

Regulation of Mu Transposition via Communication Between the  
Transposase and a Mu-Encoded Accessory Protein

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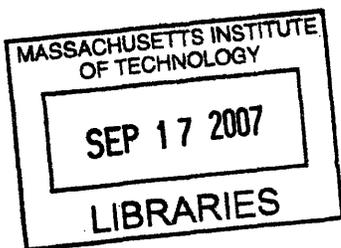
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# Regulation of Mu Transposition via Communication Between the Transposase and a Mu-Encoded Accessory Protein

by

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## ABSTRACT

Transposable elements comprise a significant portion of both prokaryotic and eukaryotic genomes. These mobile segments of DNA have helped to shape the course of evolution by generating genetic mutations and contributing to genomic rearrangements. Transposable elements often target transposition to non-random DNA sites. Ideal target sites must be common, so as to ensure efficient propagation of the transposable element, and yet non-essential, so as to protect the host cell on which the element depends. Transposable elements have devised a plethora of strategies for targeting transposition to desirable DNA.

This thesis investigates the molecular mechanisms involved in Mu transposition. We explore the interactions between the Mu transposase, MuA, and a Mu-encoded accessory protein, MuB. Together, these two proteins regulate Mu transposition and targeting. We demonstrate that MuB interacts with multiple subunits of the MuA transposase complex to stimulate transposition. These results corroborate previous theories that MuB acts as an allosteric regulator of MuA. We also investigate the mechanism by which Mu transposes into selected DNA. We find that MuB "delivers" favorable target DNA to the Mu transposase by tethering the DNA to MuA. This interaction is independent of ATP hydrolysis by MuB. The work described herein has contributed to our understanding of the protein-protein interactions involved in Mu transposition.

Thesis Supervisor: Tania A. Baker  
Title: E. C. Whitehead Professor of Biology

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# **Chapter I**

## Introduction

## Overview

Transposable elements, or transposons, are segments of DNA capable of non-homologously recombining into a target DNA molecule. These elements are often described as "selfish DNA" or as "genetic parasites" because they depend upon a host cell for replication and survival, without necessarily providing an advantage to the host. Additionally, transposons can deleteriously affect their hosts by inserting into essential genes. However, closer inspection reveals that transposons do have the potential to benefit their hosts in the long run by creating genetic variation required for evolution. Transposable elements have been detected in every organism in which their presence has been examined (Curcio and Derbyshire, 2003), and it is estimated that at least 40% of the human genome is composed of sequences related to once active transposable elements. (Lander et al., 2001). Therefore, to fully understand the evolution of modern species, one needs to consider the role that transposons have had in shaping genomes.

Transposable elements are often described as integrating "randomly" into target DNA. However, studies of transposon targeting reveal that most transposable elements chose target sites with some degree of specificity. Both the nature of the preferred target sites as well as the molecular mechanisms underlying targeting are as varied as transposons themselves. Nevertheless, some common themes do emerge. Elucidating the factors that determine transposon targeting is crucial to understanding why transposable elements have become so wide spread and how these elements have impacted evolution.

This introduction reviews where and how transposable elements target transposition. First, this chapter describes the types of preferential target sites chosen by

transposons. Second, this chapter discusses common molecular mechanisms that dictate target selection.

## **Target Site Choice**

Most transposable elements display some degree of selectivity in choosing target sites. In some cases, the transposable elements target transposition to sites that specifically benefit the element itself, such as sites that avoid disrupting existing copies of the element. In other cases, transposable elements choose sites that are less likely to harm the host. Presumably, protecting the well being of the host increases the likelihood that the transposon will be replicated and transferred to the next generation. However, not all transposable elements preferentially transpose into "safe" sites, and many seem just as likely to integrate into essential regions of the host's genome as into benign sites. Some transposons, in fact, have survived despite a penchant for integrating into coding DNA. Below, four genres of transposon target selection are described. These genres include preferential target sites that are: i) beneficial to the host, ii) benign to the host, iii) beneficial to the transposon (without necessarily being beneficial or benign to the host), and iv) neither beneficial to the host nor to the transposon.

### ***Target Sites that Benefit the Host***

All transposable elements have the ability to promote the evolution of their host species by serving as a source of genetic variation, and numerous bacterial transposons carry antibiotic resistance genes, offering their host yet another advantage. However, to date, only two transposable elements are known to target transposition to sites that will

specifically benefit the host. The Het-A and TART elements in *Drosophila* specifically target the chromosome ends (Table 1). As *Drosophila* lack telomerase, Het-A and TART are responsible for maintaining the integrity of the chromosome ends (Biessmann et al., 1992; Biessmann et al., 1997). These two transposable elements engage in a symbiotic relationship with the *Drosophila* host cells. The host cells provide the transcription machinery necessary for transposon and replicate the transposons during cell division. For their part, the transposons protect the ends of the *Drosophila* chromosomes.

|               |          |                               |   |  |
|---------------|----------|-------------------------------|---|--|
| Mu            | Bacteria | Replicative                   | Consensus site, target immunity, flanking A/T rich DNA        | Transposase recognizes consensus site & interacts with element-encoded MuB protein |
| Tn7           | Bacteria | Cut-and-paste                 | <i>glmS</i> terminator, conjugative plasmids, target immunity | Transposase interacts with element-encoded proteins: TnsD, TnsE, TnsC              |
| Ty3           | Yeast    | Retro-transposition (LTR)     | Polymerase III promoters                                      | Ty3 integrase interacts with host-encoded transcription factors TFIIIB and TFIIIC  |
| Ty5           | Yeast    | Retro-transposition (LTR)     | Heterochromatin   | Transposase interacts with host-encoded Sir4p                                      |
| Het-A<br>TART | Fly      | Retro-transposition (non-LTR) | Telomeres   | Element-encoded Gag proteins likely involved                                       |
| Tx1L          | Frog     | Retro-transposition (non-LTR) | Tx1D (a cut-and-paste transposon)                             | Tx1L endonuclease recognizes tandem repeat, PTR-1, within Tx1D                     |

**Table 1: Characteristics of Several Well-Studied Transposons.** See text for references. For review see: Craig NL, Craigie R, Gellert M & Lambowitz AM (2002) *Mobile DNA II*. ASM Press, Washington, DC.

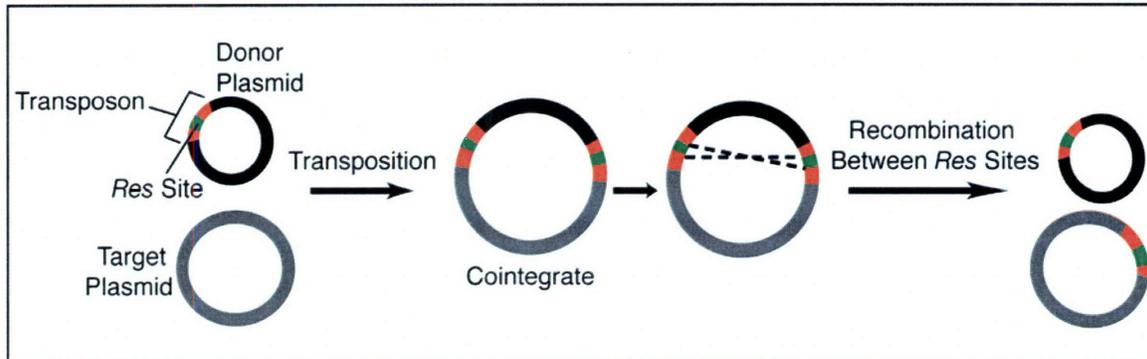
## *Target Sites that are Benign to the Host*

Several transposable elements target transposition to benign regions of the host genome. Integration into such "safe sites" is not likely to harm the host, thus increasing the chance that the transposon will be passed on to the next generation. A particularly well-studied transposon that chooses safe integration sites is the yeast retroelement Ty5 (Table 1). Ty5 preferentially inserts into heterochromatin at the telomeres and silent mating loci (HML and HMR), thereby avoiding actively transcribed regions of the yeast genome (Zou et al., 1996; Zou et al., 1995). A number of transposable elements target transposition to safe sites near or within genes. For example, the Tf1 retrotransposon from *Saccharomyces pombe* prefers to integrate into the promoter region of genes transcribed by polymerase II (Behrens et al., 2000; Kelly and Levin, 2005; Singleton and Levin, 2002). It has been demonstrated that Tf1 integration into polymerase II promoters does not affect the expression levels of the downstream gene products, confirming the benign nature of this target site (Behrens et al., 2000). The yeast retrotransposons Ty1, Ty2, Ty3 (Table 1), and Ty4 all preferentially insert upstream of genes transcribed by polymerase III (Chalker and Sandmeyer, 1992; Devine and Boeke, 1996; Kim et al., 1998). By targeting transposition to polymerase III promoters, Ty1-Ty4 leave coding regions intact. The bacterial transposon Tn7 also targets transposition to a particular site within a host gene that avoids disrupting coding DNA (Table 1). A Tn7 transposition hot-spot exists in the transcriptional terminator of the *glmS* gene, and transposition into this target site does not alter the *glmS* coding sequence (Gay et al., 1986). Interestingly, Tn7 directs transposition to the *glmS* transcriptional terminator by recognizing a sequence within the coding region of *glmS* (Qadri et al., 1989; Waddell and Craig, 1989).

Therefore, Tn7 recognizes a sequence that is well conserved but integrates into a site that prevents gene disruption. Taken together, multiple transposons have evolved mechanisms to target transposition to benign sites within the host genome.

Another strategy used by transposable elements to protect host function is to target transposition to already disrupted sites. This behavior is exhibited by numerous transposons from bacteria, plants, and insects that transpose into or near DNA that shares homology with their own sequences (Ason and Reznikoff, 2004; Galas et al., 1980; Goryshin et al., 1998; Guimond et al., 2003; Noma et al., 1997; Olasz et al., 1997; Suoniemi et al., 1997; Tower et al., 1993; Tu and Cohen, 1980; Yamazaki et al., 2001). By targeting transposition to DNA that contains a copy of the transposon's own genome, these elements avoid altering new regions of the host's genome. A dramatic example of a transposon that targets transposition to pre-disrupted sites is the Tx1L element from *Xenopus laevis* (Table 1). Tx1L targets transposition to another transposable element Tx1D, also present in *Xenopus laevis* (Christensen et al., 2000; Garrett et al., 1989). Similarly, the bacterial transposon Tn5053 targets integration to the resolution (*res*) sites of other transposable elements (Minakhina et al., 1999). Transposon *res* sites are sequences that undergo homologous recombination to resolve intermediate DNA structures formed during transposition (Figure 1). Interestingly, Tn5053 contains its own *res* site, which might compensate for the destruction of the target *res* site (Minakhina et al., 1999). Perhaps, by inserting into regions of the DNA that already contain a mobile element, transposons avoid disrupting new genes, and therefore, avoid generating new mutations that would be potentially deleterious to the host. In summary, a multitude of

transposable elements have evolved to preferentially target transposition to benign sites that are unlikely to harm the host.



**Figure 1: Resolution of transposition intermediate by recombination between *res* sites.** A donor plasmid contains a transposon with an internal *res* site. Replicative transposition into a target plasmid results in a cointegrate structure that contains the donor plasmid, the target plasmid, and two copies of the transposon. Homologous recombination between the *res* sites yields the original two plasmids, each with a copy of the transposon.

### *Target Sites that Benefit the Transposon*

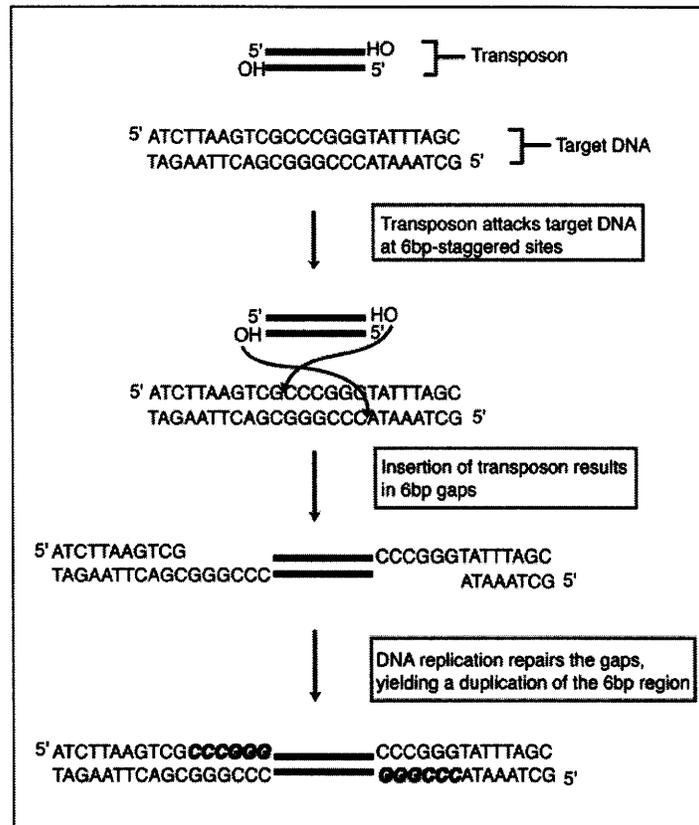
Some transposons choose target sites that benefit the transposon directly, irrespective of whether or not these sites would be harmful to the host. For example, Tn3, Tn7, and Mu all avoid inserting into already existing copies of their own genomes (Table 1) (Arciszewska et al., 1989; Lee et al., 1983; Reyes et al., 1987). This phenomenon, termed target immunity, ensures that a transposon will not commit self-destruction by integrating into and destroying its own genes or regulatory sequences. Target immunity is an important feature of Mu transposition, and this topic will be discussed throughout this thesis. Another self-beneficial targeting strategy is exhibited by transposons that preferentially integrate into DNA that is homologous to their own sequences (Ason and Reznikoff, 2004; Galas et al., 1980; Goryshin et al., 1998; Guimond et al., 2003; Noma et al., 1997; Olasz et al., 1997; Suoniemi et al., 1997; Tower et al., 1993; Tu and Cohen,

1980; Yamazaki et al., 2001). As previously explained, targeting transposition near existing copies of the transposon's own genome might benefit the host by preventing new disruptions to the host's genome. However, this targeting strategy is also likely to benefit the transposon itself by potentially promoting the creation of new, composite elements. For example, if two copies of a given transposable element are either overlapping or close to one another, it might be possible for one end from each element to be used in a recombination reaction, resulting in the creation of a novel element (Kleckner et al., 1996). Interestingly, targeting transposition to sequences that are self-similar represents the opposite goal of target immunity. However, both strategies have been employed by transposable elements to choose target sites that will benefit the transposon.

### *Target Sites with Unknown Benefits*

Many transposons target transposition to particular regions that have no known benefit for either the host or the transposon. Frequently, transposable elements prefer to integrate into DNA that conforms to a certain consensus sequence, though the significance of such consensus sequences is unclear. In many cases, the consensus motif relates to the region of the target site that occurs between the staggered insertions of the transposon ends. This is the region of the DNA that is duplicated as a result of transposition (Figure 2). Commonly, consensus sequences are palindromic, reflecting the symmetrical nature of certain transposition reactions. For example, Mu prefers to integrate on either side of the palindromic sequence 5'-C-C/T-G/C-A/G-G-3' (Haapa-Paananen et al., 2002; Mizuuchi and Mizuuchi, 1993) (Table 1), whereas Tn5 targets transposition to the palindrome 5'-G-N-T-C/T-A/T-A/G-A-N-C-3' (Goryshin et al.,

1998). Likewise, Tn10 prefers to integrate on either side of the sequence 5'-N-G-C-T-N-A-G-C-N-3' (Huisman et al., 1987). Often, the sequence of the target DNA flanking the transposon insertion sites also contributes to a consensus motif. The maize transposons PIF and mPIF choose target sites in which the sequence 5'-C-A/T-C-3' flanks the insertion sites on the target DNA (Zhang et al., 2001). Similarly, IS903 preferentially transposes into regions flanked by the sequence 5'-T/A-T-T/A-C/T-A-N-3' (Hu and Derbyshire, 1998).



**Figure 2: Schematic of insertion-site duplication.** The ends of the transposon insert into the target DNA at staggered sites. The resulting gaps in the DNA are filled-in by DNA replication. This process creates a duplication of the DNA sequence between the staggered insertion sites.

Interestingly, a few transposable elements preferentially target coding DNA. In the presence of the *E. coli* protein H-NS (histone-like nucleoid structuring protein), IS903 biases transposition toward coding regions (Swingle et al., 2004). Likewise, the Tos17 retrotransposon from rice targets transposition to low copy number regions that are enriched for genes (Yamazaki et al., 2001). Perhaps targeting transposition to coding DNA or to particular consensus sequences confers benefits that have yet to be uncovered. Alternately, these targeting mechanisms might have arisen by chance and then been maintained during evolution because they were neither detrimental to the transposon nor to the host.

## **Mechanisms of Transposon Targeting**

As discussed above, most transposable elements do not target transposition to completely random DNA sites. Rather, transposons have evolved mechanisms to direct recombination to particular regions of the host genome. Just as there are many different types of target sites used by transposons, so are there a large variety of molecular mechanisms that mediate target site choice. Some transposons identify preferred target sites with a specific nucleotide sequence, whereas other transposons identify sites with a certain DNA structure. Many factors, including nucleotide sequence, A/T-richness, DNA methylation, and histone modification, contribute to the global structure of a region of DNA. Preferred target sites, whether defined by their sequence or by the DNA structure, can be recognized by the transposase, by transposon-encoded accessory proteins that interact with the transposase, or by host-encoded proteins that interact with the transposase. Though the molecular basis of target-site choice has been elucidated for

several well-studied transposons, it remains unclear how most transposable elements choose their preferred target sites.

### *Role of DNA Sequence vs. DNA Structure in Target Site Choice*

Several of the previously discussed transposable elements identify target sites with a particular nucleotide sequence. As mentioned above, certain transposons preferentially integrate into target DNA that matches a defined consensus sequence (Goryshin et al., 1998; Haapa-Paananen et al., 2002; Huisman et al., 1987; Mizuuchi and Mizuuchi, 1993). As has been discussed, Tf1, Ty1, and Ty3 (Table 1) recognize specific promoter sequences (Behrens et al., 2000; Chalker and Sandmeyer, 1992; Devine and Boeke, 1996; Kelly and Levin, 2005; Singleton and Levin, 2002), Tn7 recognizes the *glmS* gene (Table 1) (Gay et al., 1986), and Tn5053 recognizes the resolution sites within other transposable elements (Minakhina et al., 1999). Furthermore, the Tx1L element from *Xenopus laevis* targets transposition to the Tx1D element by identifying a tandem repeat, PTR-1, within Tx1D (Table 1) (Christensen et al., 2000; Garrett et al., 1989). Additionally, transposons that target transposition to self-similar sequences, as well as those that avoid inserting into their own sequences, are able to identify DNA with homology to the transposon's own genome. Thus, transposable elements can assess the desirability of a given target site based on the nucleotide sequence at that site. As will be discussed below, recognition of target sites with particular nucleotide sequences can occur directly, via the transposase, or indirectly, via accessory proteins.

Not all target sites are selected based on nucleotide sequence. Some transposons identify target sites with particular DNA structures. For example, experiments with IS903

demonstrate that a specific consensus sequence will be targeted for transposition more or less frequently depending on the location of that consensus sequence within a plasmid (Hu et al., 2001). This result provides evidence that the DNA context contributes to target site selection for IS903. Tn7, in the presence of an element-encoded targeting protein TnsE, preferentially transposes into conjugative plasmids (Table 1) (Wolkow et al., 1996). In addition, experiments with *tnsE* mutants have exposed a propensity for Tn7 to integrate in one specific orientation with respect to the direction of DNA replication (Peters and Craig, 2001). This result is notable since transposons can generally insert in two possible orientations, given that they have non-identical right and left ends. Taken together, these data suggest that the TnsE targeting protein of Tn7 might recognize replicating DNA (Peters and Craig, 2001). Yet another example, Tn10, preferentially targets transposition to sites in which the DNA is easily bent (Bender and Kleckner, 1992b; Pribil and Haniford, 2003). Likewise, Sleeping Beauty, a reconstructed fish transposon, targets transposition to A/T-rich DNA that readily forms bent structures (Vigdal et al., 2002; Yant et al., 2005). Tc1, Tc3, and *Himar1* have also been implicated in targeting transposition to highly bendable DNA (Vigdal et al., 2002). In summary, these examples illustrate the role that DNA structure plays in determining preferred target sites. Thus, transposons evaluate both the sequence and the physical structure of DNA when selecting where in the genome to insert.

### ***Target Site Recognized by Transposase***

Transposons direct integration to particular target sites by recognizing features of the DNA sequence and/or structure. These DNA attributes can be detected by the

transposase, by transposon-encoded accessory proteins, or by host-proteins. The transposases of Tn10, Tn5, Mu, and Tc1 directly recognize DNA sites that conform to specific consensus sequences. Mutations in the Tn10 transposase can alter target specificity without affecting other aspects of transposition, indicating that the Tn10 transposase is responsible for recognizing Tn10 consensus sequences (Bender and Kleckner, 1992a). Both the Tn5 and Mu transposases target transposition to preferred sequences in *in vitro* assays that lack any additional proteins (Table 1) (Goryshin et al., 1998; Haapa-Paananen et al., 2002). Similarly, the Tc1 transposase directs transposition to TA dinucleotides *in vitro*, as it does *in vivo* (Vos et al., 1996). Hence, several transposases have been identified that directly mediate target site selection by recognizing a preferred nucleotide sequence in the DNA.

### *Target Site Recognized by Transposon-Encoded Proteins*

Several transposable elements encode accessory proteins that select target DNA. In general, these accessory proteins interact with the transposase or integrase to target transposition to the selected site. As explained above, the Het-A and TART retroelements from *Drosophila* direct insertion to telomeric DNA (Table 1). This target specificity is likely established by element-encoded Gag proteins, the retroviral versions of which are involved in packaging and exporting viral RNA (Rashkova et al., 2002a; Rashkova et al., 2002b). Comparison of Gag proteins from Het-A, TART, and non-telomeric *Drosophila* retroelements, reveals that only the Gag proteins from Het-A and TART efficiently localize to the nucleus (Rashkova et al., 2002a). Additionally, the Het-A and TART Gag proteins are associated with the chromosome ends, implicating these Gag proteins in the

overall process of targeting Het-A and TART transposition to the telomeres (Rashkova et al., 2002b).

Both Tn7 and Mu rely on element-encoded proteins to target transposition to sites with particular attributes. Tn7 encodes two different accessory proteins, TnsD and TnsE, each of which targets transposition to distinct locations described above (Table 1). The TnsD accessory protein binds to the transcriptional terminator of the *glmS* gene and directs transposition to this site via its interaction with the transposase (Waddell and Craig, 1988; Waddell and Craig, 1989). Alternately, the TnsE protein interacts with the transposase to target transposition to conjugative plasmids, possibly by recognizing replicating DNA (Peters and Craig, 2001; Wolkow et al., 1996). Mu also encodes an accessory protein, MuB, that directs target site choice. MuB's preference for A/T rich DNA creates Mu transposition hot-spots flanking A/T rich regions (Table 1) (Manna et al., 2001; Mizuuchi and Mizuuchi, 1993).

In addition to targeting transposition to particular, preferred sites, Mu and Tn7 avoid inserting into DNA that contains a copy of the transposon's own genome, a process termed target immunity (Table 1) (Arciszewska et al., 1989; Lee et al., 1983; Reyes et al., 1987). The molecular mechanisms underlying target immunity have been well studied for both Tn7 and Mu, and in both cases, transposon-encoded accessory proteins are essential. Tn7 target immunity is mediated by three Tn7-encoded proteins, TnsA, TnsB, and TnsC (Skelding et al., 2003; Stellwagen and Craig, 1997). Together, these three proteins prevent TnsD or TnsE-stimulated transposition into sites that already contain a copy of Tn7. TnsA and TnsB bind to the ends of the Tn7 genome and catalyze transposition. TnsC, in conjunction with TnsD or TnsE, binds target DNA and activates TnsA/TnsB's

catalytic activities (Bainton et al., 1993). When TnsC bound to target DNA interacts with both TnsA and TnsB, TnsC stimulates TnsA/TnsB to catalyze transposition into the bound DNA (Skelding et al., 2003). Target immunity occurs because TnsC avoids binding to DNA that is near any copies of the Tn7 genome. This binding preference stems from the fact that TnsC binds target DNA in an ATP dependent manner, and TnsB (in the absence of TnsA) can stimulate TnsC to hydrolyze ATP (Stellwagen and Craig, 1997). Therefore, when TnsC comes into contact with TnsB alone, it will hydrolyze ATP and dissociate from the DNA (Skelding et al., 2003). Since TnsB is bound to the ends of the Tn7 genome, TnsC will be cleared from regions of the DNA close to any Tn7 ends. Thus, only Tn7 hot-spots that are far from any copies of the Tn7 genome will be bound by TnsC and will be efficiently targeted for transposition. The presence or absence of TnsA plays a critical role in Tn7 target immunity. If TnsA is present, an interaction between TnsB and TnsC will result in stimulation of transposition into the TnsC-bound DNA. However, if TnsA is absent, an interaction between TnsB and TnsC will result in dissociation of TnsC from the DNA to establish target immunity (Skelding et al., 2003).

Bacteriophage Mu utilizes a similar mechanism to establish target immunity. Two Mu-encoded proteins, MuA and MuB, are important for directing transposition to non-Mu sequences (Adzuma and Mizuuchi, 1988; Maxwell et al., 1987). MuA is the transposase, and MuB binds target DNA and stimulates MuA to catalyze transposition into bound DNA (Baker et al., 1991; Surette and Chaconas, 1991; Yamauchi and Baker, 1998). Much like TnsC, MuB binds target DNA in an ATP dependent manner. MuA stimulates MuB's ATPase activity, causing MuB to dissociate from DNA near MuA (Greene and Mizuuchi, 2002a; Greene and Mizuuchi, 2002b; Greene and Mizuuchi,

2002c; Maxwell et al., 1987). Since MuA binds to the ends of the Mu genome (Craigie et al., 1984), MuB will accumulate on DNA that is far from any copies of Mu (Adzuma and Mizuuchi, 1988). Therefore, DNA that is far from copies of the Mu genome will be well-populated by MuB and will serve as an efficient substrate for transposition. It remains unclear what factors determine whether a given MuA-MuB interaction will result in stimulation of transposition into the MuB bound DNA or will result in dissociation of MuB from the DNA. It has been suggested that the oligomeric state of MuB might determine the outcome of an interaction between MuA and MuB, such that MuB monomers would be cleared from the DNA and MuB multimers would stimulate transposition into bound DNA (Greene and Mizuuchi, 2002b). However, this hypothesis has yet to be proven.

In summary, many transposable elements encode accessory proteins that mediate target site selection. These accessory proteins can act to direct transposition into favorable sites or to inhibit transposition into undesirable locations. In general, transposon-encoded accessory proteins function by recognizing particular target sites and interacting with the transposase to stimulate integration into the selected sites.

### *Target Site Recognized by Host-Encoded Proteins*

Since transposons only exist within the context of a host, both transposons and their hosts have evolved in concert. Thus, host cells have developed mechanisms to control transposition, and transposons have evolved strategies to exploit features of the host cell. Many transposons take advantage of host proteins to target transposition to preferred sites. For example, Ty5, Ty3, Ty1, Mu, and Tn7, whose preferred target sites

were described above (Table 1), all utilize host proteins to mediate targeting. Ty5, which targets transposition to heterochromatin, does so via an interaction between the Ty5 integrase and Sir4p, a host protein associated with heterochromatin (Table 1) (Xie et al., 2001). Targeting of Ty3, which preferentially integrates upstream of polymerase III transcribed genes (Chalker and Sandmeyer, 1992), is accomplished by the interaction of the Ty3 integrase with the yeast polymerase III transcription factors TFIIIB and TFIIIC (Table 1) (Kirchner et al., 1995). Ty1 also targets transposition upstream of polymerase III transcribed genes. Although the exact mechanism of Ty1 site selection has yet to be determined, preferential insertion into polymerase III transcribed genes is transcription dependent, suggesting that host transcription factors play a role (Devine and Boeke, 1996). In some cases, host factors negatively regulate transposition into particular sites. Studies have shown that Mu is less likely to transpose into sites that are efficiently transcribed, implicating the host transcription machinery in suppression of Mu transposition (Manna et al., 2004). Similarly, Tn7 transposition into the *glmS* gene, a transposition hot-spot via the TnsD pathway, is reduced upon *glmS* transcription (DeBoy and Craig, 2000). In summary, host-encoded proteins that are associated with particular DNA sites can influence the efficiency of transposition into those sites.

## Summary

Though most transposable elements display some degree of target specificity, the nature of the target sites, as well as the mechanisms governing site choice, vary widely. Whereas some transposons direct transposition to sites that are beneficial or benign to the host, others seek sites that offer advantages to the transposon itself. For several preferred

target sites, it remains unclear what, if any, benefits these sites confer upon the host or the transposon. Transposons have evolved diverse mechanisms to target transposition to favored sites. Transposable elements can identify particular target sites based on the nucleotide sequence or the structural properties of the DNA at that site. These DNA attributes can be recognized by the transposase directly or by accessory proteins that interact with the transposase. The accessory proteins can be transposon-encoded or host-encoded. Refining our understanding of transposon target site choice will clarify the factors influencing the prevalence of transposons and the impact these elements have had on host organisms.

## **Overview of Mu Transposition**

This thesis investigates the process of transposon target site choice using the Mu transposon as a model system. Mu is a bacteriophage that replicates its genome within its bacterial host via the process of replicative transposition. As discussed above, Mu preferentially transposes into DNA that is far from any pre-existing copies of the Mu genome, a phenomenon termed target immunity (Reyes et al., 1987). Since Mu is a well-studied transposon with a defined target choice mechanism, it provides an attractive model to explore the molecular mechanisms underlying transposition targeting.

The Mu transposon encodes two proteins necessary for transposition, MuA and MuB. MuA is the transposase, and it binds to specific DNA sequences located at the ends of the Mu genome (Craigie and Mizuuchi, 1987; Craigie et al., 1984). A total of four MuA subunits will bind to the two Mu DNA ends, resulting in a complex termed the transpososome (Lavoie et al., 1991; Surette et al., 1987). Following formation of the

transpososome, MuA catalyzes the DNA cleavage and joining reactions necessary for Mu transposition (Aldaz et al., 1996; Mizuuchi and Adzuma, 1991; Namgoong and Harshey, 1998; Williams et al., 1999). MuA is aided by a second Mu-encoded protein, MuB. MuB promotes assembly of the transpososome, stimulates MuA's catalytic activities, binds target DNA, and directs transposition into the bound DNA (Adzuma and Mizuuchi, 1988; Baker et al., 1991; Chaconas et al., 1985; Coelho et al., 1982; Mizuuchi et al., 1995; Surette and Chaconas, 1991; Williams et al., 1999; Yamauchi and Baker, 1998). As described in the introduction, interactions between MuA and MuB ultimately determine which DNA sequences are most likely to be bound by MuB, and therefore, which DNA sequences are most likely to serve as the target site for transposition. Thus, understanding the molecular interactions between MuA and MuB is crucial to elucidating the mechanism of Mu target site choice.

The research described herein examines the biochemical interactions between MuA and MuB that mediate target selection and transposition into selected target DNA. The first chapter dissects the MuA-MuB interactions responsible for directing transposition into selected target DNA. There, we describe the engineering of a novel MuB fusion protein that alters Mu target site specificity. The second chapter describes interactions between MuA and MuB that regulate transposase activity. Finally, the third chapter summarizes our results and suggests future experiments.

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## **Chapter II**

Dissecting MuB's role in target delivery during  
Mu transposition: ATP hydrolysis is not obligatory

## **Abstract**

A close collaboration between the MuA transposase and its activator protein, MuB, is essential for properly regulated transposition. MuB serves several roles, including activation of MuA's catalytic activity, selection of target DNA, and stimulation of transposition into the selected target DNA. MuB is an ATPase, and appropriate target DNA selection requires ATP hydrolysis by MuB. By fusing the MuB protein to a site-specific DNA binding protein (Arc repressor), we generated a version of MuB that can select target DNA independently of ATP. This Arc-MuB fusion protein, can be used to probe whether ATP-binding and hydrolysis by MuB is necessary for stimulation of transposition into selected DNA (target delivery). We find, using these fusion proteins, that MuB-dependent target delivery can efficiently occur in the absence of ATP hydrolysis. Furthermore, mapping experiments establish that the fusion protein directs transposition to regions of the DNA within 40-750 bps of its own binding site. Taken together, these results suggest that target delivery by MuB can occur as a consequence of MuB's ability to stimulate MuA while simultaneously tethering MuA to a selected target DNA. This mechanism is an attractive model to explain other examples of protein-stimulated control of transposition target site selection.

## **Introduction**

Transposable elements have been found in every species studied, and these "selfish DNAs" appear to have a tremendous impact on the evolution of their hosts (Curcio and Derbyshire, 2003). To comprehend how transposons have become ubiquitous and what effect they have had on evolution, we must understand the mechanisms that

govern their activity and their target site choice. Several transposons depend on nucleotide cofactors to regulate transposase activity or to choose appropriate target sites. For example, the bacterial Tn552 transposon requires GTP for efficient transposition, as demonstrated by *in vivo* assays with purine biosynthetic mutants (Coros et al., 2005). Likewise, GTP stimulates assembly of the initial synaptic complex of the *Drosophila* P element transposase (Kaufman and Rio, 1992; Tang et al., 2005). In contrast, GTP inhibits target DNA capture by the RAG proteins that mediate V(D)J recombination, thereby suppressing RAG-mediated transposition (Tsai and Schatz, 2003). Two well studied systems, Mu and Tn7, encode target-selection proteins that require ATP to choose the target DNA (Adzuma and Mizuuchi, 1988; Gamas and Craig, 1992; Maxwell et al., 1987; Stellwagen and Craig, 1997; Stellwagen and Craig, 1998). Thus, although it is clear that nucleotide cofactors are involved in regulating a number of transposons, it is largely uncertain the extent to which such cofactors affect the individual steps of transposition. Here, we investigate the role of ATP in target delivery during Mu transposition.

The genome of the Mu bacteriophage is a replicative transposon. Transposition is mediated by two Mu-encoded proteins: MuA and MuB. MuA, the transposase, is a member of the transposase/retroviral integrase protein family (Baker and Luo, 1994; Rice and Mizuuchi, 1995). MuA binds as a tetramer to specific sites at the ends of the Mu genome (Craigie et al., 1984), where it catalyzes the cleavage and joining reactions necessary for transposition (Aldaz et al., 1996; Mizuuchi and Adzuma, 1991; Namgoong and Harshey, 1998; Williams et al., 1999). The second protein, MuB, is an activator of MuA (Baker et al., 1991; Surette and Chaconas, 1991; Williams et al., 1999; Yamauchi

and Baker, 1998). Unlike MuA, MuB is an ATPase (Maxwell et al., 1987). As such, it exists in two distinct states: ATP-bound and ADP-bound. MuB bound to ATP binds DNA tightly ( $K_d=80\text{nM}$ ) and with little sequence preference (Yamauchi and Baker, 1998). In contrast, MuB bound to ADP binds DNA much less tightly ( $K_d=790\text{nM}$ ), as does MuB in the absence of nucleotide ( $K_d=1200\text{nM}$ ) (Yamauchi and Baker, 1998). MuB's ability to hydrolyze ATP and thereby release the DNA is essential for determining the target sites during Mu transposition, as will be explained below.

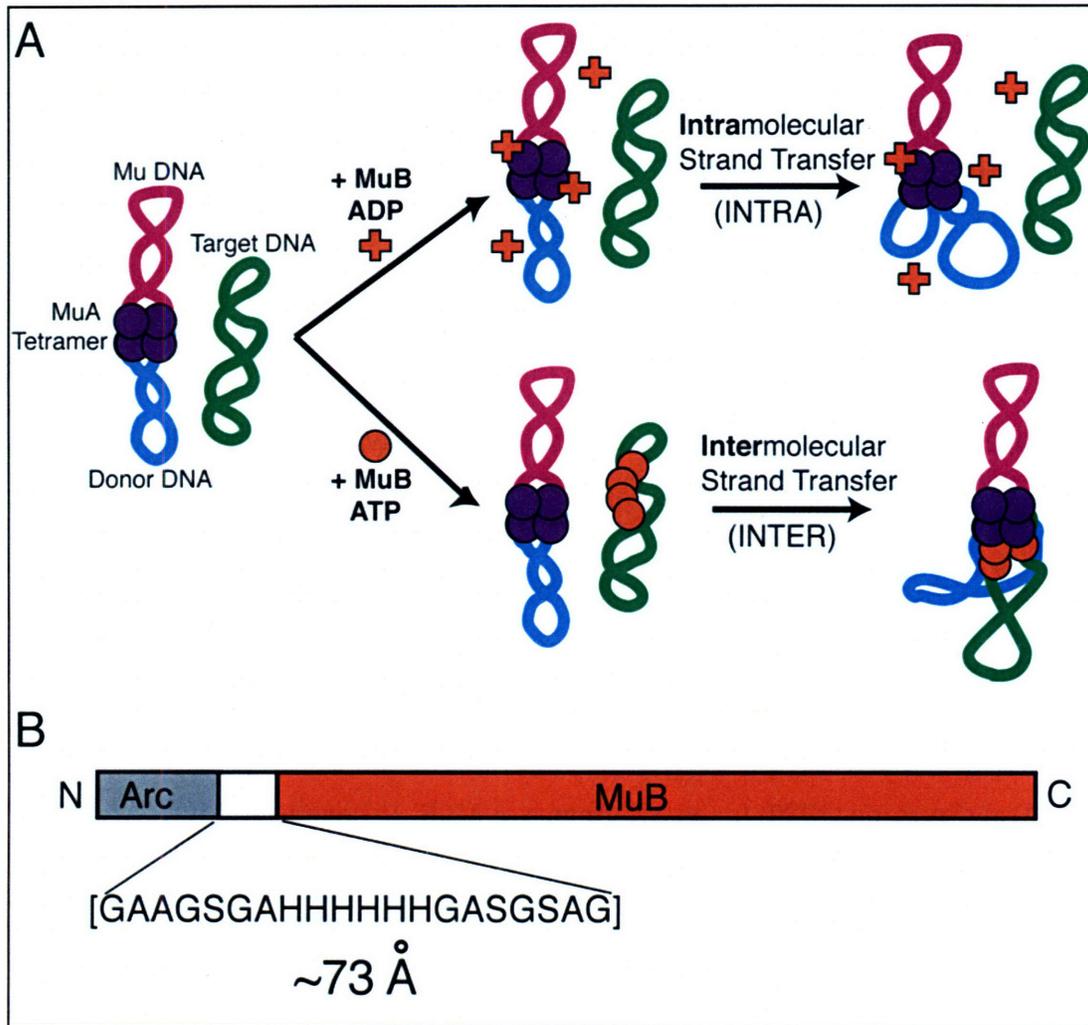
Mu avoids transposing into or near its own genome, a process termed target immunity (Reyes et al., 1987). Other transposons, including Tn7 and Tn3, also exhibit target immunity, thought to be an evolutionary strategy for avoiding self-destruction (Arciszewska et al., 1989; Lee et al., 1983; Maekawa et al., 1996; Stellwagen and Craig, 1997). In the case of Mu, target immunity is mediated by MuB and its interactions with MuA and ATP (Adzuma and Mizuuchi, 1988; Maxwell et al., 1987). Steps in this process, as currently understood, are as follows. Initially, MuB•ATP binds DNA non-specifically. MuA, which is bound to the Mu genome ends, can stimulate MuB's ATPase activity (Greene and Mizuuchi, 2002b; Greene and Mizuuchi, 2002c; Maxwell et al., 1987). Since MuB•ADP has a lower affinity for DNA, hydrolysis causes MuB to dissociate from the DNA (Greene and Mizuuchi, 2002a). As a result, MuB molecules bound to DNA near Mu genome ends, and thus near MuA, will be cleared from the DNA. Eventually, MuB will accumulate on DNA far from copies of the Mu genome (Adzuma and Mizuuchi, 1988). A second interaction between MuB and MuA must also occur, in which MuB stimulates MuA to catalyze transposition into the MuB-bound DNA. In this

interaction, MuB “delivers” the target DNA to MuA. Thus, MuB serves two key roles in Mu targeting: selecting distant DNA and promoting transposition into this DNA.

One can observe Mu target immunity *in vitro* (Maxwell et al., 1987; Mizuuchi, 1983). In such transposition assays, a donor plasmid containing a modified version of the Mu genome (pMK586 (Mizuuchi et al., 1991)) and a second, non-Mu, target plasmid are incubated with MuA, MuB, and ATP. Under these conditions, MuB preferentially selects the non-Mu plasmid and delivers this plasmid to the transposase. We will refer to this process as intermolecular transposition or “INTER” (Figure 1A). In contrast, if MuB is absent from the *in vitro* reaction, or if ADP is present in place of ATP, then target immunity fails, and transposition events occur almost exclusively into the donor plasmid’s own DNA (intramolecular transposition or “INTRA”) (Figure 1A). MuB does, however, play a role in INTRA, as demonstrated by the fact that INTRA is more efficient in the presence of MuB bound to ADP than in the absence of MuB (Surette and Chaconas, 1991; Yamauchi and Baker, 1998). Therefore, in addition to MuB’s ability to select target DNA and to deliver selected DNA to MuA, MuB can also stimulate transposition into nearby, unselected DNA.

We have described three roles of the MuB protein: to select target sites that are far from any copies of the Mu genome, to deliver selected DNA to the transposase (i.e. stimulate INTER), and to activate transposition into non-selected DNA (i.e. stimulate INTRA). These three activities differ based on their requirement for ATP hydrolysis by MuB. Target selection relies on MuB’s ATPase activity (Adzuma and Mizuuchi, 1988; Maxwell et al., 1987) , whereas stimulation of INTRA, is independent of ATP hydrolysis (since this process is supported by MuB•ADP) (Yamauchi and Baker, 1998). It remains

unknown, however, whether or not delivery of selected target DNA requires ATP hydrolysis. Therefore, we sought to determine whether ATP hydrolysis by MuB is necessary for efficient activation of INTER.



**Figure 1:** A) Diagram of Mu transposition. B) Illustration of the Arc-MuB fusion protein highlighting its flexible linker region.

In this manuscript, we describe experiments aimed at determining whether or not MuB requires ATP hydrolysis for target delivery. To this end, we have created MuB fusion proteins that bind target DNA irrespective of MuB's nucleotide state. We find that these fusion proteins can stimulate intermolecular transposition, even under conditions

that prevent ATP hydrolysis. Thus, we conclude that ATP hydrolysis by MuB is not critical for efficient intermolecular transposition and, therefore, is not essential for target delivery. We also map the location of transposition events mediated by our MuB fusion proteins. The results of these mapping experiments offer insight into the mechanism by which MuB targets transposition to bound DNA.

## Results

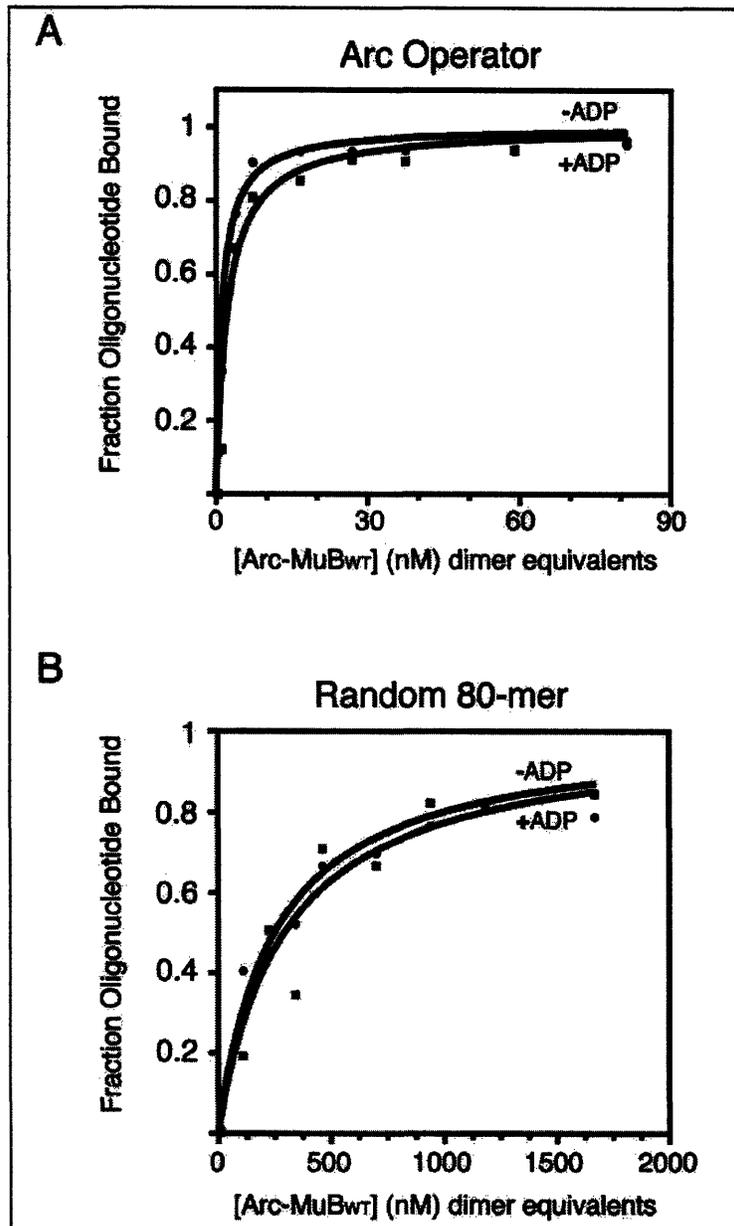
### *Design of MuB fusion proteins*

Since MuB's ATP-bound state is coupled to its ability to bind target DNA (Yamauchi and Baker, 1998), it is difficult to investigate the role of one activity without disturbing the other. Therefore, to determine whether ATP hydrolysis by MuB is fundamentally important for the mechanism of target delivery, we designed a MuB fusion protein that binds DNA independently of ATP. Specifically, we fused the Arc repressor protein (Arc) (Susskind, 1980) to the N-terminus of MuB. In contrast to MuB, Arc does not bind ATP or ADP, and therefore, its DNA binding activity is nucleotide independent. Arc is a dimer (Vershon et al., 1985), and two Arc dimers bind tightly to a specific Arc operator DNA sequence (half-maximal binding at 40 nM Arc monomer equivalents) (Robinson and Sauer, 1996). Arc also binds less tightly to non-specific DNA (half-maximal binding at 460 nM Arc monomer equivalents) (Robinson and Sauer, 1996). We made two version of the fusion protein: one with Arc fused to wild type MuB (FP<sub>WT</sub>) and one with Arc fused to MuBins101N (FP<sub>insN</sub>) (Figure 1B). MuBins101N contains an asparagine inserted into the Walker A box of the ATP binding domain (Walker et al., 1982) (Pause and Sonenberg, 1992) (Yamauchi and Baker, 1998). This previously

characterized mutant is defective in DNA binding and in ATP hydrolysis, but is still capable of stimulating MuA for INTRA (Yamauchi and Baker, 1998). By uncoupling MuB's ability to hydrolyze ATP from its ability to bind target DNA, these fusion proteins provide a tool for studying the role of ATP in target delivery.

### *Characterization of the fusion proteins*

To characterize the DNA binding activity of our MuB fusion proteins, we performed gel shift assays using 80bp DNA fragments that either did or did not contain the 21bp Arc operator sequence. These gel shift assays were performed under the same conditions as our transposition assays. Results indicate that FP<sub>WT</sub> in the presence of ADP binds Arc operator DNA half-maximally at a concentration of ~20 nM monomer equivalents (Figure 2A). Although this apparent binding affinity is weaker than that previously reported for Arc, this discrepancy is likely due to a difference in buffer conditions. Since the strength of this binding is of the same order as for MuB•ATP to DNA, we have successfully uncoupled MuB's nucleotide state from its ability to bind DNA. In the presence of ADP, FP<sub>WT</sub> bound non-Arc operator DNA half-maximally at a concentration of ~300 nM (Figure 2B). This preference of FP<sub>WT</sub> for the Arc operator site over non-specific DNA indicates that the Arc domain of the fusion protein is functional.



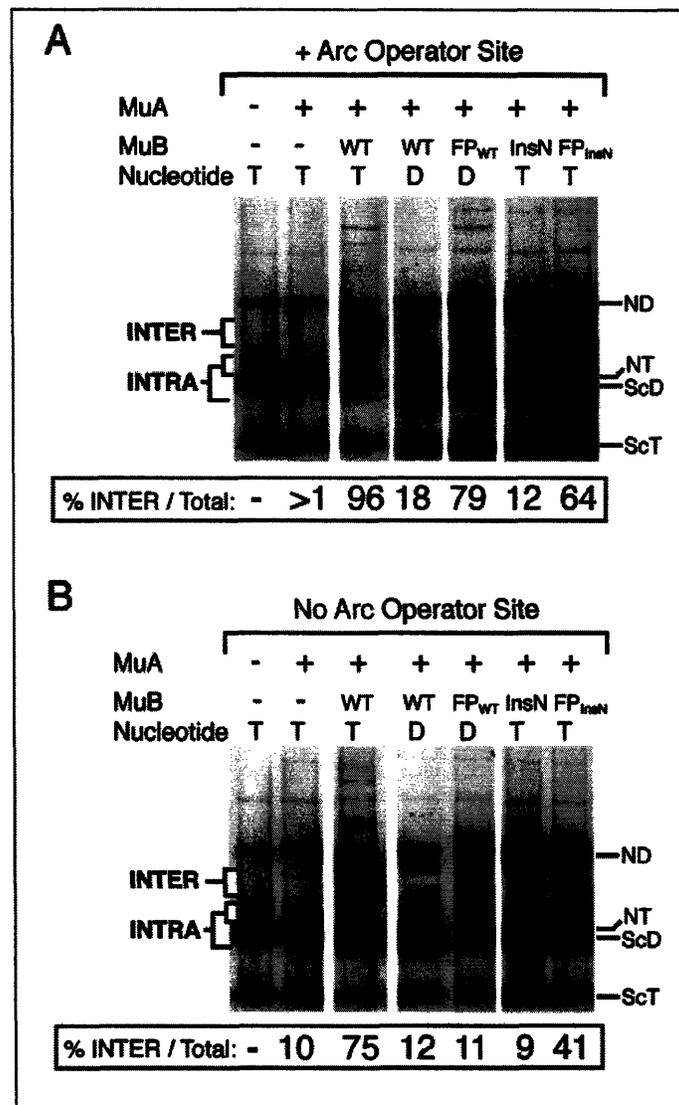
**Figure 2:** A) Gel shift assay performed with 0.1 nM oligonucleotide, FP<sub>WT</sub>, and either ADP or ATP. 80bp oligonucleotide contained a single copy of the Arc operator sequence. B) Same as in A except that oligonucleotide lacked Arc operator sequence.

FP<sub>WT</sub> binds DNA equally well whether or not ADP is present (Figure 2). As MuB binds DNA approximately one and a half times more tightly in the presence of ADP than in its absence, the fact that FP<sub>WT</sub>'s ability to bind DNA is unaffected by ADP, suggests that the fusion protein is binding principally via the Arc domain. It should be noted, however, that FP<sub>WT</sub> bound non-Arc operator DNA more tightly in the presence of ATP than in its absence (data not shown). We also tested the functionality of the MuB domain of our Arc-MuB fusion proteins by assaying its ability to stimulate formation of INTRA *in vitro*. INTRA was much more efficient in the presence of our fusion proteins, indicating that the MuB domains of our fusion proteins are indeed active (data not shown, see below).

### *Fusion proteins support INTER and do not depend on ATP hydrolysis*

To determine whether ATP hydrolysis is necessary for MuB-stimulated target delivery, we assayed the ability of our fusion proteins to direct transposition into foreign target DNA. We performed *in vitro* recombination assays, in which the MuB fusion proteins, MuA, "mini-Mu" donor DNA (pMK586), and a second, non-Mu, target plasmid were incubated for two hours at 30°C. The target plasmid either did, or did not, contain a single copy of the Arc operator. After incubation, samples were run on an agarose gel to separate the INTRA products from the INTER products. Both FP<sub>WT</sub> bound to ADP and FP<sub>insN</sub> bound to ATP are unable to hydrolyze ATP but are able to bind target DNA via their Arc domains. If MuB must hydrolyze ATP to deliver target DNA, then the fusion proteins should be inefficient at INTER. Consequently, one would expect the majority of the recombination products to be the result of INTRA. On the other hand, if ATP

hydrolysis is not necessary for target delivery, then the fusion proteins should support recombination into bound DNA. In this case, we would expect to see a preference for INTER when the target DNA contains a copy of the Arc operator site.



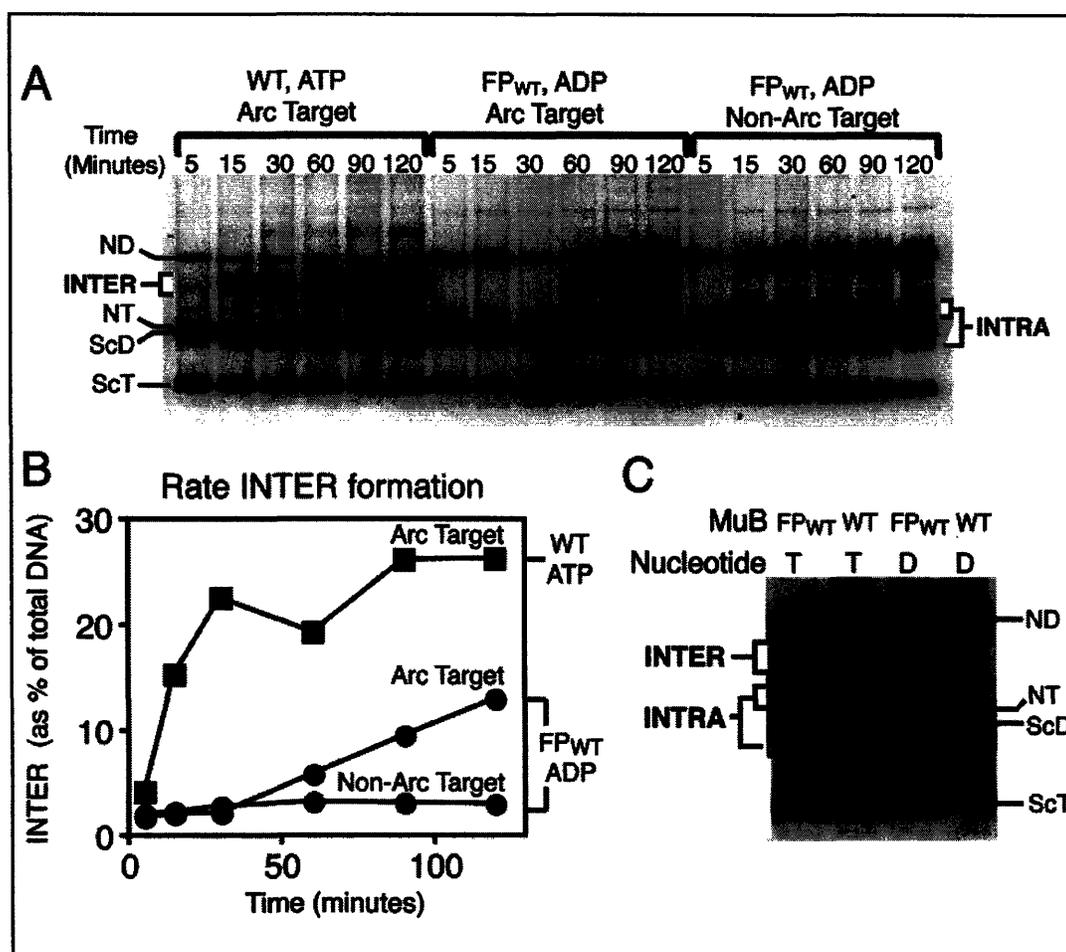
**Figure 3:** A) Agarose gel of products from *in vitro* transposition reactions. Reactions were incubated for 2 hours and contained Arc operator target plasmid (pCS13). Lanes are from the same gel with the same image contrast. B) Same as in A except target plasmid lacked Arc operator sequence (pUC19). Lanes are from the same gel with the same imaging contrast.

Both fusion proteins did support INTER into plasmids containing the Arc operator sequence (Figure 3A, lanes 5 and 7). The identity of INTRA and INTER products were verified by Southern blot (data not shown). As expected, wild type MuB•ATP also supported efficient INTER, whereas MuB<sub>insN</sub>•ATP and wild type MuB•ADP failed to generate these INTER products (Figure 3A). Moreover, when the target DNA lacked the Arc operator site, only wild type MuB•ATP efficiently targeted recombination to intermolecular sites (Figure 3B). Though the fusion proteins did support a modest level of INTER into non-Arc target DNA, this activity can be explained by Arc's relatively high affinity for non-specific DNA. We repeated the experiments in Figure 3 with a second set of Arc and non-Arc target plasmids and obtained analogous results (data not shown).

To determine whether the fusion proteins preferentially support INTER over INTRA, we calculated the percentage INTER products out of total transposition products formed in each sample (See values below gel in Figures 3A and 3B). Remarkably, with both FP<sub>WT</sub>•ADP and FP<sub>insN</sub>•ATP, more than half of the transposition products were INTER, indicating that the fusion proteins preferentially directed transposition into the intermolecular target DNA. In summary, our results indicate that FP<sub>WT</sub>•ADP and FP<sub>insN</sub>•ATP can preferentially target transposition to intermolecular DNA, and this targeting activity depends on the presence of an Arc operator site in the target DNA. From these data, we conclude that ATP hydrolysis by MuB is not mechanistically required to stimulate recombination into a distant target.

We consistently observed that FP<sub>WT</sub>•ADP and FP<sub>WT</sub>•ATP were equally efficient at stimulating INTER into Arc operator containing plasmids (Figure 4C – compare lanes

1 and 3). If our fusion proteins were binding target DNA via their MuB domains, we would expect INTER to be more efficient in the presence of ATP, since MuB binds DNA approximately ten times more tightly in the presence of ATP than in the presence of ADP. Since our fusion proteins exhibit similar activity in the presence of ATP or ADP, we conclude that the fusion proteins are primarily binding target DNA via their Arc domains.



**Figure 4:** A) Agarose gel of *in vitro* transposition products. Reactions were incubated for the length of time indicated. B) Graphical representation of the data from A. C) Agarose gel of *in vitro* transposition products. Reactions were incubated for 2 hours and contained Arc operator target plasmid (pCS13).

Although we determined that our fusion proteins can support INTER, we wanted to investigate whether these fusion proteins stimulate INTER with similar kinetics to wild type MuB. Using *in vitro* recombination reactions, we find that FP<sub>WT</sub>•ADP forms INTER at approximately 40% the efficiency of MuB•ATP (Figures 4A and 4B). The reduced activity of FP<sub>WT</sub> as compared with MuB is likely due to the difference in the number of binding sites in the target DNA for each protein. Whereas only two Arc dimers bind to the single Arc operator sequence present in the target DNA, wild type MuB can bind to any site along the entire target plasmid. To reduce the discrepancy in the number of binding sites for FP<sub>WT</sub> and MuB, we attempted *in vitro* recombination reactions using short DNA fragments as target DNA. Unfortunately, such short target molecules altered the reaction's dependence on MuB, making results difficult to compare and interpret (data not shown).

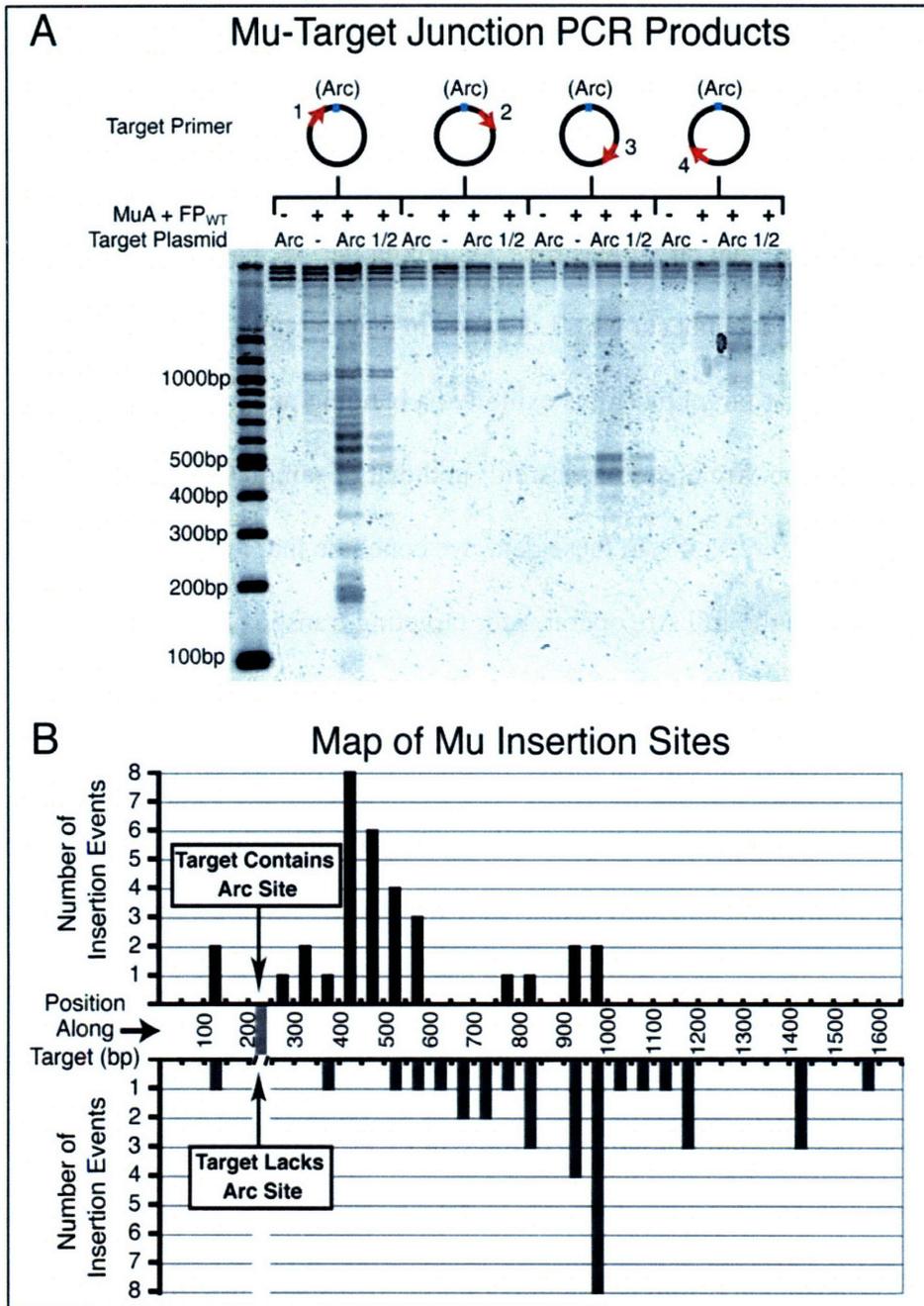
### ***Fusion proteins target transposition to DNA near the Arc operator site***

Although it is clear that the fusion proteins can target transposition to Arc operator containing plasmids, it is uncertain where on the plasmid recombination is occurring. Do the fusion proteins target transposition directly into the Arc operator site, nearby the Arc site, or throughout the plasmid? To address this question, we globally mapped transposition events from *in vitro* transposition reactions containing FP<sub>wt</sub>•ADP and either Arc or non-Arc target plasmids. Mapping was accomplished by PCR amplifying the donor-target joints using one primer specific to the Mu-end on the donor DNA and one plasmid specific to the target plasmid. The PCR products were run on an agarose gel. A given band on the gel corresponds to a transposition event at a particular

distance from the target primer. This experiment was repeated with four different target primers, spaced evenly around the target plasmid (Figure 5).

The results of our global mapping experiment demonstrate that there is a different pattern of transposition events into plasmids containing the Arc operator site compared to plasmids lacking the Arc operator site (Figure 5). Specifically, we observe a cluster of bands that correspond to insertion events ~40 bps to ~340 bps 3' of the Arc operator site. These bands only appeared in samples in which the target plasmid contained the Arc operator site (Figure 5, compare lanes 3 and 4). Another set of bands that were from insertion events ~110 bps to ~10 bps 5' of the Arc operator site also appeared in an Arc operator dependent manner (Figure 5, compare lanes 3 and 4). We did not, however, detect bands unique to the Arc operator samples at positions far from the Arc operator (Figure 5, lanes 6-17). Overall, we observed that the presence of an Arc operator site in the target DNA alters the profile of transposition events mediated by our fusion proteins. In fact, we find that regions of the DNA on either side of the Arc operator become more susceptible to transposition.

It should be noted that the samples in Figure 5 contained different amounts of total DNA. Transposition is more efficient into plasmids containing a copy of the Arc operator, and therefore, these samples contained a much larger number of donor-target joints that could be amplified during PCR. Therefore, PCR yielded a greater amount of product for the Arc operator samples than for the non-Arc operator samples. This discrepancy is apparent upon inspecting the net signal per lane for Arc operator versus non-Arc operator samples in Figure 5. It is valid, however, to compare the relative intensities of bands within a given lane.



**Figure 5:** A) Agarose gel of PCR amplified donor-target joints from *in vitro* transposition reactions containing FP<sub>wt</sub>•ADP and either pCS14 (non-Arc), pCS15 (Arc), or pCS16 (1/2 Arc) target plasmids. Cartoons above the gel indicate the position of the target primer relative to the Arc operator. B) Graph representing the frequency of transposition events at positions along the target DNA. We mapped a total of 33 events into pCS15 (Arc target) and 35 events into pCS14 (non-Arc target) from reactions containing FP<sub>wt</sub>•ADP. Individually mapped events were binned into 50bp intervals. Donor-target joints were PCR amplified using “primer 1” (see A).

To investigate the number of Arc operator-bound fusion proteins required to mediate target delivery, we created target plasmids that contained only the right half of the Arc operator. This half site should bind one Arc dimer, as compared to the tetramer-bound full site. We find that target plasmids containing the Arc half site are less efficient substrates for INTER (data not shown). Likewise, in our mapping experiments, samples containing half operator target plasmids exhibited a banding pattern between that of samples containing non-Arc operator plasmids and that of samples containing full Arc operator plasmids (Figure 5). From these data, we conclude that the half Arc operator is a less robust signal than the full Arc operator for targeting transposition into nearby DNA in reactions containing our fusion proteins.

To validate the results of our global mapping experiments, we cloned the PCR amplified donor-target joints from Figure 5, lanes 3 and 4. The clones were sequenced to map individual insertion events into target DNA that either contained or lacked an Arc operator site. The position of our PCR primer allowed us to map insertion events from ~150 bps 5' of the Arc operator site to ~1400 bps 3' of the Arc operator site. Consistent with our global mapping experiments, we find that  $FP_{WT} \bullet ADP$  targets transposition to different locations depending on whether or not the target DNA contains the Arc operator site (Figure 5B). Also, interestingly, we did not observe a single insertion event into the Arc operator itself. The data in Figure 5B was derived from multiple *in vitro* transposition reactions that were PCR amplified and cloned separately, and the results were consistent from reaction to reaction. We conclude that our fusion proteins target transposition to locations near the Arc operator site but never directly into it.

## Discussion

We have created Arc-MuB fusion proteins that effectively uncouple MuB's ATPase activity from its ability to bind target DNA. We find that these fusion proteins can target transposition to distant plasmids containing a copy of the Arc operator site. This targeting is robust even if only ADP is present in the reaction or if the MuB portion of the fusion protein is mutated to prevent ATP hydrolysis. Therefore, fusion protein-mediated stimulation of INTER can occur independently of ATP hydrolysis. These data indicate that MuB does not require ATP hydrolysis to deliver target DNA.

Though ATP hydrolysis is not necessary for target delivery, it is necessary for target site choice. The process of target immunity, by which MuB accumulates on DNA far from any copies of the Mu genome, depends on MuA stimulated ATP hydrolysis by MuB (Adzuma and Mizuuchi, 1988; Maxwell et al., 1987; Reyes et al., 1987). However, our data suggest that once a MuB gradient has formed, transposition into the MuB-bound DNA can occur independently of ATP hydrolysis. That MuB's ATPase activity is solely necessary for target selection, highlights the evolutionary importance of target site choice for transposable elements.

Our data indicate that MuB need not hydrolyze ATP to stimulate INTER. Likewise, MuB stimulation of INTRA is independent of ATP hydrolysis by MuB (Yamauchi and Baker, 1998). Therefore, we propose that MuB activates MuA to perform INTER in much the same way that it activates MuA to perform INTRA. In other words, we hypothesize that MuB stimulates INTER by activating MuA's catalytic activity (as for INTRA) while simultaneously increasing the local concentration of the bound target DNA. Thus, MuB "delivers" target DNA by tethering that DNA to the MuA tetramer.

Numerous other transposases select target DNA by interacting with proteins that are bound to the preferred target site. For example Tn7 interacts with TnsC/D to mediate target immunity in a manner similar to Mu (Stellwagen and Craig, 1997), Ty5 interacts with Sir4p to target transposition to silent DNA (Xie et al., 2001), and Ty3 interacts with transcription factor TFIIIB to target transposition upstream of RNA polymerase III promoters (Kirchner et al., 1995).

That MuB preferentially targets transposition to bound DNA is less intuitive than one might think. When MuB bound to target DNA interacts with the MuA tetramer, the local concentration of the MuB-bound DNA relative to the MuA tetramer increases. However, the local concentration of the flanking donor DNA relative to the MuA tetramer should also be quite high. Why, then, do the majority (75%-95%) of the transposition events occur into the MuB-bound DNA? One possible answer is that the flanking donor DNA is in a particularly poor orientation to be accessed by the transposase.

We mapped the locations of individual transposition events mediated by our fusion proteins into plasmids that either did, or did not, contain a copy of the Arc operator sequence. Interestingly, we found that transposition occurred into different locations depending on whether or not the Arc site was present. In particular, transposition hot-spots unique to the Arc operator containing plasmids, were observed near, but never directly into, the Arc operator site. That transposition occurs near, rather than into, the DNA to which our fusion proteins are bound, supports the model that MuB can act to tether target DNA to the transposase.

Neither the transposition hot-spots observed with the Arc operator plasmid, nor those observed with the non Arc plasmid, correspond to the Mu consensus sequence (5'-C-C/T-G/C-A/G-G-3') (Haapa-Paananen et al., 2002; Mizuuchi and Mizuuchi, 1993). It remains unclear why particular regions of the DNA are preferred target sites for Mu transposition under our reaction conditions. Perhaps, as has been previously suggested, the local structure of the DNA determines the location of Mu targeting (Manna et al., 2004).

In creating our Arc-MuB fusion proteins we have engineered a version of MuB that can preferentially target transposition to a particular DNA molecule. We hope that this system will prove a useful tool for further investigation of the mechanisms underlying Mu transposition.

## **Acknowledgements**

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## **Materials and Methods**

### ***DNA:***

The donor plasmid was pMK586 (mini-Mu) (Mizuuchi et al., 1991). Non-Arc target plasmid was either pUC19 or pCS14 (pJF122 with 180bps added to the multiple cloning site). Arc target plasmid was either pCS13 (pUC19 with atagtagagtgtcttatcat cloned into EcoR1 site) or pCS15 (pCS14 with atagtagagtgtcttatcat cloned into EcoR1 site). ½ Arc target plasmid was pCS16 (pCS14 with atagtagagtgtcttatcat cloned into the EcoR1 site). All plasmids were purified by QIAGEN Plasmid Mega kit followed by

CsCl/ethidium bromide ultracentrifugation. 80bp oligonucleotides used in our gel shift assays were ordered from Invitrogen. The sequences are as follows: Arc Operator = 5'catcaccgaaacgtccgaggcagcaagttatgatagaagcactctactatggagtcataatgtgcctgtcattgagacga3'; Non-Arc Operator = 5'catcaccgaaacgtccgaggcagcaagtttcgcacgtccgcacagcagcgtggagtcataatgtgcctgtcattgagacga3'

*Proteins:*

MuA was purified as in Baker et al., 1993 (Baker et al., 1993). HU was purified as in Baker and Luo, 1994 (Baker and Luo, 1994). Wild-type MuB and MuB<sub>ins101N</sub> were purified as described in Yamauchi and Baker, 1998 (Yamauchi and Baker, 1998).

FP<sub>WT</sub> and FP<sub>ins101N</sub> were cloned into pET20b (Novagen) and expressed in bacterial strain ER2556. Cells were grown in TB to an OD<sub>600</sub> of 0.55-0.75, induced with 0.7mM IPTG for 2 hours, centrifuged at 6,000 g for 20 minutes. Cell pellets were resuspended in 50mM Tris pH8, 10% sucrose, 2.5mM DTT, 12.5mM EDTA, plus protease inhibitor cocktail (CalBiochem). Cell were French pressed, and lysate was cleared by centrifugation at 20,000g for 30min. AmSO<sub>4</sub> was added to 35% and precipitation was allowed to occur for 1hr at 4°C. Sample was centrifuged for 15 min at ~40,000g, and the resulting pellet was resuspended in denaturing buffer (6M GuHCl, 100mM NaH<sub>2</sub>PO<sub>4</sub>, 10mM Tris, 10mM Imidazole, pH 8.0). Protein was then batch bound to Ni-NTA beads (QIAGEN), washed with denaturing buffer, and eluted with 6M GuHCl, 20mM NaH<sub>2</sub>PO<sub>4</sub>, 10mM Tris, 400mM Imidazole, pH 8.0. Eluted samples were subjected to several dialysis steps: first into MuB buffer (1M NaCl, 25mM Hepes pH 7.6, 0.1mM

EDTA, 20% glycerol, 2mM DTT) + 2M GuHCl, then into MuB buffer + 0.5 GuHCl, and finally into MuB buffer.

*Gel shift assays:*

Binding reactions contained 100mM NaCl, 0.1mM EDTA, 2.5mM HEPES-KOH pH 7.6, 3.5% glycerol, 1mM DTT, 25mM Tris-HCl pH 8, 10mM MgCl<sub>2</sub>, 0.1nM 80bp oligonucleotide (see DNA) that was P<sup>32</sup> radiolabeled at the 5' end, and FP<sub>WT</sub> (concentration varied). Some reactions also contained 2mM ADP. Reactions were incubated for 4hrs at 30°C. 0.8 volumes of a loading solution (11.25% glycerol, 2.25X loading dye) were added to each sample immediately prior to loading the sample onto a polyacrylamide gel. Samples were run on a 5% native polyacrylamide gel (19:1 acrylamide:bis-acrylamide) in 0.5X TBE at ~15 V/cm. Gels were dried at 76°C and exposed on a storage phosphor screen (Amersham Biosciences) for 2-3 days. Exposed phosphor screens were viewed using a Typhoon 9400. Bands were quantitated using ImageQuant, and curves were fit using KaleidaGraph.

*Transposition Assays:*

Transposition reactions in Figures 3A, 3B, and 4C included: 100mM NaCl, 3.5% glycerol, 1mM DTT, 25mM Tris-HCl pH 8, 10mM MgCl<sub>2</sub>, 2mM ADP or ATP (as indicated), 0.1 mg/ml BSA, 10 µg/ml pMK586 (donor), 10 µg/ml pUC19 (non-Arc target) or pCS13 (Arc target) (as indicated), 130 nM HU, 40 nM MuA, 300 nM MuB or MuBins101N or FP<sub>WT</sub> or FP<sub>insN</sub> (as indicated). Reactions were incubated for 2 hours at 30°C and stopped with 0.2 vol STOP solution (2.5% SDS, 50 mM EDTA, 30% glycerol,

bromophenol blue). Reaction products were run on a 0.9% HGT-agarose (Cambrex) gel in 1 X TAB (40 mM Tris-HCl pH 8.0, 3.6 mM EDTA, 27 mM sodium acetate) at ~5 V/cm for 2 hours at 4°C. Gels were stained with Vistra Green (Amersham Biosciences) (1:10000 dilution) and visualized on a Molecular Dynamics fluorimager. Band intensities were quantitated using ImageQuant.

Reactions in Figure 4A were performed as above, except that the MuA concentration was 30 nM, and the reactions were stopped after the indicated amount of time. Reactions for the mapping experiments in Figure 5 were also performed as above, except that the MuA concentration was 30nM, the reactions were incubated for 3 hours, and pCS14 (non-Arc), pCS15 (Arc), or pCS16 (1/2 Arc) were used as the target plasmids.

*Global mapping experiment:*

To globally map the location of Mu transposition events, transposition reactions were performed as described above. The products of these reactions were subjected to proteinase K treatment followed by Phenol:Chloroform extraction and EthOH precipitation. Samples were resuspended in H<sub>2</sub>O and used as the template in subsequent PCR. PCR was performed with a Mu specific primer (5'cccggtttttcgtacttcaagtgaatcaataca3') and one of four different target specific primers: (primer1 = 5'cgtttttgggctaacaggaggaattaacctag3'; primer2 = 5'caacttcagcagcacgtaggggac3'; primer3 = 5'cggtgtggtcgccatgatcg3'; primer4 = 5'gcattgtgcagaggtttcaccgcatc3'). PCR products were run on a 1.8% metaphore agarose (Cambrex) gel in 1X TBE at 5 V/cm at 4°C. Gels were stained with Vistra Green (Amersham Biosciences) (1:10000 dilution) and visualized on a Typhoon 4900.

***Mapping insertion sites:***

To specifically map individual insertion events, we cloned and sequenced the PCR-amplified donor-target joints generated in the global mapping experiments (above). We cloned those donor-target joints that had been "primer1" amplified from transposition reactions containing FP<sub>WT</sub>-ADP and pCS14 (non-Arc target) or pCS15 (Arc target). Donor-target joints were cloned using the TOPO TA Cloning kit (Invitrogen), and individual clones were sequence by the MIT Biopolymers Laboratory.

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## **Chapter III**

### **The Dynamic Mu Transpososome: MuB activation prevents disintegration**

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This chapter is part of a manuscript that has been submitted by Kathryn M. Lemberg, Caterina T. H. Schweidenback, and Tania A. Baker. I contributed the data concerning MuB's interaction with the transpososome (Figure 5). In addition, I was actively involved in the writing of this manuscript.

## **Abstract**

DNA transposases use a single active center to sequentially cleave the transposable element DNA and join this DNA to a new target site. Successful recombination requires controlled conformational changes within the transposase to ensure that these chemically distinct steps occur at the right time and place, and that the reaction proceeds in the net forward direction. Mu transposition is catalyzed by a stable complex of MuA transposase bound to paired Mu DNA ends (a transpososome). We find that Mu transpososomes efficiently catalyze disintegration when recombination on one end of the Mu DNA is blocked. The MuB activator protein controls the integration vs. disintegration equilibrium. When MuB is present, disintegration occurs slowly and transpososomes that have disintegrated remain able to catalyze subsequent rounds of recombination. In the absence of MuB, disintegration goes to completion. This analysis provides evidence for an Initial Joined Complex (IJC) as part of the transition to a stable transposition product. MuB controls progression of recombination by specifically stabilizing a concerted transition to the 'joining' configuration. Thus, MuB's interaction with the transpososome actively promotes coupled joining of both ends of the transpososome DNA into the target site and provides a mechanism to prevent single-end transposition products.

## **Introduction**

The multiple steps of genetic recombination are often carried out by a single nucleoprotein complex (Curcio and Derbyshire 2003; Gueguen et al. 2005; Grindley et al. 2006). To prevent formation of anomalous products—and thus potential damage to the

genome—DNA processing within these complexes is carefully orchestrated.

Recombinases of the transposase/retroviral integrase superfamily (the DDE-motif transposases) have an extra challenge as these proteins use a single active site to catalyze the two distinct chemical steps of DNA cleavage and DNA joining (Rice and Baker 2001, Mizuuchi and Baker 2002, Gueguen et al. 2005). Successful recombination requires that each active site promotes the proper chemistry at the proper time and that the reaction progresses forward, such that the substrate DNA molecules are efficiently converted to the product configuration. How transposase-DNA complexes change throughout the recombination pathway to alter the active site and ensure this forward progress is largely unknown.

Phage Mu encodes an extremely active DDE-transposase, the MuA protein, responsible for transposition of the phage genome (Baker and Luo 1994; Rice and Mizuuchi 1995). To initiate transposition, a tetramer of MuA assembles on specific DNA recognition sites (L1, L2, R1, and R2) located at the left and right ends of the Mu DNA (called the donor DNA). These assembled complexes are known as Mu transpososomes (Fig 1a).

Once the Mu transpososome has assembled to form a stable synaptic complex (SSC or type 0 complex) (Mizuuchi et al. 1992), MuA cleaves the DNA at the junction of the Mu genome and flanking DNA to generate 3'OH nicked ends (Craigie and Mizuuchi 1987). This form of the transpososome is called the cleaved donor complex (CDC or type 1 complex) (Craigie and Mizuuchi 1987; Surette et al. 1987; Lavoie et al. 1991). With the help of the ATP-dependent activator protein, MuB, the transpososome associates with the new DNA segment that will serve as the site for transposon insertion (the target DNA)

(Maxwell et al. 1987; Adzuma and Mizuuchi 1988). The 3' hydroxyl groups generated by the cleavage step then attack and join to opposite strands of the target DNA in a reaction called DNA strand transfer; this step generates the strand transfer complex (STC or type II complex) (Mizuuchi 1984; Mizuuchi and Adzuma 1991; Mizuuchi et al. 1991).

The MuB activator plays several roles in the transposition process. MuB promotes assembly of the MuA tetramer, selects target DNA, and stimulates conversion of the transpososome from the CDC to the STC. MuB interacts with MuA via direct protein-protein contact with MuA's C-terminal domain. (Adzuma and Mizuuchi 1988; Baker et al. 1991)

What drives the DNA joining process forward? For each strand transfer reaction, a phosphodiester bond in the target DNA is broken, while the new bond between the Mu DNA and the target DNA is formed. Thus, DNA joining is isoenergetic with respect to phosphodiester bonds. However, the transposition reaction proceeds in the forward direction because the STC is more stable than the CDC (Surette et al. 1987). This increased stability must be due to changed contacts within the transposase-DNA complex that serve to ensure that most CDCs progress forward to complete strand transfer. In fact, the STC is such a stable complex that the host-encoded AAA+ ATPase CIPX is required to destabilize its protein structure prior to phage DNA replication (Levchenko et al. 1995; Nakai and Kruklytis 1995; Kruklytis et al. 1996). Despite this evidence that the STC is an irreversible product of Mu transposition, little is known about specific changes that occur within the transpososome to generate the STC. Understanding this transition is essential to elucidating the overall mechanism of recombination and how transposition is regulated.

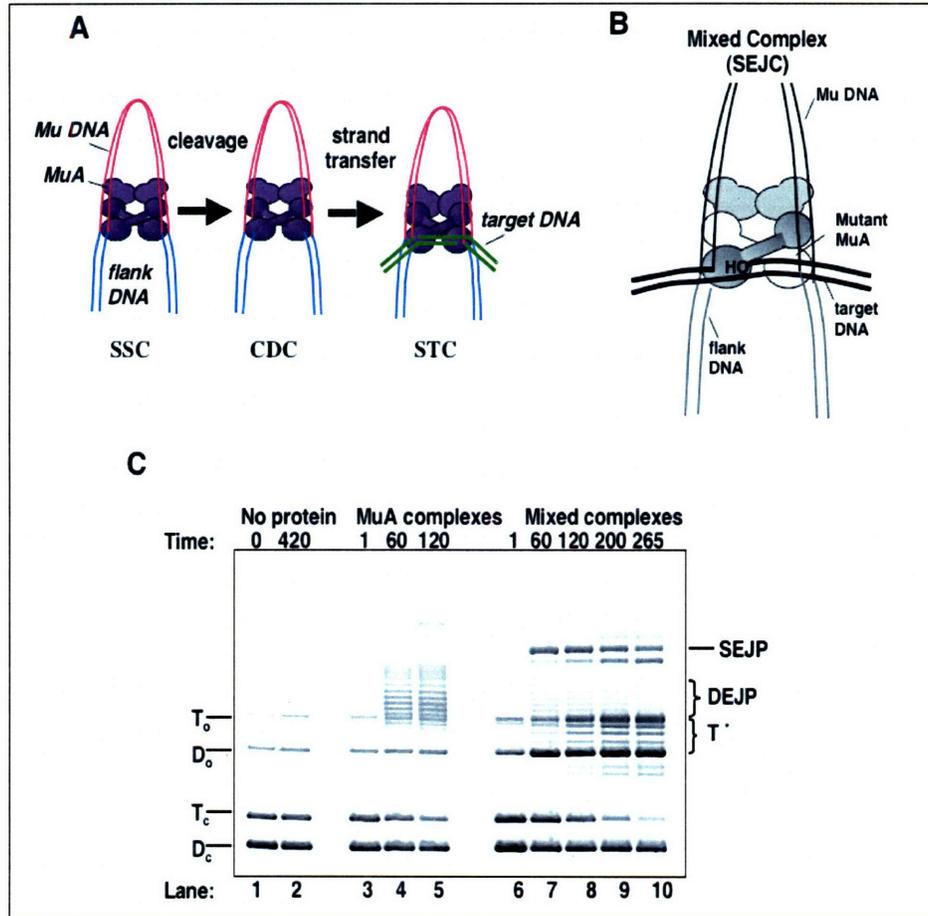
Here, we describe a state of the transpososome (the Initial Joined Complex, IJC) in which DNA joining has occurred, but the active site is still in a configuration that can readily reverse this chemistry. These complexes are abundant when processing of one end of the Mu DNA is blocked by the presence of an inactive MuA subunit within the transpososome. These results demonstrate that DNA joining and generation of the final stable strand transfer complex are separable events, dependent upon the ability of the transpososome to complete recombination of both Mu DNA ends. Furthermore, we find that MuB, by binding to MuA, has a substantial controlling influence on whether the transpososome favors integration into, or disintegration from, the target. We propose that a normal function of MuB is to stabilize the transient IJC long enough for both ends of the Mu DNA to complete strand transfer. Once these reactions are complete, the transpososome undergoes the final conformational change to render joining effectively irreversible.

## Results

### *Mixed mutant transpososomes efficiently generate relaxed target DNA*

To probe the interactions within the Mu transpososome responsible for stabilizing final strand transfer products, we studied the properties of complexes that were only able to process one end of the Mu DNA due to the presence of a subunit (or subunits) carrying an active-site mutation (Figure 1b). These mixed transpososomes were assembled using a 1:1 ratio of wild-type MuA (MuA) and MuA<sup>DE/NQ</sup>. MuA<sup>DE/NQ</sup> carries two mutations in catalytic residues (D269N and E392Q). These mutations abolish the cleavage and joining

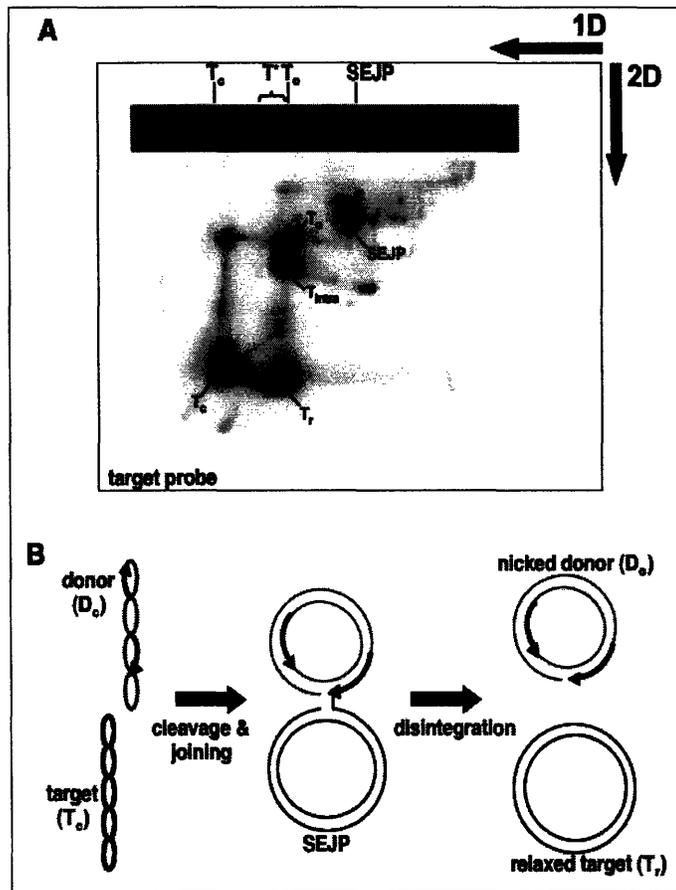
activities of the transposase, but not its ability to assemble into stable transpososomes at near wild-type efficiencies (Baker and Luo 1994).



**Figure 1: Cartoon of Mu Transposition Pathway** A) The stable synaptic complex (SSC) is formed upon assembly of the MuA tetramer at the ends of the Mu genome. MuA hydrolyzes the 3' end of each strand of the genome, resulting in a cleaved donor complex (CDC). After target DNA is delivered to the transpososome, the majority of the joined complexes undergo stabilizing changes to form final strand transfer complexes (STC). "Strand transfer" encompasses reaction steps between the CDC and STC, and the branched DNA structure stabilized within the STC is known as "strand transfer product" (STP). B) Cartoon of a strand transfer complex formed with a mixture of WT MuA and catalytically inactive MuA (DE/NQ). C) Agarose gel of plasmid recombination products. Reactions contained WT MuA or a mixture of MuA and MuA DE/NQ. Over long time courses (6 hours), the MuA reactions form double-end joined products (DEJP) (lanes 4-5.) During the same incubation period, mixed reactions form single-end joined product (SEJP) when complexes assemble with one WT MuA catalytic monomer and one MuA(DE/NQ) catalytic monomer (lanes 7-10). These reactions also yield a series of target-only products ( $T^*$ ) that migrate between  $T_o$  and  $D_o$ . Reactions were stopped at times indicated in 0.2 x vol STOP solution.  $D_c$ : supercoiled donor,  $T_c$ : supercoiled target,  $D_o$ : nicked donor,  $T_o$ : nicked target.

As observed previously, transpososomes comprised of only MuA efficiently generated products that have both ends of the Mu donor plasmid (donor DNA) joined to the target DNA (DEJP) (Fig. 1c, lanes 3-5). In contrast, transpososomes carrying a mixture of MuA and MuA<sup>DE/NQ</sup> (mixed complexes) generated substantial amounts of the single-end joined product (SEJP), as well as cleaved donor DNA (D<sub>o</sub>) (Fig. 1c, lanes 7-10) (Baker et al. 1994). Reactions with mixed complexes also generated a family of novel products, apparently absent from reactions carrying only MuA. We will refer to these products as T\* (Figure 1c). The mobility of these T\* products, as well as hybridization results, indicated that they contain only target DNA (see below, Goldhaber-Gordon et al. 2002). They are, however, formed by a process requiring MuA, MuB, Mg<sup>2+</sup>, and donor DNA (data not shown). Mixed complexes made with MuA and either MuA<sup>D269N</sup> or MuA<sup>E392Q</sup> also formed the T\* products, revealing that the double mutant was not necessary for their production (data not shown).

To investigate the structure of the T\* products, the DNA species generated by mixed transpososomes were analyzed by native two-dimensional gel electrophoresis followed by Southern hybridization with probes specific to either the target or the donor DNA (Fig. 2a and data not shown). The second gel dimension was in the presence of ethidium bromide and intercalation of this reagent into closed circular DNA molecules induces positive supercoiling, leading to rapid migration of this DNA through the gel matrix. By contrast, the conformation of nicked or linear DNA products is not greatly affected by ethidium bromide and therefore these molecules migrate slowly in the second dimension compared to their covalently closed counterparts.



**Figure 2: Identification of novel products by 2D gel and Southern analysis A)** Southern blot hybridized with radiolabeled target DNA. A photo of the first dimension gel slice separating the mixed reaction products is positioned horizontally across the top of the blot to illustrate where the reaction products migrated.  $T_c$ : supercoiled target,  $T_o$ : nicked target,  $T^*$  and  $T_r$ : closed, relaxed target,  $T_{intra}$ : intramolecular recombination product, *SEJP*: single-end joined product. B) Model for disintegration from SEJP. Supercoiled donor and target DNA molecules are joined to form SEJP. The free 3' OH group on the end of the target DNA attacks the Mu end, separating the SEJP into closed, relaxed target and nicked donor DNA.

Hybridization of a target probe revealed that the  $T^*$  products migrated slowly, near the position of nicked target DNA, during the first dimension. However, in the second dimension the majority of these molecules migrated rapidly, in fact slightly more rapidly than the unreacted, supercoiled target DNA (Fig. 2a). Hybridization with the

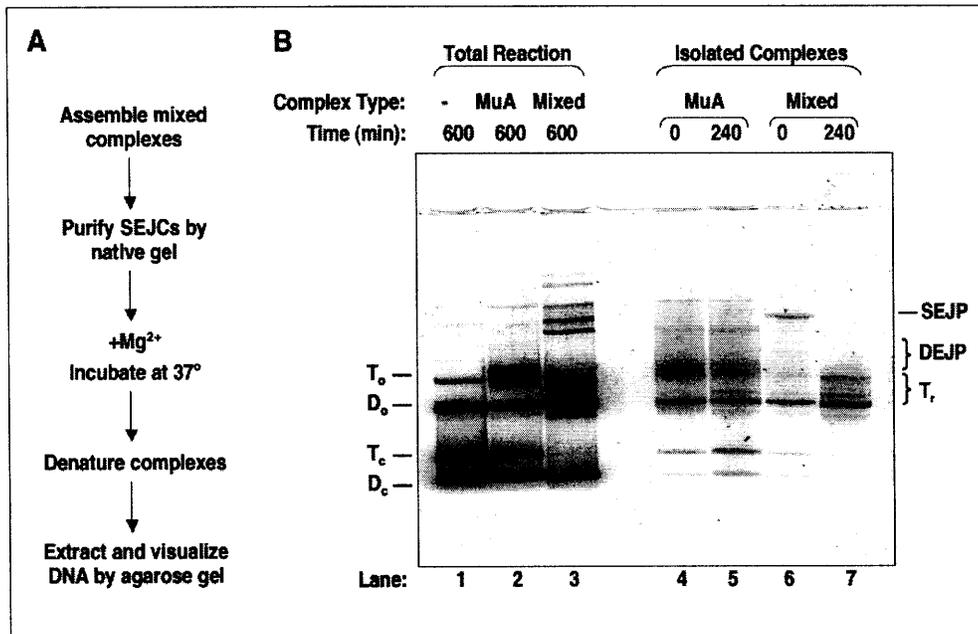
donor DNA probe confirmed that these products contained only target DNA sequences (data not shown). We conclude that the majority of the T\* DNA product is relaxed, covalently closed target DNA (marked T<sub>r</sub>). In addition to this relaxed fraction, a portion of the T\* DNA migrated between the nicked target (T<sub>o</sub>) and nicked donor DNA (D<sub>o</sub>) in both gel dimensions (marked T<sub>intra</sub>). These molecules are the intramolecular transposition product described by Goldhaber-Gordon et al. (2002), formed when mixed transpososomes assemble using one Mu DNA end and one "pseudo end" present on the target DNA.

The analysis presented above reveals that closed but relaxed target DNA is an abundant product of transposition by complexes containing a mixture of active and inactive MuA. Other abundant products include nicked donor DNA and the single-end joined product. Formation of relaxed target DNA suggests that during recombination the originally supercoiled target DNA is sometimes nicked and then re-closed. Given the requirements for both MuA and MuB in generating these products, we hypothesized that disintegration of single-end joined complexes is likely responsible for generating the relaxed target (Fig. 2b). Attack of the free 3' hydroxyl of the target DNA on the junction between the donor and target portions of the single-end joined product would lead to production of both relaxed target and nicked donor DNA.

### *Transpososomes with one joined Mu end efficiently reverse strand transfer*

To test for disintegration by transpososomes, we needed to establish a direct relationship between single-end joined complexes (SEJCs) and the T<sub>r</sub> products. Therefore, we assembled and purified SEJCs and then asked if the DNA from these

complexes could be "chased" into the  $T_r$  products upon further incubation (Fig. 3a). The experiment was set up as follows: SEJCs were generated as before using a 1:1 mixture of MuA:MuA<sup>DE/NQ</sup>, the Mu donor DNA plasmid, the target DNA, and MuB. These complexes were isolated by native gel electrophoresis from free DNA and protein. The purified complexes were then reactivated by the addition of  $Mg^{2+}$  to the gel slice, and incubated at 37°C. After different times of incubation, the reaction was stopped, and the DNA was extracted from the complexes and run on a second gel to observe how the distribution of DNA products changed over time (Fig. 3b).



**Figure 3: Isolated transpososomes chase into reversal products** A) Description of the experimental procedure. B) Denaturing gel of transposition products after second incubation. In samples 1-3, the entire reaction was incubated at 37° for 10hrs. In samples 4-7, purified complexes were incubated at 37°C in the presence of  $Mg^{2+}$  for the indicated amount of time. Small amounts of  $T_c$ ,  $D_c$  in lanes 4-7 are due to the method used to excise complexes from the native gel.

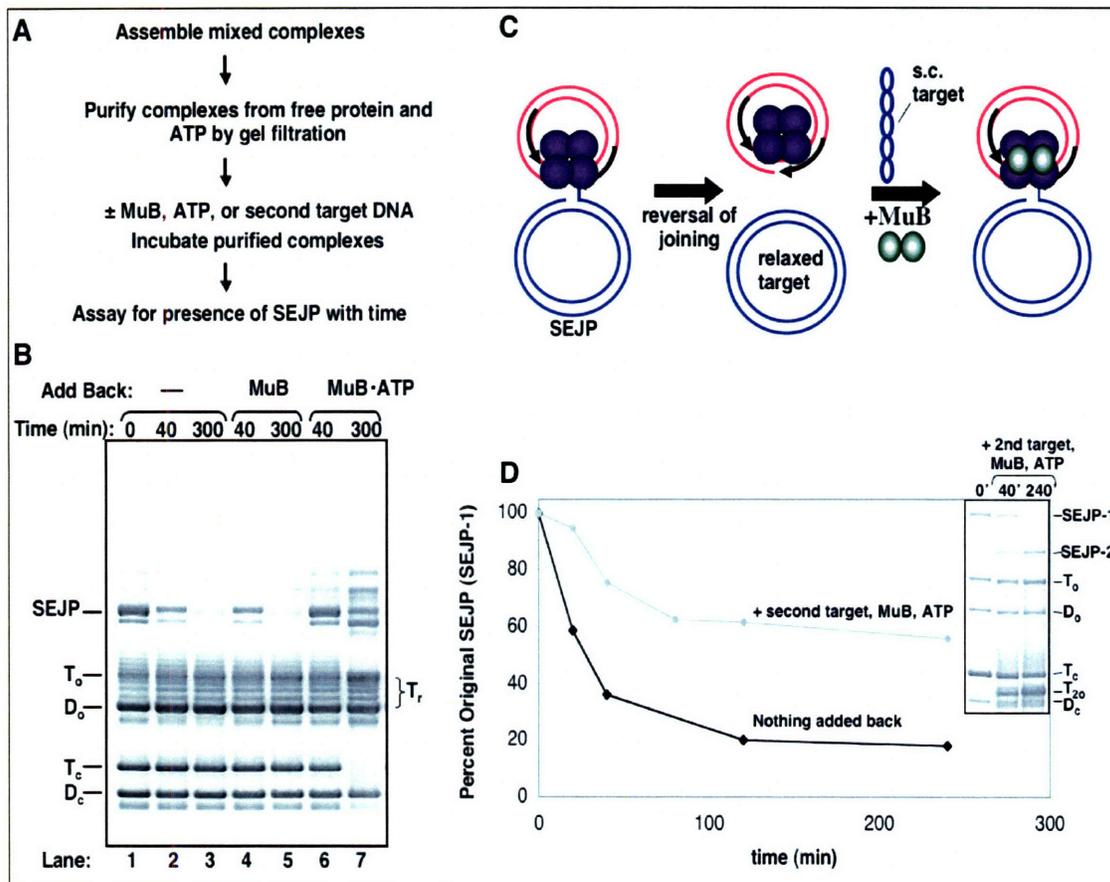
As expected, a major DNA species extracted from the purified SEJC prior to the second incubation was the SEJP. (Contamination by DEJP, and free donor and target DNA was also observed.) However, after incubating the isolated SEJC for four hours, all detectable SEJP disappeared whereas the amount of relaxed target DNA ( $T_r$ ) and cleaved donor DNA ( $D_o$ ) in this sample clearly increased. This experiment therefore strongly supports the hypothesis that the SEJC is the precursor of the relaxed target DNA and cleaved donor DNA. We conclude that mixed transpososomes that have joined one Mu DNA end to the target DNA efficiently catalyze disintegration from this target DNA. In contrast to the results observed with the mixed complex SEJCs, transpososomes that contained exclusively wild-type MuA made only the double-end joined complexes (DEJCs) and these complexes were not affected by the second incubation; little change in the distribution DNA products was observed. These data indicate that disintegration is infrequent after both Mu DNA ends have completed DNA strand transfer.

In addition to supporting the reversal of joining hypothesis, this chase experiment also revealed that the isolated SEJCs promote disintegration more completely than do non-isolated complexes (compare Fig. 1c, lane 10 to Fig. 3b, lane 7). Without isolation, the SEJP was clearly present even after 7 hours. In contrast, the isolated complexes had completed reversal in less than 4 hours. Therefore, we looked for factors in the transposition reaction that influence the partitioning between SEJCs and complexes that have undergone disintegration.

### *The MuB activator promotes joining and antagonizes reversal*

Because MuB is a strong stimulator of strand transfer complex formation (Baker et al. 1991; Naigamwalla and Chaconas 1997; Goldhaber-Gordon et al. 2003), we considered that it might have a specific role in preventing disintegration. To address this question, we developed a two-stage assay to monitor MuB's role in the fate of complexes (Fig. 4a). In the first stage, mixed complexes were incubated in the presence of MuB and ATP to generate SEJCs. These complexes were then separated from free protein and nucleotide by gel filtration. Western blotting confirmed that all detectable MuB was removed from the fractions containing the protein-DNA complexes (data not shown). The persistence of the SEJCs was then measured (by the amount of SEJP present) as a function of time during a second incubation, with or without the addition of MuB and ATP.

When MuB was not added back to the purified complexes, more than half of the SEJC originally present disappeared after 40 min (Fig. 4b lane 2). By five hours, less than 15% of these joined complexes remained (lane 3). Coincident with this disappearance in the SEJP was the appearance of the relaxed target ( $T_r$ ), as expected from disintegration. By contrast, when MuB and ATP were added back to the purified complexes, the SEJP remained for much longer time periods; after five hours approximately half of the initial level of this product was still present (lanes 6&7). ATP was required for this effect, indicating that nucleotide-bound MuB was responsible (lanes 5&7).



**Figure 4: Effect of MuB on reversal equilibrium** A) Description of experimental procedure. B) Agarose gel showing the presence of single-end joined product among complexes purified by gel filtration. “Time” refers to the time of 37°C incubation following purification of complexes. Nothing was added back to the sample in lanes 1-3. Lanes 4&5 had MuB (650nM) added back, and lanes 6&7 had MuB (650 nM) and ATP (2 mM) added back. Samples from reactions were stopped at times indicated in 0.2 x vol STOP solution. C) Illustration of a disintegration product undergoing a second round of strand transfer into a new molecule of target DNA. D) Sustained presence of SEJP with MuB-ATP addition is not entirely due to re-formation of SEJP. The original SEJP (SEJP-1) still persists in the presence of added back MuB-ATP and an excess of a second target DNA. An inset of the DNA products following gel filtration and incubation with added MuB and second target is also shown. Supercoiled second target is run off the end of the gel. *No add back: solid black line with diamonds; 2.6 uM MuB, 2 mM ATP, 90 ug/ml pUC19 add back: light grey line with circles.*

We considered two possible mechanisms to explain how MuB•ATP influenced the level of the single-end joined product: (1) MuB could interact with the Mu

transpososomes that had catalyzed single-end joining in a manner that favors the joined configuration and thereby antagonizes the disintegration reaction (*SEJP persistence*); or (2) MuB could deliver target DNA molecules to free cleaved donor complexes (CDCs) and thereby promote the continued formation of new joined products (*SEJP re-formation*, Fig. 4c).

Inspection of the product distribution from reactions containing MuB suggested that it was very likely that MuB was promoting re-formation of SEJPs. For example, in the reactions containing MuB in the second incubation, the supercoiled target DNA decreased throughout the time course, and new high-molecular weight DNA products accumulated (Fig. 4b, compare lane 3 to lane 7). Continued transposition requires the presence of active CDCs among the purified complexes. New CDC formation via transpososome assembly is prevented under these conditions because the free MuA was removed by the gel filtration. (Also note that the amount of supercoiled donor DNA ( $D_c$ ) did not change significantly during the second incubation.) Therefore, the most likely source of active CDCs is the single-end joined complexes that undergo disintegration. These complexes participate in re-formation of SEJPs.

We then sought to determine if MuB-stimulated re-formation of SEJPs was sufficient to account for the higher levels of these joined products or if MuB also influences the persistence of the SEJCs. To distinguish between the MuB-stimulated persistence and re-formation of SEJPs, we purified SEJCs and added back MuB•ATP along with a four-fold excess of a second target DNA. Because it is present at a higher concentration, the second target is more likely to be used by the CDCs and DNA joining should result in a new type of SEJP, distinguishable by gel electrophoresis since the two

target DNAs are different sizes. Therefore, the probability of re-forming the original type of SEJP (SEJP-1) was decreased substantially in this experiment, and the presence of SEJP-1 would largely be a measure of the influence of MuB on the stability/lifetime of the pre-existing single-end joined complexes.

In the presence of MuB, ATP, and a second target DNA, the original single-end joined complexes (SEJC-1) persisted longer than in the absence of MuB (Fig. 4d). Over four hours of incubation, SEJP-1 only decreased to 55% of its initial levels when both second target and MuB were added. Although some disintegration takes place over the time course, we conclude that the prolonged presence of SEJP-1 was due to MuB-stimulated persistence of SEJC-1s. Addition of the second target also prevented disappearance of the original supercoiled target DNA, and formation of a second type of SEJP was observed (SEJP-2) (Figure 4d, inset). These findings lend further support to the conclusion that SEJCs that undergo disintegration give rise to active CDCs.

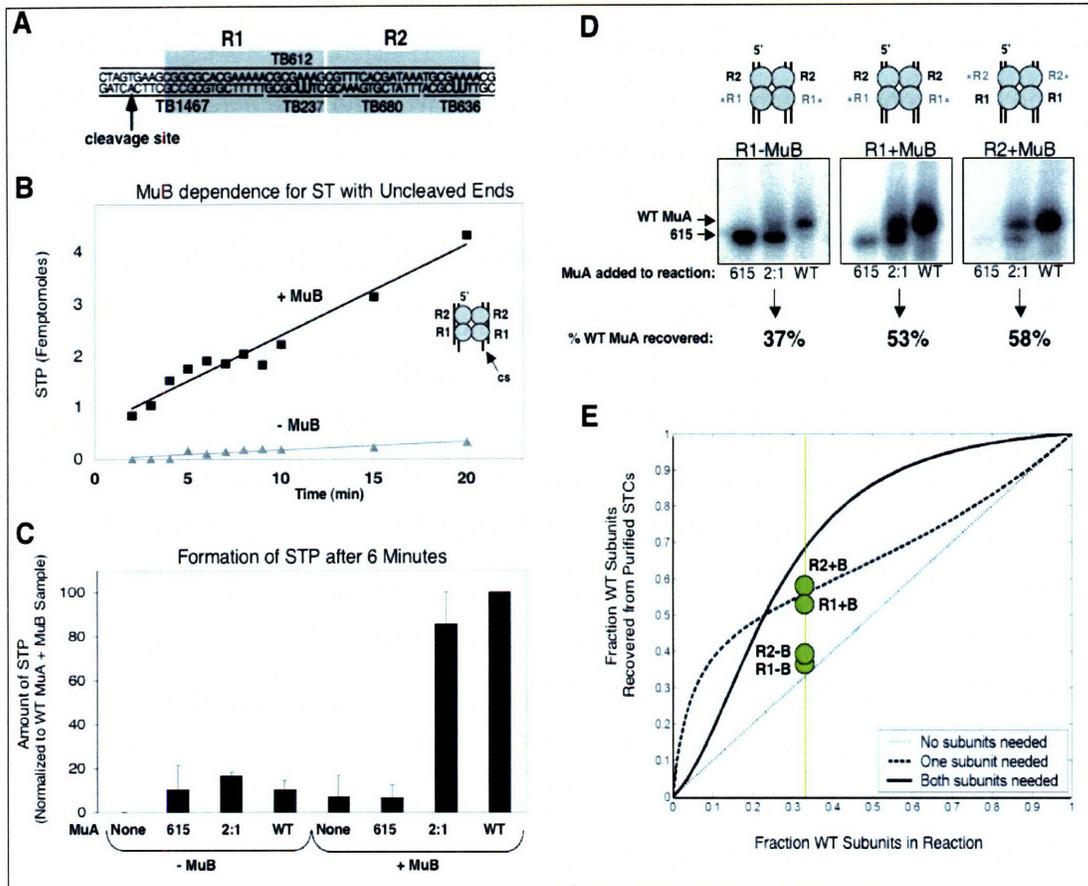
Based on this analysis, we conclude that MuB has a substantial influence on the transpososome even after the first DNA joining reaction has been completed. MuB serves both to extend the lifetime of the initial joined complexes, as well as to promote the turnover of transpososomes that have completed disintegration.

### *MuB promotes a cooperative allosteric change in the Mu transpososome*

Previous experiments (Baker et al. 1991; Surette and Chaconas 1991; Yamauchi and Baker 1998; Williams and Baker 2004) together with the analysis presented above reveal that MuB is a multifaceted activator of MuA and that it is especially important in driving the recombination reaction forward to the final strand transfer complex. To

understand how MuB functions to control the activity of the transpososome, we sought to determine if it makes preferential contacts with specific MuA subunits within the transpososome. The transpososome is a homotetramer of MuA. This tetramer contains two types of MuA subunits: (1) those bound near the cleavage sites (to the R1 and L1 binding sites), which donate their catalytic domains for the DNA cleavage and joining reactions; and (2) those bound to the distal sites (R2 and L2) which do not participate as directly in the reaction chemistry (see Fig. 1a).

To test which subunits of the transpososome interact with MuB, we modified a method developed previously which involves "marking" MuA subunits bound to specific sites using UV-induced protein-DNA crosslinking (Aldaz et al. 1996; Williams et al. 1999). This crosslinking approach is achieved by using synthetic DNA fragments as the donor DNA. These fragments carry the first 50 bp from the right end of the Mu DNA, with 5-Iodouracil (IdU) base substitutions at the R1 and R2 sites (Figure 5a). Upon exposure to UV light, the IdU bases crosslink to the MuA subunits present nearby (at the R1 and R2 sites). Since our donor DNA fragments contain nicks on either side of the IdU bases, denaturation of the DNA will yield a 10 base oligonucleotide covalently attached to a MuA subunit. By radiolabeling the 5' end of the IdU containing oligonucleotide at *either* the R1 *or* the R2 position, it is possible to distinguish MuA subunits that bind to one site or the other. Those subunits that bind to the radiolabeled site, upon crosslinking, will become covalently attached to the radiolabel. MuA subunits bound to the non-labeled site will still crosslink to the DNA, but as this DNA not radiolabeled, the subunits will be invisible in subsequent autoradiography steps



**Figure 5: Characterizing MuB's Interaction with the Transpososome** A) Diagram of crosslinkable Mu end fragment, composed of four small oligonucleotides annealed to one long oligonucleotide. B) Accumulation of strand transfer products using un-cleaved Mu end fragments +/- MuB. C) Accumulation of strand transfer product after 6 minutes with un-cleaved Mu end fragments. Reactions contained either MuA 615, a 2:1 ratio of MuA 615 to WT MuA, or all WT MuA, +/- MuB. Amount of strand transfer product was normalized to reactions containing all wild-type MuA in the presence of MuB. D) Autoradiograph of polyacrylamide gel from crosslinking experiment. Samples are purified STCs from reactions containing either MuA 615, a 2:1 ratio of MuA 615 to WT MuA, or all WT MuA, +/- MuB. Oligonucleotides in this experiment were radiolabeled at either the R1 or the R2 position, as indicated. Percentages indicate the relative amount of wt MuA recovered from purified STCs from samples initially containing a 2:1 ratio of MuA 615 to WT MuA. E) Predicted fraction of wild-type MuA / total MuA subunits to be recovered from purified strand transfer complexes as a function of the initial fraction of wild-type MuA / total MuA subunits present in the reaction. The three lines represent three different hypothetical models (that neither MuA subunit at a given position must interact with MuB, that only one MuA subunit at a given position must interact with MuB, or that both MuA subunits at a given position must interact with MuB for efficient recombination). Dots mark the observed fraction of wild-type MuA subunits recovered from reactions containing a 2:1 ratio of MuA 615 to wt MuA (i.e. an initial fraction of wt MuA subunits equal to 0.333). Reactions were done in both the presence and absence of MuB, with either the R1 or R2 position radiolabeled. Note: the data here represent the results from a single set of experiments. The experiments were repeated several times yielding similar results.

To determine which subunits within the transpososome interact with MuB, we established conditions in which efficient recombination was highly dependent on the presence of MuB. As shown in Figure 5b, strong MuB-dependence was observed with donor fragments in which the transferred strand extended substantially past the cleavage site (here 28 nucleotides). In contrast, MuB had little effect on the reaction rate or efficiency when the donor DNA was pre-cleaved (data not shown). Control experiments confirmed that MuB principally affected a post-cleavage step, rather than complex assembly or donor DNA cleavage (Goldhaber-Gordon et al. 2003; Williams and Baker 2004; and data not shown).

To decipher whether MuB stimulates transposition by interacting with MuA subunits bound to the R1 and/or R2 positions, we performed *in vitro* transposition using the crosslinkable Mu end DNA fragments described above. Transpososomes were assembled, purified and assayed as follows. The modified Mu end fragments were incubated with a 2:1 ratio of MuA<sup>1-615</sup> to full-length MuA and allowed to form mixed complexes. MuA<sup>1-615</sup> carries a C-terminal truncation that renders it unable to interact with MuB, but fully functional in transpososome assembly and catalysis of recombination (Baker et al. 1991). Target DNA, MuB and ATP were added to these mixed complexes and recombination was allowed to proceed. The reactions were then UV-irradiated and those complexes that had successfully recombined with the target DNA were isolated on a native agarose gel. Because the reaction conditions are such that recombination is strongly stimulated by MuB (Fig. 5b), this step selects for tetramers that were able to interact with MuB. Finally, the purified complexes are run on a denaturing polyacrylamide gel that separates full-length MuA from MuA<sup>1-615</sup>. The autoradiograph of

the gel reveals the ratio of MuA<sup>1-615</sup> to full-length MuA that were bound to the radiolabelled site (either R1 or R2) in the purified, active complexes. If MuB is absent from the reaction, then we would expect to recover radiolabeled MuA from the purified complexes in approximately the same ratio as the starting conditions (2:1 MuA<sup>1-615</sup>:full-length MuA). Likewise, if MuB is present in the reaction but does not interact with the MuA subunits at a given site, we expect to recover a 2:1 ratio of MuA<sup>1-615</sup>:full-length MuA. However, if the MuA subunits at the assayed position do interact with MuB, then there should be an enrichment of full-length subunits bound to that position in the purified complexes.

Full-length MuA was enriched at both the R1 and R2 positions in the purified recombination complexes when MuB was present (Fig. 5d). As a control, reactions were also performed in the absence of MuB. Although recombination was much less efficient without MuB, a small amount of product was generated under this condition (Fig. 5c). As expected, there was little or no enrichment for full-length MuA at either the R1 or R2 position in these purified complexes (Fig. 5d). Thus, we conclude that MuB-contact with R1- and R2-bound MuA subunits stimulates recombination.

In the experiments shown in Figure 5, samples that contained a 2:1 ratio of MuA<sup>1-615</sup> to full-length MuA recombined at a rate that was ~85% that seen in reactions containing only full-length MuA (Figure 5c). Since only 1.2% of the transpososomes that assembled with this 2:1 mixture of MuA are expected to contain four full-length MuA subunits (assuming random assortment during assembly, see Discussion), it is very unlikely that MuB must interact with all four MuA subunits to stimulate efficient recombination.

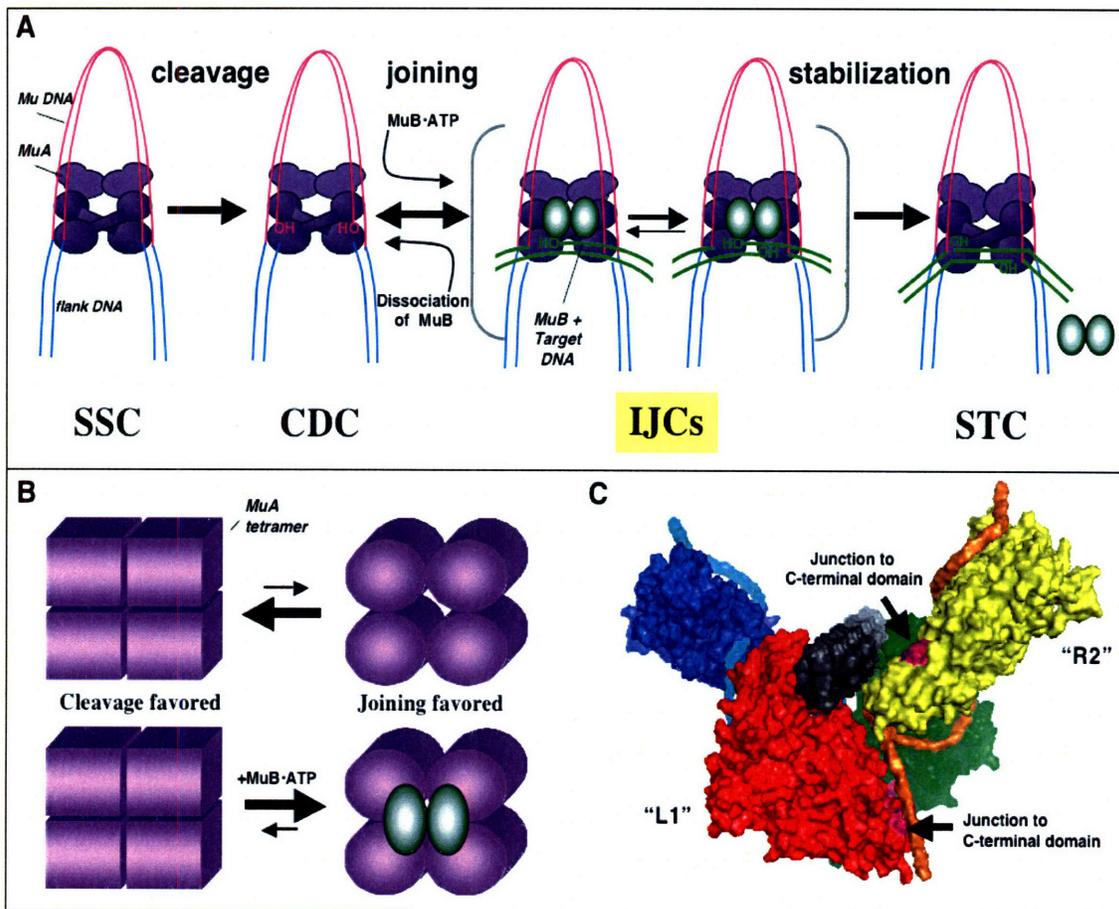
We therefore asked whether MuB must interact with either both R1-bound or both R2-bound subunits to stimulate transposition. Using the experimentally observed rates of STP formation in the presence or absence of MuB and basic probability, we were able to model the expected enrichment of full-length MuA subunits under three possible reaction scenarios (Fig. 5e). These scenarios were: (1) MuB must interact with both MuA subunits at a given position (either both R1 subunits or both R2 subunits) to stimulate recombination. (2) MuB must interact with only one MuA subunit at a given position (one R1 subunit or one R2 subunit) to stimulate recombination; or (3) MuB is not required to interact with either subunit at a given position to stimulate recombination. The points on the graph in Figure 5d indicate the experimentally observed enrichment for full-length MuA in reactions either with or without MuB at either the R1 or R2 position. In reactions without MuB, the fraction of full-length MuA recovered from the purified STPs was 0.37, was approximately the same as that of the initial reaction conditions (*e.g.* 0.33). When MuB was present, enrichment of full-length MuA was observed at both the R1 and R2 sites. However, the observed level of enrichment was lower than would be expected if both subunits at a given position were required to interact with MuB for efficient recombination. Therefore, these data indicate that MuB can stimulate recombination by interacting with MuA subunits bound to either the R1 or R2 positions, but MuB need not interact with both of the subunits at either position to be effective.

That MuB does not need to contact both MuA subunits at either position is interesting, as >80% of recombination events resulted in successful strand transfer of both Mu end fragments (data not shown). Taken with previous experiments (Mizuuchi et al. 1995; Yang et al. 1995; Mariconda et al. 2000), these results support the conclusion

that MuB can interact with the MuA transpososome to stimulate recombination of one Mu DNA end without interacting with the MuA subunit that directly catalyzes recombination of that end. Thus, this analysis supports models in which MuB acts to promote a cooperative allosteric change in the transpososome that favors the conformation of the complex that promotes DNA joining (see Discussion).

## **Discussion**

Successful transposition requires the sequential processing of multiple DNA sites bound by a single transposase complex. Whereas DNA cleavage is energetically favorable, the DNA strand transfer reaction itself is not. Therefore, to drive transposition toward recombinant products, DNA joining must be coupled to a conformational change in the transposase-DNA complex that stabilizes the recombined DNA and thus effectively prevents reversal of DNA joining. By studying reactions catalyzed by transpososomes that can process only one of the two Mu DNA ends bound within the complex, we find evidence for a new state of the transpososome, the initial joined complex (IJC), in which Mu DNA is joined to the target DNA, but the final stabilization process has not yet occurred (Figure 6a). These complexes efficiently promote disintegration, to generate relaxed, covalently closed target DNA and active cleaved donor complexes. Thus, we conclude that STC stabilization is not obligatorily coupled to DNA joining, but a separable molecular event that normally only occurs after both Mu DNA ends are successfully joined. Furthermore, our data dramatically illustrate the need for this final stabilizing conformational change in the transpososome, as we find that the joined DNA products are not long-lived in its absence.



**Figure 6: Model of How MuB Affects the MuA Tetramer** A) Model for progression of Mu transposition. The IJC is a newly-defined state of the transpososome in which Mu DNA has been joined to target DNA, but the final stable STC conformation has not yet been achieved. MuB promotes the “joined” state of the transpososome and antagonizes disintegration. B) Model of MuB’s allosteric effect on the cleavage-favored vs. joining-favored states of the MuA tetramer. C) Proposed structure of the transpososome based on Yuan *et al* (2005). R2 subunits: dark blue and yellow; R1 subunits: red and green; Mu DNA backbone: light blue and orange; target DNA: grey.

Analysis of reactions in which the IJC is well populated also revealed a new role for the transposase-activator protein MuB. Previously, MuB was known to deliver target DNA to MuA (Adzuma and Mizuuchi 1988; Naigamwalla and Chaconas 1997), stimulate DNA strand transfer (Baker *et al.* 1991 and see below), and protect transpososomes from premature destabilization by ClpX (Levchenko *et al.* 1997). We find that MuB also

antagonizes disintegration, thereby prolonging the lifetime of the IJCs. Thus, an attractive role for MuB in the normal transposition pathway is to inhibit the disintegration of one Mu DNA end, so as to allow the second Mu end a chance to join to the target DNA. Once joining of both ends is complete, the final stabilizing conformational change in the transpososome can occur, to form the STC. Once the STC is formed, with both ends joined, this complex is long-lived even in the absence of MuB.

In this study, disintegration was only convincingly detected with transpososomes containing one inactive catalytic center due to the presence of a mutant subunit. Several lines of evidence indicated that the presence of the uncleaved Mu DNA end in one active site is the principal cause for the propensity of these complexes to catalyze reversal (data not shown). However, we cannot rule out that the amino acid sequence changes in the mutant subunit also have a contributing role. These residues are undoubtedly in intimate contact with the substrate and product DNA molecules, and could therefore participate in important contacts involved in stabilizing the final STC. Mutations at these residues could also slow the conformational change needed for the final stabilization reaction, and thereby increase the lifetime of the IJC.

The retroviral integrases (Chow et al. 1992; Jonsson et al. 1993; Gerton et al., 1999), as well as several DNA transposases (Polard et al. 1996; Beall and Rio 1998; Stewart et al. 2002; Au et al., 2004), and the Rag recombinases (Melek and Gellert, 2000) have been shown to reverse DNA strand transfer. In the majority of these studies, the transposase/integrase was assembled on a synthetic DNA substrate designed to mimic the normal DNA product of this joining process. As a result of this experimental design, it has been difficult to determine if the protein assembles on these substrates in the same

way that it would be bound if it had just made the new DNA junction. In fact, in some cases the products generated by transposase acting on these disintegration substrates clearly suggest alternative modes of transposase-DNA interaction (P.A. Rice and K Mizuuchi, personal communication; Au et al., 2004; Melek and Gellert, 2000; Mazumder et al., 1994). In contrast, we observe that transpososomes that have just completed DNA cleavage and joining of one Mu DNA end clearly also catalyze the disintegration of that DNA end. This reversal reaction is efficiently observed because, by blocking catalysis on one DNA end, we "catch" the transpososomes before they undergo the stabilizing events that normally accompany the final steps of recombination. Another study reports the reversal of DNA joining by MuA using transpososomes that had joined both DNA ends to a target site (Au et al. 2004). In this study the reversal reaction was only observed after incubating the STCs at high temperature (75 °C). Similar to the conclusions of our study, these authors deduce that after DNA joining, the Mu transpososome undergoes an important stabilizing conformational change that functions to prevent reversal. By incubating at high temperature, this conformation is destabilized, making reversal a detectable reaction.

Discovery of the IJC provides new insight into how MuB modifies the activity of the Mu transpososome. When MuB is present, transpososomes reside for longer times in the conformation where the donor DNA is joined to the target site. In contrast, when MuB is removed from the mixed transpososomes, disintegration is efficient, and goes to completion. These observations strongly suggest that the transpososomes can exist in two distinct conformations: the "cleavage favored" and the "joining favored" states (Figure 6b). Protein-protein contact between MuB and MuA within the transpososome serves to

tip the balance toward the joining-favored configuration. We also investigated which MuA subunits in the homotetrameric transpososome contact MuB for MuB-stimulated recombination to occur. Our data indicate that MuB interacts with MuA subunits at both the R1 and the R2 positions. Furthermore, MuB does not need to interact with both subunits at either position to effectively stimulate recombination of both Mu ends. Therefore, MuB can stimulate recombination of a particular Mu end without directly interacting with the MuA subunit that is responsible for catalysis of that end. These data are in concert with the idea that MuB functions as allosteric activator that promotes a cooperative change in the MuA tetramer. Previous research has suggested that MuB allosterically activates MuA (Baker et al. 1991; Williams et al. 1999; Williams and Baker 2004). Thus, we propose a model in which MuB contacts MuA to promote a conformational change that favors DNA joining and disfavors disintegration. Though this conformational change can happen in the absence of MuB, contact of some MuA subunits by MuB increases the stability of the entire transpososome in the "joining favored" configuration, and consequently DNA joining will both occur and persist.

That MuB can promote an allosteric change in the MuA tetramer by interacting with subunits at either the R1 or the R2 position is especially interesting given recent insight into the structure of the transpososome. Structural analysis indicates that the MuA subunits at the R1 and R2 positions are in different relative orientations, and the two R2 subunits probably do not contact each other (Figure 6c) (Yuan et al. 2005). Therefore, it is not immediately obvious how an allosteric signal would be transmitted between these subunits; a likely solution is that the conformational signal is mediated through the R1-R2 interfaces. Furthermore, the proposed target DNA binding site within the

transpososome (grey cylinder in Figure 6c) is near the C-terminal (MuB-interacting) domains of the R2 but not the R1 MuA subunits. How, then, do MuB molecules bound to the target DNA contact the MuA subunits at the R1 position? Must the target DNA wrap around to contact these subunits? Clearly, more experiments are necessary to understand the elemental steps involved in delivering the target DNA and promoting conversion to the "joining favored" conformation of the transpososome prior the generation of the final strand transfer complex.

How do other transposases prevent disintegration? Based on limited *in vitro* analysis, the intrinsic efficiency of disintegration promoted by different transposase family members varies widely. For example, the retroviral integrases and Rag recombinases are rather efficient at disintegration, whereas the Tn10 transposase promotes this reaction only very feebly. It has been argued that efficient disintegration by the Rag proteins may be stabilizing to the genome, as it will prevent generation of Rag-induced genomic rearrangements. In contrast, the Tn10 and Mu transposases are very inefficient at catalyzing disintegration once the final strand transfer product has been created, thereby driving the reaction toward recombination, transposon movement, and its associated genome rearrangements. The cost is that the strand transfer complex is very stable, and requires energy-dependant disassembly by protein-unfolding enzymes to resolve these structures. However, based on the results presented here, we find it attractive to consider that recombination pathways promoted by transposase/integrase family members may have transient intermediate complexes in which disintegration is a robust reaction; these complexes are well positioned to be regulated by interactions with cellular or element encoded accessory proteins. Many transposases interact with other

DNA binding proteins, and these interactions are increasingly found to have regulatory consequences (e.g. Tn7 transposase is regulated by TnsC/D (Stellwagen and Craig, 1997), Ty5 integrase is regulated by Sir4p (Xie et al., 2001), and Ty3 integrase is regulated by promoter-bound transcription factor TFIIIB (Kirchner et al., 1995)). Controlling the joining versus disintegration choice may provide an important additional checkpoint to ensure the balance between successful transposition and healthy genomic integrity.

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## **Materials and Methods**

### *DNA:*

$\phi$ X174 RFI was purchased from either New England Biolabs or Invitrogen. The mini-Mu plasmid was pMK586 (Mizuuchi et al. 1991), and was purified by CsCl/ethidium bromide ultracentrifugation. Oligonucleotides for fragment assays described were synthesized by Invitrogen and purified by denaturing PAGE.

### *Proteins:*

HU was purified as described by Baker and Luo (1994). Wild-type MuA, MuA (1-615), MuA(DE/NQ), MuA(E392Q), and MuA(D269N) were purified as in Baker et al. (1993). MuA and HU were diluted into 25 mM HEPES-KOH (pH 7.6), 300 mM NaCl, 0.1 mM dithiothreitol, and 10% glycerol (Baker et al. 1991).

Wild-type MuB was purified by the method described in Yamauchi and Baker (1998). MuB dilutions were done in 1 M NaCl, 25 mM HEPES-KOH (pH 7.6), 0.1 mM EDTA, 20% glycerol, and 1 mM dithiothreitol.

### *Transposition Assays:*

Reversal products were observed following a two-step plasmid transposition assay. Blocking one end of the transpososome was achieved by a 1:1 ratio of WT MuA:MuA(DE/NQ) in the assay. The first incubation at 30°C was as described by Baker et al. (1994). Briefly, reactions included: 25 mM Tris-HCl pH8, 156 mM NaCl, 10 mM MgCl<sub>2</sub>, 1 mM dithiothreitol, 10 ug/ml BSA, 15% glycerol, 2 mM ATP, 10 ug/ml mini-Mu donor, 10 ug/ml target plasmid (usually  $\phi$ X174 RFI), 130 nM HU, 130 nM MuB, 26 nM wild-type MuA, and 26 nM MuA(DE/NQ). Addition of 0.02 vol 50% glycerol prior to a second incubation at 37°C (Goldhaber-Gordon et al. 2002) enhanced the production of reversal products. Time points were stopped in 0.2 vol STOP solution (2.5% SDS, 50 mM EDTA, 30% glycerol, bromophenol blue). Reaction products were run on a 1.0% HGT-agarose (Cambrex) gel in 1 X TAB (40 mM Tris-HCl pH 8.0, 3.6 mM EDTA, 27 mM sodium acetate) at 70V for 2.5 hours. Gels were stained using Vistra Green (1:10000 dilution) and visualized on a Molecular Dynamics fluorimager.

The fragment assay in Figure 5B contained 25 mM Tris-HCl at pH8, 30 mM dithiothreitol, 2 mM ATP, 100 ug/ml bovine serum albumin, 15% glycerol, 100 mM NaCl, 0.1% Triton X-100, 10 mM MgCl<sub>2</sub>, 48 nM donor fragment, 30 ug/ml  $\phi$ X174 RFI, 20 ug/ml MuA, 24 ug/ml MuB. Reactions were incubated at 30°, stopped in STOP solution (see above), and run on a 0.9% HGT-agarose gel. Gels were stained in Vistra Green (1:10000 dilution) and visualized on a Molecular Dynamics fluorimager. Fragment assays in Figures 5C and 5D were performed as in 5B, except that the concentrations of donor fragment,  $\phi$ X174 RFI, MuA, and MuB were all increased 5 fold. The crosslinking experiment in Figure 5D was performed as described in Aldaz et al (1996).

#### *2D Gel Analysis:*

To analyze the topologies and sequences of reversal products, two-dimensional gel electrophoresis was followed by Southern analysis. Products of mixed transposition reactions (described above) were run on a 0.4% HGT-agarose in 1X TBE (89 mM Tris, 89 mM boric acid, 2 mM EDTA) at 1 V/cm for 24 hours. The gel was stained in 0.3 ug/ml ethidium bromide and lanes of interest were excised over a UV transilluminator. Gel slices were embedded horizontally across the top of a 1.1% HGT-agarose gel with 5 ug/ml EtBr. The second dimension was run in 1xTBE and 5 ug/ml EtBr with recirculation of buffer. Products were transferred to a Gene-Screen Plus membrane in 10 X SSC (3 M NaCl, 0.3 M sodium citrate.) Products were visualized by hybridizing blots with <sup>32</sup>P labeled fragments randomly primed off either target or donor plasmid. Blots

were exposed to phosphoimager plates overnight and scanned using a Molecular Dynamics Phosphoimager.

*Chase Experiment:*

SEJCs, formed by incubating plasmid transposition reactions at 30°C for 2 hours, were isolated by native gel electrophoresis (0.85% SeaPlaque agarose (Cambrex), 80 ug/ml BSA) in 1X TAB and 1 mM DTT with recirculation of the buffer. Half of the native gel was stained in 0.3 ug/ml EtBr, and complexes were visualized on a UV-transilluminator. Unstained complexes were then excised by aligning the two halves of the gel. Gel slices were submerged in plasmid assay buffer (25 mM Tris-HCl pH8, 156 mM NaCl, 10 mM MgCl<sub>2</sub>, 1 mM dithiothreitol, 10 ug/ml BSA, 15% glycerol) to a volume that was 3x their mass and incubated at 37°C. Products were extracted from gel slices at various time points using a Qiagen gel extraction kit. Complexes were denatured by addition of 0.2 vol SDS buffer and products were run on 0.9% HGT-agarose gel.

*SEJC Purification by Gel Filtration:*

For purification of complexes by gel filtration, 5x assembly reactions were prepared (130 nM MuA and DE/NQ, 650 nM MuB, 650 nM HU, 50 ug/ml donor, 50 ug/ml target). Complexes were purified on a 1 mL column of either BioGel A15m beads (BioRad) or ABT 4% plain agarose beads (Iberagar) (Burton and Baker 2003). Peak fractions were pooled and incubated at 37°C for up to 5 hours with addition of one or more of the following components: ATP (2 mM), MuB (650 nM or 2.6 uM), pUC19 DNA (90 ug/ml). Samples of reactions were stopped at various intervals with 0.2 vol 5x

STOP buffer. DNA products were analyzed by 1.0% HGT-agarose gel and visualized as described above. Presence of SEJP was analyzed using ImageQuant.

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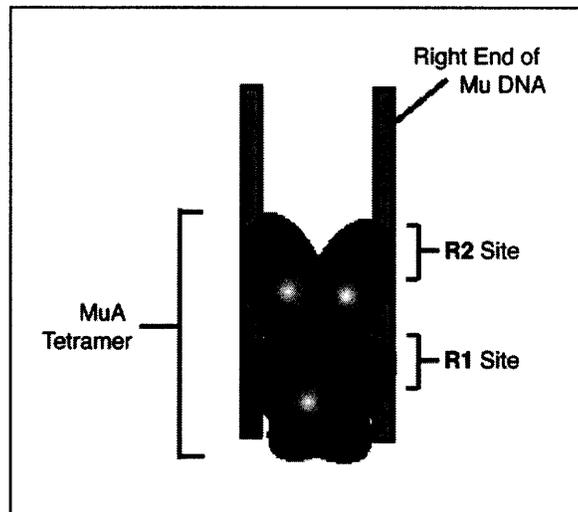
## **Chapter IV**

### **Summary and Future Directions**

Transposable elements are a ubiquitous feature of known genomes (Curcio and Derbyshire, 2003). No doubt, these mobile genetic elements have influenced the evolution of those species within whose genomes they reside. To comprehend the dynamics of transposon-host relationships, it is necessary to understand the factors governing transposition targeting. Mu provides a tractable system for the study of transposition targeting because it is well characterized and exhibits defined targeting preferences. Specifically, Mu preferentially transposes into DNA that is far from any copies of the Mu genome (Adzuma and Mizuuchi, 1988; Maxwell et al., 1987). The process by which Mu targets transposition to non-Mu DNA, termed target immunity, involves two Mu-encoded proteins, MuA and MuB. MuA catalyzes transposition, whereas MuB selects the target DNA and activates MuA-mediated transposition into the selected DNA. This thesis addresses the biochemical interactions between MuA and MuB that direct Mu transposon into selected DNA.

The MuB protein serves multiple roles during Mu transposition. MuB selects target DNA, delivers selected target DNA to MuA, and activates MuA's catalytic activity (Adzuma and Mizuuchi, 1988; Baker et al., 1991; Chaconas et al., 1985; Coelho et al., 1982; Surette and Chaconas, 1991; Williams et al., 1999; Yamauchi and Baker, 1998). MuB is an ATPase, and ATP hydrolysis by MuB is essential for the process of target selection but not for the process of MuA activation. The role of ATP hydrolysis in target delivery by MuB, however, remained unclear. To address whether ATP hydrolysis by MuB is obligatory for target delivery, we created Arc-MuB fusion proteins capable of selecting target DNA in the absence of ATP. *In vitro* assays reveal that these MuB fusion proteins can efficiently deliver intermolecular target DNA, even in the presence of ADP.

Therefore, ATP hydrolysis by MuB is not necessary for target delivery. Additionally, we mapped the location of transposition events mediated by our MuB fusion proteins. Our results indicate that the MuB fusion proteins target transposition to DNA between 40-750 base pairs from the Arc operator site to which they are bound. We did not observe any fusion protein-mediated transposition events directly into the Arc operator site. These results suggest that MuB targets transposition to a general region of the DNA, rather than to a specific location near its own binding site. We hypothesize a model in which MuB delivers target DNA by tethering the target DNA to MuA while simultaneously stimulating MuA's catalytic activity.



**Figure 1: The MuA tetramer.** A total of four MuA subunits bind to the two ends of the Mu genome, with two subunits bound to each end. The two MuA binding sites present on the right end of the Mu genome are referred to as "R1" and "R2". The R1 binding site is the site closer to the end of the Mu genome, and the MuA subunit bound at this position catalyzes the cleavage and joining reactions necessary for transposition of the Mu left end.

MuA binds as a tetramer to the ends of the Mu genome, where it catalyses the cleavage and joining reactions necessary for transposition (Aldaz et al., 1996; Craigie et

al., 1984; Lavoie et al., 1991; Mizuuchi and Adzuma, 1991; Namgoong and Harshey, 1998; Williams et al., 1999). Though it is well established that MuB stimulates catalysis by MuA (Baker et al., 1991; Surette and Chaconas, 1991; Williams et al., 1999; Yamauchi and Baker, 1998), it was unknown which subunits of the MuA tetramer were activated by MuB. We investigated whether MuB stimulates MuA subunits at the R1, R2, or R1 and R2 positions (See Figure 1) by performing *in vitro* transposition assays under conditions that were highly MuB-dependent. Our assays contained mixtures of wild type MuA and MuA<sup>1-615</sup>, a MuA mutant that is catalytically active but unable to interact with MuB (Baker et al., 1991). Using DNA crosslinking, we were able to distinguish those MuA subunits bound to the R1 position from those bound to the R2 position. Analysis of complexes that had successfully undergone transposition revealed that wild type MuA was enriched at both the R1 and the R2 positions. These results indicate that MuB contacts MuA subunits at the R1 and R2 positions. Furthermore, the level of wild type MuA enrichment suggests that MuB need not contact both MuA subunits at either position to successfully stimulate recombination of both Mu ends. Therefore, MuB can stimulate MuA-mediated recombination of a particular Mu end without directly contacting the MuA subunits responsible for catalysis of that end. Our results are in agreement with the previously suggested role of MuB as an allosteric activator of MuA (Baker et al., 1991; Williams and Baker, 2004; Williams et al., 1999). In addition, experiments analyzing the ability of Mu transposition complexes to undergo disintegration reveal that MuB inhibits the process of disintegration. Thus, we propose a model in which MuB promotes a conformational change in MuA that favors recombination.

Taken together, the data presented in this thesis have clarified the mechanisms underlying two of MuB's activities: activation of MuA and target delivery. We suggest that MuB activates the MuA tetramer by interacting with both R1 and R2 subunits to promote an allosteric change in the entire complex. We also argue that target delivery results from MuB tethering bound DNA close to the MuA tetramer, thereby increasing the likelihood that this DNA will serve as the target for transposition.

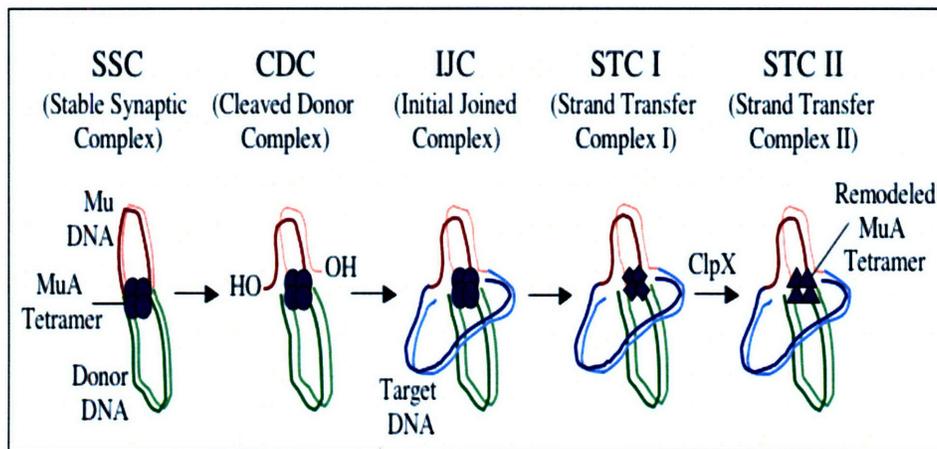
An interesting question that has not been addressed in this thesis is how the MuA-MuB interactions underlying the stimulation of Mu transposition differ from the MuA-MuB interactions involved in target selection. As discussed above, MuB interacts with MuA to activate MuA's catalytic activity. However, interactions between MuB and MuA also mediate target immunity. As a reminder, target immunity is established by the following mechanism. MuB accumulates on DNA that is far from any copies of the Mu genome, thus "selecting" this DNA as a preferred substrate for transposition (Adzuma and Mizuuchi, 1988). MuA establishes this MuB gradient by stimulating nearby MuB molecules to hydrolyze ATP and dissociate from the DNA (Greene and Mizuuchi, 2002a; Greene and Mizuuchi, 2002b; Greene and Mizuuchi, 2002c; Maxwell et al., 1987). Since MuA binds to the ends of the Mu genome (Craigie et al., 1984), MuB is cleared from DNA near copies of the Mu genome and accumulates on distant DNA (Adzuma and Mizuuchi, 1988). Though it is known that MuA-MuB interactions are important for both stimulation of transposition and for target selection, it is unclear what distinguishes these two reactions. In other words, what determines whether a given interaction between MuA and MuB will result in dissociation of MuB from bound DNA, thereby establishing target

immunity, or will result in stimulation of MuA-mediated transposition into the MuB-bound DNA?

Two possible factors that might determine the outcome of an interaction between MuA and MuB are the polymeric state of MuB and/or the conformational state of the MuA tetramer. Perhaps, when MuB monomers interact with MuA, they dissociate from the DNA, but when MuB polymers interact with MuA, they stimulate transposition. This is an attractive model since MuB does form polymers along selected DNA (Greene and Mizuuchi, 2002a). Data also indicate that MuB undergoes a conformational change upon polymer formation (Greene and Mizuuchi, 2002a; Greene and Mizuuchi, 2002b). However, it has yet to be clearly demonstrated whether the length and/or conformation of a MuB polymer directs the outcome of an interaction with MuA. Alternatively, the conformational state of the MuA tetramer might dictate how MuA interacts with MuB. It is possible that only MuA tetramers in a particular state stimulate MuB to hydrolyze ATP and dissociate from the DNA. This hypothesis could be addressed by assaying the ability of MuA tetramers that are stalled at various points along the transposition pathway to support target immunity. The experimental design of such assays will be described below.

As a reminder, the MuA tetramer, or transpososome, exists in several states throughout the process of Mu transposition (Figure 2). First, four MuA monomers bind to the ends of the Mu genome, forming the stable synaptic complex (SSC or type 0 complex) (Mizuuchi et al., 1992). MuA then nicks the DNA at the junction between the Mu genome and the flanking donor DNA, creating the cleaved donor complex (CDC or type 1 complex) (Craigie and Mizuuchi, 1987; Lavoie et al., 1991; Surette et al., 1987b).

MuA subsequently catalyzes a reaction in which the nicked Mu ends attack the target DNA, generating a branched DNA structure (Mizuuchi, 1984; Mizuuchi and Adzuma, 1991). Based on our data, the transpososome forms an initial joined complex (IJC) immediately following target joining (See chapter 2). This complex matures into the strand transfer complex (STC or type 2 complex) that is unable to undergo reversal (See chapter 2; Mizuuchi et al., 1991). Finally, the transpososome is remodeled by ClpX to create the strand transfer complex II (STC II) (Burton et al., 2001; Krukltis et al., 1996; Levchenko et al., 1995).



**Figure 2: States of the MuA tetramer during the transposition reaction.** Assembly of the MuA tetramer onto the Mu DNA ends forms the stable synaptic complex. Cleavage of the Mu ends by MuA yields the cleaved donor complex. MuA-catalyzed joining of the Mu ends to the target DNA results in the initial joined complex that undergoes a conformational change to become the strand transfer complex I. This complex is remodeled by ClpX creating the strand transfer complex II.

To test at which stage(s) during Mu transposition the MuA tetramer establishes target immunity, one could create transpososomes *in vitro* that are stalled at various points along the transposition pathway. Transpososomes can be captured in the stable synaptic complex by using MuA mutants that are defective in catalysis (Baker and Luo,

1994). Alternately, transpososomes can be stalled at the cleaved donor complex by assembling MuA active site mutants onto pre-cleaved Mu DNA. Transpososomes that have undergone both DNA cleavage and joining will remain as stable synaptic complexes in the absence of ClpX (Surette et al., 1987a), and remodeling of SSC I complexes by ClpX will result in stable SSC II complexes. Once created, the stalled complexes could be purified from free protein and DNA using gel filtration.

To investigate the influence of transpososome state on target immunity, each of the stalled complexes would be tested as potential targets in *in vitro* transposition assays containing MuA and MuB. Those complexes that effectively mediate target immunity should be poor targets for transposition. Conversely, those complexes that fail to establish target immunity should prove favorable targets. These experiments would offer insight into how the state of the MuA tetramer affects the MuA-MuB interactions responsible for target immunity. Perhaps, the results would elucidate factors that differentiate MuA-MuB interactions involved in target selection from those involved in stimulation of transposition.

This thesis has investigated the protein-protein interactions involved in Mu transposition. Specifically, the work analyses the nature of the interactions between two Mu-encoded proteins, MuA and MuB, during the processes of target delivery and recombination. These results contribute to a general body of knowledge concerning the molecular mechanisms underlying transposition targeting. It is our hope that the study of transposition targeting will uncover the factors influencing transposon-host relationships and will elucidate the roles of transposable elements in shaping modern genomes.

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