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A purified *mariner* transposase is sufficient to mediate transposition *in vitro*

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***Mariners* are a widespread and diverse family of animal transposons. Extremely similar *mariners* of the irritans subfamily are present in the genomes of three divergent insect host species, which strongly suggests that species-specific host factors are unnecessary for mobility. We tested this hypothesis by examining the activity of a purified transposase from one of these elements (*Himar1*) present in the horn fly, *Haematobia irritans*. *Himar1* transposase was sufficient to reproduce transposition faithfully in an *in vitro* inter-plasmid transposition reaction. Further analyses showed that *Himar1* transposase binds to the inverted terminal repeat sequences of its cognate transposon and mediates 5′ and 3′ cleavage of the element termini. Independence of species-specific host factors helps to explain why *mariners* have such a broad distribution and why they are capable of horizontal transfer between species.**

Keywords: host factors/*mariner*/transposase/transposition assay/transposon

Introduction

The original *mariner* element described was a small (~1.3 kb) DNA-mediated (Class II) transposable element encoding a single protein (*mariner* transposase) flanked by short inverted terminal repeat sequences (ITRs) of 28 bp (Jacobson *et al.*, 1986; Medhora *et al.*, 1991). This element is now known to be a member of a very diverse family of transposons, all of which have been called *mariners* or *mariner*-like elements (MLEs). They are known from a wide diversity of insects, as well as nematodes, flatworms and, recently, humans (for reviews, see Robertson, 1995; Robertson and Asplund, 1996). Extremely similar *mariners* can occupy the genomes of species even in different phyla, indicating that these elements recently were horizontally transferred into their genomes (Robertson, 1993; Robertson and MacLeod, 1993; Garcia-Fernàndez *et al.*, 1995; Lohe *et al.*, 1995). A particularly striking example of this phenomenon occurs in the irritans subfamily of *mariners*, where two flies in different suborders (>200 million years diverged) and a green lacewing (>265 million years diverged from the flies) each contain *mariners* whose consensus sequences encode transposases that differ from each other by no more than six amino acids out of 348 (Robertson and Lampe, 1995). From these data, we hypothesized that *mariner* transposition relies solely on the *mariner* transposase and not species-specific host factors beyond those necessary to transcribe and translate the transposase and host repair enzymes necessary to repair single-stranded gaps at the sites of transposon insertions. This situation is in contrast to what is known for some other transposable elements. A well-studied example is transposition of the P-element which involves a host-encoded 'inverted terminal repeat binding protein' that is thought to be at least one factor limiting its host range to the Drosophilidae (Kaufman *et al.*, 1989; Beall *et al.*, 1994).

Few details are available regarding the mechanism of *mariner* transposition. Progress has been made, however, towards understanding the transposition of their closest relatives, the *Tc1* family of elements, and more distant relatives. The *Tc1* family of transposons is similarly diverse, with members in nematodes, flies, fungi and fish (for reviews, see Robertson, 1995; Robertson and Asplund, 1996) and is the sister family of *mariners*, sharing 18–25% amino acid identity in their transposases. Together, these transposon families are more distantly related to the *Tec* and *TBE* transposons of ciliates and the *IS630* and *IS3* families in bacteria (Doak *et al.*, 1994). These distant relationships are based on a shared, presumed catalytic, D,D35E domain in their encoded transposases, which is also present in Tn*7*, Tn*10* and *Mu* transposases, as well as retroviral and retrotransposon integrases (see Craig, 1995; Grindley and Leschziner, 1995).

Purified transposases of both *Tc1* and *Tc3* from the nematode *Caenorhabditis elegans* bind the ITRs of their cognate elements (Vos *et al.*, 1993; Colloms *et al.*, 1994). *In vivo* data gathered for *Tc3* show that transposition is accompanied by the appearance of a linear transposition intermediate that lacks two nucleotides on the 5′ ends (van Leunen *et al.*, 1994). These intermediates also contain 3′ hydroxyl groups presumably used as nucleophiles in the strand transfer reaction that forms the covalent bond between the transposon ends and the target site when the element is integrated. These and other data led van Leunen *et al.* (1994) to propose a cut-and-paste model for *Tc3* transposition similar to that of bacterial transposons like Tn*10*, that might also be applied to *Tc1* and *mariners* (see Figure 1). Most recently, this model has been confirmed for *Tc1* where transposase prepared from *C.elegans* nuclear extracts and overexpressed in bacteria is sufficient to complete transposition *in vitro* (Vos *et al.*, 1996).

We have purified a *mariner* transposase from an element belonging to the irritans subfamily of *mariner* transposons. The purified protein is able to support all of the activities necessary for transposition *in vitro*. It binds specifically to ITR sequences and cleaves element termini at both the 5′ and 3′ ends. The ability of this transposase to mediate

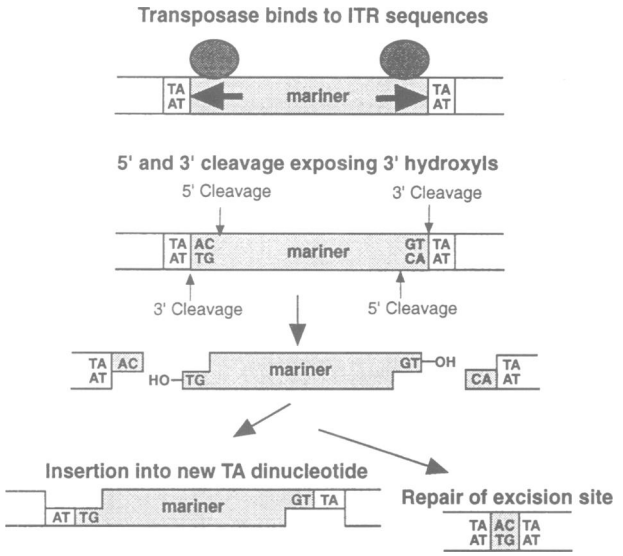


Fig. 1. Model for *Tc1/mariner* transposition. This figure is based on van Leunen *et al.* (1994) and work on other transposons (Mizuuchi, 1992b). White areas correspond to genomic DNA. Gray areas correspond to *mariner* sequences. Ovals representing transposase molecules are removed from subsequent steps for clarity but are assumed to be involved at each step of transposition.

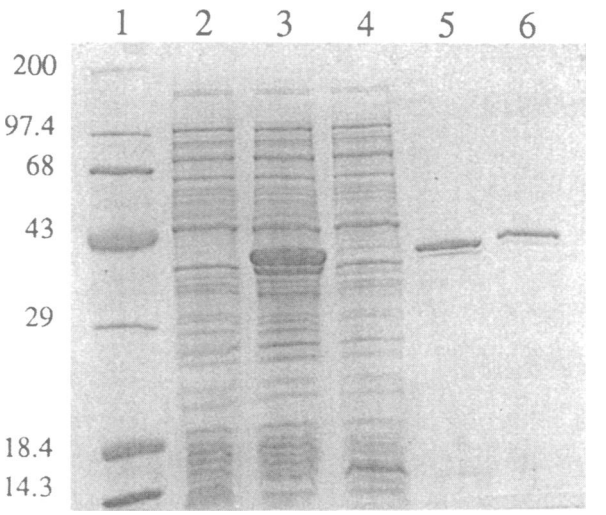


Fig. 2. Purification of *Himar1 mariner* transposase. SDS–PAGE analysis showing the steps in the purification of transposase. Molecular weight in kDa is indicated at the left. Lane 1, protein standards; lane 2, cells containing pET13a/*Himar1* before protein induction with IPTG; lane 3, cells containing pET13a/*Himar1* induced with IPTG after 1 h; lane 4, cells as per lane 3, soluble fraction; lane 5, cells as per lane 3, insoluble fraction; lane 6, purified transposase after washing, solubilization, DEAE chromatography and dialysis.

transposition *in vitro* by itself confirms the suggestion made by our distributional analysis of *mariner* elements that they are able to transpose independently of species-specific host factors. Moreover, these data suggest that *mariners* transpose by a mechanism similar to that proposed for the transposition of *Tc1* and *Tc3*. Interestingly, the substitution of Mn^{2+} for Mg^{2+} in an *in vitro* transposition assay significantly alters the target site specificity of the transposase, suggesting a role for the cation and the motif that binds it in target site selection.

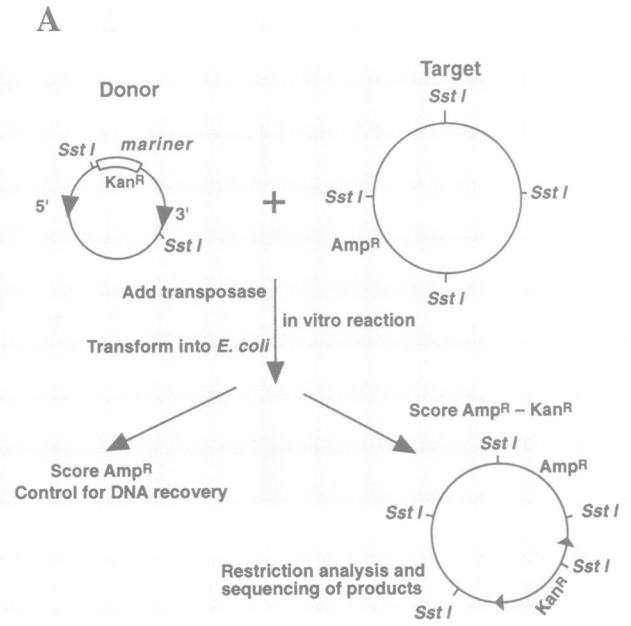
Results

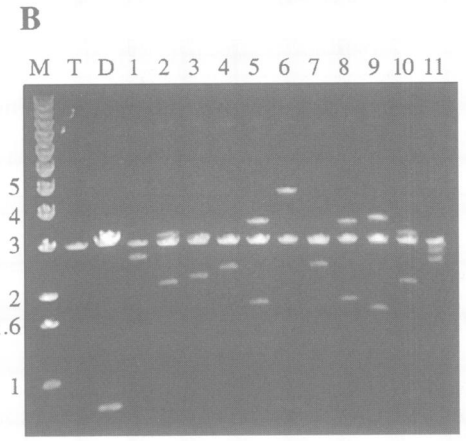
Purification and properties of Himar1 transposase

The horn fly, *Haematobia irritans*, contains ~17 000 copies of one type of *mariner* transposon (*Himar1*) implicated in a recent horizontal transfer into three insect species (Robertson and Lampe, 1995) (see Robertson and Asplund, 1996 for naming conventions for *mariners*). The copy number of *Himar1* in *H.irritans* is greater by two orders of magnitude than in either of the other two insect species. We thought it reasonable that at least some of this disparity in copy number could be due to the slight difference in amino acid sequence (and hence, perhaps, transposase activity) of *Himar1* when compared with those in the other species. *Himar1* was thus reconstructed by PCR and *in vitro* mutagenesis in order to obtain an element with high activity. The consensus created was the majority-rule consensus of the six *Himar1* copies previously described (Robertson and Lampe, 1995). The coding sequence from this consensus element was used to express and purify the *Himar1* transposase. The protein was overexpressed in *Escherichia coli* under the control of a viral T7 RNA polymerase promoter and appeared in the insoluble protein fraction (Figure 2). The transposase was purified as determined by polyacrylamide gel electrophoresis (Figure 2) through extensive washing of inclusion bodies, including washes in 6 M urea, and the performance of one simple chromatographic step. The identity of the transposase protein was confirmed by its mobility at the predicted molecular size (40.7 kDa) in polyacrylamide gel electrophoresis and N-terminal sequence analysis (data not shown). Another protein ~1–2 kDa smaller than *Himar1* transposase always co-purified with it, albeit in much lower quantity. We believe this protein is either a proteolytic cleavage product of the full-length transposase or a premature termination product of translation because it is not present in mock protein extracts made from uninduced cells in a manner identical to the transposase preparation.

An in vitro genetic assay for Himar1 transposition

To determine whether purified transposase was sufficient to mediate transposition *in vitro*, we performed an experiment similar to that used to detect and quantitate P-element transposase activity (Figure 3A) (Kaufman and Rio, 1992). This experiment was designed to genetically detect transposition products produced by transposition of a *Himar1* transposon marked with a kanamycin resistance (Kan^R) gene from a donor plasmid into an ampicillin-resistant (Amp^R) target plasmid. The target plasmid used in this assay is a tetramer of pBluescriptKS+ (pBSKS+; a gift of D.Rio). The use of such a target molecule increases the chances of recovering products of transposition because insertions into any given Amp^R gene or origin of replication in one of the monomers are still likely to produce a viable plasmid product. Transposition products are Kan^R–Amp^R and can be identified by transformation of bacteria with the reactant DNAs and selection with kanamycin and ampicillin. Potential products are analyzed in detail by restriction analysis and sequencing. Selection with ampicillin alone is used as a control for the efficiency of overall DNA recovery. Further details of this experiment are





C

			Target		
	Target	5'ITR	3'ITR	dupl	Target
donor	GGATTATA-	ACAGGTTGG...	CCAACCTGT-	TA-	TAATCTGC
1	ATAGTGTA-	ACAGGTTGG...	CCAACCTGT-	TA-	TGCGGCGA
2	TTGATTTA-	ACAGGTTGG...	CCAACCTGT-	TA-	TAAGGGAT
3	GGACTATA-	ACAGGTTGG...	CCAACCTGT-	TA-	AAGATACC
4	GAAGAGTA-	ACAGGTTGG...	CCAACCTGT-	TA-	TGAGTATT
5	GCGCATTA-	ACAGGTTGG...	CCAACCTGT-	TA-	AGCGCGGC
6	ACTCACTA-	ACAGGTTGG...	CCAACCTGT-	TA-	TAGGGCGA
7	ATACTTTA-	ACAGGTTGG...	CCAACCTGT-	TA-	GATTGATT
8	GCATCTTA-	ACAGGTTGG...	CCAACCTGT-	TA-	CGGATGGC
9	CGAAATTA-	ACAGGTTGG...	CCAACCTGT-	TA-	ACCCTCAC
10	CCCTTATA-	ACAGGTTGG...	CCAACCTGT-	TA-	AATCAAAA
11	ACTCTGTA-	ACAGGTTGG...	CCAACCTGT-	TA-	GCACCGCC

D

			Target		
	Target	5'ITR	3'ITR	dupl	Target
donor	GGATTATA-	ACAGGTTGG...	CCAACCTGT-	TA-	TAATCTGC
1	TCCTTTGA-	ACAGGTTGG...	CCAACCTGT-	GA-	TCTTTTCT
2	CATCCGTA-	ACAGGTTGG...	CCAACCTGT-	GTA-	AGATTCT
3	TTTTGCTC-	ACAGGTTGG...	CCAACCTGT-	C-	ACATGTTCT
4	AAATGCTT-	TCAGGTTGG...	CCAACCTGT-	TT-	AATACTCC
5	GCAGAAGT-	ACAGGTTGG...	CCAACCTGT-	AGT-	GGTCCTG
6	TAGAACTA-	ACAGGTTGG...	CCAACCTGT-	TA-	GTGGATCC
7	TCGCCCTA-	ACAGGTTGG...	CCAACCTGT-	TA-	TAGTGAGT
8	ACCAAATA-	ACAGGTTGG...	CCAACCTGA-	TA-	CTGTTCTT
9	CTCACTAT-	ACAGGTTGG...	CCAACCTGT-	AT-	AGGGCGAA
10	GTTATCTA-	ACAGGTTGG...	CCAACCTGT-	TA-	CACGACGG
11	CATATATA-	ACAGGTTGG...	CCAACCTGT-	TATA-	CTTTGG

Fig. 3. Genetic assay for *Himar1 mariner* transposition *in vitro*. (**A**) An overview of the *in vitro* assay for *Himar1 mariner* transposition. The details of the assay can be found in Materials and methods. The arrowheads on the various plasmids indicate the inverted terminal repeat sequences of the element containing the Kan^R gene. *Sst*I = *Sst*I restriction sites in the donor plasmid. Amp^R = ampicillin resistance gene of target plasmid. There are four of these genes per target plasmid, one per monomer. Only one is shown in this figure. A representative transposition product is shown at the bottom of the figure. (**B**) Restriction analysis of transposition products derived from the genetic assay for *Himar1 mariner* transposition. The numbers at the left indicate size in kbp. Lane M, size standard (in kbp); lane T, *Sst*I digest of target plasmid; lane D, *Sst*I digest of donor plasmid; lanes 1–11, *Sst*I digests of Kan^R–Amp^R-resistant products from the *in vitro* assay. Since the target plasmid is a tetramer, there are four *Sst*I sites, one per monomer length. Digestion of the target itself with *Sst*I results in an ~3 kb product (lane T). Insertion of the 2.3 kb *mariner*-kan sequence into the target results in the addition of one new *Sst*I site in the recombinant product. Digestion of these products will result in an ~3 kb fragment (from the three monomer lengths that are uninterrupted) plus two new fragments whose combined sizes should be 5.2 kb (*mariner*-kan plus one monomer length of target). (**C**) and (**D**) Terminal sequences of Kan^R–Amp^R products produced in the presence of either Mg^{2+} or Mn^{2+}. Sequences of the termini of the *mariner*-kan element (terminal-most nine nucleotides only) integrated into the target DNA of the products shown in Figure 4B. (C) Sequences from reactions containing Mg^{2+}. (D) Sequences from reactions containing Mn^{2+}. Dots represent sequences between the termini of the element removed for this figure; dashes bound element sequences and the target site duplication. The sequence of the donor plasmid is listed at the top for comparison.

outlined in the legend of Figure 3 and in Materials and methods.

Kan^R–Amp^R bacterial colonies were readily obtained when bacteria were transformed with DNA from reactions that used the purified transposase. The average rate of transposition for these experiments was 14.5 ± 2.5 Kan^R–Amp^R bacterial colonies per 10^3 Amp^R colonies. In no case did we recover Kan^R–Amp^R bacterial colonies from control cells transformed with reactions that used a mock bacterial extract lacking transposase, indicating that there was no recombination between target and donor plasmids in bacteria nor co-transformation of bacteria with both the donor and target plasmids that might lead to Kan^R–Amp^R products and hence false positives in the assay. Restriction analysis of Kan^R–Amp^R-resistant plasmids was always consistent with the simple insertion of a 2.3 kb Kan^R-*mariner* transposon into the 12 kb target plasmid. A product of this type is illustrated in Figure 3A. An insertion of the 2.3 kb Kan^R-*Himar1* sequence into the target and subsequent digestion of the product with *Sst*I produces a 2.9 kb fragment (corresponding to three target monomers) and two other fragments whose sizes vary but whose combined length totalled ~5.2 kb (a 2.9 kb monomer target with a 2.3 kb *Himar1*-Kan^R insertion) (Figure 3B). Sequence analysis of the transposon termini and the insertion site of Kan^R–Amp^R products confirmed that

the transposon was inserted into unique, and apparently random, target TA dinucleotides (Figure 3C). Furthermore, the target TA was duplicated as predicted by previously isolated genomic clones (Robertson and Lampe, 1995) and a model proposed for transposition of the related *Tc3* element (van Leunen *et al.*, 1994), and is thought to be a general property of the superfamily (Doak *et al.*, 1994).

Transposition also occurred when Mn^{2+} was substituted for Mg^{2+} in the assays. The rates of transposition and appearance of the products when analyzed by restriction with *Sst*I were the same with Mn^{2+} as with Mg^{2+} (data not shown). The TA dinucleotide insertion site specificity of the transposase, however, was significantly altered. Approximately half of the products sequenced had insertions of the *Himar1*-Kan^R transposon into sites other than TA (Figure 3D).

Simple insertion products are formed in vitro by Himar1 transposase

Mariners have been predicted to transpose by a cut-and-paste mechanism (van Leunen *et al.*, 1994). If so, simple insertion products should be formed *in vitro*. The genetic assay is an indirect method to observe transposase activity, so a direct observation of transposition products is necessary to eliminate the possibility that transposase is forming co-integrate structures that are then resolved by the bacteria. We tested for the formation of simple insertion products by performing the *in vitro* assay using a linearized, radiolabeled target DNA and two different sizes of donor plasmids. The two different donor constructs each carry the same 2.3 kb *mariner* transposon but differ in the length of the plasmid backbone. If co-integrate structures were the primary products produced, then transposition using donor plasmids differing in size in the plasmid backbone would lead to the production of two different sized transposition products. Alternatively, if cut-and-paste transposition occurred, simple insertion products of the same size would be produced because the two donor plasmids contain exactly the same transposon.

The results of this experiment are shown in Figure 4. No products were formed in the absence of transposase (Figure 4, lanes 1 and 2). There was one major product migrating at 5 kb using either donor plasmid, the expected size of a simple insertion of the 2.3 kb *Himar1* transposon into the radiolabeled 2.7 kb target DNA. One smaller product was also formed using only the pMarKan donor which we have not characterized. Cleavage of the reaction products with *Cla*I (which cleaves inside the transposon only) eliminated the 5 kb product (lane 5) or severely reduced its intensity (lane 6, the result of incomplete cleavage). In contrast, cleavage of reaction products with *Xho*I (which cleaves the donor plasmid backbone only) had no effect on the 5 kb product. These products and cleavage patterns would not be expected if co-integrate stuctures were being formed, nor can they be explained by the insertion of a single end of an excised transposon into the target. They are consistent, however, with the simple insertion of both ends of single transposons into the target, thus confirming that *Himar1* transposase uses a cut-and-paste mode of transposition.

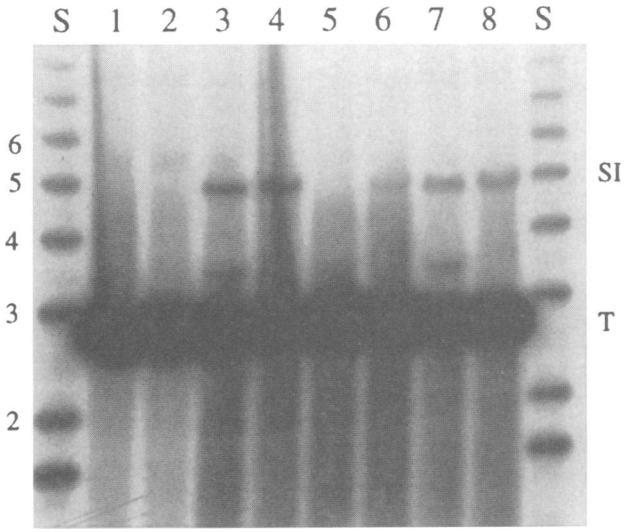


Fig. 4. Direct analysis of *in vitro* transposition products showing the presence of simple insertion products. S = standards in kilobase pairs; SI = simple insertion product; T = target. Lane 1, pMarKan donor, no transposase; lane 2, pMarKanAmp donor, no transposase; lane 3, pMarKan donor, transposase present; lane 4, pMarKanAmp donor, transposase present; lane 5, as per lane 3, *Cla*I cut; lane 6, as per lane 4, *Cla*I cut; lane 7, as per lane 3, *Xho*I cut; lane 8, as per lane 4, *Xho*I cut.

Purified transposase binds to Himar1 inverted terminal repeat sequences

One property of an active transposase is sequence-specific binding to specific transposon sequences. DNA-mediated elements like those of the *Tc1/mariner* superfamily contain ITR sequences at their 5′ and 3′ ends that can be bound by transposase (Berg and Howe, 1989). We performed a DNase I footprinting assay to determine the precise sequences to which purified transposase bound. As expected, the protein bound ITR sequences (Figure 5A) and not vector sequences. Protection from DNase I cleavage covered 28 nucleotides on both strands. The top strand was protected between nucleotides 4 and 32, and the bottom strand between nucleotides 2 and 30 (Figure 5B).

We originally defined the ITR for this element based on a strict comparison of the sequences of either end (Robertson and Lampe, 1995). The first 27 bp are perfectly inverted on both ends of *Himar1*. Taking into account a single G/A transition at position 28, however, the ITR extends to 31 bp. We believe the DNase I protection bracketing base pair 31 on both strands offers reasonable evidence that a 31bp ITR is the biologically relevant value and therefore represents the true ITR.

Himar1 transposase cleaves transposon termini

We determined the nucleotide positions at which *Himar1* transposase cleaved the *Himar1* ITR by radiolabeling on one end or the other a short double-stranded DNA fragment containing the ITR, incubating it with transposase and separating any cleavage products from the full-length DNA on a denaturing polyacrylamide gel. Purified transposase cleaved both the top and bottom strands of the ITR at a few specific sites near the end of the ITR (Figure 5A). A map of the cleavage sites and intensity is shown in Figure 5B. Cleavage of the top strand was far more pronounced in 5 mM $MnCl_2$ than in 5 mM $MgCl_2$ and was completely

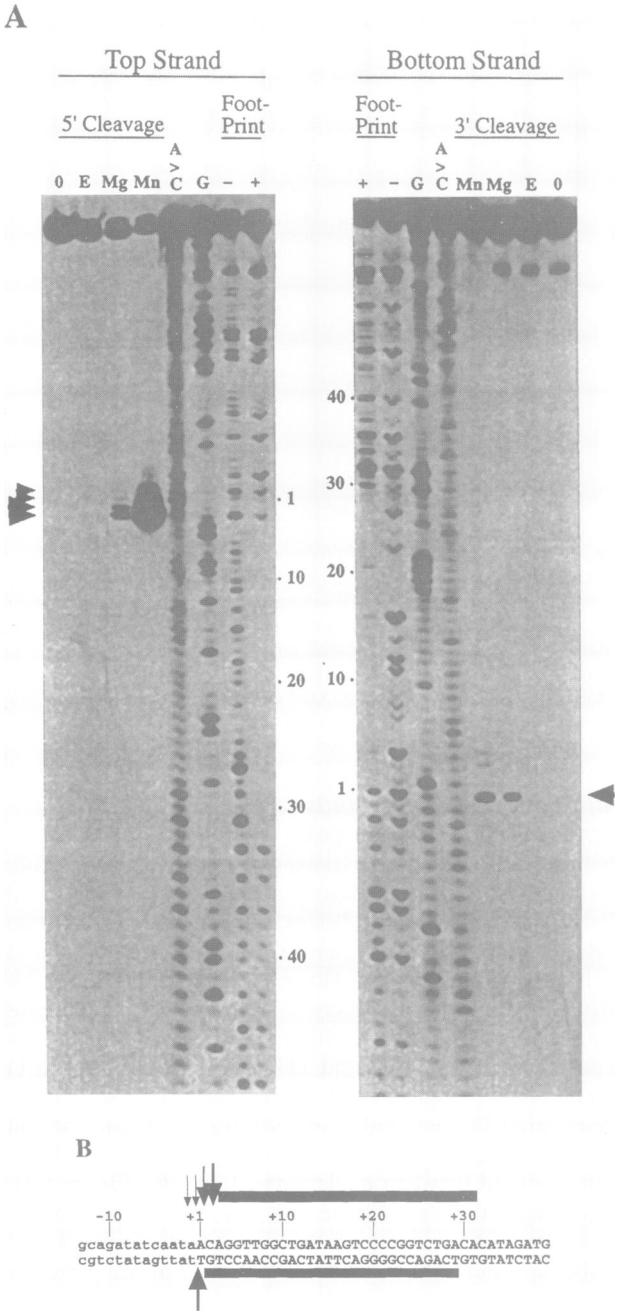


Fig. 5. Footprinting and strand-specific cleavage of *Himar1 mariner* sequences by purified transposase. (**A**) Top strand and bottom strands of the element from 5′ to 3′. The cleavage assay was performed as described in the text. 0 = cleavage reaction with no transposase; E (EDTA), Mg and Mn = cleavage reactions in the presence of those ions. A>C and G are Maxam–Gilbert sequencing reactions performed on the same labeled DNA as used in the cleavage reactions and footprinting reactions and are used here as standards (Sambrook *et al.*, 1989). The footprinting reactions were carried out as described in the text. (–) = footprinting reaction with no transposase present; (+) = footprinting with transposase. The numbers are the nucleotide position relative to the 5′ (left) end of the ITR. The arrows show the position of the Mg^{2+} cleavage pattern and are at the same positions as those in (B). (**B**) Summary of cleavage and footprinting. The partial sequence of the labeled DNA used for footprinting and strand-specific cleavage is shown above. *Himar1* sequences are in upper case and vector sequences in lower case. The ITR sequence corresponds to either the first 27 or 31 bp (see text). Filled boxes indicate sequences protected by protein on top and bottom strands, respectively. Arrows and their relative size indicate the position of strand-specific cleavage in Mg^{2+} and its relative intensity.

absent in 6.4 mM EDTA, indicating a transposase requirement for a divalent cation. More than one site on the top strand was cleaved in both Mg^{2+} and Mn^{2+}. This is somewhat unexpected but may account in part for some of the 'footprints' observed *in vivo* with the related *Mos1 mariner* (see below) which can be of variable length (Bryan *et al.*, 1990; Coates *et al.*, 1995). Cleavage of the bottom strand occurred to a nearly equivalent extent with either Mn^{2+} or Mg^{2+} primarily at one position corresponding to the bond between the terminal nucleotide of the element and the flanking DNA. Some additional minor cleavage products were seen only with Mn^{2+}. Assuming *mariners* transpose by a mechanism similar to that of other transposons, cleavage at the 3′ end of the ITR is an absolute requirement for transposition because it exposes the 3′ hydroxyl used as the nucleophile to create the covalent bond at the target site in the strand transfer reaction (Mizuuchi, 1992a).

Discussion

Himar1 transposase mediates transposition in vitro without host factors

We have developed an *in vitro mariner* transposition system. The system is able to reproduce the pattern of *Himar1 mariner* transposition predicted by sequences of genomic clones of this element and from observations on related transposons (Doak *et al.*, 1994; van Leunen *et al.*, 1994; Robertson and Lampe, 1995; Vos *et al.*, 1996). We observed the insertion of the *Himar1* transposon carrying a kanamycin resistance gene into random TA dinucleotides of the target plasmid and the duplication of the target site. No other proteins were necessary and there was no requirement for an energy source such as ATP.

Transposons move by one of two general pathways (Mizuuchi, 1992b; Craig, 1995). The first of these occurs for replicative transposons, like phage Mu, where only 3′ cleavage takes place at the transposon termini followed by strand transfer. Since no 5′ cleavage takes place, not only is the transposon DNA joined to the new target site, but also the flanking donor DNA, forming a structure called a co-integrate that resembles a Holliday junction. These structures can be resolved by multiple pathways, and many use host enzyme systems. The alternative pathway is followed by non-replicative transposons (e.g. Tn*10*) and is known as cut-and-paste transposition (Benjamin and Kleckner, 1992). Here, cleavage takes place at both the 5′ and 3′ ends, resulting in a free copy of the transposable element that is joined to the target site forming a simple insertion. *Himar1* transposase cleaves *Himar1* termini at both the 5′ and 3′ ends, consistent with a cut-and-paste mode of transposition (Figure 3). Furthermore, we recovered only simple insertion transposition products in our genetic assay and never products that would be expected if co-integrates were being formed *in vitro* and resolved by the bacteria (Figures 4 and 5B). Thus, the involvement of *E.coli* in our assay is most likely limited to the repair of the insertion site in the target molecule by filling in the single-stranded gap between the target site and *Himar1* termini. These experiments confirm the prediction that *mariner* transposition does not rely on species-specific host factors and helps to explain in part why *mariners* are so broadly distributed in animals. A

similar result has been obtained recently for *Tc1* (Vos *et al.*, 1996), so this is likely to be true for all members of the *Tc1/mariner* superfamily.

Mn^{2+} alters the target site choice of Himar1 transposase

Genomic clones from a variety of *mariner* transposons and our own transposition products produced using Mg^{2+} show insertion exclusively into a TA dinucleotide. We were surprised to find that the substitution of Mn^{2+} for Mg^{2+} in the *in vitro* transposition assay dramatically altered the target site specificity of *Himar1* transposase (Figure 3D). Approximately half of the products were inserted into sites other than TA. In one case (product # 4 with Mn^{2+}), the 5′-terminal nucleotide of the inserted element was also changed from an A to a T. We currently have no explanation for this latter phenomenon. A change in target specificity occurs when substituting Mn^{2+} for Mg^{2+} with some restriction enzymes, however. *Eco*RV, for example, shows a relaxed target specificity in reactions using Mn^{2+} (Hsu and Berg, 1978). Mn^{2+} also perturbs the activity of transposases. For example, the Mu transposase is usually able to accomplish efficient strand transfer of Mu bacteriophage only in the presence of MuB protein and Mg^{2+}. Strand transfer occurs at a similar level even in the absence of MuB, however, if Mn^{2+} is used as the cation (Baker *et al.*, 1991). Many transposable elements and retroviral integrases contain a conserved D,D35E motif or a variant of it (Kulkosky *et al.*, 1992; Doak *et al.*, 1994) that binds cations, normally Mg^{2+}. Mutation of any of the three residues in the motif is sufficient to abolish the catalytic activity of these proteins (Kulkosky *et al.*, 1992; Baker and Luo, 1994; van Leunen *et al.*, 1994; Vos and Plasterk, 1994). *Mariners* appear to have a motif related to D,D35E which is an invariant D,D34D throughout the *mariner* family (Robertson, 1995), although it has not been examined functionally. Our data indicate that this motif may be involved not only with catalysis as in other transposons and retroviral integrases, but with target site selection as well, because changing the cation which it binds changes target site specificity. Alternatively, this motif may be closely linked with a target site selection domain in the transposase that can be perturbed indirectly when the D,D34D motif binds the Mn^{2+} cation.

Himar1 transposase activity confirms a model suggested for the transposition of the Tc1/mariner superfamily of transposons

The transposases of *Tc1* and *mariner* family transposable elements share ~18–25% amino acid sequence identity and are related in structure and in some conserved amino acid positions (Robertson, 1995). To this sequence similarity we can now add a similarity in activity. van Leunen *et al.* (1994) have proposed a model for *Tc3* transposition (and, by extension, *Tc1* and *mariners*) based on *in vivo* experiments (see Figure 1 for the similar *mariner* mechanism). In this model, the transposon DNA is cleaved on both strands to produce a two nucleotide, 3′ overhang corresponding to the first two nucleotides of the element. Secondly, the target and transposon DNAs are joined via a covalent bond at a target site TA dinucleotide in a phosphoryl transfer reaction using the exposed hydroxyl on the processed transposon terminus as the nucleophile. We observed cleavage at the 5′ and 3′ end of the *Himar1* ITR in accordance with the *Tc3* transposition model. These data, in addition to those from the *in vitro* transposition assay, confirm that the model applies to *mariner* transposition as well.

Himar1 transposase cleaves the *mariner* ITR at more than one 5′ site, including a site that lies outside the boundary of the element (Figure 5). The multiple 5′ cleavage sites may be an artifact of the *in vitro* assay itself. Alternatively, *mariner* may use these sites *in vivo*, the consequences of which would be a variety of 'footprints' left in the genome after the element excises (see below). Such footprints have been noted for an active copy of the *Drosophila mauritiana mariner*, *Mos1*, although one footprint predominated over the rest and it is unclear from these experiments whether the variation in the footprints was due to the transposase cleaving at different sites within the element or was a consequence of the host's repair machinery (Bryan *et al.*, 1990; Coates *et al.*, 1995).

Purified *Himar1* transposase cleaves the 3′ end of *mariner* termini as well. The primary site of 3′ cleavage was just after the terminal nucleotide of the element, as predicted by the model for *Tc3* transposition. The 3′ cleavage activity was noticeably less than the 5′ activity in the presence of Mn^{2+}. Interestingly, the degree of 3′ cleavage was nearly equivalent in Mn^{2+} and Mg^{2+}, in contrast to that of 5′ cleavage. This lower cleavage activity in both ionic conditions suggests that this may be a rate-limiting step for transposition of this element which may help to preclude co-integrate formation.

Terminal cleavage by Himar1 resembles Tc1 more than Mos1 mariner

DNA-mediated transposable elements frequently leave small insertions when they excise from a given genomic site (e.g. Engels, 1989; Bryan *et al.*, 1990). The sequence of these insertions usually corresponds to a duplicated target insertion site and parts of the transposon ITRs. *Mariners* and *Tc1/Tc3* duplicate a target TA dinucleotide upon insertion (see Figure 1). Upon subsequent excision, staggered cutting inside the elements leaves element sequences behind and hence generates a 'footprint' (Plasterk, 1991). The specific sequences left behind as footprints are element specific. For example, the most common footprints left by *Tc1* upon excision from the *unc-54* gene were either taTGta or taCAta (where ta represents the ta dinucleotide insertion sequence and duplication and the upper case letters element sequences), although some taCATGta footprints were found as well (Eide and Anderson, 1988). Footprints resulting from *Mos1*-induced *white* alleles were either taTGAta or taCCAta (Bryan *et al.*, 1990). Similar footprints were found using a plasmid-based excision assay with *Mos1* in a variety of fly embryos (Coates *et al.*, 1995). In each case, however, footprints were also observed which included fewer nucleotides between the duplicated target sequences. Footprints like these have most often been interpreted as a consequence of exonuclease activity by the host before repair (see below) (Plasterk, 1991; Coates *et al.*, 1995).

Footprints left by *Tc1/Tc3* elements can be interpreted in light of the proposed mechanism of transposition of *Tc3* (van Leunen *et al.*, 1994). Here, the transposase produces a staggered cut at the termini of the element

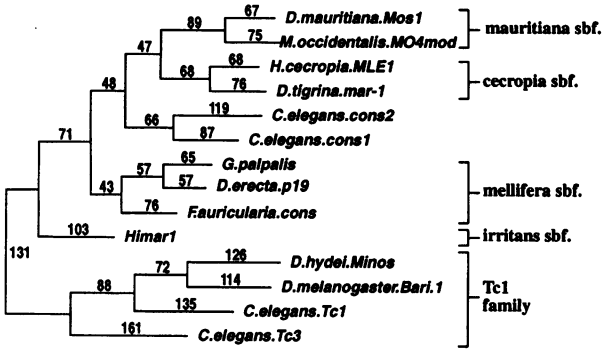


Fig. 6. Relationship of the various *mariner* subfamilies to the *Tc1* family of transposable elements. A phylogenetic tree relating various *mariners* to elements of the *Tc1* family of transposable elements and placing the *H.irritans* consensus (cons.) sequence (*Himar1*) used in this study near the root of the *mariner* clade of the tree. Numbers above the branches are numbers of amino acid changes. *Mariner* subfamilies are noted at the right, as is the *Tc1* family. The mariner subfamily designations are from Robertson and MacLeod (1993). This tree is based on full-length amino acid sequences of the various transposases. The references for *Mos1*, *H.cecropia* MLE, *D.tigrina*.mar-1, *C.elegans*.cons1, *C.elegans*.cons2, *Minos*, *Bari*, *Tc1* and *Tc3* can be found in Robertson (1995). References for the other sequences are as follows: *M.occidentalis*.MO4mod (Jeyaprakash and Hoy, 1995); *G.palpalis* (Blanchetot and Gooding, 1995); *D.erecta*.p19 (Lohe *et al.*, 1995); *F.auricularia* cons (H.M.Robertson and D.J.Lampe, unpublished results); *H.irritans* cons (Robertson and Lampe, 1995).

leaving a two nucleotide 3′ extension corresponding to terminal element sequences at the site of excision. The lesion in the chromosome is repaired by pairing of the staggered ends and mismatch repair leaving either the sequence of the first or last two nucleotides of the element. If we extend this model to the footprints that have been found for *Mos1 mariner*, then the transposase can be supposed to cleave three nucleotides inside the element. With mismatch pairing and subsequent repair, *Mos1* would leave behind a 3 bp footprint in addition to the TA insertion site duplication. Purified *Himar1* transposase in no case produces products that leave a three nucleotide terminal overhang (see Figure 5) when examined *in vitro*. Cleavage of element termini by *Himar1* transposase, therefore, more closely resembles *Tc1/Tc3* transposases than *Mos1 mariner*. Interestingly, the irritans subfamily of *mariners* to which *Himar1* belongs is a basal lineage in the *mariner* family of transposable elements, while the mauritiana subfamily, which includes *Mos1*, is more derived (Figure 6). We propose that the ancestral type of terminal processing for *mariners* is the type shown by the *Himar1* element and is shared with that of the *Tc1* family of elements. In the course of their evolution, mauritiana subfamily elements have evidently acquired a slightly different terminal processing mechanism although we are not in a position at this time to say when this event may have occurred or which amino acid substitutions may be responsible. We predict, however, that when analyzed, *Mos1* or other mauritiana subfamily *mariner* transposases will show 5′ cleavage three nucleotides within the element. As noted above, genomic footprints of *Mos1 mariner* consist primarily of 3 bp from either terminus plus a duplicated TA target site. Occasionally, smaller footprints are produced (Bryan *et al.*, 1990). This variety of footprints could be explained if *Mos1* transposase produced the same kind of multiple 5′ cleavage sites that we observed *in vitro* with *Himar1* transposase. Moreover, one footprint would dominate if cleavage occurred preferentially at one site as it does with *Himar1* transposase.

Uses for purified transposase

Purification of an active transposase representative of a large and diverse transposon family is an essential first step toward understanding its underlying biochemistry, and we are pursuing this goal. There is, however, another more practical application for purified transposase. A major goal of current molecular entomology is the development of a broad-based transformation system similar to that of the P-element used extensively in *Drosophila melanogaster*. Although *mariners* are apparently active in many genomes and can transpose without the aid of host factors, problems may arise with their use as germline transformation vectors. The standard method of germline transformation in *D.melanogaster* involves the co-injection of two plasmids based on the P-element into fly embryos. One is a 'helper' plasmid that provides a source of P-transposase mRNA but cannot be mobilized in *trans*. The other is the construct to be integrated that transposes with the aid of helper-supplied transposase. One difficulty that may arise in other insects is the construction of suitable helper plasmids that can be transcribed in the experimental organism. There are relatively few genes characterized in most insects that could provide such promoters necessary for helper plasmids. Alternatively, a general purpose promoter could be used which may be expected to operate in nearly any insect system such as the heat-inducible *hsp70* promoter from *D.melanogaster*. An alternative to the use of helper plasmids is the direct injection of purified transposase with the construct to be integrated. Such a system works for the P-element (Kaufman and Rio, 1991). We propose that purified *mariner* transposase may be used to circumvent potential problems that may arise if *mariners* were to be used as a transformation system in an organism where molecular biology is in its infancy.

Materials and methods

Recombinant DNA

PCR primers corresponding to the first 27 nucleotides of the horn fly irritans *mariner* elements (e.g. Hi2, GenBank accession #U11641) were used to amplify a full-length *mariner* from the genomic clone containing the lacewing element Cp3 (GenBank accession #U11652) using *Pfu* DNA polymerase (Stratagene) and the conditions recommended by the manufacturer. These primers change the first two nucleotides of the inverted terminal repeat sequence to CA (5′ to 3′) and add the sequence ATA to the flanking DNA. This PCR product was subcloned by TA cloning (Holton and Graham, 1991) into the vector pcDNAII (Invitrogen) at the *Eco*RV site, creating pMarPfu-10. Two changes in the coding sequence of this clone were necessary to restore the consensus coding sequence. These were introduced by *in vitro* mutagenesis using the method of Deng and Nickoloff (1992). The mutagenic primers were MAR697f (5′-CACTCCTGAGTCCAATCGACAGTCG) and MAR155F (5′-CGAGTTTTTCCGTCGATATG) and changed amino acids Lys170 and Leu151 to Asn and Phe, respectively, creating pMar27fH. The elimination of a restriction site is an integral part of this mutagenesis method, and so the *Hin*dIII site was eliminated in the vector using the primer pCDNA2HIND(–) (5′-GCTCGGTACCTAGCTTGATG).

In order to subclone the coding sequence of *Himar1* into the expression vector, an *Nde*I site was created by *in vitro* mutagenesis at the initiator ATG of the pMar27fH coding sequence using the primer MaroonedNde(+) (5′-TGTTATTGTGAACATATGGAAAAAAAGG).

Primer MaroonedNde(–) (5′-AACGGCCCCACATGAAGAAGA) was used to eliminate an internal *Nde*I site, and primer PCDNAIIKPN(–) (5′-GCTCGGTACTTAGCTTGATG) to eliminate the *Kpn*I site of the vector, creating plasmid pMar27fhNde+. The coding sequence of pMar27fHNde+ was excised as an *Nde*I–*Bam*HI fragment and inserted into the *Nde*I–*Bam*HI sites of the bacterial expression vector pET13a (a gift of Dr Mair Churchill). This placed the *mariner* coding sequence under the control of a T7 promoter.

For the footprinting assays, a subclone containing the first 59 bp of pMar27fH was created by excising the sequence as an *Nsi*I fragment containing some vector DNA. This fragment was inserted into the *Pst*I site of pK19 (Pridmore, 1987) creating pK27fH-5′.

pMarPfu-10 was the starting material for the construction of the donor plasmid used in the *in vitro* transposition assays. A *Bsp*HI–*Bgl*II fragment of pK19 containing a Kan^R gene was made blunt with the Klenow fragment of *E.coli* DNA polymerase and ligated into the *Eco*RV site of pMarPfu-10. The products from this ligation were selected on LB-ampicillin–kanamycin (100 μg/ml and 30 μg/ml, respectively) agar plates yielding pMarKanAmp.

The final donor construct for the *in vitro* transposition reactions, pMarKan, was created from pMarKanAmp by cutting with *Xho*I and *Bgl*I, repairing the ends with Klenow, and religating the plasmid to itself. This procedure removed most of the Amp^R gene and half of the *lacZ* gene.

Purification of Himar1 transposase

One liter of 2× YT containing 30 μg/ml kanamycin was inoculated with 700 μl of an overnight culture of the pET13a/*mariner* construct in BL21(DE3) *E.coli* cells. The culture was shaken at 200 r.p.m. at 37°C until the OD_{600} was ~0.9 (3.5–4 h). Protein expression was induced by adding IPTG to a concentration of 0.5 mM. The cells were allowed to grow for 1 h and then harvested by centrifugation for 15 min at 1000 *g* at 4°C. Growing the cells for longer periods of time did not significantly increase the amount of protein produced. Cells were resuspended in 10 ml of resuspension buffer [20 mM Tris–HCl (pH 7.6 at room temperature), 25% sucrose, 2 mM $MgCl_2$, 0.6 mM phenylmethylsulfonyl fluoride (PMSF), 1 mM benzamidine (BZA), 1 mM dithiothreitol (DTT)]. At this point, the resuspended cells typically were divided into 1 ml aliquots, quick frozen on dry ice, and stored at –80°C.

One ml of stored cells was thawed on ice and 0.25 mg of lysozyme was added. This mixture was left at room temperature for 5 min, after which 1 ml of detergent buffer [20 mM Tris–HCl (pH 7.6), 4 mM EDTA, 200 mM NaCl, 1% deoxycholate, 1% NP-40, 0.6 mM PMSF, 1 mM BZA, 1 mM DTT] was added and left at room temperature for an additional 15 min. $MgCl_2$ was then added to a final concentration of 10 mM followed by the addition of 60 μg of DNase I. The mixture was pipeted up and down with a blue pipet tip until it was no longer viscous and it was allowed to incubate at room temperature for 20 min. The lysate was then spun in a microfuge for 2 min and the supernatant withdrawn and discarded. *Himar1* transposase was expressed exclusively in inclusion bodies. The pellet containing inclusion bodies was washed three times by resuspension and repelleting in 1 ml of 0.5% NP-40, 1 mM EDTA at 4°C. The final two washes of the pellet were performed using 1 ml of 6 M deionized urea.

The washed pellet was resuspended in 500 μl of column buffer [4 M guanidine–HCl, 1 mM PMSF, 1 mM BZA, 20 mM Tris–HCl (pH 7.6), 50 mM NaCl, 5 mM DTT]. The solution was spun for 2 min in a microfuge to remove any insoluble material and then applied to a DEAE Sephacryl column equilibrated in column buffer at 4°C. The column was made in a 10 ml syringe by stopping it with glass wool and applying DEAE Sephacryl until the bed volume was 8 ml. Fifteen 500 μl samples were collected, beginning immediately after sample application. Because of its relatively high pI (9.7), transposase eluted at the front, typically in fractions 7–9. Samples of each fraction were checked on a 14% SDS–PAGE gel to locate the transposase definitively.

Transposase-containing fractions were pooled and placed in dialysis tubing (Spectrapore 6000–8000 MWCO). Samples were dialyzed against 200 ml of dialysis buffer I [10% glycerol (v/v), 25 mM Tris–HCl (pH 7.6), 50 mM NaCl, 2 mM DTT, 5 mM $MgCl_2$] for 5 h. A second dialysis was performed against 200 ml of dialysis buffer II (dialysis buffer I except containing 0.5 mM DTT) for an additional 8 h to overnight. The sample then was removed from the dialysis tubing, spun for 2 min in a microfuge at 4°C to remove the precipitate, and the supernatant aliquoted, frozen on dry ice and stored at –80°C. Purified transposase could be thawed and refrozen at least twice without any noticeable effect on activity. The concentration of protein in the purified sample was measured by means of a micro BCA analysis (Pierce) and typically was 250–300 μg/ml.

Genetic assay for in vitro transposition

In vitro transposition assays were carried out in 10% glycerol (v/v), 25 mM HEPES (pH 7.9 at room temperature), 250 μg of acetylated bovine serum albumin (BSA), 2 mM DTT, 100 mM NaCl and 5 mM $MgCl_2$, and contained ~12.5 nM purified transposase in a final volume of 20 μl. The donor plasmid was pMarKan described above. The target plasmid was a naturally occurring tetramer of pBSKS+ (a gift of D.Rio). We used ~12 fmol (~100 ng) of target DNA and 12 fmol of donor DNA (~32 ng) per each 20 μl reaction. The reactions were allowed to incubate for 1 h at room temperature. They were then stopped by the addition of 80 μl of stop solution (50 mM Tris–HCl, pH 7.6; 0.5 mg/ml proteinase K; 10 mM EDTA; 250 μg/ml yeast tRNA), and allowed to incubate at 37°C for 30 min after which they were phenol/chloroform extracted and precipitated using standard techniques. The precipitated DNA was resuspended in 10 μl of TE and 1 μl was electrotransformed into TOP10 F′ *E.coli* cells (Invitrogen) using a BRL electroporation device following the manufacturer's instructions. One ml of SOC (0.5% yeast extract, 2% tryptone, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 20 mM $MgSO_4$, 20 mM glucose) was added to the transformed cells and the suspension incubated at 37°C with vigorous shaking for 45 min. One μl of the cells was plated on LB-ampicillin (100 μg/ml) agar plates to test for DNA recovery and 500 μl were plated on LB-ampicillin (100 μg/ml)–kanamycin (30 μg/ml) agar plates to detect transposition products. DNA from potential transposition products was prepared by a boiling miniprep method (Sambrook *et al.*, 1989) and examined by restriction digestion and sequencing. Reactions containing Mn^{2+} were performed identically except 5 mM $MnCl_2$ was substituted for $MgCl_2$ in the *in vitro* assay. Controls were performed by adding a mock transposase extract in place of purified transposase. This extract was made from uninduced *E.coli* cells carrying the pET13a/*mariner* construct in a manner identical to that of induced cells.

Direct analysis of in vitro transposition products

To test whether simple insertions were present in the *in vitro* reaction prior to transformation into bacteria, we performed the following experiment. A 2.7 kb plasmid was linearized with *Eco*RI, end-labeled with Klenow fragment, and used as the target (~10 ng) under the reaction conditions outlined above, except that the reactions were performed at 37°C and using a 5-fold greater amount of transposase. Two separate reactions were performed: one with the 3.8 kb pMarKan donor plasmid and another with the 5.3 kb pMarKanAmp plasmid from which the former donor was derived.

The experimental reactions containing transposase were divided into three parts after phenol extraction and ethanol precipitation. One-third was left untreated, one-third cut with *Cla*I, which cleaves in the *mariner* transposon but not in the target, and another third cleaved with *Xho*I which cleaves at the very end of the target molecule and in the 5.3 kb donor outside the *mariner*. The reactants were separated on a 0.5% 1× TAE agarose gel which was dried and autoradiographed.

Radiolabeling and purification of Himar1 DNA fragments

The footprinting and cleavage assays were performed using pK27fH-5′ which contains the first 59 bp of *Himar1*. The top strand was labeled by cutting 3 μg of the plasmid with 15 U of *Xba*I in a 25 μl reaction volume for 1 h. After heating the reaction to 65°C for 20 min, the following were added: 2.5 μl of React 2 buffer (BRL), 8 μl of 3000 Ci/mmol [^{32}P]dCTP, 8 μl of 3000 Ci/mmol [^{32}P]dATP, 1 μl of 1.5 mM dTTP, 1 μl of 1.5 mM dGTP, 5 U of Klenow fragment of *E.coli* DNA polymerase I, and water to a final volume of 50 μl. The labeling reaction was left at room temperature for 25 min and then heated to 65°C for 20 min to stop the reaction. The buffer in the reaction was exchanged for that of New England Biolabs restriction buffer 3 by ultrafiltration through a Millipore UltrafreeMC30 ultrafiltration unit. The final volume of the retentate was 30 μl. To the retentate was added 15 U of *Spe*I; the reaction was placed at 37°C for 1 h and then stopped by heating to 65°C for 20 min.

Radiolabeled DNA was isolated by electrophoresis on an 8% (30:1, acrylamide: bisacrylamide) polyacrylamide gel, 0.5× TBE, run at 200 V for 1.25 h. After a brief exposure to autoradiographic film to locate the desired band, a gel slice was removed and the DNA electroeluted onto a pad of 10 M NH_4OAc in 0.5× TBE at 150 V for 1 h. The DNA in the 10 M NH_4OAc was precipitated by the addition of 2.5 vol of EtOH and spun in a microfuge for 30 min at 4°C. The DNA was resuspended in 100 μl of TE and any contaminating acrylamide monomers removed by passing the solution over a G50 Sephadex spin column in TE (Sambrook *et al.*, 1989). The specific activity of the purified solution was determined by scintillation counting. The bottom strand was labeled

and purified in an identical manner except that the DNA was first cut with *Spe*I, labeled, and cut a second time with *Xba*I.

DNase I footprinting assay

DNase I footprinting assays were performed in activity buffer [10% glycerol (v/v), 25 mM HEPES (pH 7.9), 250 μg of acetylated BSA, 100 mM NaCl, 100 ng poly(dI)–poly(dC), 2 mM DTT, 2.5 mM $MgCl_2$] containing 70 000 c.p.m. of end-labeled pK27fH-5′ fragment (labeled on the top or bottom strand) and ~50 nM purified *mariner* transposase in a volume of 20 μl. This reaction was allowed to equilibrate at room temperature for 30 min, after which 5 U of DNase I was added and the reaction was allowed to proceed for exactly 2 min. The reaction was stopped by the addition of 100 μl of 25 mM EDTA, 0.01% SDS followed by extraction with 100 μl of phenol:chloroform:isoamyl alcohol (24:24:1). The reactions were extracted once more with chloroform:isoamyl alcohol alone and the DNA precipitated by the addition of 0.1 vol of 3 M NaOAc (pH 5.3) and 3 vol of EtOH followed by spinning in a microfuge for 30 min at 12 000 *g*. The resulting pellet was washed with 100% EtOH, dried, and resuspended in 5 μl of sequencing gel loading buffer (US Biochemicals). Size standards were generated from the same labeled DNA using standard Maxam–Gilbert sequencing techniques for the G and A>C reactions (Sambrook *et al.*, 1989). Products from the footprinting assays were resolved on an 8% urea sequencing gel using 3 μl of the resuspended DNA.

Strand-specific cleavage assay

Strand-specific cleavage assays were performed in activity buffer as per the DNase I footprinting assays except using ~12.5 nM purified transposase. The cation was varied between either 5 mM $MnCl_2$, 5 mM $MgCl_2$ or 6.5 mM EDTA. Reactions were allowed to incubate at room temperature for 2 h, after which they were stopped and treated as per the DNase I footprinting assays.

Phylogenetic analysis of mariner sequences

Transposase amino acid sequences were aligned based on the alignment of Robertson (1995). The sequences were obtained as indicated in the legend of Figure 6. Aligned transposase sequences were analyzed using PAUP v3.1.1 (Swofford, 1993) for the Macintosh using the heuristic search mode, random addition of sequences and tree bisection–reconnection branch swapping.

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