Analysis of Specific Cis-acting DNA Sequences of the Himar1 mariner Transposon

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ANALYSIS OF SPECIFIC CIS-ACTING DNA SEQUENCES
OF THE HIMARI MARINER TRANSPOSON

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ABSTRACT

*Himar1*, found in the horn fly *Haematobia irritans*, belongs to the *mariner* family of transposable elements. *Mariners* are a diverse and widespread family of eukaryotic transposons that transpose via a cut-and-paste mechanism. *Mariner* transposition not only requires the transposase, but also specific cis-acting DNA sequences known as inverted terminal repeats (ITRs). Very little is known about the fine structure of *mariner* ITRs, particularly which bases are important for transposition. A *mariner* family ITR sequence logo representing 20 different *mariners* from six different subfamilies suggests that more conserved regions of the logo are required for transposase binding while the more variable regions of the logo are likely to be involved in secondary transposition functions such as DNA cleavage and element insertion. To test this hypothesis, point mutations were introduced at 27 positions of the *Himar1* ITR plus the two flanking nucleotides, “T” and “A”. An *Escherichia coli* based genetic screen yielded results that lacked a clear pattern similar to that of the sequence logo. Therefore, gel mobility shift DNA-binding, strand-specific cleavage, and *in vitro* transposition assays were performed to test certain positions in the ITR that exhibited null phenotypes in the genetic screen. Positions 2, 12, 15, 22, and 24 of the ITR are required for DNA-binding. Positions 5, 8, 11, 20, 26, 27, and the second flanking nucleotide (A) exhibited inefficient DNA cleavage in comparison to the wild type ITR. Position 26 of the ITR is required for element insertion into the target DNA. Overall, my results indicate that the transposase is making base-specific contacts with the DNA on one face of the *Himar1* ITR. However, these contacts appear to be occurring along the variable regions of the *mariner* ITR sequence logo.
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INTRODUCTION

Transposable elements are segments of DNA that move from one chromosomal site to another within the genome of the host or sometimes between genomes of unrelated species. Barbara McClintock first discovered transposable elements in maize in the early 1940s (Comfort, 1995). Twenty years later, transposable elements were discovered in bacteria and today they have been found in essentially all organisms including insects, nematodes, flatworms, and humans (Snyder and Champness, 1997).

Transposition is the movement of a transposable element that is facilitated by a protein that is usually encoded by the transposon known as the transposase. Transposable elements are divided into two main classes based on their mode of transposition and structural organization. Class I elements use an RNA-mediated mechanism of transposition and encode a reverse transcriptase. Class I elements, also known as retrotransposons, behave like retroviruses and use an RNA intermediate (Zupunski, Gubensek, et al. 2001). An RNA copy of chromosomal DNA template is made and then it is copied back into DNA by a reverse transcriptase. The DNA intermediate then integrates elsewhere in the genome. Class I elements include LINEs, SINEs, and gypsy/Ty3-like elements (Curcio and Derbyshire, 2003). Class II elements use either a replicative or a cut-and-paste mechanism of transposition. Transposons such as Tn3 and phage Mu use the replicative mechanism, in which the entire transposon replicates during transposition resulting in two copies of the transposon (Snyder and Champness, 1997). The cut-and-paste mechanism of transposition is promoted by a transposase (Robertson and Lampe, 1995a). This particular mechanism will be discussed in more detail later. P
elements in *Drosophila*, *Ac* in maize, and *mariner* elements transpose via the cut-and-paste mechanism (Curcio and Derbyshire, 2003).

The result of transposition is the transposon has been placed in a different location in the genome from where it was originally. Therefore, the mechanism of transposition has been likened to recombination because it helps to create variety within genomes by causing insertions, deletions, inversions, and chromosomal fusions. These mutations caused by transposons can lead to evolutionary changes (Goryshin *et al*. 2000) and can also be the causative agents of some diseases (Reznikoff, 1993).

**DNA-mediated (Class II) Transposable Elements**

Unlike class I elements, class II elements do not require an RNA intermediate to transpose. Class II elements are also quite diverse. They are found in a broad range of species from protozoa, bacteria, plants, and most animals.

The structure of class II elements is usually very similar. The transposon is generally flanked by short inverted terminal repeat (ITR) DNA sequences. The transposon generally encodes a protein known as a transposase. The transposase is required to catalyze a multi-step process referred to as the cut-and-paste mechanism of transposition. The ITR sequences that flank the transposable element are also absolutely required for transposition (Zhou *et al*. 1997).

![Figure 1. The structure of a class II DNA-mediated transposable element. The arrows represent the ITRs that flank the transposase-coding sequence shown in the green box.](image-url)
**Molecular Evolution and the Impact of Transposable Elements**

Transposable elements research has determined that genomes are not simple collections of DNA sequences that code for proteins. Instead, genomes are comprised of these parasitic elements that mobilize themselves at the expense of the host. Due to their abundance in virtually all domains of life, many questions have been raised concerning whether mechanisms exist to counter transposable elements and if transposable elements ever benefit the host (Freeman and Herron, 2001).

Evidence over the last decade shows two hypotheses may be responsible for limiting the spread of transposable elements. The first is based on the idea that when multiple copies of a transposable element are found on the same chromosome, errors in meiosis such as ectopic recombination frequently occur. Ectopic recombination can occur between these elements located at different sites on homologous chromosomes. Half of the resulting chromosomes will lack certain loci and therefore, will be strongly selected against. Sniegowski and Charlesworth (1994) tested this hypothesis by quantifying the frequency of transposable elements in inversions in the *D. melanogaster* genome. They found that transposable elements are much more common inside inversions than they are in homologous noninverted chromosome segments suggesting that ectopic recombination slows down the spread of transposable elements.

The second hypothesis is based on the idea that organisms methylate their DNA as a way to deter transposable elements. Waugh O’Neill *et al.* (1998) tested this hypothesis. They analyzed the chromosomes from a hybrid offspring of a mating between a swamp wallaby and a tammer wallaby. They found that the offspring’s DNA was unmethylated. They also found the retrotransposon KERV-1 was quite abundant in
the chromosomes of this offspring supporting the host-defense by DNA methylation hypothesis.

Recent work has also shown that the host may benefit from transposition events. Agrawal et al. (1998) suggest that a key feature of the vertebrate immune system, V (variable), D (diversity), and J (joining) recombination, originated from an insertion event by a transposable element several hundred million years ago. Furthermore, it has been demonstrated that the reaction mechanism of V(D)J recombination is identical to the chemical events that occur during transposable element mobilization.

**Mariner Family Transposable Elements**

*Mariners* are a large family of eukaryotic DNA-mediated transposable elements. *Mariner* elements are approximately 1.3 kilobase (kb) pairs in size and are flanked by ITRs that are between 27-30 base pairs (bp) in length. *Mariner* transposons encode a protein of approximately 350 amino acids (Lampe et al. 1996; Hartl, Lohe et al. 1997). Although they are widespread among eukaryotes, their mechanism of transposition is not entirely understood. Only three known active *mariner* elements have been identified, *Mos1* from *Drosophila mauritiana*, the consensus sequence of the *Himar1* element isolated from the horn fly, *Haematobia irritans* (Lampe et al. 1998), and *Famar1* isolated from the European earwig, *Forficula auricularia* (Barry et al. 2004).

Due to their similarity to other transposable element families, *mariners* are now considered to be members of a much larger superfamily of transposable elements. This superfamily includes prokaryotic members such as the bacteriophage Mu, the transposons Tn5 and Tn7, and many bacterial insertion sequences such as *IS2, IS3, IS4, and IS30*.
Members of this transposable element superfamily encode transposases that can be distinguished by a conserved D,D35E motif in the catalytic domain of the protein. *Mariners*, however, contain a slightly modified motif, D,D34D (Lampe et al. 1999). This catalytic domain is located in the C-terminal half of the encoded transposase in *mariners*. The DNA-binding domain, which includes a helix-turn-helix motif required for the recognition of the ITRs, is located in the N-terminal region (Zhang et al. 2001). Since part of the active site of this motif serves as a binding domain for a divalent cation (e.g. Mg$^{2+}$ or Mn$^{2+}$) necessary for catalysis, it is thought that the D,D34D motif plays a key role in the transposition process (Hartl, Lohe et al. 1997).

Phylogenetic studies of *mariner* family elements have led to several conclusions. First, *mariners* are widespread among eukaryotes from insects to humans (Lampe et al. 1996). Second, *mariners* are quite diverse. The *mariner* family of transposable elements can be grouped into at least 13 different subfamilies based on transposase sequence homologies ranging on average from 25 to 35% identity (Robertson and Lampe, 1995a). Finally, *mariners* appear to be capable of horizontal transfer. Because very similar *mariners* have been found in highly divergent species, this suggests that horizontal transfer has occurred. An example of this phenomenon occurs in the irritans subfamily of *mariners*. Here, horizontal transfer of a *mariner* element has occurred between two flies in different suborders and also a green lacewing that belongs to a different order than the flies. All three of these organisms contain *mariners* that encode transposases that differ from one another by no more than six amino acids (Lampe et al. 1996).

*Mos1* (mosaic factor 1) was the first *mariner* element to be discovered. *Mos1* was isolated from an unstable mutation in the white gene of *D. mauritiana* (Jacobson,
Medhora, et al. 1986). When the white insertion was crossed into a background containing Mos1, the white insertion became mobile in both the germ and the somatic cell lines. Both the white insertion and Mos1 were determined to be mariner transposable elements through various cloning experiments. Since its discovery, Mos1 has been transferred to Drosophila melanogaster where it was also found to be active (Garza et al. 1991).

**Himar1: An Active Mariner Element**

Himar1, the second mariner identified as active, belongs to the irritans subfamily of mariner elements (Lampe et al. 1996). Himar1 was discovered using homology-based PCR in H. irritans. The active copy is a reconstructed consensus sequence from a series of genomic clones (Robertson and Lampe, 1995b). Himar1 comprises approximately 1% of the H. irritans genome (Robertson and Lampe, 1995a).

Like other mariner elements, Himar1 is approximately 1.3 kb in size. Deletion analysis has demonstrated that the Himar1 ITRs that flank the transposon are 27 bp in length (D. Lampe, unpublished). The transposon contains a central coding sequence of 1,047 bp that encodes a transposase of 348 amino acids (Robertson and Lampe, 1995a).

Himar1, like other mariners, transposes via a cut-and-paste mechanism of transposition. Lampe et al. (1996) demonstrated that the requirements of Himar1 transposition are simple. Transposition in vitro requires only a purified transposase, a suitable donor transposon construct, and a target DNA molecule. Due to their widespread occurrence in most eukaryotic phyla and their ability to horizontally transfer, it is inferred that Himar1 transposition does not require species-specific host factors.
Finally, *Himar1* does not randomly insert itself into the target DNA. Similar to other *mariner* elements, such as *Mos1*, *Himar1* inserts itself into target TA dinucleotide sequences within the genome (Lampe *et al*. 1996).

**Himar1 Transposase**

The *Himar1* transposase is a complex protein that catalyzes the transposition reaction both *in vivo* and *in vitro*. The transposase is capable of recognizing and binding to the ITR sequences of its cognate element, of cleaving the transposon at the 5’ and 3’ ends, and of transferring the transposon from the donor DNA to the target DNA ultimately resulting in the insertion of the transposon at the target TA site (Plasterk *et al*. 1996; Lampe *et al*. 1996).

![“D,D34D Motif”](image)

Figure 2. A schematic diagram of a model of the *Himar1* transposase illustrating the functional domains. HTH refers to the helix-turn-helix identified in the specific DNA-binding domain. "D" indicates the positions of the aspartic acid residues in the catalytic domain. NLS refers to the nuclear localizing signals found in the catalytic domain (Lampe *et al*. 1999).

Figure 2 represents a model of the *Himar1* transposase in which the DNA-binding domain lies within the first 115 amino acids in the N-terminal region (Chakraborty, 2000). Sequence comparisons of *mariner* and other *Tc1*-like elements show a DNA-
binding domain containing three blocks of conserved amino acids (Pietrekowski and Henikoff, 1997). One of the blocks, located near the proposed non-specific binding domain, encodes a helix-turn-helix (HTH) motif. The HTH motif has been found to be essential for transposition in Tc1/mariner family members such as Tc3, pogo, and Mos1 (van Pouderoyen, 1997; Wang et al. 1999; Zhang et al. 2001). A general non-specific binding domain is proposed to lie between residues 116 and 173. The catalytic domain appears to lie between residues 158 and 287. The D,D34D motif characteristic of Himar1 lies within this region (Lampe et al. 1998). Two nuclear localizing signals (NLS) are also present within this region, beginning at positions 184 and 243. The NLS is responsible for signaling the transport of the transposase from the cytoplasm to the nucleus after translation. However, it is unclear if one or both of the NLS is utilized for this process (Lampe et al. 1996). The C-terminal region between residues 288 and 348 is of unknown function. It is speculated that this region could be involved in the dimerization of the transposase and the ITR (Lampe et al. 1999).

**A Model for the mariner Mechanism of Transposition**

Mariners are thought to transpose via a mechanism common to many bacterial transposons such as Tn5 and Tn10 known as the cut-and-paste mechanism. This conservative mechanism of transposition is unlike the mechanism required to mobilize transposons such as Tn3 and phage Mu, which require replication of the element (Curcio and Derbyshire, 2003). Instead, the cut-and-paste mechanism is a multi-step process that is catalyzed by the transposase.
Although a great deal remains to be learned about the transposition mechanism of eukaryotic transposable elements such as mariners, a significant amount of research has been done on bacterial elements such as Tn5, Tn10, and Tn7. Tn5 is the best model for understanding the molecular mechanism behind DNA-mediated transposition. Similar to Tn5 transposition, the process of mariner transposition begins with transposase monomers that bind to specific DNA recognition sequences, the ITRs (Figure 2). In Tn5, the end sequences are brought together to form a complex nucleoprotein structure known as the synaptic complex (Bhasin et al. 1999). The transposase then makes double-stranded breaks at the ends of the element. Dawson et al. (2003) have proposed that cleavage in mariners such as Mos1 occurs independently of the synaptic complex on the nontransferred strand.

Unlike Tn5 where the first step in cleavage is the nick generated at the 3’ end of the transferred strand, it has been demonstrated in Mos1 that the 5’ end of the nontransferred strand is cleaved initially (Reznikoff, 2003; Dawson et al. 2003; D. Lampe, unpublished). Cleavage of the transferred strand occurs at the 3’ end generating single-stranded overhangs with reactive 3’ hydroxyls. The excised transposon then integrates itself 5’ of the thymidine nucleotide at a TA dinucleotide site in the target DNA (Plasterk et al. 1996; Dawson et al. 2003). During integration, the transposase causes another staggered DNA cut at the TA target site. Duplicated TA dinucleotides flank the inserted transposon after the gap in the target DNA is repaired.
Figure 3. A model for the cut-and-paste mechanism. This model outlines the steps thought to occur during mariner transposition. The blue areas of the figure represent the mariner DNA. The green ovals represent transposase molecules and their theoretical roles in transposition. Although the figure does not depict the transposase after initial binding, the transposase does remain bound to the DNA through cleavage and target site insertion (Lampe et al. 1996).
After the transposon has been excised from the donor DNA, a double-stranded break in the DNA is left. The host repairs the excision site in the donor DNA. In some cases it has been proposed that the terminal nucleotides of the inverted repeats left in the gap are regenerated by direct ligation of the broken DNA ends by non-homologous end-joining (NHEJ). The element-cleaved sequences that are left are called transposon footprints (Plasterk et al. 1999).

**Fine Structure of Cis-acting DNA Sequences**

In addition to the transposase, transposition requires specific cis-acting DNA sequences, the ITRs. *Mariner* ITRs are 27-30 bp in length and are generally divergent from one another between different elements. At the start of transposition, transposase monomers bind to the ITRs as illustrated in figure 2. The transposase cleaves the nontransferred strand of the ITR three base pairs from the end of the ITR followed by transferred strand cleavage at the end of the transposons (Lampe et al. 1996). While work has been done in *Mos1* and the *Tc1/mariner* family member *Sleeping Beauty* to characterize the structure and function of their ITRs as well as to determine the minimum sequence required to enable transposition, much more remains to be learned about these cis-acting sequences. In other systems, not only are these specific sequences required for transposase binding, but they also contain properties necessary for secondary steps in transposition. These properties could include but are not limited to host protein interaction sites, and programming synaptic complex formation and cleavage events.

Bacterial transposition systems such as Tn5 serve as excellent models in order to understand the role of ITRs in the transposition mechanism. Tn5 is a composite bacterial
transposon belonging to the IS4 family of DNA-mediated transposable elements (Naumann et al. 2002). Tn5 consists of two insertion sequences IS50L and IS50R that flank a DNA region encoding three antibiotic resistance genes. IS50L and IS50R are flanked by 19 bp sequences termed the outside end (OE) and the inside end (IE) (Steiniger-White et al. 2002). Both the OE and the IE contain 12 identical bases (Zhou et al. 1997). The transposase is encoded by IS50R. The Tn5 transposase recognizes and binds to the OE of each insertion sequence (Steiniger-White et al. 2002). Analyses such as hydroxyl radical, missing-nucleoside, and dimethyl sulfate interference experiments have been used to determine which sites in the Tn5 OE are involved in transposase-DNA binding. The Tn5 transposase binds initially to positions 6 to 9 and 13 to 19 of the OE (Jilk et al. 1996). Positions 1 to 5 and 10 to 12 appear to be involved in secondary transposition functions. Mutations in positions 1 to 3 cause significant decreases in transposition suggesting an inhibition event that follows the transposase-DNA binding such as synapse formation, cleavage, or strand exchange (Makris et al. 1998; Jilk et al. 1996). Additional studies of the transposase-DNA complex have determined that the transposase binds to all positions of the OE except position 1 in the synaptic complex. However, hydroxyl radical footprinting and interference experiments have demonstrated that positions 2 to 5 are required for synaptic complex formation even though they are not involved in the initial monomer complex formation (Bhasin et al. 2000). Cleavage appears to occur at position 1. Mutagenesis studies of Tn10 tell a very different story from Tn5. Nucleotide substitutions at any one of the first three base pairs of the Tn10 OE allow synaptic complex formation and excision, but these mutants are unable to carry out strand exchange (Jilk et al. 1996).
There is also evidence to suggest that not all ITR sequences are optimal for transposition. Zhou et al. (1997) found that hypertransposing transposase mutants, such as E54K, from Tn5 bind to DNA much more efficiently than the wild type transposase. The transposase mutant E54K shows a preference for the OE versus the IE compared to the wild type transposase. Genetic and gel mobility shift DNA-binding assays of mutated OE and IE DNA revealed that positions 10 to 12 of the OE are required for the specific recognition by the E54K transposase. Although this data contradicts the research described earlier involving Tn5 OE, it is important to bear in mind that the Tn5 transposase is a very inactive protein. Therefore, the combination of a hypertransposing transposase mutant and a mutated OE sequence not only results in a higher transposition frequency, but also suggests that the wild type sequence is not always optimal in order for transposition to occur (Zhou et al. 1998).

Fortunately, a large amount of sequence data has been compiled for mariner ITRs. Figure 4 represents the sequence alignments of 20 different mariner ITRs from six different subfamilies. Interestingly, some positions within the ITRs are more conserved than others. Positions 4-8 and 14-18 appear to be the most conserved regions, suggesting that the transposase may be making specific contacts within these regions of the mariner ITR. The more variable regions of the ITR could potentially be involved in secondary functions such as cleavage or element integration.
Figure 4. A sequence logo of mariner family ITRs. The height and the order of the bases in a particular stack are representative of their frequency at that position in the alignment.

Mariner Evolution and the Cis-acting DNA Sequences

Extensive horizontal transfer between species dominates the phylogenetic history of mariner family transposable elements. An example of this phenomenon has recently been studied in the closely related members of the mellifera subfamily of mariners. These insects include the European earwig, Forficula auricularia, the European honeybee, Apis mellifera, the Mediterranean fruit fly, Ceratitis capitata, and a blister beetle, Epicauta funebris (Lampe et al. 2003). But it is also evident that multiple different kinds of mariners can be found occupying the same genome. For example, the human genome contains two distinct mariner elements, Hsmar1 from the cecropia subfamily and Hsmar2 belonging to the irritans subfamily (Robertson and Martos, 1997; Robertson and Zumpano, 1997).

Because multiple distinct kinds of mariners can occupy the same genome, the likelihood that they interact with one another seems slight. As a result, investigations into the divergence of mariners over evolutionary time have begun in order to determine if divergent elements are capable of interacting (Lampe et al. 2001). There is also
interest in determining how much divergence elements in a genome can undergo before they fail to interact.

Lampe et al. (2001) suggested a model for the evolutionary fate of *mariners*. This model consists of two paths that an element containing neutral or functionally different mutations either in the transposase-coding sequence or in the ITRs can proceed through. The first path involves the horizontal transmission of a divergent element to another species. This, of course, implies that the invading element is active. The intrinsic activity of an invading element, however, can only be determined by the number of copies of itself it can generate in that genome. Therefore, any mutations that can increase an elements activity will ultimately benefit the element. For example, a mutation in the transposase-coding sequence of the transferred copy that causes a decrease in activity may be reversed by a cis-acting suppressor mutation in the ITR. A suppressor mutation such as this may also reduce interaction with the parental sequence. If this process occurred repeatedly, the descendents from the original parental element would be expected to diverge to the point where they could no longer interact with the parent if contact were ever reestablished.

The second path involves the divergence within a single species. This path involves the formation of a noninteracting element within the same species as the parent element. If this model were accurate, slight coevolutionary changes in the transposase-coding sequence and the ITRs would be sufficient to enable the element to preferentially mobilize itself. Although this event is rare, the fact that many different *mariners* occupy the genomes of many different species could enable this situation to occur (Lampe et al. 2001). Also, the host may be capable of performing gene shuffling of transposable
elements, therefore, causing a greater diversity of changes compared to point mutations. The ultimate fate of mariners in a species is inactivation due to point mutations or emergent regulatory processes (Hartl, Lohe, Lozovskaya et al. 1997). Therefore, there may be a selective advantage to any element that can proceed down path two (Lampe et al. 2001). Introducing mutations into the Himar1 ITR that decrease or eliminate transposition can test this path by conducting an Escherichia coli based genetic screen to look for transposase mutants that suppress these changes.
RESEARCH OBJECTIVES

Transposition not only requires transposase, but also specific cis-acting DNA sequences known as ITRs. Very little is known about the fine structure of mariner ITRs, particularly which specific bases are important for transposition. Research performed on the bacterial transposon Tn5 has shown that the outside ends of the inverted repeat are essential to transposase binding. However, regions within the Tn5 outside ends have other specific roles (Jilk et al. 1996). Research on the Tn5 outside ends has also determined that the wild type sequence is not the optimal sequence for transposition. These features of ITRs have not been determined for Himar1. Based on prior work done in systems such as Tn5, my objective is to determine which sites in the ITR are important to Himar1 transposition. The mariner ITR sequence logo found in figure 4 serves as a good starting point. Based upon the information in this logo, I hypothesize that the more conserved regions; positions 4-8 and 14-18 are involved in specifically contacting the transposase. I hypothesize that the more variable regions found at the ends of the sequence and in between the conserved regions contain secondary functions in the transposition process such as DNA cleavage and element insertion.

To begin testing this hypothesis, I mutagenized the 3’ Himar1 ITR. I introduced point mutations at 27 positions of the ITR and at the flanking DNA (ta). The affect on transposition was tested in an E. coli based genetic screen known as a papillation assay. Colonies were scored for varying phenotypes following 48 hours of incubation at 32 °C. Colonies representing normal transposition were scored as wild type. Colonies representing transposition that occurred at a slower rate were scored as hypomorph. Colonies that exhibited a lack of transposition were scored as null. Once all of the data
was collected, I determined which positions were affected most by the mutations. Those positions that tested null for transposition were tested in a gel mobility shift DNA-binding assay to determine if these mutations caused a failure in binding to the transposase. Those mutated ITRs that bound to the Himar1 transposase were tested in strand-specific cleavage assays to determine whether or not the transposase could cleave the mutated ITR when these mutations were present.

Finally, a transposase suppressor screen was performed to determine if mutations in the Himar1 transposase could compensate for mutations in the Himar1 ITR that disrupt transposition. The purpose of this screen was to learn more about the coevolution of the transposase and its cognate ITRs and to test path two in the evolutionary model proposed by Lampe et al. (2001). Once again a papillation assay was performed to carry out this particular part of the research.
# MATERIALS AND METHODS

Table 1. List of Plasmids Used in this Study.

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Description</th>
<th>Reference or Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>pACYC184</td>
<td>low copy number 4,245 bp plasmid with a rho 15a origin of replication</td>
<td>Chang and Cohen, 1978</td>
</tr>
<tr>
<td>pBB1.2</td>
<td>pACYC184 digested with BamHI and SalI; 5' Himar1 wild type ITR</td>
<td>This study</td>
</tr>
<tr>
<td>pOJ427</td>
<td>Contains apramycin resistance gene (aminoglycoside N(3')-acyetyltransferase IV gene)</td>
<td>J. McCormick, Duquesne University</td>
</tr>
<tr>
<td>pBB3</td>
<td>pGEM-3zf(+) digested with XbaI and SmaI to remove apramycin resistance gene obtained from pOJ427</td>
<td>This study</td>
</tr>
<tr>
<td>pBB6.4</td>
<td>pBB1 digested with BamHI, apramycin resistance gene in reverse orientation flanked by 5' WT ITR</td>
<td>This study</td>
</tr>
<tr>
<td>pBB9</td>
<td>pBB6.4 digested with BamHI I and Hind III; WT 3' Himar1 ITR</td>
<td>This study</td>
</tr>
<tr>
<td>pBB10</td>
<td>3' Himar1 ITR contains an &quot;A&quot; at position 12 instead of the WT &quot;G&quot;</td>
<td>This study</td>
</tr>
<tr>
<td>pBB11</td>
<td>3' Himar1 ITR contains a &quot;T&quot; at position 20 instead of the WT &quot;C&quot;</td>
<td>This study</td>
</tr>
<tr>
<td>pBADmar1</td>
<td>Himar1 WT transposase coding sequence under the araBAD promoter</td>
<td>Lampe et al., 1999</td>
</tr>
<tr>
<td>pBB12</td>
<td>Both 5' and 3' Himar1 ITRs contain an &quot;A&quot; position 12 instead of the WT &quot;G&quot;</td>
<td>This study</td>
</tr>
<tr>
<td>pBB13</td>
<td>Both 5' and 3' Himar1 ITRs contain a &quot;C&quot; position 12 instead of the WT &quot;G&quot;</td>
<td>This study</td>
</tr>
<tr>
<td>pBB14</td>
<td>Both 5' and 3' Himar1 ITRs contain a &quot;T&quot; position 12 instead of the WT &quot;G&quot;</td>
<td>This study</td>
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<tr>
<td>pBB15</td>
<td>Both 5' and 3' Himar1 ITRs contain an &quot;A&quot; position 20 instead of the WT &quot;C&quot;</td>
<td>This study</td>
</tr>
<tr>
<td>pBB16</td>
<td>Both 5' and 3' Himar1 ITRs contain a &quot;G&quot; position 20 instead of the WT &quot;C&quot;</td>
<td>This study</td>
</tr>
<tr>
<td>pBB17</td>
<td>Both 5' and 3' Himar1 ITRs contain a &quot;T&quot; position 20 instead of the WT &quot;C&quot;</td>
<td>This study</td>
</tr>
<tr>
<td>pBB18.1</td>
<td>pGEM-3zf(+) digested with EcoRI; WT 3' ITR</td>
<td>This study</td>
</tr>
<tr>
<td>pBB19</td>
<td>3' Himar1 ITR contains &quot;C&quot; at position 5 instead of WT &quot;G&quot;</td>
<td>This study</td>
</tr>
<tr>
<td>pBB20</td>
<td>3' Himar1 ITR contains &quot;A&quot; at position 8 instead of WT &quot;G&quot;</td>
<td>This study</td>
</tr>
<tr>
<td>pBB21.1</td>
<td>3' Himar1 ITR contains &quot;T&quot; at position 11 instead of WT &quot;C&quot;</td>
<td>This study</td>
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<tr>
<td>pBB22</td>
<td>3' Himar1 ITR contains &quot;T&quot; at the second flanking DNA instead of WT &quot;A&quot;</td>
<td>This study</td>
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<tr>
<td>pBB23</td>
<td>3' Himar1 ITR contains &quot;T&quot; at position 20 instead of WT &quot;C&quot;</td>
<td>This study</td>
</tr>
<tr>
<td>pBB24</td>
<td>3' Himar1 ITR contains &quot;C&quot; at position 27 instead of WT &quot;T&quot;</td>
<td>This study</td>
</tr>
<tr>
<td>pBB25</td>
<td>3' Himar1 ITR contains &quot;G&quot; at position 26 instead of WT &quot;C&quot;</td>
<td>This study</td>
</tr>
</tbody>
</table>

Abbreviations: ITR = inverted terminal repeat; WT = wild type
Table 2. List of *E. coli* Strains Used in this Study.

<table>
<thead>
<tr>
<th><em>E. coli</em> strain</th>
<th>Genotype</th>
<th>Reference or Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>DH5α</td>
<td><em>deoR, endA1, gyrA96, hsdR17(rK mK'), recA1, relA1, supE44, thi-1, Δ(lacZ YA-argF V169), φ80ΔlacZΔM15, F', λ</em></td>
<td>Clontech</td>
</tr>
<tr>
<td>DL6</td>
<td>DH5α lysogenized with λNK1295</td>
<td>D. Lampe; Bender &amp; Kleckner, 1992</td>
</tr>
<tr>
<td>DL8</td>
<td>pBADmar1/DL6</td>
<td>This study</td>
</tr>
<tr>
<td>Top10 F'</td>
<td>F' lacI, Tn10(Tet&lt;sup&gt;R&lt;/sup&gt;)/mcrA Δ(mrr-hsdRMS-mcrBC), (φ80lacZΔM15), ΔlacX74, *deoR, recA1, araD139, Δ(ara-leu)7697, galU, galK, rpsL(Str&lt;sup&gt;R&lt;/sup&gt;) endA1, nup6</td>
<td>Invitrogen</td>
</tr>
</tbody>
</table>

<p>| | | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
</tr>
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<tbody>
<tr>
<td>BB2</td>
<td>pBB12/DL6</td>
<td>This study</td>
</tr>
<tr>
<td>BB3</td>
<td>pBB13/DL6</td>
<td>This study</td>
</tr>
<tr>
<td>BB4</td>
<td>pBB14/DL6</td>
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<td>BB5</td>
<td>pBB15/DL6</td>
<td>This study</td>
</tr>
<tr>
<td>BB6</td>
<td>pBB16/DL6</td>
<td>This study</td>
</tr>
<tr>
<td>BB7</td>
<td>pBB17/DL6</td>
<td>This study</td>
</tr>
</tbody>
</table>

### Media and Antibiotics

Plasmids were grown in Luria-Bertani (LB) broth or on LB agar plates with the appropriate antibiotic at 37 °C. Cultures were grown overnight in an orbital shaker at 250 rpm at 37 °C unless otherwise indicated. Papillation assays for the inverted terminal repeat (ITR) mutagenesis work were performed on plates containing 50 ml of MacConkey lactose agar and ampicillin (Amp) and apramycin (Apr). The suppressor screens were performed on larger plates (150 mm) that contained 100 ml of MacConkey lactose agar and ampicillin and chloramphenicol (Cam). MacConkey lactose agar plates were incubated for greater than 48 hours at 32 °C unless otherwise indicated. The following antibiotics were commonly used during this research: Ampicillin (Amp; 100 µg/ml), Apramycin (Apr; 40 µg/ml), and Chloramphenicol (Cam; 100 µg/ml).
<table>
<thead>
<tr>
<th>Oligo</th>
<th>Sequence 5' --&gt; 3'</th>
<th>Purpose</th>
</tr>
</thead>
<tbody>
<tr>
<td>Beth5'ITRTOP</td>
<td>/5' Phos/TCGACTAACAGGGTGGCTGATAAGTC</td>
<td>Construct plasmid with WT ITRs</td>
</tr>
<tr>
<td>Beth5'ITRBOT</td>
<td>/5' Phos/GATCCGCTAGCAGACCAGGGGACTTAT</td>
<td>Construct plasmid with WT ITRs</td>
</tr>
<tr>
<td>Beth3'ITRTOP</td>
<td>/5' Phos/GATCCAGACCCGGACTTTACAGCCA</td>
<td>Construct plasmid with WT ITRs</td>
</tr>
<tr>
<td>Beth3'ITRBOT</td>
<td>/5' Phos/CAGACCGGGGACTTTACAGCCAACCT</td>
<td>Construct plasmid with WT ITRs</td>
</tr>
<tr>
<td>BethSeqSal</td>
<td>TTAAGAAGCAGCCCAGTAGTAGG</td>
<td>To sequence 5' ITR</td>
</tr>
<tr>
<td>BethSeqHin</td>
<td>ATGTAGCACCTGAACTGCGC</td>
<td>To sequence 3' ITR</td>
</tr>
<tr>
<td>BethSeqSal2</td>
<td>GAAGGCTCTCAAGGGCATCGGT</td>
<td>To amplify aprR gene from pSP2.1</td>
</tr>
<tr>
<td>Apr-5'</td>
<td>ATGGATCCGAGATTCACTCAGGTCC</td>
<td>To amplify aprR gene from pSP2.1</td>
</tr>
<tr>
<td>Apr-3'</td>
<td>TAAGATCTGAGGCTCCAGGCTACAAAT</td>
<td>Sequence 3' ITR from within transposon</td>
</tr>
<tr>
<td>AprR-revseq</td>
<td>GATATGAAAACGCTGAACCCACC</td>
<td>Sequence 3' ITR from within transposon</td>
</tr>
<tr>
<td>20fh-ITR</td>
<td>TAAACAGTCCTGATGAACTGAA</td>
<td>Sequence aprR gene</td>
</tr>
<tr>
<td>WTdoped4-8</td>
<td>TAAAGCTTAACAGGTTGGCTGATAAGTCCCCGGTCTTACGGA</td>
<td>Doped experiment</td>
</tr>
<tr>
<td>BethSeqHin2</td>
<td>GGTAGCTCAGAGAACCTT</td>
<td>PCR with GeneMorph</td>
</tr>
<tr>
<td>AprRseq2</td>
<td>ATGATATGCAGGCTCAATG</td>
<td>PCR with GeneMorph</td>
</tr>
<tr>
<td>ITR12aBAM</td>
<td>TTGGATCCCTAAACAGGGTTGGCTAATAAGTCCCCGG</td>
<td>Mutate both ITRs</td>
</tr>
<tr>
<td>ITR12cBAM</td>
<td>TTGGATCCCTAAACAGGGTTGGCTAATAAGTCCCCGG</td>
<td>Mutate both ITRs</td>
</tr>
<tr>
<td>ITR12tBAM</td>
<td>TTGGATCCCTAAACAGGGTTGGCTAATAAGTCCCCGG</td>
<td>Mutate both ITRs</td>
</tr>
<tr>
<td>ITR20aBAM</td>
<td>TTGGATCCCTAAACAGGGTTGGCTAATAAGTCCCCGG</td>
<td>Mutate both ITRs</td>
</tr>
<tr>
<td>ITR20gBAM</td>
<td>TTGGATCCCTAAACAGGGTTGGCTAATAAGTCCCCGG</td>
<td>Mutate both ITRs</td>
</tr>
<tr>
<td>ITR20tBAM</td>
<td>TTGGATCCCTAAACAGGGTTGGCTAATAAGTCCCCGG</td>
<td>Mutate both ITRs</td>
</tr>
<tr>
<td>pBAD24seqR</td>
<td>ATCAGGCTGAAAATCTCTTTCTCAGT</td>
<td>Randomly mutagenize transposase</td>
</tr>
<tr>
<td>Arg101R</td>
<td>TTCCTTTGTGTTTTTTTGTTTT</td>
<td>Directed mutagenesis of transposase</td>
</tr>
<tr>
<td>Arg101RandomF</td>
<td>NNNNTTGGTCTACATACATCC</td>
<td>Directed mutagenesis of transposase</td>
</tr>
<tr>
<td>Met86R</td>
<td>TTTACGGTCTTAAAATCTTTGT</td>
<td>Directed mutagenesis of transposase</td>
</tr>
<tr>
<td>Met86RandomF</td>
<td>NNNAAGTTGGTCTCTGGGTTTTGTTG</td>
<td>Directed mutagenesis of transposase</td>
</tr>
<tr>
<td>mar186R</td>
<td>GGCTTTGTTTTGTTTTGTTTTGTTTGT</td>
<td>Directed mutagenesis of transposase</td>
</tr>
<tr>
<td>BethSpeF Eco</td>
<td>ATGAATTTCATGGAATATGAGACCTGGAAGTCA</td>
<td>Cleavage Assay</td>
</tr>
<tr>
<td>BethNcoREco</td>
<td>ATGAATTCCCATGGAACGTGTCTAGGATCCGAGAG</td>
<td>Cleavage Assay</td>
</tr>
</tbody>
</table>
I. ITR MUTAGENESIS

A. Plasmid Construction

A plasmid was constructed that allowed for the simple mutagenesis of one inverted terminal repeat of Himar1 (Figure 5). Figure 6 shows an overview of plasmid construction for this study. pACYC184 was initially cut with restriction enzymes Sal I and BamHI at 37 °C for 1 h. One microliter of CIP (Calf Intestinal Alkaline Phosphatase; New England BioLabs) was added to dephosphorylate the ends. The reaction was incubated for an additional 30 min at 37 °C and the reaction was gel purified in a 1X TAE 0.5% agarose gel. The approximately 4 kilobase pair (kbp) band was excised from the gel. The DNA was extracted from the gel slice using the Zymoclean DNA Recovery Kit and eluted in 8 µl of 0.2 µm filtered water.

Annealed 5' ITR oligos (Table 3) were ligated to the cut pACYC184 using T4 DNA Ligase (New England BioLabs) overnight at 16 °C. The ligations were terminated the following day by adding 20 µl of water at 65 °C for 20 min. One microliter of the ligations were transformed into 40 µl of E. coli Top10 F’ competent cells at 1.8 KV using a Bio-Rad electroporator. The transformed cells were added to 960 µl of LB. The transformed cells were incubated for 1 h in the orbital shaker at 250 rpm at 37 °C. After

Figure 5. The Himar1 wild type ITR. The flanking nucleotides are in lower case letters. The 27 bp of the ITR are in upper case letters.
Figure 6. Overview of plasmid construction. The methods used to construct these plasmids are described briefly next to each arrow. More detailed descriptions can be found in the Materials and Methods.
1 h, 100 µl of transformed cells were spread onto LB agar plates containing chloramphenicol (Cam) and incubated at 37 ºC overnight. The next day three colonies were picked from the plate and grown in 5 ml of LB and Cam overnight at 37 ºC at 250 rpm. The DNA from the overnight cultures was purified using the Sigma GenElute Plasmid Miniprep kit and eluted in 50 µl of water. The purified DNA was sequenced using the BethSeqHin primer (Table 3) to confirm that the 5’ ITR was in the plasmid. This created plasmid pBB1 (Table 1).

B. Cloning the Apramycin Resistance Gene DNA Fragment

The apramycin resistance gene DNA fragment (from the aminoglycoside N (3’)-acetyltransferase IV gene) used in this study was obtained from pOJ427. Plasmid pOJ427 was digested with BamH I and Xba I to remove the fragment that was approximately 1.2 kbp. Plasmid pGEM-3Zf(+) was digested with BamH I. Both digests were gel purified on a 1X TAE 0.5% agarose gel. The apramycin resistance gene DNA fragment was ligated into the BamH I site of pGEM-3Zf(+). This created plasmid pBB3 (Table 1).

Plasmid pBB3 containing the apramycin resistance gene from pOJ427 was cut with Xba I and Sma I to excise the apramycin gene. Plasmid pBB1.2 that contains the 5’ ITR was cut with BamH I so that the apramycin gene could be ligated into this site. Both reactions were incubated at 37 ºC for 1 h. To create blunt ends in both reactions, 5 Units of Klenow Large Fragment DNA Polymerase I (New England BioLabs) and 1 µl of 10 mM dNTPs was added to each reaction and incubated for an additional 30 min at 37 ºC. The reactions were terminated in a 70 ºC water bath for 20 min. After 20 min 1 µl of CIP
was added to pBB1.2 and incubated for 30 min at 37 °C. The restriction digests were gel purified on a 1X TAE 0.5% agarose gel. An approximately 1.2 kbp band was excised from the gel from the pBB3 digest and a 4 kbp band was excised from the pBB1.2 digest. The DNA from both gel slices was extracted as before.

The apramycin resistance gene DNA fragment was ligated to pBB1.2 overnight at 16 °C. The following day the ligations were terminated and transformed into Top10 F’ competent cells as before. The DNA from overnight cultures of transformed cells was purified following the Boiling Miniprep protocol. The DNA was cut with both BamH I and Hind III to determine the orientation of the apramycin gene. The DNA from the appropriate culture was again purified using the GFX Micro Plasmid Prep Kit (Amersham Biosciences) and eluted in 50 µl of filtered water for sequencing analysis. To further verify that the apramycin gene was in the correct orientation in the plasmid, the primer 20fhR (Table 3) was used. This created plasmid pBB6.4 (Table 1).

C. Mutagenesis of the 3’ Inverted Terminal Repeat: Several Alternative Methods

Once a clone was obtained in which the apramycin resistance gene was in the correct orientation (pBB6.4), the wild type 3’ ITR and the mutagenized ITRs could now be cloned into the plasmid. Plasmid pBB6.4 was cut with BamH I and Hind III and the vector DNA was purified. Both the 3’ wild type ITR and the mutagenized 3’ ITRs could be ligated into these sites. Three different approaches were used to try to introduce mutagenized ITRs into pBB6.4.
1. A Doped Oligonucleotides Approach at Mutagenesis

The first approach at mutagenesis of the ITR involved using doped ITR oligonucleotides. The doped ITR sequences would contain one nucleotide substitution per oligonucleotide molecule. This would allow the ITR to be mutagenized one position at a time within a given region. These ITRs were doped in specific regions such as 4 – 8 of the ITR, 9 – 13, and 14 – 18 of the ITR. Doping the ITRs in this region meant that a certain percentage of colonies would have the wild type ITR while another percentage of colonies would have a mutation specifically in this region. The wild type oligo was synthesized at Integrated DNA Technologies (IDT, Inc.). The other doped oligos were synthesized at the University of Illinois Biotechnology Center. To convert these oligos into double stranded oligos, 2 µl (1 to 2 µg of a 1 mg/ml concentration) of the oligo and 5 µl of filtered water were combined in a microcentrifuge tube. The reaction was incubated for 5 min at 70 °C. After 5 min, 1 µl of 10X DNA Polymerase I Buffer (New England BioLabs) was added to the reaction. The reaction was allowed to cool to room temperature and remained there for 1 h. This allowed the 3' ends of the oligos to anneal to each other (Sambrook and Russell, 2001). After 1 h, 2 µl of 10 mM dNTPs and 5 Units of Klenow DNA polymerase I were added to the reaction in order to convert the oligos to double stranded DNA. The reaction was incubated at room temperature for an additional hour. Finally, 1 µl of Klenow was added and the reaction was incubated overnight at room temperature.

The following day the reaction was terminated with 1 µl of 0.5 M EDTA. The volume of the reaction was adjusted to 50 µl with TE (10 mM Tris-HCl, 1 mM EDTA, pH 8.0). Five microliters of 0.3 M sodium acetate was added to the reaction. Phenol
extraction of the DNA from the reaction was performed using phenol:chloroform:isoamyl alcohol (24:24:1). Following extraction, the DNA was precipitated at 4 °C with three volumes of 100% ethyl alcohol, 10 % 3 M sodium acetate, and 10 mg/ml of glycogen (Sigma). The reaction was placed at –20 °C for 20 min and centrifuged at 13,200 rpm at 4 °C for 10 min. The supernatant was decanted and the pellet was washed with 100 µl of 70% ethyl alcohol and centrifuged at 4 °C. Following precipitation and resuspension in 20 µl of TE, the annealed oligos were cut with BamH I and Hind III for 2.5 h at 37 °C. After 2.5 h, an additional 0.5 µl of each enzyme was added to the reactions. Digestion continued overnight at 37 °C. The following day the DNA from the digestion was precipitated as before. The DNA was run on a 1X TAE 2% agarose gel at 60 V for 3 h at 4 °C. After three hours the gel was stained in a 1:10,000 dilution of SYBR Green I Nucleic Acid Stain (Molecular Probes) on an orbital platform at 50 rpm for three hours. A band that was less than 100 bp was excised from the gel. The DNA was extracted from the gel slice by electroelution. The double stranded doped ITRs were ligated to a gel-purified pBB6.4 (Table 1).

2. Mutagenesis of the ITR with GeneMorph

A second method was employed to mutagenize the ITR that involved performing PCR on pBB9 using the GeneMorph PCR Mutagenesis Kit (Stratagene, Inc.). Mutazyme DNA polymerase supplied with this kit randomly introduces errors more frequently than Taq DNA polymerase without a transition/transversion bias. To achieve single mutations with this kit, three different amounts of pBB9 were used for the PCR: 50, 5 and 0.5 ng were used as starting concentrations of the template. The primers BethSeqHin2 and
AprRseq2 (Table 3) were used to amplify the apramycin resistance gene flanked by both ITRs. A 50 µl reaction was set up for each amount of pBB9 used and the reactions were placed in the RoboCycler. The program used was 95 °C for 1 min for 1 cycle; 95 °C for 1 min, 52 °C for 1 min, 72 °C for 1 min for 30 cycles.

The reactions were purified using G-50 Sephadex. The DNA from the PCR reactions was lyophilized for 10 min in order to achieve a volume of 20 µl. Each PCR product was digested with 1.5 µl each of BamH I and Hind III for 1 h at 37 °C. After 1 h the digested DNA was run on a 4.5% NuSieve GTG (FMC BioProducts) low melting temperature agarose gel at 4 °C for 2.5 h at 75 V. After 2.5 hrs the gel was stained with a 1:10,000 dilution of SYBR Green I Nucleic Acid stain for 1 h on the orbital shaker at 70 rpm. Bands that were approximately 40 bp in length were excised from the gel. The DNA from each gel slice was extracted using the crush-and-soak method. One ml of TE was added to each microcentrifuge tube that contained a gel slice and rocked at 4 °C overnight. The following day the TE was removed from each tube and stored at –20 °C. Another 1 ml of TE was added to each tube and the tubes were placed back on the Nutator at 4 °C. Later that day the TE was removed from the tubes and combined with the TE that had been stored at –20 °C. The TE from each extraction was placed into 5,000 MWCO concentrator spin tubes (VivaScience). The tubes were spun at 5,000 x g in 5 min intervals for 45 min. The solution (approximately 25 µl) that could not be filtered through was washed in 50 mM TE. Approximately 25 µl was collected from each tube. Dilutions ranging from 1:10 to 1:1000 of the extracted DNA were ligated to gel purified pBB9 that had been cut with BamH I and Hind III.
3. ITRs Containing Single Nucleotide Substitutions

The third and final method used to introduce ITR mutations is a "brute" force method that consisted of constructing each single nucleotide mutation individually. Two 96 deep-welled microplates containing synthesized mutated ITR oligos were ordered from IDT. Each well of these plates contained both the top and bottom strands of the ITR mutated at the same position. Since there could be three possible mutations at each of the 27 ITR positions, each position used three wells. The flanking nucleotides (TA) were mutagenized as well. To anneal the synthesized oligos, 50 µl of 1X annealing reaction buffer (10 mM Tris-HCl pH 8.0, 1 mM EDTA, 50 mM NaCl) was added to each well. The plate was vortexed and allowed to incubate at room temperature for 15 min in order to hydrate the oligos. The plate was placed in a 95 °C water bath for 2 min. After 2 min the water bath was turned off while the plate remained in the bath in order for the plate to slowly cool to room temperature (approximately 5 h). To force the condensation down into the bottom of the wells, the plate was centrifuged for 15 min at 1,000 rpm in an Eppendorf Centrifuge 5810. Since there were annealed oligos for one position in three different wells, 10 µl was taken from each well for a given position and combined into one microcentrifuge tube marked specifically for that position. As a result, there were a total of 29 tubes containing 30 µl of 1X double stranded oligos. These were stored at −20 °C. The plates were tightly wrapped for long-term storage at −80 °C.

Each mixture of mutated ITRs was ligated to pBB6.4 (Table 1). Undiluted and diluted oligos ranging from 1:10 to 1:100,000 nM-fold dilutions were used in the ligations.
D. A Papillation Assay To Determine the Effects of a Mutated Himar1 ITR on Transposition

To look at the effects of the mutated positions on transposition, a papillation assay was performed. Plasmid pBB9 that contained wild type ITRs was used as a positive control each time the assay was performed. Ligations were transformed into 40 µl of DL8 electrocompetent cells at 1.8 KV using the Bio-Rad electroporator. The transformed cells were grown and plated as stated earlier. Beginning at 48 h and every 1-2 h thereafter, the colonies were examined under a dissecting microscope to determine the extent of papillation. By hour 51-53 colonies were identified as wild type based on the abundance of papillae located in the center of the colony. Particular colonies were tracked further during incubation. If a plate containing a particular position exhibited little to no activity during the first 55-60 h of incubation, it was allowed to incubate further at 32 °C. If colonies still exhibited no activity between 70-72 h, they were categorized as null or if they had papillated at a slower rate then they were identified as hypomorphs. Colonies ranging in phenotypes from null to wild type were chosen for sequence analysis.

E. Sequence Analysis

To determine the nucleotide substitution responsible for a particular phenotype, sequence analysis was performed on purified DNA from colonies collected during the papillation assays. Big Dye Sequencing Terminator Version 3.0 (ABI) and the ABI Prism 310 Genetic Analyzer were used to aid in determining nucleotide substitutions. Once the substitutions were identified, the sequences were catalogued and cell stocks were saved for future experiments.
F. Polymerase Chain Reactions

The Biometra Personal T and the Stratagene Robocycler thermalcyclers were used to perform various polymerase chain reactions (PCRs). The Biometra was primarily used to conduct sequencing PCR while the Stratagene Robocycler was used for other PCRs. The primer used to sequence and to confirm the mutations in the ITR was BethSeqHin (Table 3). Sequence reactions were always run in the Biometra using the conditions: 95 °C for 1 min for 1 cycle; 95 °C for 10 s, 50 °C for 5 s, 60 °C for 4 min for 54 cycles.

II. TRANSPOSASE SUPPRESSOR SCREEN

A. Constructing Plasmids that Contain Single Nucleotide Substitutions in Both ITRs

Plasmids that contained single nucleotide substitutions at positions 12 and 20 that affected transposition during the ITR Mutagenesis experiments were selected for a transposase suppressor screen. Six PCRs were set up using the primers ITR12aBAM, ITR12cBAM, ITR12tBAM, ITR20aBAM, ITR20gBAM, and ITR20tBAM (Table 3). These primers were designed to amplify the region of pBB9 that contained both ITRs flanking the AprR gene while introducing a specific nucleotide substitution into both ITRs. The PCRs were set up with 1X Pfí amplification buffer, 0.3 mM dNTPs, 1 mM MgSO4, 1 Unit of Platinum Pfí DNA polymerase, 0.3 μM of the appropriate primers, and 10 ng of template DNA. The PCR was run using the following conditions: 95 °C for 2 min; 95 °C for 1 min, 52 °C for 45 s, 75 °C for 2 min for 30 cycles. After the PCR was complete, 5 μl of each PCR was loaded into a 1X TAE 0.5% agarose gel to verify that a band approximately 1.2 kb in size had been obtained. Each PCR was purified using G-50 Sephadex columns.
Each PCR was digested with *Bam*H I in order to obtain a fragment that could be ligated to pACYC184 that had also been digested with *Bam*H I and gel purified. Once the amplified fragment had been ligated to pACYC184, the ligations were transformed into Top10 F’ electrocompetent cells as before. Boiling minipreps were performed on overnight cultures from transformed cells. The DNA was cut with *Ava* I to look for a predicted restriction pattern and the same orientation was chosen for each.

**A. Competent Cell Preparation of Strains Containing Two Mutated ITRs**

Ten nanograms of DNA containing the two mutated ITRs were transformed in to DL6 electrocompetent cells. The cells were grown in LB broth for 30 min at 37 °C in the orbital shaker. The pelleted cells (100 µl) were plated onto LB agar plates containing Apr. The plates were incubated at 37 °C overnight. The following day a single colony was picked from each plate and placed into a flask containing 50 ml of LB broth and 20 µl of Apr. The cultures were grown overnight at 37 °C in the orbital shaker. The following day flasks containing 500 ml of prewarmed LB broth and 200 µl of apramycin were inoculated with 5 ml of the appropriate culture.

The cultures were incubated in the orbital shaker at 37 °C until an O.D.₆₀₀ of 0.6 was reached. The flasks were placed on ice for 30 minutes. After 30 min the cultures were divided into 250 ml centrifuge bottles and centrifuged for 12 min at 4,000 rpm at 4 °C. The supernatant was decanted and any remaining liquid was removed using vacuum aspiration. The pellets were resuspended in 250 ml of 10% glycerol and centrifuged at 4,000 rpm for 12 min at 4 °C. The supernatant was again decanted and the remaining liquid was removed using vacuum aspiration. The cell pellets were resuspended
second time in 10% glycerol. The remaining pellet was resuspended in 1.5 – 2 ml of GYT (10% glycerol, 0.125% yeast extract, and 0.25% tryptone). Forty microliter aliquots were prepared from the resuspended pellet. These aliquots were stored at –80 °C. A total of six different strains of competent cells were made, BB2-BB7. Descriptions of these strains can be found in Table 2.

**B. Transforming pBADmar1 into BB2 – BB7: Control Experiment**

As a control experiment, 10 ng of pBADmar1 was transformed into each of the competent cell strains, BB2-BB7. The cells were grown in LB broth at 37 °C for 45 min in the orbital shaker. 100 µl of a 1:100 dilution of transformed cells were plated on MacConkey lactose agar plates containing Amp and Cam. The plates were incubated at 32 °C for 55-60 h.

**C. Transposase Mutagenesis**

Two approaches were taken to introduce mutations into the wild type Himar1 transposase coding sequence in pBADmar1.

1. **Random Mutagenesis**

The first approach involved randomly mutagenizing the transposase using Taq DNA polymerase and small amounts of MnCl₂. A PCR was performed that consisted of 1X PCR buffer containing 2 mM MgCl₂, 0.2 mM dNTPs, 0.4 µM each of pBAD24seqF and pBAD24seqR primers, 1.25 Units of Taq DNA polymerase, 10 ng of pBADmar1, and 50 µM MnCl₂. The PCR was run as was previously described in the preceding section, however, the number of cycles was extended to 40 with a 1.5-min extension time. The PCR was purified using G-50 Sephadex columns.
Both the PCR product and pBADmar1 were digested with EcoRI and PstI for 1 h at 37 °C. CIP was added to pBADmar1 as before. Both restriction digests were run on a 1X TAE 0.5% agarose gel. A band from the PCR restriction digest of approximately 1.1 kbp was excised from the gel. A band from the pBADmar1 digest that was between 4-5 kbp in length was also excised from the gel and purified. The PCR fragment was ligated to the purified pBADmar1 overnight at 16 °C. Ligations were terminated the following day as before.

The ligations were transformed into BB2-BB7 (Table 2) to see what affect there would be on transposition. The cells were grown in LB broth for 45 min in the orbital shaker. Three hundred microliter of cells were plated onto large plates containing MacConkey lactose agar as well as Amp and Cam. The plates were incubated at 32 °C for 55-60 h.

2. **Direct Mutagenesis of Transposase Using PCR-ligation-PCR**

Two amino acids were targeted in the transposase to be mutagenized, methionine at position 86 and arginine at position 101. Four primers were designed to target these areas in pBADmar1 in a PCR-ligation-PCR technique. Table 3 contains a description of each of these primers. Met86RandomF and Arg101RandomF contain three “N” at the 5’ end so that any of the 64 possible codon substitutions could be introduced. Met86R and Arg101R were designed to abut these primers (Figure 7). Two initial PCRs were set up for each group of primers. The first PCR involved pBAD24seqF flanking primer and Arg101R. The second PCR utilized mar186R flanking primer and Arg101RandomF. Both flanking primers were also used to conduct PCR with the methionine primers. Platinum Pfx DNA polymerase was used to carry out the PCR. The PCR was run using
the following conditions: 94 °C for 2 min; 94 °C for 15 s, 55 °C for 30 s, 68 °C for 1 min for 30 cycles. After the PCR was complete all 25 µl of each was loaded into a 1X TAE 1% agarose gel. The bands were excised from the gel and purified using the Zymoclean DNA Recovery kit. The DNA was eluted in 25 µl of filtered water.

Each of the PCR products was kinased in a 50 µl reaction using T4 polynucleotide kinase (PNK; New England BioLabs). Four separate reactions were prepared using 1X PNK buffer, 100 mM ATP, 5 µl PCR product, and 1 Unit of T4 PNK. The reactions were incubated at 37 °C for 30 min and were terminated at 65 °C for 15 min. The kinased products were ligated to each other by mixing 5 µl of each product and adding 0.3 µl T4 DNA ligase. The ligations were incubated at room temperature for 15 min after which the ligations were terminated at 65 °C for 15 min.

A final PCR was set up utilizing the ligated PCR products as a template, and the flanking external primers, pBAD24seqF and mar186R. The PCR was run as before. After the PCR was complete it was run on a 1X TAE 1% agarose gel. Bands at approximately 660 bp were excised from the gel and purified as before.

The purified PCR products were digested with EcoR I and Sac I at 37 °C for 1 h. The digests were run on a 1X TAE 1% agarose gel following digestion. Bands at approximately 375 bp were excised from the gel and the DNA was extracted as before. The digested PCR products were ligated to gel purified pBADmar1 that had previously been digested with EcoR I and Sac I. The ligations were incubated and terminated as before. The ligations were transformed into BB2-BB7 (Table 2) as before.
Figure 7. Schematic overview of PCR-ligation-PCR. This technique utilizes four primers, two fixed flanking primers and two primers that abut one another to introduce mutations into a specific sequence. One of the two latter primers contains the mutation of interest as is indicated by an "*". A more detailed description of this technique can be found in the Materials and Methods.
III. GEL MOBILITY SHIFT ASSAY TO MEASURE DNA-BINDING

A. DNA Isolation and Radiolabeling with $^{32}$P

The plasmid containing the wild type ITR, pBB9 was used as a positive control. Ten micrograms of pBB9 and plasmids containing mutated ITR DNA were digested with the restriction enzymes *Bam*HI and *Xba*I to remove the ITR. The restriction digests were run on a 1X TAE 0.5% agarose gel. Bands approximately 134 bp in length were excised from the gel and purified as before.

One hundred and fifty nanograms of DNA was end-labeled with 3 µl of 3000 Ci/mm mol $[^{32}P]$ dATP and 3 µl of 3000 Ci/mm mol $[^{32}P]$ dCTP. In addition to the radioactive materials, the labeling reactions also consisted of 10 mM dGT, NEB buffer 2, and 5 units of Klenow DNA polymerase I. The reaction was incubated at room temperature for 20 min. Following incubation, the reaction was chased with 0.75 µl of unlabeled 10 mM dNTPs for 10 min. The reactions were terminated in a 65 °C water bath for 10 min. After the reactions were terminated, the unincorporated label was removed using G-50 Sephadex columns. The columns were centrifuged for 2 min at 750 x g and the eluate was saved. After the labeled DNA was purified, 6 µl of 6X agarose gel loading buffer was added to each labeled DNA. The labeled DNA was loaded into an 8% native acrylamide gel (40% Acryl:Bis, 5X TBE). Five microliters of 6X agarose gel loading buffer was also loaded into the gel to provide orientation. The gel was run for 2 h at 238 V at room temperature. After 2 h the gel was removed from the cassette and exposed to Kodak autoradiographic film for 2 min. The film was developed as per the manufacturer’s recommendation. After the film was dried, the gel and the film were aligned and the proper bands were excised from the gel. The gel slices were placed in 1.5 ml centrifuge tubes and 1 ml of TE was added to each tube. The tubes were placed on a
Nutator at 4 °C overnight to extract the DNA from the gel slices. The extracted DNA was ethanol precipitated as described above. The pelleted DNA was resuspended in 50 µl of TE.

B. DNA-Protein Incubations

The labeled ITRs were incubated with wild type MBP-\textit{Himar1} transposase in a 20 µl reaction that consisted of 1 ng of DNA, 1X Triton reaction buffer (40mM sodium phosphate pH 7.5, 200 mM potassium glutamate, 2mM DTT, 1mM EDTA, 400 µg/ml BSA, 0.2% Triton X-100 in ddH$_2$O), 250 ng of poly(dI)-(dC), and a 1:100 dilution of transposase in 1X Triton reaction buffer. The reactions were incubated at room temperature for 45 min. Reactions were also set up for each position that consisted of only DNA.

C. Gel Electrophoresis and Exposure

After 45 min of protein-DNA incubation, 20 % glycerol was added to each reaction. Each reaction was loaded into a 6.5 % native acrylamide gel. Five microliters of 6X agarose gel loading buffer was loaded into the gel for orientation purposes. The gel was run at 238 V for 2 h at 5 °C. After 2 h the gel was removed from the Bio-Rad gel cassettes. A piece of blotting paper was placed on the gel in order to carefully remove it from one of the glass plates. The gel was covered with saran wrap and placed in the gel dryer for 1.5 h. After the gel was dried, it was placed in the Bio-Rad GS-505 Sample Exposure Platform with a piece of Mylar covering it. The imaging screen cassette-BI, 20 x 25 was also placed in the exposure platform overnight. Fourteen to eighteen h later the cassette was removed from the exposure platform and placed into the Bio-Rad GS-525
Molecular Imager System. Using the Molecular Analyst software the image was scanned and then analyzed.

IV. STRAND-SPECIFIC CLEAVAGE ASSAYS TO TEST TRANSPOSITIONAL MUTANTS

A. Cloning the 3’ WT ITR and Mutated ITRs into pGEM-3zf(+)

PCR was performed on pBB9 and those plasmids containing point mutants using the primers BethSpeFEco and BethNcoREco (Table 3). The PCRs were set up and run under conditions similar to those PCRs conducted during the PCR-ligation-PCR described earlier. Once the PCRs were complete, they were run on a 1X TAE 1.5% agarose gel. Bands that were greater than 100 bp were excised from the gel. The DNA was extracted from the gel slices using the Zymoclean DNA Recovery Kit and eluted in 20 µl of water.

The purified PCR products were digested with EcoR I at 37 °C for 1 hour. Plasmid pGEM-3zf(+) was also digested with EcoR I at 37 °C for 1 h. After 1 h, 1 µl of CIP was added to pGEM-3zf(+) and incubated for 30 min at 37 °C. Both the digested PCR products and the vector were run on a 1X TAE 0.5% agarose gel. The appropriate bands were excised from the gel and the DNA was extracted using the Zymoclean DNA Recovery Kit. Using T4 DNA ligase the PCR products were ligated to pGEM-3zf(+) overnight at 16 °C as before. The following day the ligations were terminated as before and were transformed into Top10 F’ electrocompetent cells as before. Colonies were picked and purified. The purified DNA was sequenced with the BethSpeFEco primer in order to determine if the ITR contained the correct mutation.
B. Radiolabeling Himar1 3’ ITRs with $^{32}$P

Once it was determined that the ITR DNAs contained the correct sequence, the top strand of each was labeled first. Ten micrograms of each plasmid was cut with 15 U of Nco I for 1 h at 37 °C. After 1 h the digestion was heated to 65 °C for 20 min. Half of the digest was then end-labeled with 4 µl of 3000 Ci/mmol $[^{32}$P] dATP and 4 µl of 3000 Ci/mmol $[^{32}$P] dCTP. In addition to the radioactive materials, the labeling reactions also consisted of 1X NEB buffer 2, 1 µl of 10 mM dGT, 5 U of Klenow DNA polymerase I, and water to a final volume of 40 µl. The reaction was incubated at room temperature for 20 minutes. Following incubation, the reaction was chased with 0.4 mM of unlabeled dNTPs for 10 min. The reactions were heated to 65 °C for 10 min. The unincorporated label was removed from the reactions using G-50 Sephadex columns. The flow through, approximately 40 µl was cut with 15 U of Spe I for 1 h at 37 °C and heated to 65 °C for 20 min.

The reactions were loaded onto a 1% modified TAE-agarose gel. Bands that were greater than 100 bp in size were excised from the gel. The DNA was extracted from the gel slices using the Millipore Montage DNA Gel Extraction kit. Gel slices were centrifuged for 10 min at 5000 x g. An equal volume of phenol:chloroform:isoamyl alcohol (24:24:1) was used to extract the DNA from the TAE solution. The DNA was extracted once more using an equal volume of chloroform:isoamyl alcohol. The DNA was then precipitated with 3 volumes of ethanol, 0.1 volume of sodium acetate (pH 5.3), and 10 mg/ml of glycogen. The reactions were centrifuged for 15 min at 13,200 rpm at 4 °C. The pellet was then washed with 100% ethanol, dried, and then resuspended in 50 µl of water. The specific radioactivity of each labeled ITR fragment was measured by
scintillation counting. The bottom strand was labeled in an identical manner except that the DNA was first cut with Spe I, labeled, and then cut with Nco I.

B. Construction of Size Standards Using the Maxam-Gilbert Sequencing Techniques

Standard Maxam-Gilbert sequencing techniques were used for A>C reactions for both the top and bottom strands of the labeled wild type ITR DNA (pBB18.1). One hundred microliters of 1.2 N NaOH, 1 mM EDTA and 4 µl of sonicated herring sperm DNA was mixed with 200,000 c.p.m of labeled pBB18.1 ITR DNA. The reaction was incubated at 90 °C for 7 min and chilled to 0 °C. The following were added to the reaction, 150 µl of 1 M acetic acid and 5 ng of tRNA and then mixed. Ice-cold ethanol was added to the reaction and stored on ice for 5 min. The reaction was centrifuged at 4 °C for 5 min at 12,000 x g. The supernatant was removed and the DNA was precipitated with 300 µl of ice-cold 0.3 M sodium acetate (pH 5.2) and 900 µl of ice-cold ethanol. The reaction was kept on ice for 5 min and centrifuged as before. The supernatant was removed and the pellet was washed with 1 ml of ethanol. The pellet was lyophilized for 5 min. Following lyophilization, 100 µl of 1 M piperidine was added to the pellet, mixed, and heated to 90 °C for 30 min. The pellet was lyophilized for 1 h. Twenty microliters of water was added, vortexed, and lyophilized for 30 min. This step was repeated a second time. Ten microliters of formamide-loading buffer was added to the standard (Sambrook and Russell, 2001). The specific radioactivity of the standard was measured by scintillation counting.
C. Strand-specific Cleavage Assay

Wild type MBP-Himar1 transposase (60 nM) was incubated with 100,000 c.p.m. of labeled DNA, *in vitro* transposition buffer (10% glycerol (v/v), 25 mM HEPES (pH 7.9), 250 µg BSA, 100 mM NaCl, 2 mM DTT, 5 mM MnCl₂), 100 ng poly (dI)-(dC), and water to a volume of 20 µl. The reactions were incubated at 30 °C for 2 h. The reaction was stopped with 100 µl of 25 mM EDTA, 0.01% SDS followed by extraction with an equal volume of phenol:chloroform:isoamyl alcohol (24:24:1). The reactions were extracted once more with chloroform:isoamyl alcohol. The DNA was precipitated as before and centrifuged for 30 min at 12,000 x g at 4 °C. The pellet was washed with 100% ethanol, dried, and resuspended in 10 µl of formamide loading buffer. After the reactions were resuspended in the formamide-loading buffer, the specific activity of each labeled DNA fragment was measured by scintillation counting.

D. Gel Electrophoresis and Exposure

A 6% denaturing polyacrylamide gel (39:1 acrylamide:bisacrylamide) was prewarmed to 50 °C. During this time both the cleavage reactions and the A>C standards were heated to 90 °C for 2 min and placed on ice until the gel had reached the appropriate temperature. Once the gel was prewarmed, 30,000 c.p.m. of each reaction and the A>C standard were loaded onto the gel. The gel was run at approximately 55 W in order to maintain a constant gel temperature of 50 °C for 2 h. After 2 h the Bio-Rad glass plates were separated and piece of blotting paper was used to carefully remove the gel from the inner glass plate. The gel was covered with saran wrap and dried in the gel drier for 1.5 h. After the gel was dried, it was exposed to autoradiographic film at -80 °C for 16 to 18 h.
V. DETECTING TRANSPOSITION INTERMEDIATES

Plasmid pGEM-3zf(+) was first digested with EcoR I, was labeled as before with 4 µl of 3000 Ci/mmol [\(^{32}\)P] dATP, and digested with Sap I in order to obtain a fragment 317 bp in size. The labeled DNA was run on a 1% modified 1X TAE-agarose gel. A band approximately 317 bp were excised from the gel and purified as mentioned in section IV part B. Once the DNA was purified, the DNA was extracted with phenol:chloroform and then precipitated as before with ethanol and glycogen. The DNA was resuspended in 50 µl of water. The specific activity of the labeled pGEM fragment was measured by scintillation counting.

VI. IN VITRO TRANSPOSITION ASSAYS TO TEST TRANSPOSTIONAL MUTANTS

An in vitro transposition assay was set up similar to the strand-specific cleavage assays except that the poly(dI)-(dC) was left out of the reactions. \(10^5\) c.p.m. of end-labeled DNA containing the Himar1 ITR was incubated with 450 ng of pGEM-3zf(+) and 60 nM MBP-Himar1 transposase. Control reactions were also set up without transposase. The reactions were incubated at 30 °C. After 2 h an aliquot of the reaction was removed and the remainder of the reaction was incubated an additional 2 h. The addition of 60 mM EDTA in 6X agarose gel-loading buffer terminated the reactions. The reactions were loaded onto a 0.5% 1X TAE-agarose gel. The gel was run 119 V for 55 min. The gel was placed on a piece of exposed film and placed in a drying oven at 55 °C for 5 h. The gel was exposed to autoradiographic film for 5 h.
RESULTS

Plasmid pBB9

Plasmid pBB9 (Figure 8) and a complete series of plasmids containing 3’ ITRS with all possible single nucleotide substitutions were constructed to ascertain the effects of these changes on transposition. Because pBB9 contained the wild type ITRs that flanked the AprR gene fragment it was used as a positive control in the papillation assays, and in the gel mobility shift DNA-binding assays.

![Figure 8. The physical map of pBB9 that contains wild type ITRs.](image)

Apr = apramycin resistance; Cam = chloramphenicol resistance; ITR = inverted terminal repeat

Papillation Assay: Control Experiment

I used pBB9 in a papillation assay to determine whether or not transposition could be detected by delivering a promoter to a chromosomal promoterless lacZ gene. The
Figure 9. Overview of the papillation assay. The transposon donor was pBB9 or plasmids containing the mutagenized ITR. The transposon donor was transformed into the transposase source strain, DL8. The transposase binds to the inverted repeats in the transposon donor and inserts the transposon into the target site, activating lacZ.
papillation assay illustrated in figure 9 is part of a screen based on the ability of the Himar1 transposase to mobilize a transposon (antibiotic resistance gene) into a chromosomal target site that flanks a promoterless lacZ gene (Lampe et al. 1999). The promoterless lacZ gene target site for the transposon insertion is flanked by a four tandem rrnB terminator sequences (TTTT) which isolates lacZ from any insertion outside the target sequence. Once the transposon insertion is inserted in the correct orientation with the lacZ gene, β-galactosidase can be produced. Therefore, cells in an otherwise Lac^- E. coli colony are converted to Lac^+ cells. Lac^+ cells are capable of metabolizing the lactose contained within the MacConkey agar. The Lac^+ cells will grow much faster than Lac^- cells producing small bumps, or papillae, on the top of the colonies. The papillae will also turn red due to the neutral red in the MacConkey agar that detects lactic acid production in the Lac^+ cells (Lampe et al. 1999).

At approximately 48 hours of incubation at 32 °C the pBB9 colonies showed a large number of papillae located in the center of the colonies. This indicated that the inverted repeats were in the construct and that it was capable of carrying out transposition. Figure 10 is a picture taken with a dissecting microscope of colonies on a plate containing pBB9 at 48 hours.

**Papillation Assay: Testing Mutagenized 3’ ITRs**

Three methods were employed to attempt to introduce mutagenized ITRs into pBB6.4. The first method involved using doped ITR oligonucleotides which are pools of oligonucleotides randomized at certain positions (Sambrook and Russell, 2001). In principle, the doped ITR sequences would contain one nucleotide substitution per
oligonucleotide molecule allowing the ITR to be mutagenized one position at a time within a given doped region. Studying the Himar1 mutations in this manner would have been straightforward because the number of different sequence combinations that would have been screened would have been $3 \times 27 = 81$.

![Image](image1.png)

**Figure 10.** DL8 strain colonies carrying pBB9 on a MacConkey lactose agar plate after 48 hours of incubation during the control papillation assay. Papillae are located in the center of each colony.

However, little data was obtained from this method. Many problems were encountered in obtaining single nucleotide mutants. In many cases more than half of the colonies that were analyzed exhibited more than one mutation in the doped regions, and single and multiple nucleotide deletions near the end of the inverted repeat were also common. As a result, this method was abandoned.
The second method involved using the GeneMorph PCR Mutagenesis kit (Stratagene, Inc.) to mutagenize the 3’ ITR in pBB9 via error-prone PCR. Although the Mutazyme DNA polymerase supplied with this kit is intended to randomly introduce errors more frequently than Taq DNA polymerase, I had trouble specifically targeting the ITR in the PCR or getting many mutations. As a result, only a single mutant was identified using this method. This single nucleotide substitution was found at position one in the ITR. A “C” was randomly substituted for the wild type “A” producing a null phenotype. This method was also abandoned.

The third method used proved to be the most successful. This method involved pooling together three annealed oligos containing a single nucleotide substitution at any given position in the inverted repeat plus the flanking DNA (TA). The pool of mutated positions thus had all three substitutions, but never the wild type nucleotide at any given position. Mutated ITRs were cloned into the BamH I and Hind III site in pBB6.4. This ligation was repeated 29 times, with each ligation containing all of the mutants at one site in the ITR. The MacConkey lactose agar plates were incubated for more than 48 hours at 32 °C. Beginning at 48 hours of incubation, colonies were examined every one to two hours to determine the extent of papillation. At approximately 48 hours the control plate containing pBB9 began papillating. As a result, if colonies on plates containing the mutated positions began papillating at the same time as pBB9, these colonies were scored as having a wild type phenotype. However, if colonies began to papillate after 50 hours they were identified as hypomorphs. By 70 to 72 hours if colonies still exhibited no activity they were scored as null. Figure 11 represents the various phenotypes obtained during the genetic screen.
Figures 12 – 14 exhibit the data obtained from this method. Figure 12 depicts those substitutions that did not effect transposition. Out of all 29 positions that were tested, only one position exhibited no effect on transposition with all three substitutions. This position is the first flanking nucleotide (t). Six other positions that were tested also exhibited no effect on transposition when two out of the three nucleotides were substituted for the wild type. These positions are 4, 10, 14, 17, 19, and 21. Seven of the positions exhibited no effect on transposition when only one of the three-nucleotide substitutions were made. These positions are 2, 5, 6, 7, 16, 18, and 23. What is interesting about four of these seven positions is that transposition is not affected when a chemically similar substitution is made.

Figure 11. Pictures taken with a dissecting microscope of three different colonies representing the three phenotypes seen during the genetic screen of the Himar1 ITR. The pictures of the wild type colony and the hypomorph were taken after 54 hours of incubation. The picture of the null colony was taken after 70 hours of incubation.
Figure 12. Graph depicting the single nucleotide substitutions made in the Himar1 3' ITR that did not effect transposition. Nineteen nucleotide substitutions out of a possible 81 (23%) in the ITR show no effect on transposition. All nucleotide substitutions made in the first flanking nucleotide (t) also had no effect.

Figure 13. Graph depicting single nucleotide substitutions in the Himar1 3' ITR that caused a hypomorphic phenotype during the papillation assays. Only nine nucleotide substitutions in the ITR out of a possible 81 (11%) caused hypomorphs.
Figure 14. Graph depicting the single nucleotide substitutions made in the Himar1 3’ ITR that caused a null phenotype during the papillation assays. Fifty-three nucleotide substitutions out of a possible of 81 (65%) in the ITR caused a null phenotype.

Figure 13 depicts the hypomorphs that were isolated. As can be seen, relatively few hypomorphs were isolated during the genetic screen. When the wild type nucleotide at position 1 was substituted for two out of three nucleotides a hypomorphic phenotype occurred. Only seven out of the 29 positions exhibited a hypomorphic phenotype during the screen when only one of the three-nucleotide substitutions were made. Three of these seven positions exhibited slower transposition when a chemically similar substitution was made.

Figure 14 shows the null mutations that were isolated. Nine out of the 29 positions tested were greatly affected by all three nucleotide substitutions. Surprisingly, five of these positions are located in a cluster toward the internal end of the ITR, between positions 20 – 27, where there is the least amount of sequence conservation among mariner ITR. Ten out of the 29 positions tested exhibited a lack of transposition when two out of the three-nucleotide substitutions were made. Five out of these ten positions
are located in the more conserved positions of the mariner ITR where DNA-binding was hypothesized to occur (Lampe et al. 2001).

**Transposase Suppressor Screen**

The genetic screen revealed which positions in the Himar1 ITR affected transposition when single nucleotide substitutions were made. To further study the Himar1 ITR, a transposase-ITR mutation suppressor screen was conducted. The purpose of this screen was to study the coevolution of the Himar1 transposase and its cognate ITRs by determining if changes in the ITRs could be compensated for by changes in the Himar1 transposase protein. In other words, if a change in the ITR is made, can it be compensated for by a change in the transposase? From an evolutionary perspective this is important because it may shed light on element divergence over time and thus the generation of new mariners.

To begin the transposase suppressor screen, two positions from the genetic screen were chosen. The data were examined to determine which positions in the ITR affected transposition the most when nucleotide substitutions were made. Since eight positions exhibited a loss of transpositional activity when all three nucleotide substitutions were made, I chose two positions for the screen. Hydroxyl radical footprint studies conducted by other researchers helped me determine which positions to choose. Research conducted by Lipkow and Chalmers (personal communication) showed that the Himar1 transposase binds to two regions of the Himar1 ITR on the same face of the helix. These regions are not conserved and are found between positions 6-12 and positions 16-22. Therefore, I chose two positions from the variable regions of the logo that are bound to
the transposase. Positions 12 and 20 were chosen not only because they both lie within these regions of DNA-transposase binding, but also because null phenotypes were produced when all three nucleotide substitutions were made during the genetic screen.

Once two positions in the Himar1 ITR were chosen for the screen, I wanted to see what effect a mutated transposase would have on transposition. For instance, could a mutated transposase actually “rescue” transposition when used in a papillation assay where the target construct contained two mutated ITRs? An overview of the transposase suppressor screen is presented in figure 15. PCR was performed on pBB9 with a specific primer that would insert one of the three-nucleotide substitutions in the ITRs at position 12 or position 20. Transformed cells were then used to make six different competent cell strains, BB2 – BB7.

A control experiment was performed initially by transforming pBADmar1 into these six strains to make sure that transposition would not occur. As expected, after 48 hours the colonies exhibited a null phenotype (Figure 16). However, after 68 hours large papillae formed over the entire surface of the colonies which is quite different than normal (Figure 17). It is unclear what caused this. Perhaps after 48 hours the DNA sequence reverted to wild type, which led to the appearance of papillae on the surface of the colonies. To deter the appearance of these papillae, I substituted Cam for Apr that was originally used in addition to Amp. However, this did not seem to make a difference because the large papillae still appeared. Therefore, plates were incubated for no longer than 60 hours at 32 °C. This amount of time is well within the time period in which “normal” transposition can be detected.
Figure 15. Overview of the transposase suppressor screen. Plasmid pBADmar1 (control) or plasmids containing mutagenized transposase-coding sequences were transformed into electrocompetent cell strains containing two mutagenized ITRs (positions 12 and 20) flanking the apramycin resistance gene fragment. If transposition occurs, the apramycin gene will insert into the transposition target site which can be visualized on MacConkey agar plates containing lactose.
Following the control experiment, the Himar1 transposase was randomly mutagenized using Taq DNA polymerase and MnCl₂. The PCR product was cloned into pBADmar1 and this construct was transformed into each one of the competent cell strains. Approximately, 20,000 colonies were screened for each strain (120,000 colonies total) for phenotypic variation after 48 hours of incubation at 32 °C. All colonies were null indicating that any mutations randomly made in the Himar1 transposase were not capable of “rescuing” transposition. I would have had to screen 6,593 different amino acid substitutions in order to screen every substitution possible in the transposase. Although I screened 120,000 colonies, none of these was sequenced to determine if any substitutions had occurred simply because all of the colonies exhibited a null phenotype.

Since no change in phenotype was visualized by randomly mutagenizing the transposase, a more directed approach was taken. PCR primers were designed specifically to target the DNA coding sequence of Himar1. Two positions in the first 115 amino acids of the Himar1 transposase were chosen for mutation; these were methionine at position 86 (M86) and arginine at position 101 (R101). These positions were chosen for two reasons. First, sequence alignment analysis of different mariner transposase reveals that both of these positions are highly variable among mariners. Therefore, these sites may represent selection of diverse amino acids for binding to different ITR sequences in the course of mariner evolution. Secondly, these sites are located within the helix-turn-helix (HTH) of the first 115 amino acids, the DNA-binding domain of Himar1 transposase. Mutagenesis studies of this domain have revealed that this motif contains amino acid residues critical for DNA-binding. These two sites in particular were null for
Figure 16. A scanned image of a plate from the suppressor screen. Plasmid pBADmar1 was transformed into cell strain BB3. Colonies after 48 hours of incubation exhibited a null phenotype. This plate is representative of the results found for the other five strains.
Figure 17. A single colony captured by a dissecting microscope exhibiting the formation of large papillae over the colony’s surface after 68 hours of incubation. This figure is quite unlike the colonies in figure 10 where the papillae are confined to the middle of the colony. Although this colony is from the BB4 strain it represents the phenotype for all strains after 68 hours.

DNA-binding when alanine substitutions were made (D. Lampe, unpublished). Therefore, I thought it would be interesting to see if any codon substitutions at these positions could “rescue” transposition. By using the PCR-ligation-PCR technique to introduce mutations into the Himar1 coding sequence, it was possible to screen all 63 possible codon substitutions. Approximately 400 colonies were screened for each competent cell strain. Once again all of the colonies that were screened were null for transposition following 48 hours of incubation at 32 °C. To verify that codon substitutions were introduced, a few colonies were chosen at random from each screen and sequenced using the pBAD24seqF primer. A total of 20 colonies were sequenced
from the various transformed strains for each amino acid. All colonies sequenced exhibited codon substitutions at these positions (data not shown).

**Mobility Shift DNA-Binding Assay**

After the data were collected from the papillation assays involving the mutagenized 3’ ITRs, I decided to test several positions in a mobility shift DNA-binding assay. A null phenotype for transposition might be due to either a lack of DNA-binding or due to the inability of the transposase to carry out additional steps in the transposition mechanism such as DNA cleavage.

The DNA-binding assay utilizes a native polyacrylamide gel to provide a simple, yet sensitive method to analyze DNA-protein interactions. The DNA-binding assays involve the incubation of an end-labeled DNA fragment with a specific protein at room temperature. Following incubation, the assays were loaded into a native polyacrylamide gel. During electrophoresis, proteins that bind specifically to the labeled fragment retard the mobility of the fragment through the gel. This results in distinct bands that represent the DNA-protein complexes. However, unbound fragments will travel faster through the gel (Ausubel *et al*. 1999).

The DNA-binding assays performed during this study involved incubating 1 ng of end-labeled 3’ mutated ITR fragment with approximately 50 ng of wild type *Himar1* transposase. Plasmids containing chemically similar mutations were chosen for these tests. The wild type ITR excised from plasmid pBB9 was always used as a positive control for these assays.
Since the null data collected from the papillation assays lacked a clear-cut pattern, I chose to test those positions that were affected by all three nucleotide substitutions (positions 8, 11, 12, 20, 22, 24, 26, 27, and the second flanking nucleotide). I also tested specific positions found in the different regions of the mariner ITR sequence logo (positions 2, 5, and 15). Position 2 for instance is found at the external end of the ITR, which is a variable region of the sequence, whereas positions 5 and 15 are the most conserved positions in the entire sequence logo. Not all of the data is found in a single figure because I decided to test particular positions based on the results of the gel shifts that I performed presuming I would find some pattern in the data. Figure 18 shows a 6.5% native polyacrylamide gel exhibiting both bound and unbound wild type ITR from pBB9. At position 12 (lane 4) when an “A” is substituted for the wild type “G” the MBP-Himar1 transposase does not bind to the ITR indicating that this substitution creates a DNA-binding mutant. However, when a “T” is substituted for a “C” at position 20 (lane 6) the MBP-Himar1 transposase binds to the mutated ITR indicating that this position is likely to be involved in another step of the transposition process.

Figure 19 contains the DNA-binding assays of those ITRs mutated at positions 2, 8, and 11. Since the chemically similar substitution of “T” at position 2 did not affect transposition, I chose to test a mutated ITR that contained a “G” at this position (lane 4). When a “G” is substituted for the wild type “C”, the MBP-Himar1 transposase does not bind to the ITR, indicating that this substitution causes the ITR to be deficient in binding to the transposase. However, when chemically similar substitutions were made at positions 8 and 11 (lanes 6 and 8) the MBP-Himar1 transposase binds to the mutated ITR.
suggesting that these positions may be involved in other steps in the transposition process.

Figure 20 contains the DNA-binding assays of those mutated ITRs at positions 5, 15, 26, 27, and the second flanking DNA (a). Since an “A” substituted for a “G” at position 5 did not effect transposition, a mutated ITR containing a “C” at this position was tested. The other positions tested contained chemically similar substitutions. Only position 15 (lane 6) exhibited a lack of DNA-binding.

Figure 21 contains the DNA-binding assays of positions 22 and 24. When ITRs containing chemically similar substitutions at these positions were tested in this assay, DNA-binding did not occur (lanes 4 and 6). Figure 22 summarizes the results obtained during these studies. Positions 2, 12, 15, 22, and 24 failed to bind to DNA when mutated while positions 5, 8, 11, 20, 26, 27, and the second flanking nucleotide were unaffected.
Figure 18. Gel shift showing a DNA-binding mutant at position 12 in the Himar1 ITR. Lanes 1 and 2 contain the WT ITR control; unbound and bound DNA respectively. Lanes 3 and 4 contain an ITR with a G to A transition at position 12. Lane 4 shows that the MBP-Himar1 transposase fusion protein does not bind to the ITR mutated at position 12. Lanes 5 and 6 contain an ITR with a C to T transition at position 20. Lane 6 shows that the MBP- Himar1 transposase does bind to this mutated ITR.
Figure 19. Gel shift showing a DNA-binding mutant at position 2 in the Himar1 ITR. Lanes 1 and 2 contain the WT ITR control; unbound and bound DNA respectively. Lanes 3 and 4 contain an ITR with C to G transversion at position 2. Lane 4 shows that the MBP-Himar1 transposase fusion protein does not bind to the ITR mutated at this position. Lanes 5 and 6 contain an ITR with a G to A transition at position 8. Lanes 7 and 8 contain an ITR with a T to C transition at position 11. Lanes 6 and 8 show that the MBP-Himar1 transposase does bind to both mutated ITRs.
Figure 20. Gel shift exhibiting a DNA-binding mutant at position 15 in the Himar1 ITR. Lanes 1 and 2 contain the WT ITR control; unbound and bound DNA respectively. Lanes 3 and 4 contain an ITR with a G to C transversion at position 5. Lanes 5 and 6 contain an ITR with an A to G transition at position 15. Lane 6 shows that the MBP-Himar1 transposase fusion protein does not bind to the ITR mutated at position 15. Lanes 7 through 12 contain ITRs with chemically similar substitutions at positions 26, 27, and the second flanking nucleotide. Lanes 4, 8, 10, and 12 show that the MBP-Himar1 transposase does bind to these mutated ITRs.
Figure 21. Gel shift showing DNA-binding mutants at positions 22 and 24 in the Himar1 ITR. Lanes 1 and 2 contain the WT ITR control; unbound and bound DNA respectively. Lanes 3 and 4 contain an ITR with a C to T transition at position 22. Lanes 5 and 6 contain an ITR with a G to A transition at position 24. Both lanes 4 and 6 show that the MBP-Himar1 transposase fusion protein does not bind to these mutated ITRs.
Figure 22. Summary of the results obtained from the gel shift mobility DNA-binding assays. This figure includes the mariner ITR sequence logo. The Himar1 ITR sequence is located below the logo. The arrows above the sequence represent those positions that were tested in the assay. The green arrows point to those positions in the ITR that bind to DNA. The red arrows point to those positions that were null for DNA-binding.

**Strand-Specific Cleavage Assays**

Once the mobility shift DNA-binding assays were complete, those mutated ITRs that still exhibited DNA-transposase binding were tested in a strand-specific cleavage assay. Since these mutated ITRs bind to the transposase, it is possible that the mutations inhibit additional steps in the transposition mechanism, such as DNA cleavage, from occurring.

The strand-specific cleavage assay utilizes a denaturing polyacrylamide sequencing gel to visualize sites of DNA cleavage catalyzed by the transposase. The Maxam-Gilbert sequencing standards also run on the gel enable one to determine where in the sequence cleavage is occurring. Similar to DNA-binding, these assays involve the
incubation of an end-labeled DNA fragment with a specific protein at 30 °C for 2 hours. Following incubation, the reaction is stopped and the DNA is extracted using phenol:chloroform. The DNA is precipitated, dried, and resuspended in formamide loading buffer. Prior to loading the reaction on the gel the specific radioactivity of each reaction was measured to ensure that the same number of counts were being loaded into each lane.

During this study, 100,000 c.p.m. of end-labeled 3’ mutated ITR top strand fragments and bottom strand fragments were incubated with 60 nM of wild type MBP-

Himar1 transposase. The second flanking nucleotide and positions 5, 8, 11, 20, 26, and 27 were chosen for these tests. All positions except for positions 5 and 26 contained chemically similar mutations at their respective positions. Plasmid pBB19 containing a “C” instead of the wild type “G” at position 5 in the ITR was chosen because an “A” at this position did not effect transposition during the genetic screen. Plasmid pBB25 containing a “G” instead of the wild type “C” at position 26 in the ITR was chosen because the original mutant used would revert to the wild type “C” from “T”. The wild type 3’ ITR was used as the positive control for these tests as well as the Maxam-Gilbert sequencing standard.

Figure 23 shows the cleavage assays of the top strand of DNA for those ITRs mutated at the second flanking nucleotide and positions 5, 8, 11, 20, and 27. This analysis shows that the MBP-Himar1 transposase cleaves at two sites near the end of each of the mutated ITR similar to the wild type ITR (Lampe et al. 1996). However, the degree of cleavage varies in the mutants when compared to the wild type. For instance, cleavage of the wild type ITR is approximately 4x more intense than those of the ITRs
mutated at positions 5, 8, and 11, indicating that cleavage of these ITRs is not as efficient. Cleavage of the ITR containing the mutation in the second flanking nucleotide and a mutation at position 20 is comparable to the cleavage of the wild type ITR.

Figure 24 shows the cleavage assays of the bottom strand of DNA for those ITRs mutated at the second flanking nucleotide and positions 5, 8, 11, 20, and 27 as well as the wild type ITR. This analysis shows that the MBP-

Himar1 transposase cleaves the bottom strand at one specific site near position 1 of each of the mutated ITRs like the wild type ITR. Once again, cleavage of the wild type ITR is strongest. Using the Molecular Analysis software, the densitometry of the bands was measured. There appears to be a 4-fold decrease in cleavage seen in the second flanking DNA and at positions 8, 11, and 27. Whereas there is a 2-fold decrease in cleavage seen in the ITRs containing mutations at positions 5 and 20 when compared to the cleavage of the wild type ITR. Although the differences in DNA cleavage are interesting, figure 16 presented additional features that were of great interest. Above the bright bands were the DNA fragment begins to run on the gel is a single band for each fragment. This band is not in the well and could potentially be a hairpin intermediate formed as the bottom strand was cut by the transposase. Further work is currently being done to begin characterizing this band.

Figure 25 shows the cleavage assays of the top and bottom strands of the Himar1 ITR containing a single point mutation at position 26. An ITR containing a "G" substituted for the wild type "C" was used in the assay as opposed to the chemically similar mutation "T", because during the PCR of this DNA fragment, the mutation reverted back to the wild type "C". Dozens of colonies were sequenced and they
Figure 23. Top (nontransferred) strand cleavage of the Himar1 ITR. A>C is the Maxam-Gilbert sequencing standard used. The sequencing reaction was performed on the top strand of the wild type ITR. The numbers are the nucleotide position relative to the 5’ end of the ITR. The reactions were performed in buffer containing Mn^{2+}.
Figure 24. Bottom (transferred) strand cleavage of the Himar1 ITR. A>C is the Maxam-Gilbert sequencing standard. The sequencing reaction was performed on the bottom strand of the WT ITR. The numbers are nucleotide position relative to the 5' end of the ITR. The reactions were performed in buffer containing Mn$^{2+}$. The arrow at the top right of the picture is pointing to a possible transposition intermediate formed, perhaps a hairpin.
consistently exhibited a "C" in this position. Finally, it was decided to try an ITR containing a different mutation. This analysis shows that the MBP-\textit{Himar1} transposase cleaves the top strand of the mutated ITR at two specific sites similar to the wild type (figure 25A). Cleavage of the top strand of the mutated ITR is significantly less than that of the wild type. Figure 25B shows that the MBP-\textit{Himar1} transposase cleaves the bottom strand as well near position 1 of the ITR. Once again, cleavage of the mutated ITR is not as strong as the wild type.

A G sequencing standard was also run on this gel because it was determined in figure 26 that the "G" was also present in the A>C standard of the pGEM-3zf(+) fragment. I wanted to see if this was also occurring when I conducted the A>C reactions of the \textit{Himar1} ITR fragment. I found that the "G" was also appearing in the A>C reactions of the \textit{Himar1} ITR fragments for reasons that I do not understand.

**Preliminary Investigation of \textit{Himar1} Hairpins**

The presence of products of a higher molecular weight than the input DNA on the gels exhibiting bottom strand cleavage could be potential hairpins. A set of strand-specific cleavage assays were performed to determine the size of these bands. Based on the size of the PCR product used for the cleavage assays, the size of a hairpin, if it exists, should be between 166-169 bp. Figure 26 represents a gel run in order to determine the size of the band in question and if this band was present when Mg$^{2+}$ was substituted for Mn$^{2+}$. This analysis shows that the band in question appears only in the presence of Mn$^{2+}$. The A>C and G standards of the 317 bp pGEM-3zf(+) fragment loaded into the
Figure 25. Strand-specific cleavage of the Himar1 ITR mutated at position 26.

A. Top (nontransferred) strand cleavage of the Himar1 ITR mutated at position 26. The cleavage reactions were performed in the presence of Mn$^{2+}$.
B. Bottom (transferred) strand cleavage of the Himar1 ITR mutated at position 26. The cleavage reactions were performed in the presence of Mn$^{2+}$. 
Figure 26. Bottom (transferred) strand cleavage of the Himar1 ITR to determine the size of the hairpin. A 317 bp EcoRI/SacI fragment from pGEM-3zf(+) was used as a size standard. The reactions were performed on the bottom strand of the ITR in the presence of Mn$^{2+}$ or Mg$^{2+}$. The positions along the standard indicate the size of the intermediates formed.
Figure 27. Strand-specific cleavage of the top and bottom strands of the Himar1 ITR by the mutated transposase E100A. The cleavage reactions were performed in the presence of Mn$^{2+}$. A>C and G are the Maxam-Gilbert sequencing standards used.
gel helped to determine the size of the band. The band of interest appears to be slightly smaller than it should be if this is indeed a hairpin. The band is approximately 163 bp. This means that if this is a hairpin, it is formed too far out in the flanking DNA to be a product of normal transposition.

To further characterize the higher molecular weight products, the bottom strand of the wild type Himar1 ITR was incubated with Himar1 mutant transposase E100A. E100 is located in the helix-turn-helix motif of the DNA-binding domain. Previous work has demonstrated that when this position is mutated to an alanine, DNA-binding still occurs but DNA cleavage only occurs on the top strand of the ITR (D. Lampe, unpublished). Therefore, I wanted to determine if the higher molecular weight product still forms when the ITR is incubated in the presence of E100A. Figure 27 is an analysis of the mutated transposase E100A and its effect on the bottom strand of the wild type Himar1 ITR. This analysis shows that E100A transposase cleaves the top strand, but not the bottom strand. Even though the mutated transposase does not cleave the bottom strand, the higher molecular weight product still forms. The data illustrated in figures 26 and 27 suggest that if this product is a hairpin, it is an intermediate that forms outside of the normal transposition process.

**In vitro Transposition Assay**

Although positions 5, 8, 11, 20, 26, 27, and the second flanking nucleotide tested in the strand-specific cleavage assays exhibited inefficient cleavage compared to the wild type, they were for the most part relatively unaffected by the mutations at these positions. It seemed that this level of activity should have produced papillae in the genetic screen.
Therefore, the final step to test for in the transposition mechanism was insertion of the element into the target site.

The in vitro transposition assay is used to measure the ability of the transposase to insert a radiolabeled DNA fragment containing the Himar1 ITR into a supercoiled target DNA. The transposition event linearizes the target DNA and labels it with \(^{32}\)P. Transposition can easily be detected by autoradiography or phosphorimaging (Lampe et al. 1999). The assay is performed in the same manner as the strand-specific cleavage assays except that the poly(dI)-(dC) were removed from the reaction. MBP-Himar1 transposase was mixed with a radiolabeled DNA fragment containing the 3’ ITR mutated at position 26 (plasmid pBB25; Table 1) and pGEM-3zf(+). The reactions were incubated at 30 °C for 2 and 4 hours. Control reactions were set up that contained the radiolabeled wild type 3’ ITR. Additional controls were prepared for both sets of reactions that contained no transposase. Following incubation the reactions were terminated and loaded into a 0.5% 1X TAE-agarose gel.

Figure 28 shows the in vitro transposition assay for pBB25 run on a 0.5% 1X TAE-agarose gel. After the gel was dried, it was exposed to autoradiographic film for five hours. A "G" substituted for the wild type "C" at position 26 causes this mutant to be null for target site insertion. The wild type control was positive for target site insertion. Since a radiolabeled DNA ladder was not run on this gel, an UV fluorescent ruler was used to determine if the transposition product obtained was the correct size. Prior to drying the gel, the gel was photographed under UV light in order to visualize the 1 kb ladder and the bands obtained from the reactions. After the gel image was developed on autoradiographic film, the ruler was used to measure the locations of the bands obtained.
for the wild type reactions. They were found to be in the same location as the DNA bands visualized under UV light. Therefore, it was inferred that the transposition products were the correct size. Although the other sites that were tested in the strand-specific cleavage assays that were also unaffected have yet to be tested in the in vitro transposition assay, these mutants are also expected to be null for target site insertion.

Figure 28. In vitro transposition assay showing an integration mutant at position 26 of the Himar1 ITR. Wild type (control) and mutant ITR DNA was incubated in the presence of 60 nM MBP-Himar1 transposase for 2 and 4 h. DNA incubated without MBP-Himar1 transposase also served as controls. The 3' wild type ITR incubated with transposase exhibited bands approximately 3.0 kb in size.
DISCUSSION

Mariners are a diverse and widespread family of eukaryotic transposons. The mariner transposon encodes a single protein, the transposase, and is flanked by short inverted terminal repeat (ITR) sequences. Mobilization of mariners occurs via a cut-and-paste mechanism, which is catalyzed by the transposase. Transposition in vitro requires only a purified transposase, a suitable donor transposon construct, and a target DNA molecule. Due to their ability to horizontally transfer and their occurrence in many eukaryotic phyla mariner transposition does not seem to require species-specific host factors.

Although the mechanism of mariner transposition is not entirely understood, progress has been made in understanding transposition in their closest relatives, the Tc1 family of transposons (Lampe et al. 1996). A significant amount of work has also been done to understand transposition in bacterial elements such as Tn5 and Tn10. Transposition in each case not only requires the complex regulatory protein, the transposase, but also the specific cis-acting DNA sequences, the ITRs. ITRs are important in transposition for several reasons. ITRs function as the site of transposase binding as well as the DNA breakage and joining reactions that constitute transposition.

Much of the information that exists on mariner ITRs has been determined by deletion analysis or by mutational analysis of the terminal nucleotides. The objective of this research was to mutagenize the entire Himar1 3’ ITR in order to determine which positions within the ITR are important to transposition. The results obtained from this study have provided a significant amount of information concerning the role of the
Himar1 ITRs in transposition. The interpretation of this data has been based upon comparisons made to a mariner ITR sequence logo as well as research done in other mariner elements such as Mos1 and in other systems such as Tn5 and Tn10.

**Mutagenesis of the Himar1 3’ Inverted Terminal Repeat**

Single nucleotide substitutions were introduced at each position of the 27 bp Himar1 3’ ITR and the two flanking nucleotides (ta). The effect that these substitutions had on transposition was tested in an *Escherichia coli* based genetic screen known as the papillation assay. Twenty-three percent of the nucleotide substitutions made had no effect on transposition suggesting that other sequence combinations can exist that still promote transposition similar to that of the wild type. When the graph of positions that exhibited a wild type phenotype (Figure 12) was compared to the mariner ITR sequence logo, these substitutions were found not only in variable regions of the logo but also in conserved regions such as positions 4 to 8 and 14 to 18. Only 11% of the substitutions made caused a hypomorphic phenotype. Sixty-five percent of substitutions made in the ITR caused a null phenotype. It appears that in general those positions that were most affected by single nucleotide substitutions lie within regions of the ITR sequence logo that are highly variable, for instance, positions 11, 12, 20, and 24 – 27.

The data obtained during the genetic screen did not appear to fit my original hypothesis on the structure of the Himar1 ITR (Figure 29). For instance, two highly conserved positions in the ITR sequence logo, positions 5 and 15 exhibited null phenotypes when only two out of the three-nucleotide substitutions were made. When a chemically similar mutation at position 5 was made transposition was not affected where
as transposition was affected when a chemically different substitution was made at position 15. Since positions 5 and 15 are one helical turn away from each other, it was suggested that the transposase might be contacting the DNA on one face of the ITR in two locations of the major groove (Lampe et al. 2001). Why there is a difference between the two positions is unclear, but obviously the alteration of the chemical makeup at one position has a greater effect on transposition than it does at the other position.

Figure 29. A sequence logo of mariner family ITRs. The height and the order of the bases in a particular stack are representative of their importance at that position in the alignment.

Wild Type Himar1 ITR is Optimal for Transposition

Unlike other systems such as Tn5 that have isolated hyperactive mutations in the inverted terminal repeats, hyperactive mutations were not isolated in the Himar1 ITR during the genetic screen. Mutations in the ITR that cause it to bind to the transposase better than the wild type ITR would suggest that the wild type ITR has sub-optimal
binding capabilities. However, this does not appear to be the case in Himar1. From an application standpoint this is unfortunate in regards to utilizing Himar1 as a genetic tool in other organisms. Greater activity would allow Himar1 to be utilized more easily. Lampe et al. (1999) identified two hyperactive mutations in the Himar1 transposase. Mutants A7 (H267R) and C9 (Q131R/E137K) were found to be significantly more active in E. coli than the wild type transposase when mating-out assays were performed. The level of activity increased as much as 50-fold in comparison to the wild type. When Q131R and E137K were combined they acted synergistically rather than additively increasing transposition frequency 50-fold. Clearly, the combination of a hyperactive transposase with ITRs containing hyperactive mutations would make Himar1 a more efficient genetic tool.

Although the wild type Himar1 ITR is optimal for transposition, this is not the case in the closely related Mos1 element. It has been demonstrated in Mos1 that the wild type ITRs are sub-optimal for transposition. The inverted repeats that flank the Mos1 transposon differ in sequence at four positions. Each of these positions has an impact on transposition. In vitro transposition assays demonstrate that Mos1 transposase binds to the 3’ ITR better than the 5’ ITR. When the Mos1 transposon is flanked by two 3’ ITRs transposition is enhanced and stable transposition products are obtained (Augé-Gouillou et al. 2001).

Identification of DNA-binding Mutants

Gel mobility shift DNA-binding assays were performed on those ITRs that were mutated at positions where transposition was greatly affected. Three additional ITRs containing mutations at positions 2, 5, and 15 were also chosen. The results of these
assays showed that transposase specifically binds to positions 2, 12, 15, 22, and 24 of the Himar1 ITR since binding was eliminated by mutations at these positions. Four out of five of these positions are found in variable regions of the ITR sequence logo suggesting that nonconserved positions in the ITR strongly influence transposase binding. This data is supported by gel retardation experiments conducted on natural Mosl ITR mismatches at positions 1, 16, 18, and 26. DNA-binding assays here showed that the transposase bound with only moderate affinity when nucleotide substitutions were made at positions 1, 18, and 26 in the Mosl 3’ ITR. However, when a guanine was substituted for a thymine at position 16 of the 3’ ITR, a decrease in transposase binding similar to that obtained for the wild type 5’ ITR was observed. According to the mauritiana mariner-like element sequence data, these positions are not highly conserved, suggesting that these positions also strongly influence DNA binding (Augé-Gouillou et al. 2001). Two of these positions are found in variable regions (e.g. positions 1 and 26) and two of these positions are found in conserved regions (e.g. positions 16 and 18) of the mariner ITR sequence logo. Despite the range in sequence comparisons both Mosl and Himar1 data sets demonstrate that variable regions of the ITR strongly influence DNA binding.

Position 15, one of the most conserved positions in the ITR sequence logo, requires the wild type nucleotide (A) at that position in order for the Himar1 transposase to bind and transposition to occur. When the ITR containing a mutation at position 5, (another strongly conserved position), was tested in the DNA-binding assay no mutation affected DNA binding suggesting that this position is not required for the formation of the initial monomer complex and may be involved in secondary transposition functions such as DNA cleavage or target site insertion.
Even though most positions in the Himar1 ITR were not tested directly for DNA-binding, it is apparent that the results obtained do not correspond to my initial hypothesis. The hypothesis stated that those regions of the mariner ITR sequence logo that were highly conserved were involved in base-specific contacts with the transposase and those regions of the logo that were highly variable were involved in secondary functions of transposition. However, some of the DNA-binding mutants that were isolated were found near the internal end of the Himar1 ITR. This is interesting because one of the regions that the Tn5 transposase binds to is located near the internal end of its outside end (OE). Jilk et al. (1996) reported that the Tn5 transposase binds to two nonsymmetrical regions of the OE. These include positions 6 to 9 and 13 to 19. Positions 13 and 19 lie toward the internal end of the Tn5 inverted terminal repeat. Positions 22 and 24 also lay near the internal end of the Himar1 ITR suggesting that this area of the ITR have a specific role in DNA-transposase binding. However, if this were true, this role would not extend passed position 26 of the ITR because it was determined during the DNA-binding assays that this position is not directly involved in specific transposase binding. The same results were obtained for the ITR mutated at position 27. Similar to the ITR mutated at position 5, these results suggest that positions 26 and 27 could be involved in secondary transposition functions. It can be inferred that the bases adjacent to positions 22 and 24 could also be involved in making specific contact with the transposase. The same could be said of positions 12 and 15. Another region of the ITR that could potentially be involved in making specific contact with the transposase could lie between bases 12 and 15.
The ITR mutated at position 2 was also found to be a DNA-binding mutant. This result does not fit with results obtained in Tn5 that suggest that position 2 in this system is required for synaptic complex formation. In Tn5, hydroxyl radical footprinting and interference techniques have demonstrated that all positions except for position 1 are contacted by the transposase in the pre-cleavage synaptic complex. However, positions 2 – 5 were found to be specifically required for the formation of the synaptic complex even though these positions are not required in the formation of the initial monomer complex (Bhasin et al. 2000). In the case of Himar1, position 2 is required in the formation of the initial monomer complex. Studies done in Mos1 suggest that the synaptic complex exists but only under catalytic conditions. Dawson et al. (2003) demonstrated that top strand cleavage occurs independently of the synaptic complex in a manner similar to V(D)J recombination. If this is the case, then positions at the external end of the Himar1 ITR could potentially be required for initial monomer complex formation. Figure 30 is a summary of the proposed mechanisms for V(D)J recombination and the Tn5 transposition mechanism discussed here.

The pattern of DNA-binding mutants in figure 22 suggests a possible physical explanation for my results. As was previously mentioned, positions 2, 12, 15, 22, and 24 were null for DNA-binding. These positions happen to be approximately 10 bp apart from each other, which is approximately one helical turn of DNA. These results suggest that the transposase is making contact with the DNA on one face of the Himar1 ITR over two turns. It was previously thought that since positions 5 and 15 (also one helical turn from each other) of the mariner ITR sequence logo were highly conserved, the transposase was making specific contacts at these positions (Lampe et al. 2001).
However, my results indicate that these base-specific contacts are occurring in the more variable regions of the ITR sequence logo.

**Himar1 Transposase Cleaves Mutated ITRs Inefficiently**

Strand-specific cleavage assays were performed on those mutated ITRs that were not DNA-binding mutants in order to determine if they were deficient in the DNA cleavage step of transposition. All of the mutated ITRs were cleaved by the Himar1 transposase, but they were not cleaved as efficiently as the wild type ITR. This was particularly evident in those ITRs mutated at positions 5, 8, and 11 as well as in the bottom (transferred) strand for each mutated ITR that was tested. These results for the most part suggest that these positions when mutated do effect DNA-transposase binding and strand cleavage, but they do not eliminate either step. Dawson *et al.* (2003) suggested that each strand might possess different requirements for cleavage as is indicated by the lack of coupling between the first and second strand cleavage seen in *Mos1*. Because there were differences in band intensity between the top and bottom strands of the Himar1 ITR, differing requirements in cleavage could account for this.

The results obtained from the all the assays performed on the ITR mutated at the second flanking nucleotide are interesting because its apparent role in transposition has yet to be determined. During the genetic screen when all three nucleotide substitutions
Figure 30. The proposed mechanisms for V(D)J recombination and Tn5 transposition. It has been proposed that **mariners** such as *Mos1* and *Himar1* transpose in a mechanism similar to V(D)J recombination where cleavage of the nontransferred strand occurs independently of the synaptic complex formation.

were made at this position a null phenotype was exhibited. The ITRs mutated at this position also demonstrated that the transposase could bind to the DNA and that the ITR could be cleaved. In *Tc1*, when the flanking TA dinucleotides are mutated are mutated on one side, excision of the element is not detectable. However, in *Tc3* the TA dinucleotides are not required for efficient transposition of the element. This suggests a
difference in requirements for target site selection between Tc1 and Tc3 (Fischer et al. 1999). According to the genetic data for Himar1, transposition still occurs despite the presence of point mutations at the first flanking nucleotide. It is unclear in Himar1 why there is a dramatic effect on transposition when mutations were introduced at the second flanking nucleotide in comparison to the first flanking nucleotide. Both DNA-binding and strand-specific cleavage assays have exhibited that the transposase binds and cleaves the ITR despite the presence of a mutation at the second flanking nucleotide. However, element insertion at the target site has yet to be tested on this mutant. The role of the first flanking nucleotide in transposition is unclear. We do know that the presence of the TA dinucleotide is a key component of efficient transposition. Perhaps the first flanking nucleotide’s role in transposition in vitro differs from its role in vivo.

Formation of a Hairpin Intermediate

The strand-specific cleavage assays of the bottom (transferred) strand of Himar1 ITR exhibited a single, band of a higher molecular weight than the input DNA on the denaturing gels. The presence of this band suggested that some intermediate or additional product was being formed during Himar1 transposition. Both Tn5 and Tn10 cleavage reactions proceed via a hairpin intermediate (Bhasin et al. 1999; Kennedy et al. 1998). A great deal of biochemical and structural information has been collected regarding the three catalytic steps involved in DNA cleavage in bacterial elements such as Tn5. The three catalytic steps are 3’-strand nicking, hairpin formation, and hairpin resolution (Reznikoff, 2003). The proposed model for DNA cleavage via a hairpin (Figure 31) in bacterial elements first involves a 3’ nick at the OE. The free 3’ hydroxyl
attacks the 5’ end forming a hairpin at the transposon end. The flanking donor DNA is released. In order for strand transfer to occur, the hairpin must be resolved. Resolution of the hairpin occurs by hydrolytically cleaving the hairpin in a step similar to the initial 3’ nick that was initially made. A 3’ hydroxyl is freed and then joins to the target DNA in a strand transfer reaction similar to hairpin formation (Bhasin et al. 1999). The model of hairpin formation in Himar1 is similar to that of Tn5 however, the 5’ end of the top (nontransferred) strand is nicked first. The 3’ hydroxyl then attacks the 3’ end of the bottom strand forming a hairpin of the flanking DNA. It was proposed that cleavage of Himar1 proceeds through a hairpin intermediate in a manner that is similar to that of V(D)J recombination but unlike Tn5 and Tn10 (Lipkow and Chalmers, unpublished; D. Lampe, unpublished).

Dawson et al. (2003) failed to detect the formation of a hairpin in Mos1 and suggest that second strand cleavage occurs via hydrolysis rather than through a hairpin. They were, however, able to detect an abnormal hairpin that was missing the sequence immediately flanking the cleavage site at the ends of the element. Dawson et al. (2003) were able to detect hairpins under conditions that would enhance the levels of cleavage (i.e. Mn$^{2+}$ was used). The potential hairpins that I discovered were also detected in the presence of Mn$^{2+}$ but not with Mg$^{2+}$ supporting the results obtained by Dawson et al. (2003). Similarly, the potential hairpin discovered during this study also appears to be
Figure 31. The proposed mechanisms for the formation of a hairpin intermediate during cleavage of the ITR in both Tn5 and Himar1. Tn5 on the left is initially cleaved at the 3’ end of the bottom (transferred strand) and forms a hairpin on the element sequences. Himar1 on the right is initially cleaved at the 5’ end of the top strand (nontransferred strand) and forms a hairpin on the flanking DNA.

Smaller than expected. If the hairpin consists of only the flanking DNA it would be approximately 166 bp. If the hairpin includes the flanking DNA plus the three terminal nucleotides of the ITR then it would be 169 bp. The hairpin I have found is approximately 163 bp.

Site E100 of the Himar1 transposase is located in the helix-turn-helix (HTH) motif of the DNA-binding domain. When E100 is mutated to an alanine, Lampe et al.
(unpublished) have demonstrated that this mutant is capable of DNA binding, but it is deficient in transferred strand cleavage. Therefore, I chose to test E100A under the conditions used in the strand-specific cleavage assays to determine if a single, higher molecular weight band would be present even though transferred strand cleavage does not occur. I found that even though E100A does not cleave the transferred strand, an intermediate of a higher molecular weight, the hairpin, was formed at approximately the same intensity as the wild type. If hairpins were normal intermediates of transposition, I would have expected them to accumulate using this mutant. These results suggest the hairpin is an intermediate that forms that is not part of the normal transposition process.

**A Himar1 Mutant ITR Fails to Integrate into the Target Site**

The genetic screen demonstrated that when the three-nucleotide substitutions were made at position 26 of the Himar1 ITR, transposition did not occur. However, both the DNA-binding and strand-specific cleavage assays demonstrated that DNA binding and cleavage are not affected by mutations at this position. An in vitro transposition assay was performed to test for element insertion into the target site. It appears that position 26 is required for insertion of the element into the target DNA. This particular result does fit with the initial hypothesis that stated that variable regions of the mariner ITR sequence logo are involved in secondary transposition functions. Although the other ITRs that contain mutations at positions 5, 8, 11, 20, 27, and the second flanking nucleotide have yet to be tested in this assay, it is likely that these positions are also involved in target site insertion.
Coevolutionary Changes Between the Transposasae and the ITR Appear to be Rare

In order to determine the degree to which minor changes in the ITRs can disrupt transposase binding and whether these changes can be compensated for by mutations in the transposase, a transposase-ITR mutation suppressor screen was performed. Thousands of colonies were screened and transposition products were not recovered when the Himar1 transposase was randomly mutagenized nor was transposition detected when two positions in the transposase (M86 and R101) were randomly mutagenized. These results suggest that coevolutionary changes occurring in the same element are rare and that mariner family diversity probably occurs as a result of the first path of the horizontal transfer model proposed by Lampe et al. (2001).

This screen may not have been successful because of the mutated ITRs chosen (e.g. ITRs mutated at positions 12 and 20). One mutant was a DNA-binding mutant and the other is a transpositional mutant that has yet to be determined. Perhaps if ITRs that had been mutated at positions 1 or 14 (particular mutations at these positions only decrease transposition) had been chosen, different results would have been observed. While interactions between mariner elements have been investigated, element lineages within a single species have not been examined. This type of analysis could provide useful information regarding coexisting elements that contain particular fixed ITR changes and fixed transposase-coding sequence changes (Lampe et al. 2001). This information then could enable us to determine which positions are best in both the ITR and the transposase to test in the transposase-ITR mutation suppressor screen.
REFERENCES


