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Resident aliens

the Tc1/*mariner* superfamily of transposable elements

Ronald H.A. Plasterk
rplas@nki.nl

***Zsuzsanna Izsvák**
zizsvak@mdc-berlin.de

***Zoltán Ivics**
zivics@mdc-berlin.de

Division of Molecular Biology, Netherlands Cancer Institute and Center for Biomedical Genetics, Division of Molecular Biology, Plesmanlaan 121, Amsterdam 1066CX, The Netherlands.
*Present address, the Max Delbrück Center for Molecular Medicine, Robert Roessle Strasse 10, 13122 Berlin, Germany.

Transgenic technology is currently applied to several animal species of agricultural or medical importance, such as fish, cattle, mosquitos and parasitic worms. However, the repertoire of genetic tools used for molecular analyses of mice and *Drosophila* is not always applicable to other species. For example, while retroviral enhancer-trap experiments in mice can be based on embryonic stem (ES) cell technology, this is not currently an option with other animals. Similarly, the germline transformation of *Drosophila* depends on the use of the *P-element* transposon, which does not jump in other genera. This article analyses the main characteristics of Tc1/*mariner* transposable elements, examines some of the factors that have contributed to their evolutionary success, and describes their potential, as well as their limitations, for transgenesis and insertional mutagenesis in diverse animals.

When David Hirsch and Scott Emmons discovered the Tc1 transposable element in 1983 as a repeat sequence in the genome of *Caenorhabditis elegans* (Ref. 1), they probably did not realize that this was the tip of a very large iceberg. We now know that homologs of Tc1 and those of the related *mariner* transposon found in *Drosophila mauritiana* (Ref. 2) are probably the most widespread DNA transposons in nature; they can be found in fungi, plants, ciliates and animals, including nematodes, arthropods, fish, frogs and humans. Together with related *pogo* transposons[3,4], Tc1 and *mariner* elements are members of a large superfamily of transposable elements, the Tc1/*mariner* superfamily[5–8], which is named after its two best-studied members. Tc1/*mariner* elements are about 1300–2400 bp in length and contain a single gene encoding a transposase enzyme that is flanked by terminal inverted repeats. Although they are divergent in primary sequence (about 15% amino acid identity

between the transposases of the different families)[6], members of the Tc1/*mariner* superfamily are probably monophyletic in origin[6,9] (Fig. 1), and have similar structures and molecular mechanisms of transposition. As shown in Fig. 1, a more-remote similarity exists between these transposons and several bacterial IS elements, long terminal repeat (LTR) retrotransposons and retroviruses[10]. The recombinase proteins encoded by these diverse genetic elements are all related and contain a signature of three acidic amino acids (DDE or DDD, Fig. 2) with a characteristic spacing[9,10].

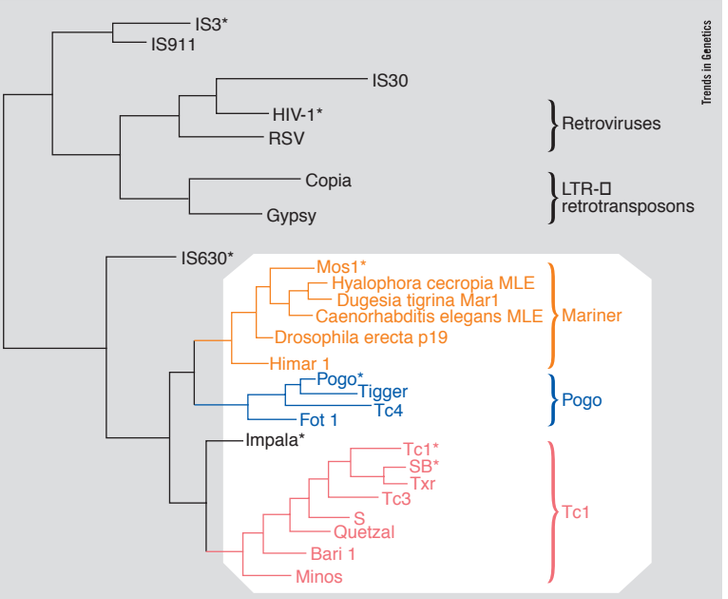
The transposase

Transposition is mediated by the transposase protein that is encoded by the transposon. Evidence for this came from work on the Tc1 and *Himar1 mariner* elements; transposase activity alone is sufficient to mediate full excision and reintegration *in vitro*[17,18]. Note that this evidence does not exclude the involvement of host proteins in transposition *in vivo*, because test-tube reactions might have been forced by non-physiological conditions.

The main structure–function analysis has focused on the N-terminal DNA-binding domain of the transposase. Tc1-like transposases contain bipartite DNA-binding domains[19] that, on the basis of sequence alignments and secondary-structure predictions, have been proposed to consist of two helix–turn–helix (HTH) motifs[20] (Fig. 2). The first of these HTH motifs, which is similar to the paired domain of some transcription factors[21,22] (Fig. 2a), has been crystallized in a complex with double-stranded DNA that corresponds to the termini of Tc3 transposons in *C. elegans* (Ref. 11). The crystal structure did, indeed, show an HTH fold, forming a dimer that brings together the two DNA ends; it is not certain whether the dimer is physiologically relevant. The paired-like domain is followed by a second HTH motif that is embedded in a homeo-like DNA-binding domain (Fig. 2a). Secondary structure predictions indicate that *mariner* transposases might also contain such a bipartite DNA-binding domain, consisting of two HTH motifs (Fig. 2b). Some bacterial transposases[23,24] and *Pogo* (Ref. 25) contain 'solo' HTH motifs (Fig. 2b). A GRPR-like sequence between the two HTH motifs is conserved in Tc1/*mariner* transposases (Fig. 2). The GRPR motif is characteristic to homeodomain proteins[26] and mediates interactions with DNA in the Hin invertase of *Salmonella* sp. (Ref. 13), and in the RAG1 recombinase that mediates V(D)J recombination of immunoglobulin genes in vertebrates[14,15]. The existence of a relationship between DNA binding by Tc1 transposase and RAG1 recombinase is further supported by DNA sequence similarities between their binding sites[27]. Members of the retroviral integrase family carry a combined motif of a zinc-binding domain[28,29] and an HTH motif (Fig. 2b) that resembles the Tc3 paired-like structure[30].

As shown in Fig. 2, a nuclear localization signal (NLS) overlaps partially with the DNA-binding domain in Tc1/*mariner* transposases[22]. A single amino acid replacement in the NLS of the *Mos1 mariner* transposase is detrimental to overall transposase function[31]. The transposase NLS is flanked by phosphorylation target sites of casein kinase II. Phosphorylation of these sites is a potential checkpoint in the regulation of transposition. The NLS indicates that these transposons, unlike murine retroviruses, can take advantage of the receptor-mediated transport machinery of host cells for the nuclear uptake of their transposases.

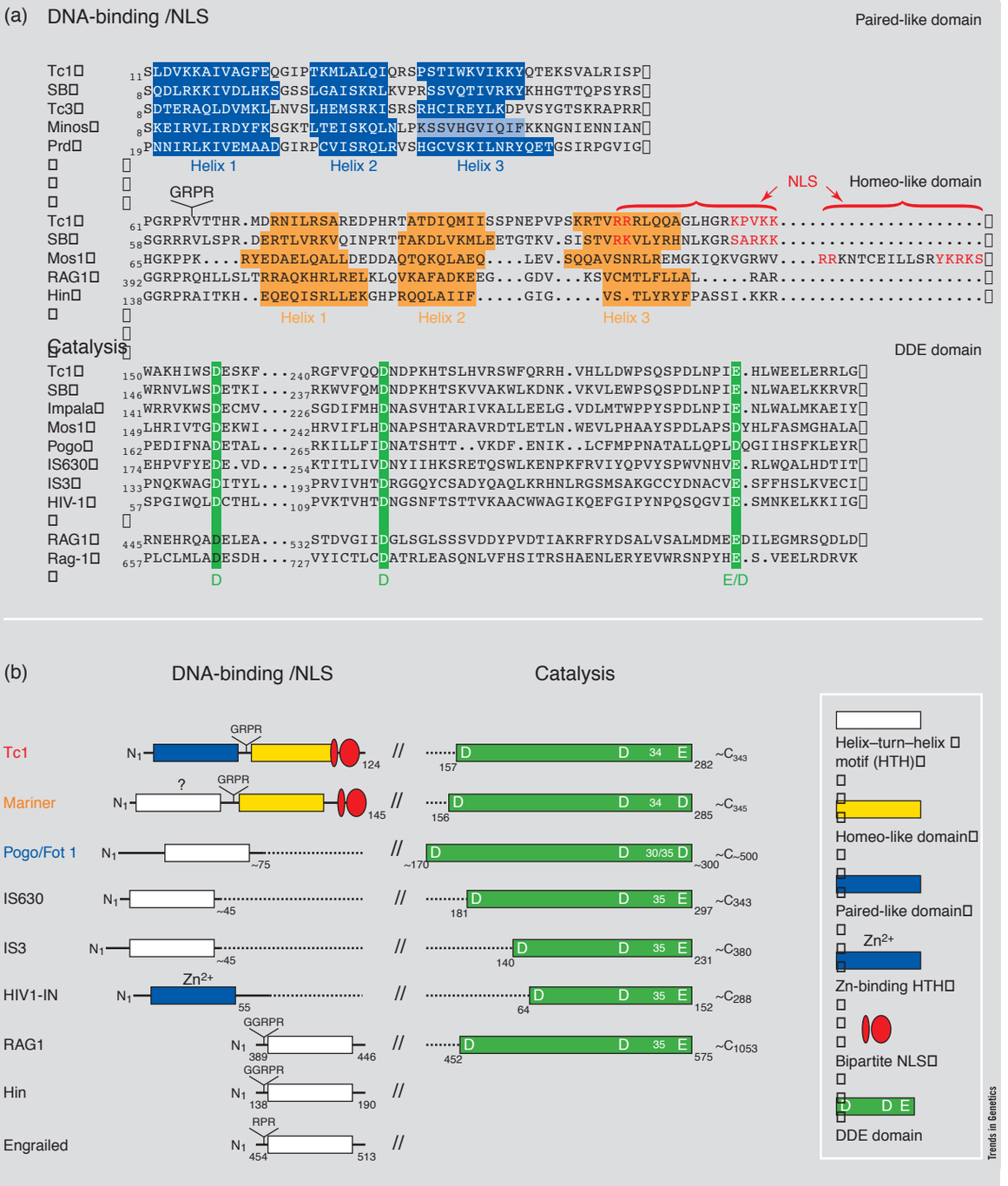
FIGURE 1. Phylogeny of the Tc1/*mariner* superfamily



Recombinases that contain the DDE amino acid motif are grouped into two major clusters: a DNA-transposon group and a retroelement group. Bacterial transposable elements (called IS elements) are DNA-transposons but certain elements, such as IS3, IS911 and IS30, are grouped together with the retroelement group, whereas the position of IS630 is close to the Tc1/*mariner* superfamily (white box) in the phylogenetic tree. The Tc1 (pink), *mariner* (orange) and *pogo* (blue) transposon families are probably monophyletic. Relationships within each family are not well supported by bootstrap analysis, in agreement with Robertson[6]. The maximum parsimony tree was generated by PAUP using a multiple sequence alignment of regions containing the DDE motif, as in Capy *et al.*[9] Elements included in the analysis represent only a subset of all known DDE-containing recombinases and those elements whose DDE domains are shown in a sequence alignment in Fig. 2a are marked with asterisks.

The third major domain of the transposase has been referred to as the catalytic domain because it is responsible for the DNA cleavage and joining reactions of transposition. Owing to the presence of the characteristic DDE (or DDD in the case of *mariner* and *pogo*) motif found in some other transposases and recombinases[10] (Fig. 2), this region of the transposase was initially proposed to be the catalytic domain. Site-directed mutagenesis of these positions in the Tc3 transposase has confirmed that these three amino acids are essential for all catalytic activities[32]. Interestingly, a change of the exceptional third D of *mariner*, which turns the DDD motif into the canonical DDE motif, inactivates the transposase[31]. This is most-easily explained by assuming that the catalytic role of either aspartic or glutamic acid is similar, but that the precise spatial position within the transposase fold requires the presence of the correct residue. The catalytic domain of RAG1 might be related to the DDE recombinases (Fig. 2). The presence of a putative DDE motif in RAG1 was noted by Dreyfus[16] and his observation is now extended by the detection of an alternative potential DDE signature (Fig. 2a). Both putative DDE signatures are in a core region of the RAG1 recombinase that is essential for V(D)J recombination[33,34]. However, it must be noted that the DDE residues can occur in a protein sequence by chance; therefore, the potential homology between the

FIGURE 2. Functional domains of Tc1/*mariner* transposases



(a) Conservation of amino acid sequences between Tc1/*mariner* transposases and other proteins. Functional protein modules and their conservation between transposases and the paired DNA-binding domain of transcription factors (top), homeodomain DNA-binding domains of recombinases (middle) and the DDE motif-containing catalytic domains of recombinases (bottom). Top, an HTH motif within the DNA-binding domains of transposases and the paired domain, consisting of three α-helices (blue) that are either derived from actual crystal structures for Tc3 (Ref. 11) and prd (Ref. 12), or predicted by an algorithm provided by the PredictProtein server at EMBL. Middle, a GRPR-like amino acid motif, together with a second HTH motif, is shown in the homeo-like DNA-binding domains of transposases and RAG1 and Hin recombinases. Helices (yellow) are derived from actual structure in Hin (Ref. 12), or are based on experimental observations[14,15] and predicted for the transposases. The third helix is overlapping with, or followed by, a nuclear localization signal in Tc1 and *mariner* transposases, respectively. Bottom, catalytic regions of transposases containing the DDE motif are shown, with the conserved aspartate and glutamate residues indicated. The DDE motif is conserved between Tc1/*mariner* and IS element transposases and retroelement integrases. The first putative DDE motif in RAG1, starting at position 445, was proposed by Dreyfus *et al.*[16] (b) Modular structure of Tc1/*mariner* transposases and topology of their major functional domains, in comparison with other DDE-motif recombinases and DNA-binding proteins. Tc1/*mariner* transposases have an N-terminal DNA-binding domain that is followed by a nuclear localization signal and a C-terminal catalytic domain. DDE recombinases have a DDE motif-containing core that catalyzes polynucleotidyl transfer reactions. The catalytic core has acquired different DNA-binding modules during evolution to give rise to a diverse family of recombinases. The DDE domain in RAG1 is hypothetical.

catalytic domains of RAG1 and the DDE superfamily of recombinases needs to be verified by site-specific mutagenesis of the proposed DDE residues, or by comparison of their crystal structures, or both. In addition to the DDE-containing transposases and integrases[35], crystallographic analyses of the catalytic domains of proteins whose functions are not obviously related to transposition, such as RNAase H (Ref. 36) or RuvC (Ref. 37) have revealed a remarkably similar overall fold.

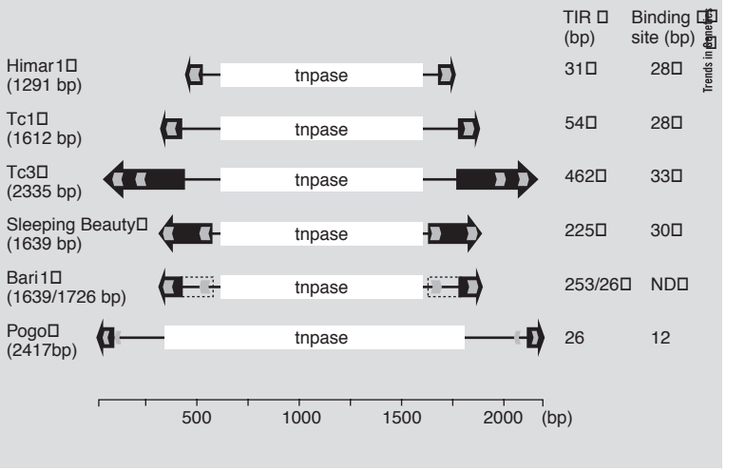
The emerging picture reinforces the idea of a common structural motif that catalyses polynucleotidyl transfer reactions in diverse biological contexts[28,35]. The different specificities in binding to DNA might have evolved by the apparent acquisition of different DNA-binding domains, and combinations thereof, in the evolution of DDE recombinases[10].

The transposon inverted repeats

The transposons are framed by terminal inverted repeats that contain binding sites for the transposase. Inverted repeats vary in length and contain transposase-binding sites in different numbers and patterns in the Tc1/*mariner* family (Fig. 3). Tc1 and *mariner* elements are the simplest and have repeats of <100 bp and a single binding site per repeat[18,19]. Tc3 elements have inverted repeats of >400 bp in length, each of which contains two binding sites, but the internal pair is not required for transposition (S. Fischer *et al.*, unpublished). A third group named IR–DR has a pair of binding sites containing short, 15–20 bp direct repeats (DRs) located at the ends of inverted repeats (IRs) that are 200–250 bp long[38]. This structure can be found in several elements whose inverted repeats are not significantly similar at the DNA-sequence level, such as: *Minos* and *S* elements in flies[39,40]; *Quetzal* elements in mosquitos[41]; Txr elements in frogs[42]; and at least three Tc1-like transposon subfamilies in fish[22], including *Sleeping Beauty*, a reconstructed transposon of the salmonid subfamily[43] (Fig. 3). There are two types of *Bari* elements in *Drosophila*; those that have short inverted repeats similar to Tc1, and those that have IR–DR structure[44]. However, both types of *Bari* element have two putative transposase-binding sites flanking their transposase genes (Fig. 3). This suggests that it is not the long inverted repeats *per se*, but the multiple binding sites for the transposase that are essential for the mobility of these elements. Indeed, we have found that both the outer and the inner pairs of transposase-binding sites are required for transposition of *Sleeping Beauty* (Zs. Izsvák *et al.*, unpublished).

Similar to the DNA-binding domains, the ~30 bp binding sites for Tc1-like transposases (Fig. 3) have a bipartite structure, in which the 5′-part of the binding site is recognized by the homeo-like domain, whereas 3′-sequences interact with the paired-like domain of the transposase[19]. The binding sites for *mariner* transposase are also around 30 bp in length, supporting the hypothesis that these transposases also have bipartite DNA-binding domains. By contrast, *pogo* elements have binding sites of 12 bp within their short inverted repeats[25], consistent with the predicted single HTH motif in their DNA-binding domains (Fig. 2b). These binding sites are repeated either in direct or in inverted orientation at the ends of the element (Fig. 3), but it has not been determined whether they are required for the mobility of *pogo* elements. Taken together, the Tc1/*mariner* superfamily contains some elements of simple structure, in which the transposase gene is flanked by a pair of transposase binding sites, and some elements of a more sophisticated structure, with multiple binding sites that might impose some control over the timing and specificity of the transposition reaction.

FIGURE 3. Structure of Tc1/*mariner* transposons



The central transposase genes (*tnpase*) are flanked by terminal inverted repeats (TIR; black arrows) that contain binding sites for the transposase. TIRs come in different lengths and contain binding sites in different numbers and patterns in the Tc1/*mariner* superfamily. Dotted lines in *Bari* elements indicate that certain versions of these transposons have long inverted repeats. Actual or putative transposase-binding sites are indicated as grey arrows near the ends of the elements.

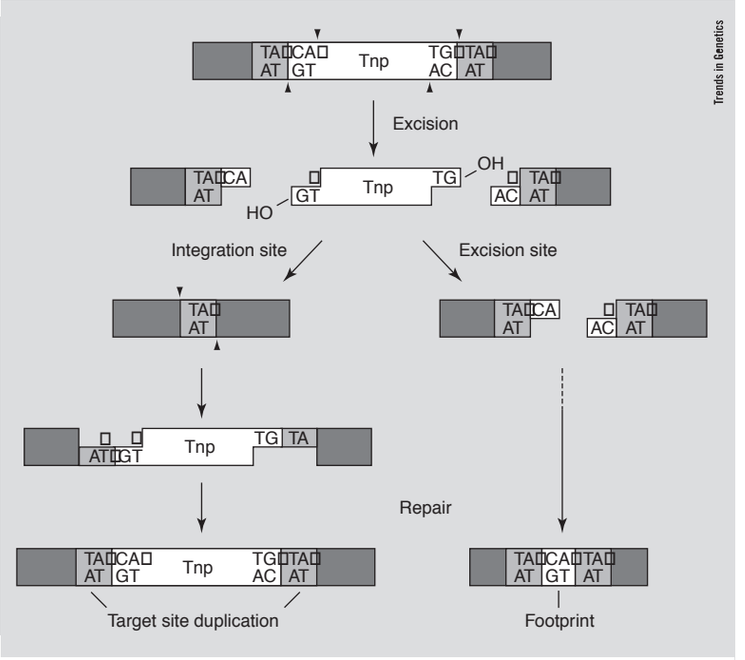
Mechanism of transposition

The transposase protein and the inverted repeats engage in a series of molecular events that lead to the excision of the element from its DNA context and its reintegration into a different locus (cut-and-paste transposition). Figure 4 shows the mechanism of jumping of the Tc3 element[32]. Similar mechanisms have since been found to apply to Tc1 (Ref. 17) and the *Himar1 mariner* elements[18].

The elements are all thought to undergo excision by a pair of staggered double-strand DNA breaks at the ends of the transposon (Fig. 4). The staggered cuts result in some of the sequences of the transposon inverted repeats being left behind, which might form transposon 'footprints' when the gap in the chromosome (or other DNA) is sealed by cellular repair processes. In some cases, the footprints can be the result of direct ligation of the staggered broken DNA ends[45]. Tc1/*mariner* transposons generally leave 2 bp (Refs 18, 32) or 3 bp (Refs 46, 47) footprints. It is reasonable to assume that elements that leave three, rather than two, nucleotides in the footprint probably excise via staggered cuts of 3 bp. Because the subsequent integration reaction is carried out by the 3′ end, the precise position of the 5′ cleavage only affects the size of the single-stranded gap that needs to be repaired after strand transfer (Fig. 4) and, thus, does not affect the final product of the transposition reaction.

The majority of Tc1/*mariner* transposons integrate into the sequence TA. There are a few exceptions, such as the ciliate TBE1 elements that prefer the target TNA instead[10]. In addition, transposases appear to recognize some bases next to the TA sequence, because sequences flanking the target TA determine the frequency at which transposition into a particular TA will occur[48,49]. Apart from the primary sequence, DNA structure at the insertion

FIGURE 4. Model for the mechanism of jumping of Tc1/*mariner* transposons



The Tc3 element is excised by transposase-mediated double-stranded breaks at the ends of the inverted repeats[32]. The DNA cut is staggered, which generates single-stranded transposon termini of two overhanging nucleotides with reactive 3′-hydroxyl groups (OH) and leaves two nucleotides of the transposon ends at the site of excision. Some other Tc1/*mariner* elements probably excise via a 3 bp staggered cut. The excised element integrates into a TA dinucleotide site in the target DNA. During integration, another staggered double-stranded DNA break is introduced by the incoming transposon at the TA target site, so that the TA will be duplicated and flank the inserted element after the single-stranded gap in the DNA is sealed by cellular repair processes. The excision site is also subject to DNA repair that can, in some cases, regenerate the terminal nucleotides of the transposon inverted repeats left in the gap, resulting in transposon footprints[45].

site also appears to influence mobility of *mariner* elements[50].

In summary, although biochemical evidence has mainly been obtained for Tc1, Tc3 and *mariner*, on the basis of similarity in sequences of the transposon ends, the target sequences and the transposase proteins as well as the similarity of the reaction products, it seems safe to assume that all Tc1/*mariner* transposons jump via the reaction mechanism proposed by van Luenen *et al.*[32] It has been proposed that there are mechanistic links between different recombination reactions, such as the cut-and-paste transposition used by Tc1/*mariner* elements, bacteriophage Mu transposition and retroviral integration, all of which proceed via Mg^{2+}-dependent transesterification reactions[51,52]. Similarly, V(D)J recombination is a transposition-like direct transesterification reaction, the chemistry of which resembles that of transposition of the bacterial elements Tn7 and Tn10 (Ref. 53). The Tn7 and Tn10 transposases both have DDE domains[54,55], supporting the proposition that the RAG1 recombinase might also have a DDE-containing catalytic core (Fig. 2). The similarity between V(D)J recombination and transposition is further supported by the recent discovery that the RAG proteins can mediate the transposition of DNA that is flanked by recombination signal sequences, suggesting that the V(D)J recombination machinery evolved from an ancient RAG transposon[56,57].

Tc1/*mariner* transposons in new hosts

Many prokaryotic elements require specific host proteins for transposition, which limits their mobility outside their natural hosts. Similarly, *P-elements* do not seem to jump outside the genus *Drosophila*[58]. The demonstration that, apart from the transposase, the transposition of Tc1/*mariner* transposons requires no other protein (or at least no species-specific protein) suggests that these elements do not have severe host restrictions. Indeed, on an evolutionary time scale, there is an indication that Tc1/*mariner* elements are promiscuous. Sequence comparisons of some transposons in species that are thought to have diverged more than 100 million years ago show elements that are virtually identical[22,59]. This observation imples that the elements spread recently from one species to the other by horizontal transfer[60], an idea that is supported by the absence of these elements in species that are related to the ones containing these elements (although selective loss is an alternative explanation for this).

The natural process of horizontal transfer can be mimicked in the laboratory. The Tc1/*mariner* experiments predict that expression of transposase in any host should be sufficient to trigger transposition of the corresponding transposon. Over the past few years a series of papers has been published that confirm this prediction. Table 1 summarizes experiments in which transposition of various Tc1/*mariner* elements was shown in species other than their natural hosts. Not only can these transposons be shuttled from one fly species to another[61–65] and from flies into mosquitos[66], bacteria[67] and protozoa[68], but they can also be used as transgene vectors for vertebrates. Transgenic animals have been generated by microinjection of transposon-containing plasmid vectors into eggs of zebrafish[69,70] and chicken[71] (Table 1). Furthermore, successful transposition in cell lines has been obtained for mouse ES cells[47] and human cells[43,72,73], and we have found the transposition of the *Sleeping Beauty* element into cells of many different species of fish, frog and mammals (Zs. Izsvák *et al.*, unpublished).

Tc1/*mariner* elements found in vertebrate genomes; *Sleeping Beauty* kissed to life

Tc1/*mariner*-like elements have been found in several vertebrate genomes[22,38,42,74–76], including the human genome[3,77,78]. All of the transposon copies isolated to date from vertebrates are clearly dead remnants of once active transposons[22,79–81] that, after successfully colonizing genomes, have become inactivated by mutations.

We recently reported how an alignment of different defective copies of Tc1-like elements from fish was used to reconstruct the sequence of an ancestral element that was presumed to be active more than 10–15 million years ago. A series of swaps and site-directed mutagenesis have brought it back to life; it was named *Sleeping Beauty*[43]. Interestingly, recent experiments show that, when tested under identical experimental conditions, the jumping activity of *Sleeping Beauty* was ~25-fold that of Tc1, Tc3 and *mariner* elements in human cells (S. Fischer, pers. commun.). In a sense, this seems counterintuitive, because this element was dead until two years ago. However, a plausible explanation for the difference in activity is related directly to the reconstruction event: whereas most transposons, as effective parasites, have settled for a low level of activity that is compatible with viability of the host, the reconstruction of *Sleeping Beauty* could have

TABLE 1. Horizontal transfer in the laboratory

	Host			Mobilization		
Transposon	**Original**	**New**	**Method of transfer**	**From**	**To**	**Ref.**
Tc1	Nematode (*C. elegans*)	Human	Transfection	Plasmid	Chromosome	81
Tc3	Nematode (*C. elegans*)	Zebrafish	Microinjection	Plasmid	Chromosome	78
Sleeping Beauty	Fish (reconstructed)	Fish*‡	Microinjection and transfection	Plasmid	Chromosome	47
		Frog*‡				47
		Mammals*‡				
		Human*				
		Mouse ES*	Transfection	Chromosome	Chromosome	53
Minos	Insect	Insect				
	(*D. hydei*)	(*D. melanogaster*)	Microinjection and *P-element*	Plasmid	Chromosome	72
		(*C. capitata*)	transformation	Plasmid	Chromosome	68
Himar1	Insect	Bacteria	Transformation	Plasmid	Chromosome	68
	(*H. irritans*)	(*E. coli*)				75
		(*M. smegmatis*)				75
		Human*	Adenoviral infection	Plasmid	Plasmid	12
Mos1	Insect	Insect				
	(*D. mauritiana*)	(*D. melanogaster*)	Microinjection	Plasmid	Chromosome	69
		(*D. virilis*)	Microinjection	Plasmid	Chromosome	70
		(*Ae. aegypti*)	Microinjection	Plasmid	Chromosome	73
		(*L. cuprina*)	Microinjection	Plasmid	Chromosome	71
		(*D. tryoni*)	Microinjection	Plasmid	Plasmid	71
		Protozoa (*L. major*)	Transformation	Plasmid	Chromosome	76
		Chicken	Microinjection	Plasmid	Chromosome	79
		Zebrafish	Microinjection	Plasmid	Chromosome	77

A summary of genetic transformation experiments using Tc1/*mariner* transposons in heterologous species. New hosts marked with asterisks are tissue-culture cell lines.
‡Unpublished work by Zs. Izsvák, Z. Ivics and R.H. Plasterk. Abbreviations: *C. elegans*, *Caenorhabditis elegans*; *D. melanogaster*, *Drosophila melanogaster*; *C. capitata*, *Ceratitis capitata*; *D. hydei*, *Drosophila hydei*; *H. irritans*, *Haematobia irritans*; *E. coli*, *Escherichia coli*; *M. smegmatis*, Mycobacteria smegmatis; *D. mauritiana*, *Drosophila mauritiana*; *D. virilis*, *Drosophila virilis*; *Ae. aegypti*, *Aedes aegypti*; *L. cuprina*, *Lucilia caprina*; *L. major*, *Leishmania major*; *D. tryoni*, *Dacus tryoni*.

resulted in an archetypal transposon at the prime of its activity, as it was when it had just started invading the new (fish) hosts and before it was slowly silenced by mutations.

Tc1/*mariner* elements as genetic tools

Transposons can be harnessed as vehicles for bringing new phenotypes into genomes by transgenesis (gain-of-function mutations), as well as destroying endogenous genes by insertional mutagenesis (loss-of-function mutations), in order to determine the function and importance of genes in cellular pathways. However, until recently, most animal species of agricultural or medical importance, as well as vertebrate models, lacked transposon technology.

Tc1/*mariner* elements have some potential advantages over existing viral and nonviral gene-delivery technologies. Transposon vectors mediate stable, single-copy integration of transgenes into chromosomes, which forms the basis of long-term expression throughout many generations of transgenic cells and organisms. Although the frequency of transposition decreases with larger insert size[50] (Zs. Izsvák *et al.*, unpublished; S. Fischer *et al.*, unpublished), elements of up to 14 kb have been seen to jump[62]. Thus, in experiments where the highest transposition frequency is not crucial, these elements might have advantages over retroviruses, adenoviruses and adeno-associated viruses that have strict maximal insert sizes. The *cis*-requirements for transposition are modest: fewer than 250 bp of transposon DNA are sufficient for jumping, which compares favourably with the LTRs of retrotransposons, and which could be useful when gene-trap vectors are to be constructed (especially for translational fusions). Another useful feature is that transposition requires only the transposase protein; thus, the site and moment of jumping can be regulated simply by controlling the expression of the transposase. So far, the tagging of retroviral oncogenes in mice has been limited to tissues that are accessible to retroviruses, such as MoMLV and MMTV, but Tc1/*mariner* elements might allow similar experiments for tumours of organs other than mamma and the lymphoid compartment.

As far as gene therapy applications in humans are concerned, Tc1/*mariner* elements have the same problem as retroviral vectors: random integration into chromosomes is undesirable. However, random insertion can be a clear advantage for gene identification through insertional mutagenesis. In this situation, a limiting factor can be the frequency of transposition, which might not be high enough for forward mutagenesis screens. It will certainly be important to establish whether the *Sleeping Beauty* element can be made to jump efficiently in vertebrate models, such as zebrafish, *Xenopus* and mice. An important area of research will be to derive the most-efficient transposition system, by comparison of Tc1/*mariner* elements, and possibly by screens for more-active transposase mutants.

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