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Review

DNA transposons in vertebrate functional genomics

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Abstract. Genome sequences of many model organisms of developmental or agricultural importance are becoming available. The tremendous amount of sequence data is fuelling the next phases of challenging research: annotating all genes with functional information, and devising new ways for the experimental manipulation of vertebrate genomes. Transposable elements are known to be efficient carriers of foreign DNA into cells. Notably, members of the Tc1/*mariner* and the hAT transposon families retain their high transpositional activities in species other than their hosts. Indeed, several of these elements have been successfully used for transgenesis and insertional mutagenesis, expanding our abilities in genome manipulations in vertebrate model organisms. Transposon-based genetic tools can help scientists to understand mechanisms of embryonic development and pathogenesis, and will likely contribute to successful human gene therapy. We discuss the possibilities of transposon-based techniques in functional genomics, and review the latest results achieved by the most active DNA transposons in vertebrates. We put emphasis on the evolution and regulation of members of the best-characterized and most widely used Tc1/*mariner* family.

Key words. Transposon; mutagenesis; transgenesis; functional genomics; gene trapping; zebrafish; mouse.

The Tc1/*mariner* transposon family

Transposable elements (TEs) are discrete DNA sequences that possess an intrinsic capability to change their genomic position. TEs are widespread in all organisms from bacteria to humans, and form a major fraction of eukaryotic genomes [1, 2].

TEs are distinguished whether their movement involves reverse transcription of an RNA intermediate (retroelements) or relies exclusively on DNA intermediates (DNA transposons). The vast majority of DNA transposons use a 'cut-and-paste' mechanism for moving [3]. These TEs are excised by an element-encoded transposase enzyme, and can be reinserted in a variety of sites in the host genome.

Members of the Tc1/*mariner* family are probably the most widespread DNA transposons in nature, represented in ciliates, plants, fungi and animals [4]. This monophyletic family is defined on the basis of transposase sequence homologies and a similar molecular mechanism of transposition [5]. These transposons are generally 1.3–2.4 kb long, and encode a transposase gene flanked by terminal inverted repeats (IRs) (fig. 1A). Tc1/*mariner* elements follow the cut-and-paste mechanism of transposition without overt target preference, except that they always integrate into TA target dinucleotides of host chromosomes (fig. 1B) [4].

The active invertebrate Tc1/*mariner*s were isolated from *Caenorhabditis elegans* (Tc1, Tc3) and from the *Drosophila* genus (Mos1, *Minos*). The active Himar1 element is a majority rule consensus of cloned genomic copies obtained from the horn fly *Haematobia irritans*

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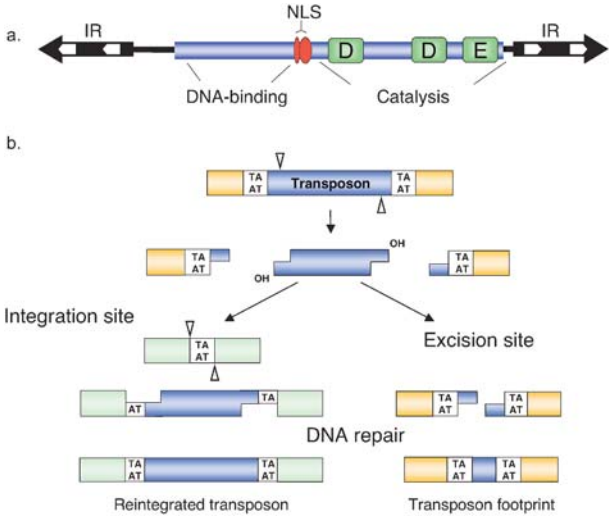

Figure 1. Structure and mechanism of transposition of Tc1/*mariner* elements. (*a*) Schematic representation of a Tc1/*mariner* transposon. The terminal inverted repeats (IR, black arrows) contain one or two binding sites for the transposase (white arrows). The element contains a single gene encoding the transposase (blue box). The N-terminal part of the transposase contains a DNA binding domain, followed by a nuclear localization signal (NLS). The C-terminal part of the protein is responsible for catalysis, including the DNA cleavage and rejoining reactions. The DDE amino acid triad is a characteristic signature of the Tc1-like transposases; *mariners* have DDD. (*b*) Cut and paste mechanism of transposition. The transposase initiates the excision of the transposon with staggered cuts and reintegrates it at a TA target dinucleotide. The single-stranded gaps at the integration site as well as the double-strand DNA breaks in the donor DNA are repaired by the host DNA repair machinery. After repair, the target TA is duplicated at the integration site, and a small footprint is left behind at the place of excision.

[6]. However, extensive search for active vertebrate transposons has so far failed to yield an active vertebrate Tc1/*mariner*-like transposon, for reasons that are discussed in the next section.

Evolutionary history of Tc1/*mariner* transposons in natural hosts

Phylogenetic relationships between very closely related Tc1/*mariner* elements are often inconsistent with those of their hosts [7, 8]. For instance, the closest relatives of a *mariner* subfamily in humans can be found in insects and worms [9]. It has been suggested that 'horizontal transfer' accounts for the spreading of elements across distantly related phyla [10]. Because TEs themselves are not infectious, it is not exactly known how they can invade new genomes. Potential vectors of horizontal transmission include viruses, and external and intracellular parasites [11, 12]. Once a transposon is transferred to a new host, it has to colonize its germline to persist in a population or, ultimately, in the entire species. At this initial phase, transposons can explosively amplify themselves [13]. However, transposable elements are not under positive selection, and thus mutations may accumulate in them in a time-proportional manner, resulting in partially or completely inactive transposon copies. This process is termed 'vertical inactivation' [10]. In parallel, the mutated transposase copies might become dominant-negative regulators of transposition. Thus, with time, the rate of propagation slows down, and finally, due to random genetic drift, transposons start to be extinct from their host genomes. The phenomenon is known as stochastic loss [10]. Therefore, in order to survive, transposons have to be horizontally transferred to new germlines and start their life cycle over again (fig. 2). DNA transposons are believed to be horizontally transferred more often than retroelements, possibly because the endurance of DNA intermediates of transposition within cells offers a better chance for hitchhiking transfer vectors [14]. Indeed, in some retrotransposition reactions the RNA intermediate is directly reverse transcribed into the integration site [15], thereby offering little chance to be horizontally transferred.

Due to the above mechanisms, Tc1/*mariner* transposons are extraordinarily widespread in nature. But the vast majority of these elements are defective in all eukaryotic genomes, and no active element has been identified so far from vertebrates. The ability of recombinant transposase proteins to catalyze complete transposition reactions in vitro [6, 16] implies that the broad distribution of these elements is due to the lack of highly specific host factors required for their transposition. However, Tc1/*mariner* elements are not truly promiscuous. For example, the reconstructed vertebrate *Sleeping Beauty* (SB) and *Frog*

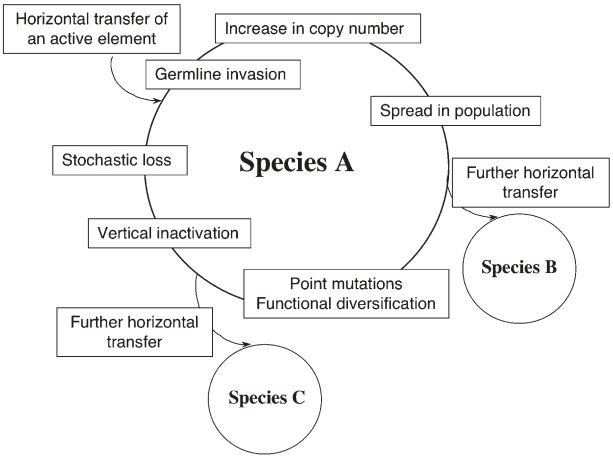

Figure 2. Evolutionary life-cycle of Tc1/*mariner* elements in natural hosts. The main events of the life cycle are depicted (for details, see text). The cycle was proposed to describe the evolution of *mariner* elements [26], but is probably also valid for the Tc1 family. Horizontal transfer of active transposons into new species can occur before or after functional diversification. Modified after [26] and [9].

Prince (FP) elements are active in essentially all vertebrate classes [17, 18], but there are indications that they might not be active outside vertebrates. Similarly, the activity of the nematode transposons Tc1 and Tc3 and the insect *mariner* elements in vertebrate species is not comparable with transposition frequencies observed in their original hosts [19]. Accumulating evidence indicates that certain host factors are needed for transposition, which can set barriers for the successful colonization of a new species by any given element.

Regulation of Tc1/*mariner* transposons

Any TE that can regulate its activity either by its intrinsic features or by interaction with host factors can override evolutionary factors acting towards its elimination [13]. The general presence and long-term persistence of TEs in genomes provoked regulatory mechanisms to evolve both in the transposons themselves and in the host genomes. Host-encoded regulatory mechanisms include transcriptional and post-transcriptional silencing processes that can downregulate factors required for transposition. For example, RNA interference (RNAi) has been found to be a major mechanism for transposon silencing in nematodes [20], and perhaps contributes to transposon regulation in other animals as well.

One of the transposon-derived regulatory mechanisms acts when the functional ('wild-type') transposase is present in the cell at a higher-than-optimal concentration. In this case, the overall transposition activity decreases. The phenomenon, termed overproduction inhibition (OPI), has been described for bacterial, plant and vertebrate transposons [21–24], and the best studied during transposition of the Mos1 and Himar1 *mariner* elements [25–27]. The mechanism of OPI is not clearly understood, but it has been suggested that it acts on the post-translational level. Given that transposases function in multimeric complexes, the high number of available transposase molecules can shift the equilibrium towards less active multimers [25].

A decline in overall transposition frequency can also be observed in the presence of missense mutations and truncated versions of the transposase proteins [28]. In the case of Mos1 transposition, transposases mutated in their catalytic domain have the most profound effect. Such transposases are not only impaired in their abilities to catalyze the transposition reaction, but can also downregulate the activity of the active transposase [29, 30]. The most likely explanation for the phenomenon is dominant-negative complementation by inactive transposase subunits that 'poison' the activity of wild-type transposase in multimeric complexes [25]. Catalytically inactive transposases might also compete with wild-type transposase in substrate binding, thereby lowering transpositional frequencies.

Finally, transposons that no longer encode functional transposases but still retain transposase binding sites can function as substrates for transposition, thereby soaking up wild-type transposase. The process called transposase titration was first suggested for P elements [31]. These non-autonomous transposons can move and multiply, but they do not contribute to propagation of the transposase gene. As a result, this process will lead to a decrease of the ratio of autonomous to nonautonomous elements in a genome over evolutionary time [26]. The latter two regulatory mechanisms imply that inactive elements can be positively selected for their repressing activities [25], and can explain the prevalence of many defective elements in the same genome. Recent data suggest the intimate involvement of various host factors in the regulation of DNA transposition in vertebrates.

Host factors in SB transposition

Structural factors in SB transposition

Dependence of the transposition process on host factors is best studied in the case of SB, a reactivated Tc1/*mariner* transposon from fish [32]. Although SB can jump in cells of all the major vertebrate classes, the efficiency of transposition can significantly vary between different cell types [17]. Even in the same organism, depending on whether transposition occurs in somatic, germinal or embryonic stem cells, the frequency of transposition can differ by up to three orders of magnitude [33]. One possible explanation for this phenomenon is differential expression of cellular factors required for transposition.

Recent results indicated that conformational properties of the transposon and those of its genomic context have a profound influence on the efficiency of SB transposition. Zayed et al. (2003) found that the high-mobility group protein HMGB1 is a host factor of SB transposition. HMGB1 is a non-histone, chromatin-associated protein which is thought to be recruited to specific DNA sequences by protein-protein interactions [34]. HMGB1 has been implicated in gene regulation, DNA replication and recombination processes mainly through its ability to alter DNA architecture [34]. The transposition frequency of SB dropped dramatically in HMGB1 knockout mouse cells. Moreover, overexpression of this protein in wild-type mouse cells resulted in an elevated level of transposition, suggesting that HMGB1 is a limiting factor of SB transposition. The role of HMGB1 is most likely manifold, but its main contribution to transposition seems to apply to the pre-excision phase of transposon movement. HMGB1 is recruited to the IRs by the SB transposase, where it facilitates transosase binding [35]. In addition, due to its pronounced DNA bending ability, HMGB1 was proposed to regulate the proper assembly of the catalytically active synaptic complex at the transposon ends [35].

Similarly to SB transposition, HMG proteins are involved in other transposition-like mechnisms, such as V(D)J recombination [36] and retroviral integration [37].
Another host mechanism regulating SB transposition is CpG methylation, and subsequent heterochromatin formation at the transposon donor site [33]. Expression of a transgene from concatameric transposon units was found to be silenced [38], suggesting that the transposon arrays had been epigenetically modified. Interestingly, methylated transposons from both episomal and genomic contexts excise approximately 100-fold more efficiently than unmethylated transposons [33]. Chromatin immunoprecipitation experiments revealed that the hyperactive genomic donor sites have the characteristics of a heterochromatic structure. Thus, similarly to the effect of HMGB1, it seems that conformational changes of the excising transposon greatly influence the efficiency of transposition. Nevertheless, differences in chromatin state of mouse ES cells and germ cells can only partly explain the pronounced differences in transposition rates in these cells [38–40]; thus, alternative explanations, e.g. specific cellular factors, are yet to be sought.

SB and host DNA repair

Since SB, like any other DNA transposon, is not equipped with innate DNA repair activity, it remains the duty of the host cell to heal the chromosomal wounds introduced by transposition. Based on studies performed on the P element [41] and *mariner* [42], it has been proposed that both nonhomologous end joining (NHEJ) and homologous recombination (HR) play a role in repairing DNA damage induced by cut-and-paste transposons. Two recent studies examined the relationship between SB transposition and host DNA repair in mammalian cells [43, 44]. The fate of transposon excision sites was monitored in wild-type and mutant mammalian cells lacking components of the major pathways of double-strand break repair. It has been found that the NHEJ pathway is the predominant way of repairing transposon-induced DNA breaks; however, in the absence of these factors, HR pathways can also participate in the process. Izsvák et al. (2004) showed in vivo interaction between the SB transposase and Ku, the DNA binding subunit of the DNA-dependent protein kinase, a major factor in NHEJ. This observation suggests an intimate relationship between SB transposition and this particular host repair machinery.
Taken together, several lines of research indicate that SB can interact with various proteins of its hosts, and some of them are limiting factors of its activity. The host factors of SB transposition identified so far are all abundant components of cells. They are also highly conserved proteins among (but not necessarily outside) vertebrates, providing an explanation why SB is able to transpose in all vertebrate classes, but not in invertebrates. It is also tempting to speculate that the abundance of these proteins and the capability of SB to interact with them can contribute to the varying transpositional activity of SB in different vertebrate cells [17]. Understanding these interactions offers the opportunity to further develop the system as a research tool.

Transposons as genetic tools

Insertional mutagenesis

Alongside computational approaches and gene expression studies, mutational analysis is the most straightforward way of identifying gene function. One approach of creating mutants is to target and disrupt a gene of interest by homologous recombination; also referred to as reverse genetics. However, in spite of our growing acquaintance with protein domains, protein-protein interactions and molecular structures, our knowledge is still inadequate to reliably predict the biological process that will be affected by knocking out a particular gene.
Another approach of obtaining mutant phenotypes is to introduce loss-of-function mutations into genomes of model organisms in a random and genome-wide fashion, termed forward genetics. Mutagenesis efforts have been carried out mainly based on X-ray irradiation and chemicals. However, it turned out that X-ray irradiation can cause a variety of chromosomal rearrangements affecting several genes simultaneously, which makes identification of the functions of individual genes difficult. Ethylnitrosourea (ENU) is a potent chemical mutagen that primarily introduces point mutations into DNA [45]. Two large-scale mutagenesis screens have been performed in zebrafish (*Danio rerio*) using ENU, and it is routinely used in functional genetic analyses of the mouse genome [46–48]. The major advantages of ENU are easy use and highly efficient mutagenic rates in high-throughput screens. Nonetheless, a common disadvantage of these mutagenesis approaches is the time-consuming and labor-intensive molecular identification of the affected genes by positional cloning. While in some cases mutant phenotypes implicate certain signal transductional or developmental processes or genes, such a candidate gene approach can only be used in a fraction of the mutants. There are ~35,000 genes in mammals [2], which necessitates the development of methods for rapid identification and functional annotation of genes.
An alternative approach of introducing mutations into the genome is insertional mutagenesis. Discrete pieces of foreign DNA can be harnessed to disrupt host gene function by creating random insertions in the genome. As opposed to chemical mutagenesis, inserting DNA fragments into genes simultaneously provides a molecular tag which can be used to rapidly identify the mutated allele. Viral and non-viral technologies have been devised

to facilitate the penetration of transgenes through biological membranes. Non-viral methods, including naked DNA injection, electroporation, liposomes and 'gene guns' can be useful to introduce DNA into the cells, but chromosomal integration of the introduced DNA is still very inefficient. Moreover, a common drawback of the integration created by these techniques is the concatamerization of the foreign DNA at the insertion locus. Such events can facilitate chromosomal rearrangements [49], aberrant splicing, heterochromatin formation, gene silencing [50], and can interfere with cloning.

The above problems can be circumvented by using retroviruses. The overt advantage of using viruses as vehicles for delivering DNA into cells is their capability to penetrate membranes and to catalyze the integration of single copies of the proviral DNA into chromosomes. However, retroviruses have pronounced preferences for their sites of integration [51], thereby limiting the spectrum of mutations. Moreover, retroviral vectors have limited packaging size, and due to their long terminal repeats, they can induce gene silencing [50] and ectopic reporter gene expression. Additionally, the observations coming from mutagenesis screens in zebrafish suggest that virus-based techniques are labor intensive, and achieving high throughput requires a large facility for screening [52]. Therefore, as an alternative approach to viruses, techniques of transposon-based whole-genome manipulation launched a new wave of research in functional biology.

DNA transposons have been routinely used for studying bacterial, fungal and plant genes in forward genetic screens. Similarly to the retrovirus-based methods, transposons can be utilized for insertional mutagenesis, followed by easy identification of the mutated gene. However, DNA transposons have several advantages compared to the above approaches. For example, unlike proviral insertions, transposon insertions can be remobilized by supplying the transposase activity in trans. Thus, instead of performing time-consuming microinjections, it is possible to generate transposon insertions at new loci by simply crossing stocks transgenic for the two components of the transposon system (transposon + transposase). This scenario is especially useful when transposition events are directed to the germline of experimental animals in order to mutagenize germ cells. Also, transposase expression can be directed to particular tissues or organs by using a variety of specific promoters. In addition, remobilization of a mutagenic transposon out of its insertion site can be used to isolate revertants, and if transposon excision is associated with a deletion of flanking DNA, it can be used to generate deletion mutations. Furthermore, since transposons are composed of DNA and can be maintained in simple plasmids, they are much safer and easier to work with than highly infectious retroviruses. The transposase activity can be supplied in the form of DNA, messenger RNA (mRNA) or protein in the desired experimental phase.

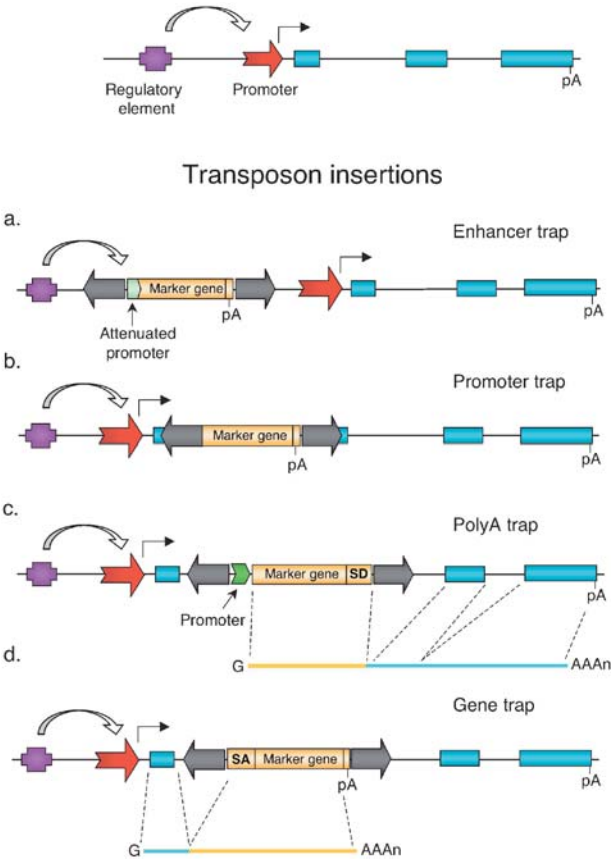

Figure 3. Transposon-based gene-trapping vectors. On top, a hypothetical transcription unit is depicted with an upstream regulatory element (purple box), a promoter (red arrow), three exons (blue boxes) and a polyadenylation signal (pA). Major classes of transposon-based trapping constructs and spliced transcripts are shown below. Transposon inverted repeats are indicated by black arrows, different promoters are depicted as green arrows, and SD and SA represent splice donor and slice acceptor sites, respectively. Modified after [53].

When transposons are used in insertional mutagenesis screens, transposon vectors often comprise four major classes of constructs to identify the mutated genes rapidly (fig. 3). These contain a reporter gene, which should be expressed depending on the genetic context of the integration. In *enhancer traps* (a), the expression of the reporter requires the presence of a genomic *cis*-regulator to act on an attenuated promoter within the integrated construct. *Promoter traps* (b) contain no promoter at all. These vectors are only expressed if they land in-frame in an exon or close downstream to a promoter of an expressed gene. In *polyA traps* (c), the marker gene lacks a polyA signal, but contains a splice donor (SD) site. Thus, when integrating into an intron, a fusion transcript can be synthesized comprising the marker and the downstream exons of the trapped gene. *Gene traps* (or exon

traps) (d) also lack promoters, but are equipped with a splice acceptor (SA) preceding the marker gene. Reporter activation occurs if the vector is integrated into an expressed gene, and splicing between the reporter and an upstream exon takes place. The gene trap and polyA trap cassettes can be combined. In that case, the marker of the polyA trap part is amended with a promoter so that the vector can also trap downstream exons, and both upstream and downstream fusion transcripts of the trapped gene can be obtained [53]. The above constructs also offer the possibility to visualize spatial and temporal expression patterns of the mutated genes by using *LacZ* or fluorescent proteins as a marker gene.

Transgenesis

The other major field of applications of transposon-based technologies is somatic and germline transgenesis. Transposon-based technologies can be exploited for gene transfer in cultured cells. Once integrated, transposase-deficient non-autonomous transposons are stable in the absence of the transposase. Transposons can be harnessed to integrate plasmid-based siRNA expression cassettes into chromosomes to obtain stable knockdown cell lines by RNAi [54]. Also, TEs hold potential for generating transgenic model organisms, or animals of agricultural and biotechnological importance. Nevertheless, the far end on the scale of transposition-based somatic gene transfer is human gene therapy. Indeed, a large body of work has already been done in mice investigating possibilities of transposon-based human gene therapy. These issues have recently been reviewed [55], and are therefore not considered in this article.

Tc1/*mariner* and hAT superfamily transposons in vertebrate functional genomics

The invertebrate P element and Tc1 transposon-based vectors have been extremely valuable in exploring gene function in the invertebrate model organisms *Drosophila melanogaster* and *Caenorhabditis elegans*, respectively [56, 57]. However, manipulating vertebrate genomes with TEs was until recently not feasible. Unfortunately, vertebrate model organisms seem to lack active, endogenous transposons such as P and Tc1; the only exception so far is the *Tol2* element of the medaka fish (*Oryzias latipes*) (see below). To address this problem, a variety of invertebrate TEs were adopted for gene transfer in vertebrates. However, invertebrate transposons tend to have moderate activity in vertebrates [19], most likely due to restricting activities, or to the lack of specific cofactors. Another way out of the problem is resurrecting vertebrate transposons from their once active genomic remnants. Indeed, the two, probably most powerful, vertebrate transposon systems SB and FP are results of molecular reconstructions performed on defective fish and frog transposon sequences, respectively (see below).

The following sections focus on the latest genetic applications of members of the two most promising transposon families so far: the Tc1/*mariner* and the hAT superfamily elements.

Minos

The Tc1 family *Minos* element [58] has been used for gene transfer in cultured human cells [59] and in mouse tissues [60]. Transfection of 10^6 HeLa cells with *Minos* vectors resulted in ~4×10^4 transgenic cells with an average of two insertions per cell. In a larger-scale gene trap experiment the authors proved for the first time that a TE is potentially capable of disrupting all genes of a mammalian organism [59]. The transposition activity of *Minos* in mammalian tissues was assessed in a double transgenic mouse line expressing the transposase in the thymus and in the spleen. The ~0.6% transposition frequency per thymus cell was low compared to that detected in HeLa cells. However, directing transposase expression into the female germline with an oocyte-specific promoter resulted in an 8.2% transpositional frequency [61].

Encouraging results were obtained when *gfp*-marked *Minos* transposons were coinjected with *Minos* transposase mRNA into fertilized eggs of the basal chordate model system *Ciona intestinalis* [62]. Founder animals transmitted the tissue-specifically expressed transgene to every third of their progeny. In two of the green fluorescent protein (GFP) positive lines irregular GFP patterns were observed. Molecular analysis revealed that the integrated transposon disrupted genes. Therefore, the authors concluded that these insertions were in fact enhancer trap events, and the promoter of the marker gene was influenced by enhancer sequences [62].

These results indicate the usefulness of the *Minos* system in different model organisms with various gene identification approaches. However, the potential of *Minos* based-vectors in high-throughput screens still has to be determined.

Sleeping Beauty

SB exhibits high transpositional activity in a variety of vertebrate cultured cell lines [17], embryonic stem cells [39] and in both somatic [63] and germline [19, 38, 40, 64] cells of the mouse in vivo. However, the major bottleneck of any TE-based application is overall transpositional activity. Therefore, considerable effort has been made to improve the transposition efficiency of SB by means of modifying its IRs and systematically mutating the transposase gene [23, 24, 65]. The combined effect of these modifications is an almost 10-fold enhancement of trans-

position in human cells as compared to the first-generation transposon system [24]. It was recently reported that profound changes introduced into the IR structure of the transposon significantly extended the transgene-carrying capacity of SB vectors [24]. Furthermore, SB can be equipped with gene-trap cassettes [64, 66, 67], which significantly enhances its utility as a tool for functional genomics in vertebrate models.

Insertional mutagenesis in mouse with SB

SB has been successfully used for forward genetics approaches in the mouse. Double transgenic mouse lines were generated bearing chromosomally present transposons and an either ubiquitously [38, 40, 64, 66] or male germline-specifically [19] expressed transposase gene. Segregating the transposition events by mating the founder males to wild-type females revealed that up to 80% of the progeny can carry transposon insertions [38], and a single sperm of a founder can contain, on average, two insertion events [40]. Additionally, a recent paper elegantly showed that the germline of such a founder can harbor ~10,000 different mutations [64].

These experiments revealed that the transposons in the double-transgenic animals are efficiently mobilized from an array of multiple transposon units at a chromosomally resident donor site. Indeed, excision frequencies of *gfp*-marked transposons in founder mice were as high as 1 per 1.5 cells in the tail, and 1 per 15 cells in the blood [38]. Experimental evidence suggests a correlation between frequencies of element excision and integration [68], and that most of the excised transposons get reintegrated [39]. Thus, it was an unexpected finding that no GFP expression was detected in founder animals in which transposon excision apparently occurred efficiently. These data indicate that the transgenes carried by SB can be subjected to positional effects and expressional downregulation. However, when the doubly transgenic founders were crossed with wild-type mouse, the frequency of GFP-active mice reached up to 80% [38]. As an explanation the following scenario is suggested. Multiple arrays of transposons in a head-to-tail orientation (created by transposase-independent integration) are subject to mechanisms of epigenetic modifications, such as methylation and heterochromatin formation, but these DNA alterations do not hinder element excision. Nevertheless, the integrated elements retain their repressed chromatin state in their new locus; thus no GFP expression is detected. On the other hand, when the insertions at new loci are passed through the germline, they are freed of the repressed state. Thus the marker gene can be expressed. Transposition of gene-trap transposons identified mouse genes with ubiquitous and tissue-specific expression patterns, and mutant/lethal phenotypes were easily obtained by generating homozygous animals [64, 66].

Local Hopping

The studies described above established very efficient transposition of SB in the mouse germline, showed no integration preference with respect to gene structure [66], but revealed that SB tends to reintegrate to sites that are relatively close to the donor locus, a phenomenon called local hopping. Local hopping of SB was first described by Luo et al. (1998) in mouse embryonic stem (ES) cells, and then observed in the mouse germline, indicated by cosegregation of new transposon insertions with their donor sites [19, 40]. Additional data showed that most of the reintegration events occur within 3 Mb [64], and that the total transposition interval of local hopping is between 5 and 15 Mb, which is significantly broader than the 100-kb local hopping interval of the P element [69]. Given that *Minos* has also been found to exhibit preference for local transposition in mice [61], the phenomenon seems to be a general property of the Tc1/*mariner* family.

Local hopping offers the possibility to direct extensive insertional muatagenesis to gene clusters and particular chromosomal regions. The feasibility of such application has recently been demonstrated by generating four mutant mice having different transposon insertions in a single gene. Does local hopping interfere with the intention to perform whole-genome transposon mutagenesis from a limited number of donor sites? The high number of transposition sites in the germ cells of founder mice and the fact that approximately every fourth excised transposon can be randomly reintegrated into chromosomes other than the donor chromosome suggest that whole-genome mutagenesis is feasible [64]. Alternatively, the problem of local hopping can be circumvented by injecting SB transposons and transposase mRNA into one-cell mouse embryos. In this case, integration into any chromosome has equal likelihood [70]. These results may also indicate that local hopping is not an intrinsic feature of the transposition machinery, but is due to unequal availability of the different chromosomes as a transposition target in the nucleus.

Transgenesis with SB in fish

SB has been used for transgenesis in both of the most important fish model systems: zebrafish and medaka. Fertilized zebrafish eggs were coinjected with SB mRNA together with fluorescent protein-marked transposons [71]. A transgenesis rate of 30% was obtained; ~90% of the total integration events were transposase mediated, and at least 80% of these expressed the cargo transgene.

A similar experimental setup was used to determine the efficiency of SB transposition in medaka [72]. It was found that the presence of SB IRs alone was able to enhance promoter-dependent transient expression in the injected F0 fish. Transgenesis frequencies in the presence and absence of the transposase were also very similar (31 and 29%, respectively). The reason for this has not been

elucidated. The authors tested whether the SB system can be used to detect enhancer trap events in medaka, and temporally and spatially restricted GFP expression was observed in F1 transgenic fish lines, presumably due to integration of the enhancer trap SB construct near chromosomal regulatory sequences.

Together, SBs can efficiently be harnessed for reporter gene integrations in fish, with transgenesis rates comparable to those obtained with the non-transposon based I-*Sce*I meganuclease approach in medaka [73].

Frog Prince

As discussed above, SB is not equally active in different model species. Consistently, transposition assays in a variety of vertebrate cultured cells revealed an extensive variation in the efficiency of transposition [17]. Therefore, it was expected that the availability of other, highly active transposons of different vertebrate origins could widen the possibilities of transposon-based genetic manipulations.

FP is a Tc1/*mariner*-like element that was recently reactivated from genomic transposon copies of the Northern Leopard Frog (*Rana pipiens*) [18]. An open reading frame trapping method was used to identify uninterrupted transposase coding regions, and the majority rule consensus of these sequences revealed an active transposase gene. Thus, in contrast to the 'resurrection' procedure of SB, the relatively young state of genomic elements in *Rana pipiens* made it possible to ground the majority rule consensus on transposon copies derived from a single species. The SB and FP transposons are clearly distinct, sharing only ~50% identity in their transposase sequences [18].

The transposition activity of FP was determined in cultured cell lines of major vertebrate taxa. FP shows similar activity to SB, but interestingly, it exhibits 70% higher transposition efficiency in zebrafish cells. In considering explanations for this finding, the intrinsic activity of FP can possibly be ruled out, since the two systems have similar activities in mammalian cells. However, it is tempting to speculate that the difference in transposition activity is due to the lack, or inefficiency, of repressing activities that would interfere with the FP transposition machinery. Being a fish element, SB can be subject to inhibitory mechanisms acting originally on numerous endogenous copies of the Tdr1 element, a zebrafish transposon very similar to SB [74]. On the contrary, the amphibian FP transposon, significantly different from SB, seems to be less vulnerable to such inhibitory mechanisms.

The ability of FP to efficiently trap expressed genes was tested using a gene-trap transposon in cultured HeLa cells [18]. To our surprise, up to 30% of the selected FP insertions hit human genes so that correct splicing could occur with 5′ exons and the marker gene. The reasons for this exceptional gene-trapping efficiency and the activity of FP in embryos and in the germline of different model systems are yet to be determined. The lack of detectable interaction between SB and FP offers the possibility to use the two systems simultaneously and complementarily in genetic analyses in vertebrates.

The *Tol2* element: an active member of the hAT transposon family in medaka

A recessive mutation causing an albino phenotype of the Japanese medaka (*Oryzias latipes*), a small freshwater fish of East Asia, was isolated from a wild population [75]. It was found that the mutation is due to a 4.7-kb-long TE insertion into the fifth exon of the tyrosinase gene [76]. The DNA sequence of the element, named *Tol2*, is similar to transposons of the hAT family, including *hobo* of *Drosophila*, *Ac* of maize and *Tam3* of snapdragon [77].

Two lines of evidence suggest that the *Tol2* element invaded the medaka fish genome recently. First, only 2 out of 10 medaka species tested, *Oryzias latipes* and *Oryzias curvinotus*, possess the *Tol2* elements in their genomes [78]. Second, the transposon copies found in these species are highly homogeneous in their structures [79]. It is not known where the *Tol2* element was horizontally transferred from and when it was captured by the medaka genome.

Although the *Tol2-tyr* element, the particular copy found at the tyrosinase gene locus, can be excised during medaka embryogenesis at low frequencies [76], it had not been known whether it is autonomous (i.e., capable of expressing an active transposase). To address this question, a simple assay system to detect transposition activity in zebrafish embryos was developed [80]. When plasmid DNA containing a non-autonomous transposon vector is injected into fertilized zebrafish eggs together with the transposase mRNA synthesized in vitro, the transposase protein catalyzes excision of the transposon from the plasmid. The excision site on the plasmid is healed by the host repair machinery, resulting in characteristic transposon footprints, whereas the excised transposons can integrate into the genomes of future germ cells during embryogenesis, and the insertions can be identified in the offspring from the injected founder fish [80]. *Tol2* insertions are flanked by 8-bp duplications of the integration site and do not cause any chromosomal rearrangement at the target locus [80]. *Tol2* is the only natural transposon in vertebrates from which an autonomous member encoding a fully functional transposase has ever been found.

Tol2-mediated transgenesis and a gene-trap approach in zebrafish

Transposition of *Tol2* in zebrafish is highly efficient. Using optimized experimental conditions, ~50% of the fish injected with a transposon-containing plasmid and transposase mRNA can transmit transposon insertions to the

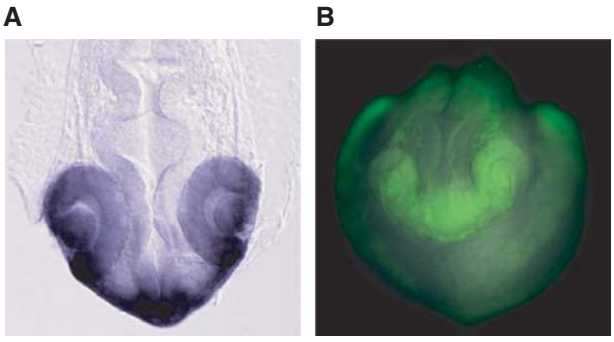

Figure 4. Transgenesis in zebrafish using *Tol2*. (*A*) Expression of *six3.2* in a 24-h zebrafish embryo as revealed by whole-mount in situ hybridization using a *six3.2* RNA probe. (*B*) GFP expression in transgenic fish with an insertion of the transposon construct carrying the GFP gene under the control of *six3.2* promoter.

next generation, thereby becoming founder fish [81]. This frequency is higher than that observed with any other method used to generate transgenic fish, including injection of naked plasmid DNA (5–9% [82]), the *Tc3* transposon system (7.5% [83]), a pseudotyped retrovirus (10% [84]), the I-*Sce*I meganuclease system (30.5% [73]) and the SB transposon system (5–31% [71]). The germ cells of the founder fish are highly mosaic with respect to transposon insertions. In one extreme case, 100% of F1 fish had transposon insertions, and in total, more than 25 different insertions were transmitted by a single founder fish. The average number of transposon insertions transmitted per founder fish is currently between five and six [81].

An important application of transgenesis in zebrafish is to establish transgenic lines expressing GFP in a specific tissue or organ. The *Tol2* transposon system was applied to construct transgenic fish expressing GFP under the control of the promoter of the *six3.2* gene, which is expressed in the anterior neural plate and in the eye [85]. Embryos containing a single transposon insertion expressed GFP in the forebrain and eyes, indicating that regulated gene expression can be recapitulated by transgenesis using the *Tol2* transposon system (fig. 4) [81]. Importantly, specific expression patterns are observed through several generations (currently up to F4), indicating that transgenic lines can be established with persistent reporter gene expression.

The *Tol2* transposon system has been applied to gene trapping [81]. When a gene trap transposon vector containing a splice acceptor, the GFP gene, and the SV40 polyA signal are integrated in the zebrafish genome, a variety of GFP expression patterns can be observed in F1 embryos: i.e. some are weak and some are strong, or some are ubiquitous, and some are temporally and spatially restricted (fig. 5). This indicates that the gene-trap construct is inserted at various loci, and GFP is expressed under the control of endogenous promoters [81]. In a pilot experiment, 36 unique GFP expression patterns at the first day of development were identified out of 156

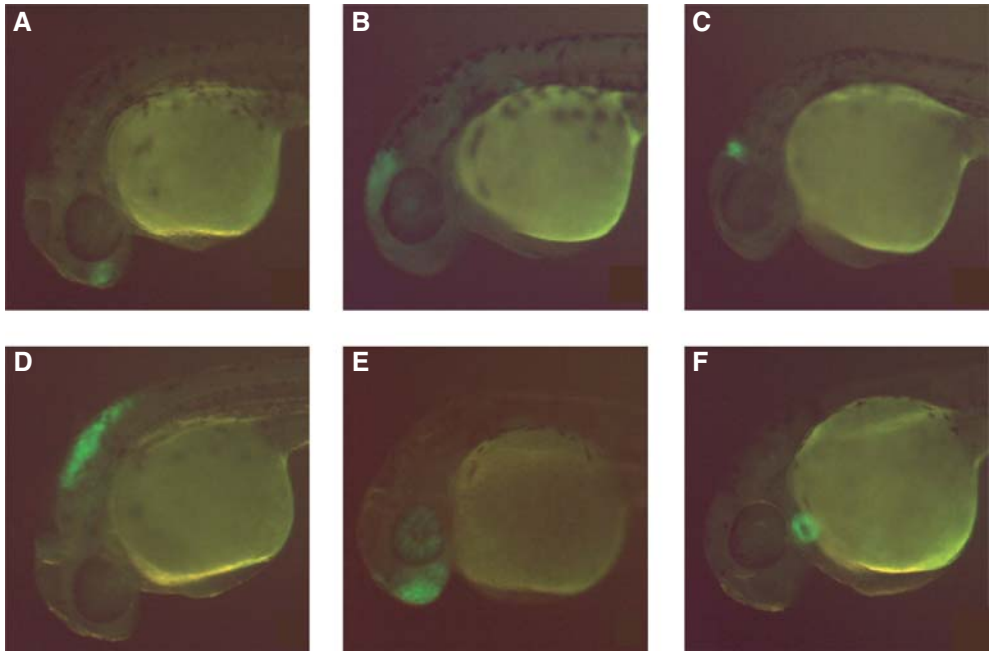

Figure 5. Gene traps using *Tol2* in zebrafish. Unique GFP expression patterns observed in embryos carrying insertions of the gene-trap transposon construct. GFP expression in (*A*) forebrain, (*B*) midbrain, (*C*) midbrain-hindbrain boundary, (*D*) hindbrain, (*E*) forebrain and eye, (*F*) heart.

injected fish. 5′ RACE analysis revealed fusion transcripts of endogenous upstream exons and the GFP gene precisely at the splice acceptor within the transposon. The GFP gene in the gene-trap construct contained an ATG codon for translational initiation, so that gene trapping could occur by insertion either upstream or downstream of the endogenous initiation codon of a gene. Indeed, one of the transposon insertions trapped the *hoxc3a* gene by fusing the first non-coding exon of the gene to the GFP coding region. The amount of the *hoxc3a* transcript in homozygous embryos was decreased to less than 25% of that synthesized in wild-type embryos. Thus, although the insertion did not abolish the wild-type transcript completely, it markedly interfered with the synthesis of the normally spliced transcript. The leakiness of the gene-trap vector may be consistent with the finding that no lethal phenotypes have been observed to date in animals that are homozygous for transposon insertions. The next goal, therefore, is to develop methods that couple gene trapping with efficient insertional mutagenesis.

In the pilot screen for gene traps, transgenic zebrafish lines with 36 unique GFP expression patterns were established by screening offspring from 156 injected fish, and it is estimated that about 8% of the chromosomal insertions of the gene-trap construct can cause such unique GFP expression patterns [81]. Currently, construction of one transgenic fish line with specific GFP expression usually takes more than a year. The gene trap approach will be an alternative. Since one unique expression pattern can be isolated in every four or five injected fish (36 patterns out of 156 injected fish), a small lab can collect hundreds of fish with different expression patterns within 1 year, possibly including the desired one. Collaborative work by several laboratories could produce thousands of gene trap lines, which would represent a useful resource. The transposon-mediated gene trap approach in zebrafish should facilitate studies on the function of vertebrate developmental genes, and provide a basis for further development of useful genetic methodologies in zebrafish.

Tol2 in other vertebrates

The *Tol2* transposon system is also active in vertebrates other than zebrafish. Chromosomal transposition has been demonstrated in medaka [86]. Transposase-dependent excision of a transposon vector form plasmids injected into embryos has been shown in *Xenopus laevis* and *tropicalis* [87]. Furthermore, the element can undergo excision in mouse and human cells [88], and chromosomal transposition from donor plasmids has been demonstrated in mouse ES cells [89]. Thus, although host factors necessary for transposition of *Tol2* have not yet been elucidated, such factors should be conserved from fish to mammals.

Conclusions

Transposable elements belonging to the Tc1/*mariner* superfamily and the *Tol2* element are both being applied in a variety of experimental approaches for transgenesis and insertional mutagenesis in vertebrate model systems. It will be important to determine whether these diverse transposons have different characteristics in both efficiency and preference for integration. All transposons display some level of preference for integration sites, and target site selection has been shown to be governed by, among other factors, primary DNA sequence and structural characteristics of the target DNA. For example, all Tc1/*mariner* elements integrate into TA dinucleotides within bendable regions of DNA [90]. Although the *Tol2* element does not show a pronounced specificity for inserting into a particular sequence [81], it is expected that it nevertheless will show a non-random insertion profile. It is likely that, similar to the *hobo* element in *Drosophila* [91], target selection of *Tol2* is influenced by structural characteristics of the DNA and chromatin. Thus, the preferences of these elements to integrate into expressed genes versus non-coding DNA, and preferences for integration sites within genes may be substantially different. If so, the different patterns of integration of these transposon systems can be exploited in a complementary fashion. For instance, one could use different transposon systems to introduce several transgenes into cells sequentially, without accidental and unwanted mobilization of already integrated transgenes. In addition, the number of target loci that can be mutagenized by transposon vectors could dramatically increase by combining different transposon systems in genome-wide screens. Undoubtedly, these transposon systems will be of great utility as genetic tools to develop novel gene transfer, transgenesis and insertional mutagenesis strategies in mouse and other vertebrates, and possibly to develop novel, non-viral vectors for gene transfer in humans.

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