Mobile Genetic Elements 4, e28807; January–December 2014; © 2014 Landes Bioscience

One to rule them all

A highly conserved motif in mariner transposase controls multiple steps of transposition

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Keywords: transposition, DNA recombination, human genome, gene therapy, genetic engineering, hyperactive transposase, conformational change, overproduction inhibition

Submitted: 03/11/2014

Revised: 04/01/2014

Accepted: 04/07/2014

Published Online: 04/14/2014

Citation: Claeys Bouuaert C, Tellier M, Chalmers R. One to rule them all: A highly conserved motif in mariner transposase controls multiple steps of transposition. Mobile Genetic Elements 2014; 4:e28807; http://dx.doi.org/10.4161/mge.28807

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Commentary to: Liu D, Chalmers R. Hyperactive mariner transposons are created by mutations that disrupt allosterism and increase the rate of transposon end synapsis. Nucleic Acids Res 2014; 42:2637–45; PMID:24319144; http://dx.doi.org/10.1093/nar/gkt1218

The development of transposon-based genome manipulation tools can benefit greatly from understanding transposons' inherent regulatory mechanisms. The Tc1-mariner transposons, which are being widely used in biotechnological applications, are subject to a self-inhibitory mechanism whereby increasing transposase expression beyond a certain point decreases the rate of transposition. In a recent paper, Liu and Chalmers performed saturating mutagenesis on the highly conserved WVPHEL motif in the mariner-family transposase from the Hsmar1 element. Curiously, they found that the majority of all possible single mutations were hyperactive. Biochemical characterizations of the mutants revealed that the hyperactivity is due to a defect in communication between transposase subunits, which normally regulates transposition by reducing the rate of synapsis. This provides important clues for improving transposon-based tools. However, some WVPHEL mutants also showed features that would be undesirable for most biotechnological applications: they showed uncontrolled DNA cleavage activities and defects in the coordination of cleavage between the two transposon ends. The study illustrates how the knowledge of inhibitory mechanisms can help improve transposon tools but also highlights an important challenge, which is to specifically target a regulatory mechanism without affecting other important functions of the transposase.

Regulation of DNA Transposons in Bacteria and Eukaryotes

The life-style of a transposon has many of the hallmarks of a classical host-parasite relationship. The host has adaptations, such as RNAi, to suppress the parasite, while the parasite has adaptations to spare the fitness of the host. Indeed, it appears that transposons are so well adapted that they have invaded all branches of the tree of life to the extent that they are now the numerically dominant family of genes in nature.[1] Transposons can be divided into two types depending on whether they mobilize via an RNA or a DNA intermediate. Here we will focus on the latter, which are the dominant type in bacteria but still contribute several percent of the genomic sequences in higher eukaryotes.

DNA transposons don't persist indefinitely in a given genome and their evolutionary success relies on horizontal transfer into new hosts. From an evolutionary perspective, the life cycle of a transposon can be considered as the period between its appearance in a virgin genome and its extinction. Theoretical considerations suggest that the selective pressure on the transpositional activity of the element will vary at different stages of a genomic invasion. At the start of the invasion, when there are only one or few copies of the transposon, a high rate of transposition is advantageous because it will protect against genetic drift. Once the element is established in the genome, selection will favor a progressively lower rate of transposition (per copy) to protect

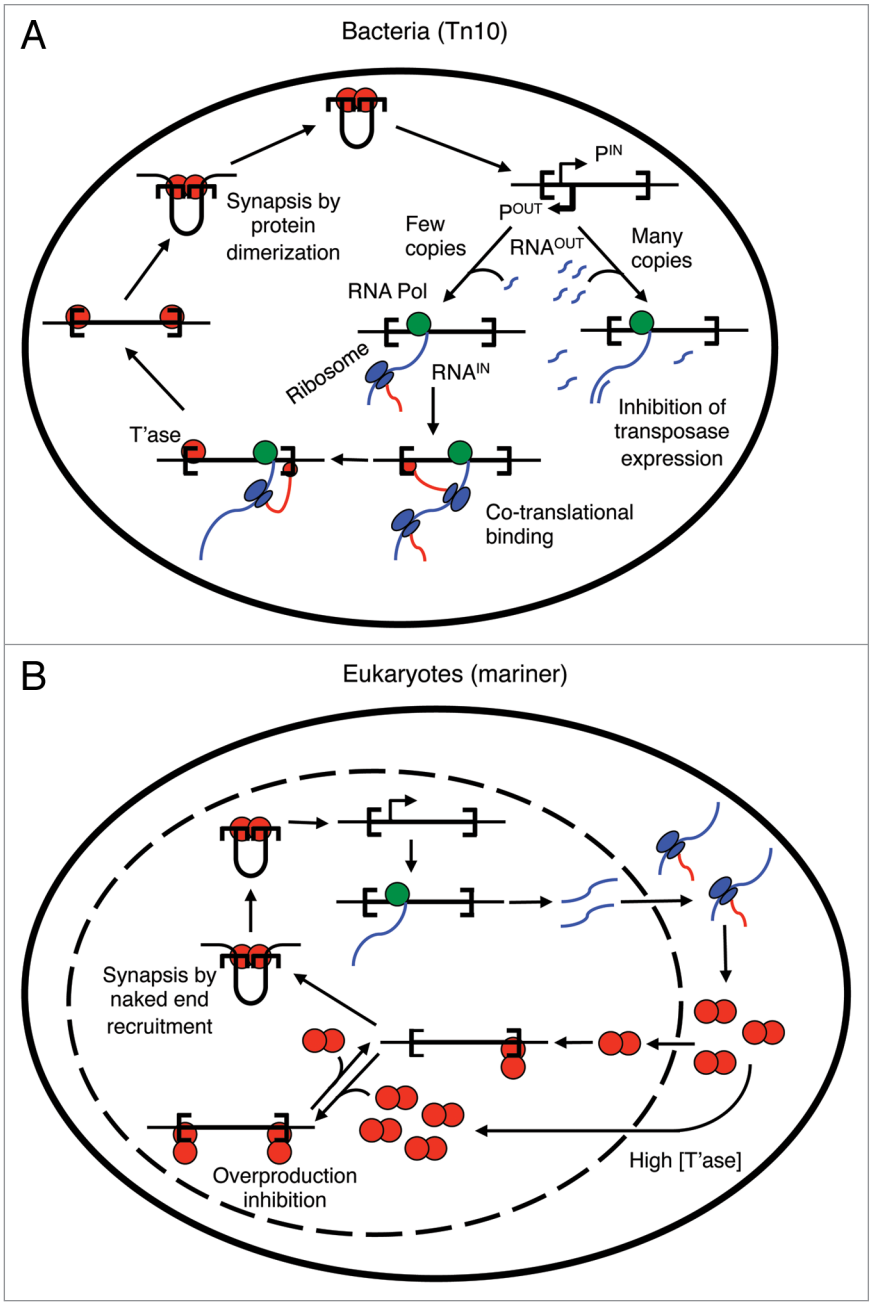

Figure 1. Autoregulation of transposition in bacteria and eukaryotes. (**A**) Regulation of transposon copy number in bacteria relies on the expression of a cis-active transposase and a trans-active inhibitor. Illustrated is an example of bacterial transposon where the trans acting inhibitor is an antisense RNA, as in Tn10. P^{IN}, promoter controlling the expression of transposase mRNA (RNA^{IN}). P^{OUT}, promoter controlling the expression of the antisense RNA (RNA^{OUT}). The cis-activity of transposase relies on the continuity of transcription and translation. The N-terminal DNA binding domain has the opportunity to bind the transposon end during transposase synthesis. DNA-bound transposase monomers dimerize to form the synaptic complex, in which transposition takes place. As the transposon copy number rises, the elevated levels of antisense RNA prevent ribosome binding on the mRNA and inhibit transposase expression. Thus, as the copy number rises, the concentration of transposase and the rate of transposition falls. (**B**) In eukaryotes, Tc1/mariner transposons are subject to overproduction inhibition. When the genome harbors few transposon copies, transposase expression is low. After being synthesized in the cytoplasm, transposase enters the nucleus and binds one end of a transposon. The second (naked) transposon end is then recruited to form the synaptic complex. When the transposon copy number is high the transposase concentration rises. The probability of double occupancy of the two transposon ends rises accordingly. The excess transposase competes for free transposon ends, which inhibits their recruitment into the developing transpososome. Green sphere, RNA polymerase; blue oval, ribosome; red spheres, transposase; black lines, DNA; blue lines RNA; red lines, polypeptide.

the host against an exponential explosion in numbers. Consistent with this idea, transposons appear to have acquired sophisticated regulatory mechanisms (below). Indeed, computer modeling of the dynamics of a genomic invasion suggests that unregulated transposition quickly leads to the demise of the transposon or the host.[2,3] Autoregulation is thus likely to be an essential feature of a successful molecular parasite such as a transposon.

Autoregulation has been studied in some detail in the bacterial transposons. The best-known strategy involves the combination of a cis-acting transposase and a trans-acting inhibitor. As the number of copies of the element rises, the power of the cis-acting transposase to mobilize each element remains constant, while the power of the trans-acting inhibitor increases progressively (**Fig. 1A**). This defuses the exponential amplification and is thought to make the total rate of transposition largely independent of the copy number. This strategy has been documented in Tn*10* and Tn*5* where the inhibitor takes the form of an anti-sense RNA and a truncated transposase isoform, respectively.[4,5] The cis-activity of the transposase relies on the fact that transcription and translation in prokaryotes are simultaneous. This has been most clearly demonstrated by studies on IS*911*.[6] During protein synthesis, the N-terminal DNA-binding domain has an opportunity to fold and bind the nearest transposon end available. Once translation is completed, the N-terminal DNA binding domain is occluded by an inhibitory motif located in the transposase C-terminus. This provides a very narrow window of opportunity for the transposase to diffuse away from its site of synthesis, which is the basis for its cis-action.

While this mechanism is effective in bacteria, it is not compatible with eukaryotic cell biology, where transcription and translation are separated. Eukaryotic

transposases are therefore necessarily trans-acting and the transposons were assumed to have evolved alternative mechanisms to preempt their exponential amplification. Working in *Drosophila*, Daniel Hartl's group saw the first indications that such a mechanism may exist in Mos1.[7] They found that the rate of transposition decreased when transposase was overexpressed under the control of a heat-shock promoter or when the gene was present in multiple copies. This phenomenon was termed overproduction inhibition (OPI), but the underlying mechanism remained unknown. Recent in vitro experiments with the closely related Hsmar1 transposon, also a member of the mariner family, revealed the mechanism responsible for OPI.[8] The mechanism is simple and elegant, and is likely to have been adopted by many other eukaryotic elements, as suggested by in vivo experiments with Sleeping Beauty and the more distantly related transposon piggyBac.[8]

It turns out that Hsmar1 autoregulation is provided by a competition between active multimers of the transposase for their binding sites on the transposon ends (**Fig. 1B**).[8] Before any of the chemical steps of transposition can take place, a transposase multimer must first bind one transposon end and then recruit a second, naked, transposon end to form the synaptic complex within which the reaction takes place. As a genomic invasion progresses, each additional copy of the transposon contributes transposase to the pool of multimers. This tends to saturate the binding sites, reducing the number of naked transposon ends available for productive synapsis (**Fig. 1B**, bottom left). Note that the mechanism does not depend on the actual multimeric state of the transposase, only on the fact that the second transposon end is naked when it is recruited into the developing complex. This ensures that an increase in the transposase concentration beyond a certain point will always lead to a reduction in the rate of transposition.

This contrasts markedly with the situation in the bacterial transposons Tn*10* and Tn*5*. Their unbound transposases are monomeric and a productive synapsis arises from the dimerization of a pair of bound transposon ends (**Fig. 1A**). In this case, an increase in transposase concentration will always increase the rate of transposition. The cis-action of the transposases helps to slow the exponential increase in the rate of transposition. However, it cannot be eliminated even if cis-action was 100% effective.[8] Hence the need for a trans-acting inhibitor.

Mariner Autoregulation Relies on Communication between Transposase Subunits

The model described above for mariner autoregulation was described as an assembly-site occlusion (ASO) mechanism. In its simplest form, as illustrated in **Figure 1B**, inhibition will not become significant until the free transposase concentration reaches a significant fraction of its affinity for the transposon end.[8] This is because the transposase must search a much greater volume of the nucleus to find the first transposon end than to find the second, which can never be too far away owing to the continuity of the DNA connecting them. This simple version of the ASO mechanism is therefore ineffective until there are hundreds of thousands of transposons contributing to the pool of transposase.

Biochemical analysis revealed that mariner has evolved a mechanism, based on conformational coupling between the transposase subunits, that greatly enhances the effectiveness of ASO.[8] During the initial binding phase of the reaction, the transposase behaves like a typical helix-turn-helix DNA-binding protein and interacts quickly with the first transposon end it encounters. In other words, it has a high on-rate. However, at this point it seems to experience a conformational change that alters its behavior quite markedly. First, it has a very slow off-rate and its $t_{1/2}$ for transposon-end binding is about 20 min. This is much slower than the value for a typical helix-turn-helix protein such as the lac repressor, which is about one minute. Second, the transposase's affinity, or on-rate, for the other transposon end is reduced by a factor of about 10^5 compared with the first. This slows synapsis and favors formation of the unproductive OPI complex over the productive synaptic complex. Taken together these factors enhance the ASO mechanism such that a pseudo-steady-state rate of transposition is established very early in a genomic invasion, when only a few copies of the transposon are present.[8]

The conformational change, and the resulting low affinity of the developing complex for the second transposon end, also serves another role. It provides the reaction with a topological selectivity that favors transposon ends in the inverted-repeat configuration over those in a direct-repeat configuration or those located on different molecules.[9] This "topological filter" requires free negative-supercoiling, which overcomes slow synapsis by providing the transposon ends with a high relative concentration and favorable angular distribution in the plectosome (**Fig. 2A**). When the transposon ends are in the direct-repeat configuration, or when the inverted-repeat ends experience positive supercoiling, the unfavorable angular distribution gives slow synapsis despite the high relative concentration in the plectosome (**Fig. 2B and C**). In the absence of supercoiling, or when transposon ends are on different molecules, synapsis is slow due to their low relative concentrations (**Fig. 2D and E**).

The authors speculated that the topological filter might suppress genomic instability, which arises from the promiscuous synapsis of transposon ends (e.g., ref. 10). They also suggested that the system is driven by the free negative supercoiling that exists transiently in eukaryotic DNA behind transcription and replication bubbles and during chromatin remodeling, and that this synchronizes transposition with cellular events.[9] It is worth noting that topological selectivity has been documented in several prokaryotic recombination reactions, such as phage Mu transposition, Tn3/γδ resolvase, and the *hin*/*gin*/*cin* DNA inversion systems.[11-15] However, to our knowledge this is the first suggestion that it may operate in higher eukaryotes. In addition, the topological filter of mariner is unique in that it involves only the two transposon ends and does not depend on the recruitment of a third site.

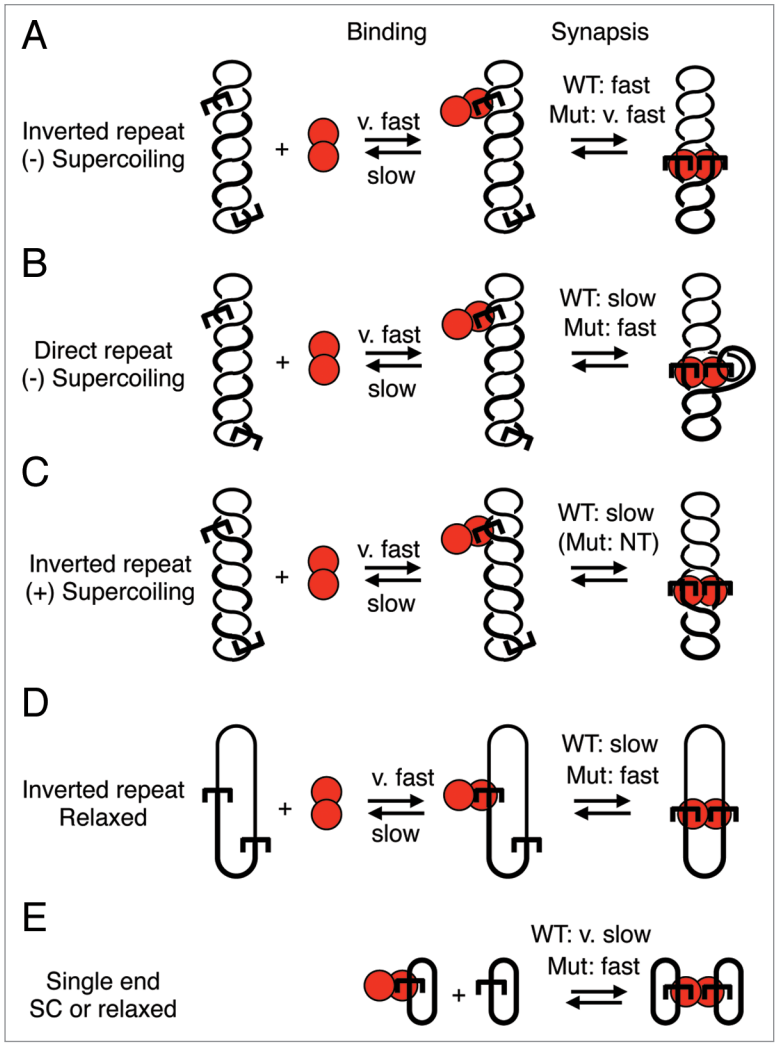

Figure 2. Topological selectivity of transposon end synapsis in *Hsmar1* transposition. Binding of a transposon dimer to the first transposon end is rapid. Recruitment of the second transposon end is slow with wild-type transposase, but is faster with the WVPHEL mutants. Wild-type transposase is subject to topological selectivity of the transposon ends: synapsis is most efficient with a negatively (–) supercoiled (SC) substrate that has transposon ends in the inverted repeat configuration. In contrast, when transposon ends are in direct repeat, or on different molecules, or when the substrate is positively supercoiled or relaxed, transposon ends synapsis is very slow with the wild-type transposase. The WVPHEL mutants have a relaxed topological selectivity for the transposon ends: synapsis is much faster with direct-repeat substrates, nicked inverted repeat and single ended substrates. NT, not tested.

Disrupting Inter-Subunit Communication Provides Hyperactive Transposases

In a recent paper published in Nucleic Acids Research, Liu and Chalmers reported a detailed analysis of mutants that disrupt the coupling between transposase subunits.[16] The study focused on the WVPHEL amino acid motif, which is highly conserved in the mariner transposases. It is located in the "linker" region between the N-terminal DNA-binding domain and the C-terminal catalytic domain (**Fig. 3A**). In the crystal structure of the Mos1 post-cleavage transpososome the WVPHEL motif is seen to occupy a central position in the dimer interface.[17] An unstructured "clamp-loop" feature emerges from the catalytic core of one transposase subunit, crosses the dimer interface, and interacts with the transposon end and the WVPHEL motif on the other side of the complex (**Fig. 3B and C**). These interactions account for more than 70% of the dimer interface.

Liu and Chalmers were motivated by a previous alanine scanning mutagenesis study of the WVPHEL motif in the related Himar1 transposase. This showed that mutations at 4 out of the 6 positions provided hyperactive transposases in a bacterial transposition assay.[20] This was an intriguing result because mutations in a conserved motif would be expected to reduce the activity of a protein, rather than enhance it. One possibility is that the motif performs a regulatory function that balances the selfish amplification of the transposon against it detrimental effects on host fitness. To address this issue, and to search for more active transposase variants, Liu and Chalmers generated almost all possible single amino-acid substitutions in the WVPHEL motif of Hsmar1.[16] In a bacterial assay, the great majority of the mutants at the W, V, E, and L positions had activities ranging from just above wild type to more than 60-fold higher. Most of those tested were also hyperactive in a HeLa cell transposition system, although the advantage enjoyed over wild type was lower than in the bacterial assay. In contrast, almost all mutations at the P and H positions were hypoactive, suggesting that these residues are important for the reaction, over-and-above their role in a regulatory function.

Liu and Chalmers went on to characterize a representative subset of mutants biochemically. The reaction provided by Hsmar1 is by far the most efficient and reliable in vitro system available for a eukaryotic transposon and it offers unique opportunities for detailed mechanistic studies. The standard in vitro assay relies on gel-electrophoretic analysis of reactions using plasmid substrates.[21] By analyzing the kinetics of the reactions using supercoiled or relaxed substrates that encode either one or two transposon ends, Liu and Chalmers showed that the WVPHEL mutants are hyperactive because they increase the rate of the synapsis step.[16]

Faster synapsis was most evident when the reaction was performed using a single ended substrate as illustrated in **Figure 2E**. With this substrate synapsis is by far the slowest step of the reaction.

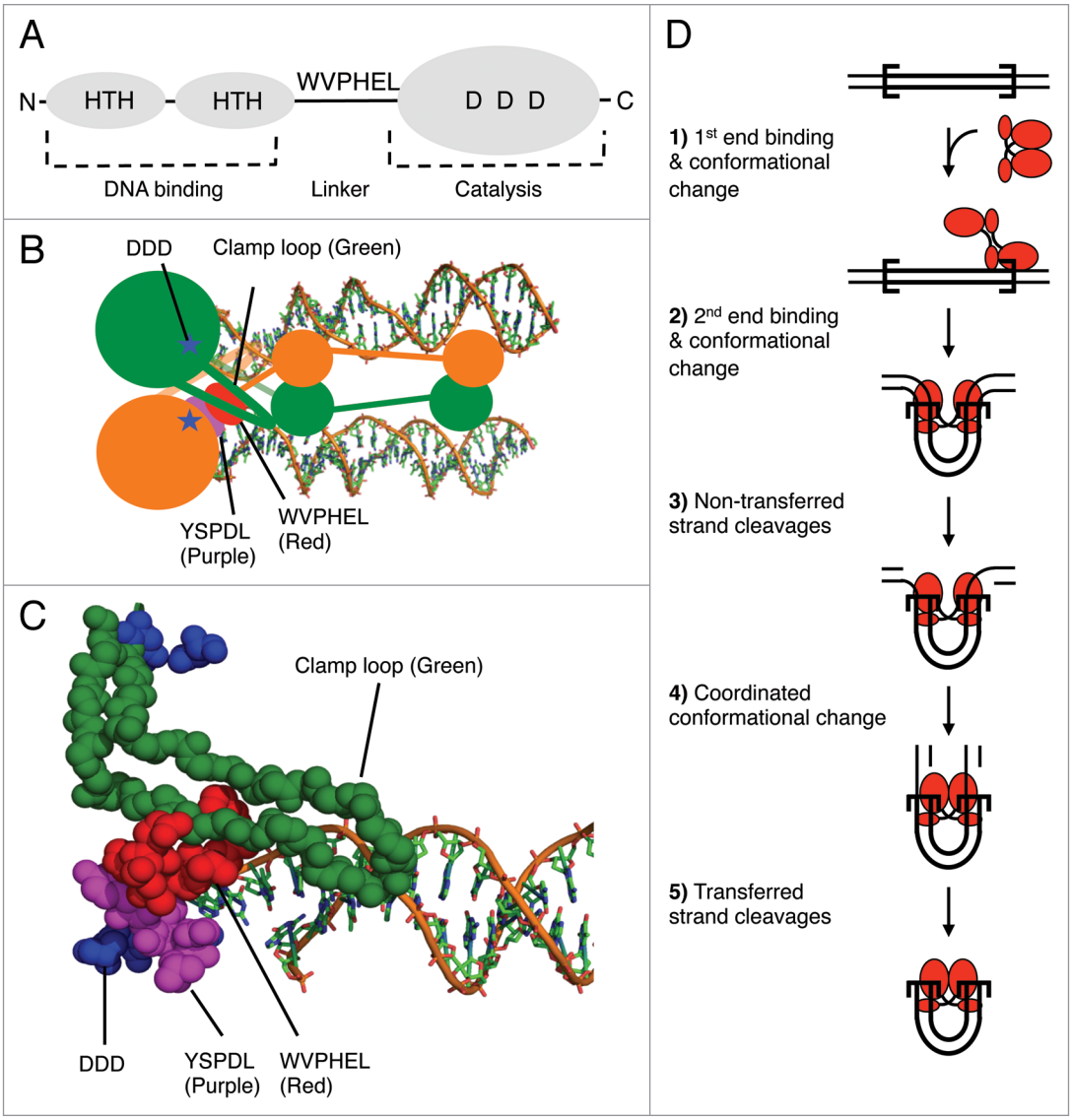

Figure 3. Structural relationship of the WVPHEL motif within the mariner transpososome and conformational changes involved during transposition. (**A**) Transposase has an N-terminal DNA binding domain (amino acids 1–115 approximately) with two helix-turn-helix motifs (HTH). The catalytic domain (amino acids 125–343) has a triad of conserved aspartate residues (DDD), which coordinate the catalytic Mg^{2+} ions. The domains are connected by a proteolitically sensitive linker region, which harbors the conserved WVPHEL sequence motif. (**B**) The trans architecture of the transposome as visualized in the crystal structure of the post-cleavage intermediate.[17] Transposase is represented as green and orange blobs; active site, blue; clamp loop, green; WVPHEL motif, red; YSPDL motif, magenta. (**C**) A space filling representation for the interactions between the clamp loop and the WVPHEL motif at the dimer interface. Coordinates from PDB HOT3. (**B and C**) are reproduced from reference 18, available under Creative Commons Attribution License. (**D**) Mariner transposition involves multiple conformational changes. (1) Binding of a transposase dimer to the first transposon end reduces the affinity of the unbound subunit for the second end. This appears to be associated with an elongation of the transposase dimer, where subunits are held head-to-head with the N-terminal DNA binding domain.[19] (2) Second end binding is associated with a conformational change that prepares transposase for catalysis. (3) Non-transferred strand cleavages expose 5′-phosphates at the transposon ends. (4) A coordinated conformational change then prepares the complex for (5) transferred strand cleavages that expose the 3′-hydroxyl groups at the transposon ends.[18]

This is because of the low relative concentration of transposon ends and the low affinity of the developing complex for the second transposon end.[9] Under these stringent conditions, the WVPHEL mutants reacted up to 10 times faster than wild-type. In a set of experiments that was not included in the original study, the authors found that the mutants were also much more active on the negatively-supercoiled direct-repeat substrate (Liu and Chalmers, unpublished data). The WVPHEL mutations thus both increased the rate of synapsis and relaxed the topological selectivity for the transposon ends.

The WVPHEL Motif Plays Multiple Functions

Disrupting the autoregulatory mechanism of transposons is valuable to generate hyperactive variants for biotechnological and medical applications.[22] The finding that

the WVPHEL motif is central to autoregulation therefore provides important clues for improving mariner-based tools. However, Liu and Chalmers also found that the WVPHEL mutants have another, unanticipated feature, that would not be desirable for transposon-based genome manipulations. Transposases usually avoid uncontrolled cleavage events, which are unproductive for the transposon and harmful to the host. To prevent unnecessary damage, cleavage is therefore normally only supported within the context of the synaptic complex. The transposase active site must therefore be restrained until the transpososome is fully assembled. However, the WVPHEL mutants failed to obey this precept. Indeed, they exhibited significant cleavage activities under OPI conditions, where synapsis is precluded by the double occupancy of transposon ends. In fact, non-specific nuclease activity was also significant on plasmid substrates that entirely lacked a transposon end. This indicates that the mutants were defective in the control of the initiation of catalysis.[16]

So far, we have described how the WVPHEL motif controls the rate and topological selectivity of synapsis and the initiation of catalysis. However, it has yet another function. It is also important for the communication between transposase subunits during catalysis.[18] Mariner transposases cleave the two DNA strands at each transposon end by sequential hydrolysis reactions. The precise mechanism remains unknown, but it involves a structural change between hydrolysis of the non-transferred and the transferred strand.[21,23,24] This transition is coordinated within the transpososome.[18] Thus, both non-transferred strands must be cleaved before a coordinated structural change prepares the transpososome for cleavage of the two transferred strands. Two observations suggested that the coordinated transition was affected in the WVPHEL mutants. First, one of the few hypoactive mutants was unable to perform the transition at all and the reaction stalled after non-transferred strand cleavage. Second, a hyperactive mutant was able to complete the transition but was less robust than wild type at coordinating the transition between subunits.

Conclusion

DNA transposition is a complex, multi-step reaction, which involves six strand breaking and joining reactions. In the case of mariner, and other elements, there are also in-built control mechanisms that balance the transposon's fitness against the fitness of the host. Important insights into the mechanism of mariner transposition have been gained from recent biochemical and structural studies. In particular, it has become clear that transposition involves multiple conformational changes, during which transposase subunits are in close communication. However, structural data remains sparse and we still lack a detailed view of the various steps. Indeed, the post-cleavage transpososome is the only intermediate for which structural data exists.[17]

The classical view of a transposition reaction is that it comprises four main stages: 1) free transposase, 2) synaptic complex, 3) post-cleavage complex, and 4) target capture/integration complex. However, biochemical analysis suggests that there are several additional sub-steps (**Fig. 3D**). The free transposase is presumably a symmetrical entity because this is the lowest energy conformation for a homodimer. However, first end binding must necessarily break the symmetry (**Fig. 3D**, step 1). In the biochemical experiments, this corresponds to the conformational change that reduces the affinity of the developing complex for the second transposon end. In the next stage, synapsis restores the symmetry of the complex. This in turn must be associated with a structural change that prepares the two active sites for catalysis (step 2). This step also significantly destabilizes the subunit interface because the synaptic complex falls apart into two single-end complexes during electrophoresis.[8] Finally, the transition between non-transferred-strand and transferred-strand cleavage is also associated with a structural change that is coordinated between the two sides of the complex and is presumably required for the double strand cleavage of the transposon ends (step 4). While we await structural data to shed light on these fascinating conformational transitions, one thing at least seems clear: the WVPHEL motif rules them all.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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