Materials and Methods

DNA for Transposition and Cloning. All altered-specificity MuA variants and the miniMu constructs were produced using the Quikchange kit (Stratagene). The sequence of each altered Mu DNA binding site can be found in Namgoong and Harshey (18). For construction of the double right-end miniMu plasmid, pMK586 was digested with ClaI and EcoNI to remove the left-end binding sites, treated with calf intestinal phosphatase, and ligated to 5'-phosphate annealed oligonucleotides containing R1 and R2 MuA binding sites with appropriate DNA overhangs.

Protein Purification. Unlabeled MuA variants (29), HU protein (30), ClpX (31) and ClpP (32) were purified as described. ³⁵S-MuA, ³⁵S-MuA^{Δ8}, and ³⁵S-MuA^{R146V} were purified using the same protocol as the unlabeled MuA variants, with the several modifications included in *SI Text*.

Transpososome Assembly. Transpososomes (formed as intramolecular strand transfer complexes) were assembled *in vitro* in 25 mM Hepes (pH 7.6), 1 mM MgCl₂, 140 mM NaCl, 1 mM DTT, 15% glycerol, 20 μg/mL BSA and 12% DMSO. Transposition reactions contained 30 μg/mL circular miniMu or miniMu altered-specificity variant (4,415 base pairs) and 130 nanomolar (nM) *E. coli* HU protein. To assemble mixed transpososomes, 300 nM ³⁵S-MuA^{R146V} was preincubated with miniMu DNA for 5 min at 30 °C, 50 nM of unlabeled MuA or MuA^{Δ8} was added, and the mixture was incubated at 30 °C for 90 min. Preincubation of MuA R146V was necessary to prevent wild-type MuA from binding to the altered-specificity Mu sites (14, 18). Transposomes were purified prior to disassembly by passage through ~100 μL of phosphocellulose resin packed into a minispin column (Pierce) and equilibrated in 25 mM Hepes (pH 7.6), 0.1 mM EDTA, 5 mM DTT, 10% glycerol and 300 mM KCl.

Disassembly Assays and Visualization. A master mix (14 μ L) containing ClpX and ATP regeneration mix (ATP, creatine phosphate, and creatine kinase) was preincubated for 90 s at 30 °C and added to 40 μ L of transpososomes assembled as described above (final concentrations: $[\text{ClpX}_6] = 0.2 \mu\text{M}$; $[\text{ATP}] = 8 \text{ mM}$; $[\text{creatine kinase}] = 50 \mu\text{g/mL}$; $[\text{creatine phosphate}] = 10 \text{ mM}$). Samples (12 μ L) were removed from the reaction mix at different times and stopped by addition of 2 μ L of 500 mM EDTA. For each time point, 1 μ L was removed, diluted into 25 mM Hepes (pH 7.6), 0.1 mM EDTA, 5 mM DTT, 10% glycerol, 300 mM KCl, and 100 mM EDTA, and used to monitor the rate of disassembly by appearance of DNA disassembly product; the rest of the sample was used for labeled subunit release analysis on a separate agarose gel. Samples were electrophoresed on 0.9% high gelling temperature (HGT)-Agarose gel (Lonza) containing 10 $\mu\text{g/mL}$ BSA and 10 $\mu\text{g/mL}$ heparin. Gels containing samples for storage-phosphor quantification were first stained with Sybr Green I (Invitrogen/Molecular Probes) or Vistra Green

(GE/Amersham), pressed and dried using a Biorad Dryer and placed into phosphorimager cassette. Gels containing samples for DNA-product appearance quantification were stained with Sybr Green I or Vistra Green and visualized using a Typhoon 4100 imager. Rates of disassembly were quantified using Imagequant (GE) using the rolling-ball background subtraction method (radius = 200). DNA-product appearance was quantified as previously described (21). K_M and V_{max} values for ClpX disassembly of double right-end transpososomes were determined as described (21).

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