2004

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Chapter 16

Applications of Transposable Elements in Fish for Transgenesis and Functional Genomics

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Transgenic fish were first made more than 30 years ago. Since then a variety of methods and constructs have been tested for introducing genetic sequences into fish for scientific investigations as well as commercial purposes. Here we review transposable elements and their applications in fish. Transposons can be used to deliver genes to chromosomes to confer new traits or as insertional agents and traps to uncover the functions and expression patterns of natural genes in chromosomes. Two DNA transposons have been characterized for transposon-based gene transfer and insertional mutagenesis. The first is the *Sleeping Beauty* transposon system that was reconstituted from a *Tc1/mariner*-like relic in salmonid genomes after more than a 10 million year evolutionary sleep. The second is a naturally occurring transposon from medaka, the *Tol2* transposon that belongs to the *hAT* family of mobile elements. In comparison with random integration of plasmid sequences and pseudotyped retroviral genomes, transposons have several advantages for genetic studies in fish. These include introduction of a single, defined DNA sequence into a cellular chromosome, stable expression from the integrant for multiple generations, no absolute

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size restrictions on the transferred gene, ease in construction and use, and safety. Early experiments have validated the versatility of the *Sleeping Beauty* transposon for all of these purposes. The applications of transposon systems surpass use just in fish; the *Sleeping Beauty* transposon system is being used in mice to discover functions of genes and is being developed for gene therapy in humans.

1. Introduction

Research involving small model fish such as zebrafish and medaka are being increasingly used for a two broad applications as the 21st century dawns. The first application is for basic research. Here the goal is to discover the functions of vertebrate genes in order to uncover their coordinated expression patterns that take a single cell through a myriad of developmental programs to produce a multicellular adult capable of producing more such single cells. A second broad set of applications is using fish as model vertebrates for testing a variety of biomedical treatments such as drugs and other biopharmaceuticals. Here we review development of transgenic procedures that led to the construction and discovery of active transposable elements that could be used to deliver precise units of genetic information to vertebrate cells.

1.1. Transgenesis in Fish

The advantages of small fish in general, and zebrafish in particular, as model vertebrates are well documented.[1–12] Essentially they are: (1) developmental rates of small fish are rapid;[13] (2) the embryos of many species develop outside the mother so that non-invasive analysis of living embryos can be performed; (3) fish share approximately 90% of their genes with other vertebrates — this leads to common physiological pathways and responses to drugs;[14,15] (4) large numbers of embryos and fish can be efficiently generated at low cost so that large sample sizes can be examined during genetic screening of mutants;[4,16–26,230] (5) transgenic procedures have been developed for delivering genes to many species of fish (reviewed by Hackett;[27] Gong and Hew;[28] Maclean;[29] Hyatt and Ekker;[30] Meng *et al.*;[31] Hackett and Alvarez;[6] and

Zbikowska[32]); (6) a wide range of mutations in medaka[33] and zebrafish[1,2,34–36] have been characterized in hundreds of genes so that a myriad of genetic interactions can be examined, many of which have medical relevance; and (7) the optical clarity of the zebrafish, medaka, and other fish is especially suitable for visualizing activities in developing embryos.[37,38] Furthermore, the optical clarity allows efficient visualization of the expression of fluorescent proteins[39] under the control of specific promoters,[8,31,40–52,228,231,232] fluorescent proteins fused to other polypeptide sequences,[53–55] and other fluorescent marker macromolecules.[9,56,57]

The powerful advantages listed above place fish in a prime position to address problems in vertebrate development, physiology that often center on the following questions: (1) What are the functions of the approximate 35,000 genes in vertebrate genomes? (2) How are these genes coordinately regulated — that is, what are the regulatory elements for these genes? To answer these questions, geneticists have developed a number of transgenic tools to investigate several aspects of gene expression and its control in fish.[218]

1.2. Retroviruses and Transposons for Gene Delivery in Fish

Gene delivery to fish chromosomes is not new, and in fact predated the recognition of zebrafish as a model system. The first study of gene transfer was reported by Vielkind *et al.*,[58] who introduced heterologous fragments of *E. coli* DNA. Later large fragments of chromosomal DNA containing a locus that caused formation of tumors in Xiphorphorine fish were transferred by microinjection.[59,60] The first report of integration of a specific gene into a fish genome was by Zhu *et al.*[61] wherein a single gene was microinjected into fish that appeared able to transmit the transgene through the germline. Since then there have been several hundred papers on fish transgenesis that have reported wide variations in efficacy of gene delivery.[6] Nearly all of these studies used purified DNA that entered genomes by random integration. The process worked, but was highly inefficient. As genome projects were initiated, the emphasis in the 1990s shifted from production of transgenics for single gene analysis to high frequency insertion of DNA for random insertional mutagenesis

screens. For this two techniques were developed. The first was the use of pseudotyped retroviruses that have a broad host range.[62] This form of retrovirus is encapsulated in an envelope glycoprotein that permits the virus to infect most cell types rather than the normal species/tissue-specific manner that most retroviruses employ. The second was the development of DNA-based transposons.

Viruses are attractive for introducing DNA sequences into vertebrate chromosomes; indeed, they are the primary delivery tools for human gene therapy.[63] Retroviruses are able to circumvent three barriers, cellular and nuclear membranes and chromosomal integrity; these steps are necessary before DNA sequences can be integrated into a genome. In general, viruses are very picky about the cells they infect. The specificity resides in particular glycoproteins on their exterior membranes, which they pick up as they bud from their host cell. There are only a few known fish retroviruses[64–66] so a common mouse retrovirus was developed that had a replacement of its normal species/tissue-specific *env* protein gene by the G-protein of vesicular stomatitis virus.[62] These retroviruses can infect any cell (fish or human) and once inside the infected cell, the viral integrase protein carried inside the virus catalyzes the integration of a DNA copy of the retroviral genome (called a provirus) into a chromosome of the host cell. However, the pseudotyped virus cannot penetrate fish chorions so they must be injected. In zebrafish, customarily about $10–20 \times 10^4$ viral particles are injected into blastula-stage embryos (512–2000 cells), leading to several insertions into zebrafish genomes.[67] This leads to embryos that are highly mosaic with from 5 to 22 proviral insertions transmitted to F_1 fish. For screening of mutations that affect growth and development, this is a useful strategy because it allows a number of mutations to be screened simultaneously per fish.[68–70] A large-scale retroviral screen has isolated approximately 500 insertional mutants[20,71] with an estimated mutagenic frequency of 1 mutation in 70 insertions.[19]

There are several drawbacks of using pseudotype retroviruses as vectors for gene delivery. First, pseudotyped retroviruses are difficult to prepare at the high titers required for efficient integration. Second, the high-titer retroviruses apparently do not express transgenes.[72] As a result, to date they have only been effective as insertional mutagens for tagging genes,

after molecular analysis has detected their presence in chromosomes. Third, they do not integrate randomly; rather they have a preference for transcriptional control motifs close to the 5' ends of expressed genes.[73] Fourth, for some purposes, such as genetically engineering food fish, retroviruses had distinct disadvantages because these elements involve retroviral-like integration and therefore had the potential of being misunderstood by the public due to their association with cancer- and leukemia-causing viruses. Lastly, there are questions about safety of using these retroviruses because they can infect the human conducting the experiments (e.g., Smith *et al.*[74]). Consequently, we developed an alternative vector system, the *Sleeping Beauty* transposon system.[75]

In this review, we will examine the application of DNA-based transposons for transgenesis and functional genomics in fish. We will concentrate on three advantages of DNA transposons for genetic investigations in fish. They are: (1) ease of delivery and detection; (2) integration of precise sequences; (3) a relatively high efficiency of

The *Sleeping Beauty* Transposon System:
A Tool for Gene Delivery and Gene Discovery

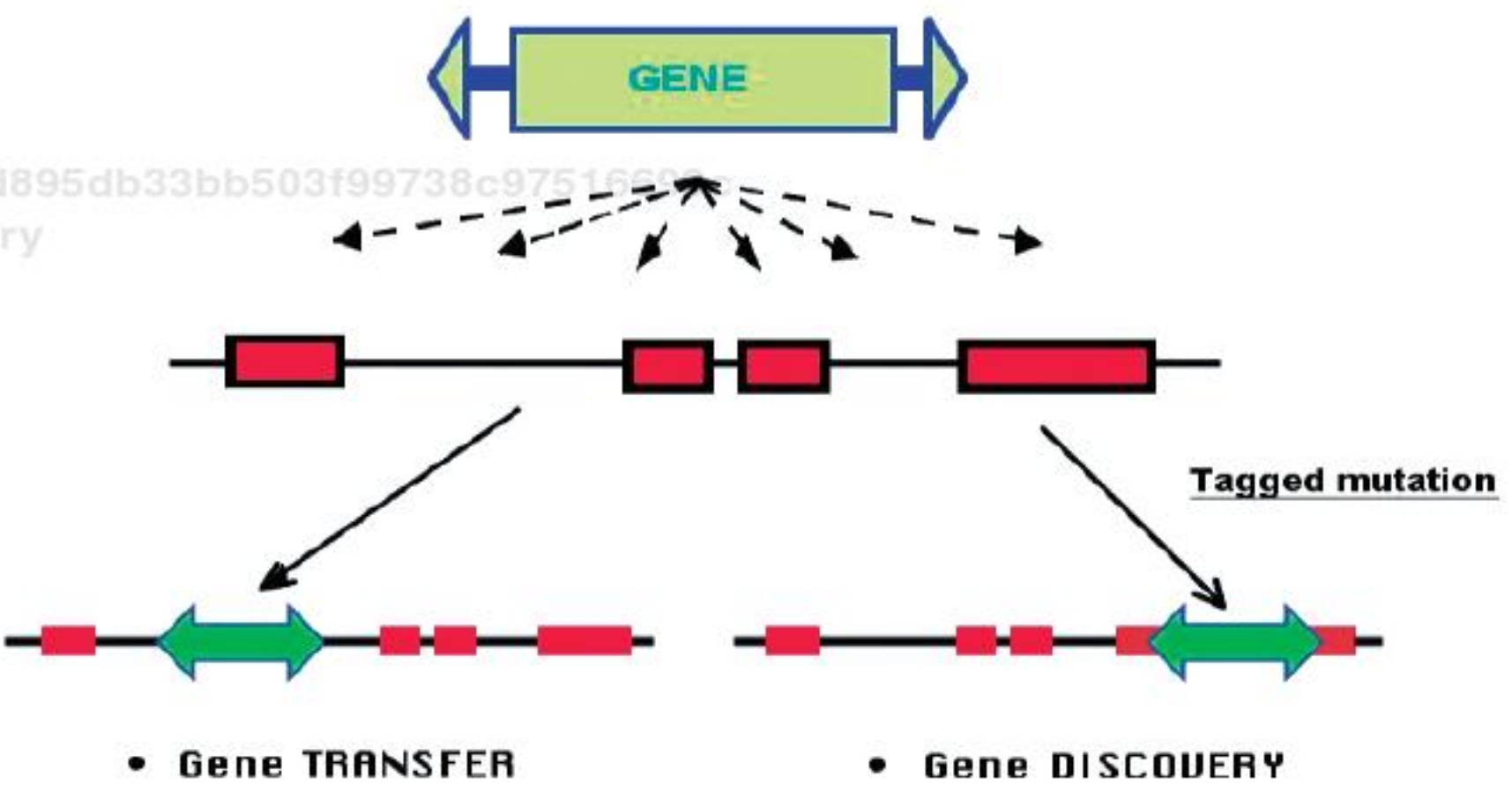

Fig. 1 The transposon containing a gene, with an appropriate transcriptional regulator, is shown flanked between the inverted terminal repeats of the transposon, which are indicated by the inverted arrowheads.

gene transfer into chromosomes; and (4) random integration. Fig. 1 summarizes the activities of the transposons discussed in this review.

2. Transposons

This review concentrates on DNA-based transposons. However, these mobile elements are just one type of mobile element, in fact a minority group in most vertebrate (and plant) genomes. About 90%[76] of mobile elements in fish genomes are retro-elements, so called because they operate as intracellular retrovirus in which an mRNA copy of the element is copied by cellular RNA-dependent DNA polymerase into a double-stranded DNA that invades the chromosome using a cellular integrase activity. In humans, the LINE and SINE families comprise the largest number of retro-elements, approximately 33% of the genome.[77] Furthermore, these elements are still active and cause random mutations in humans.[78] In zebrafish, the same family of SINEs has been given the names DANA[79] and *mermaid*.[80] Although they are distributed throughout the genomes of vertebrates, LINE and SINE elements often appear in nests that have likely resulted from repeated integration into or close to each other over the course of millions of years.[81] This clustering may also be a consequence of selection against deleterious integrations.[82]

2.1. Structures and Mechanisms of Transposition of DNA-Type Transposons

Attractive candidates for introducing DNA into fish chromosomes are DNA transposases that were responsible for the widespread distribution of transposons in fish and other animal genomes.[76,83] DNA transposons move in a simple, cut-and-paste manner (Fig. 2) in which a precise DNA segment is excised from one DNA molecule and moved to another site in the same or different DNA molecule.[84] The protein that catalyzes this reaction, the transposase, is encoded within the transposon for an autonomous element or can be supplied in *trans* by another source for a non-autonomous element. *Tc1/mariner*-type transposases require a

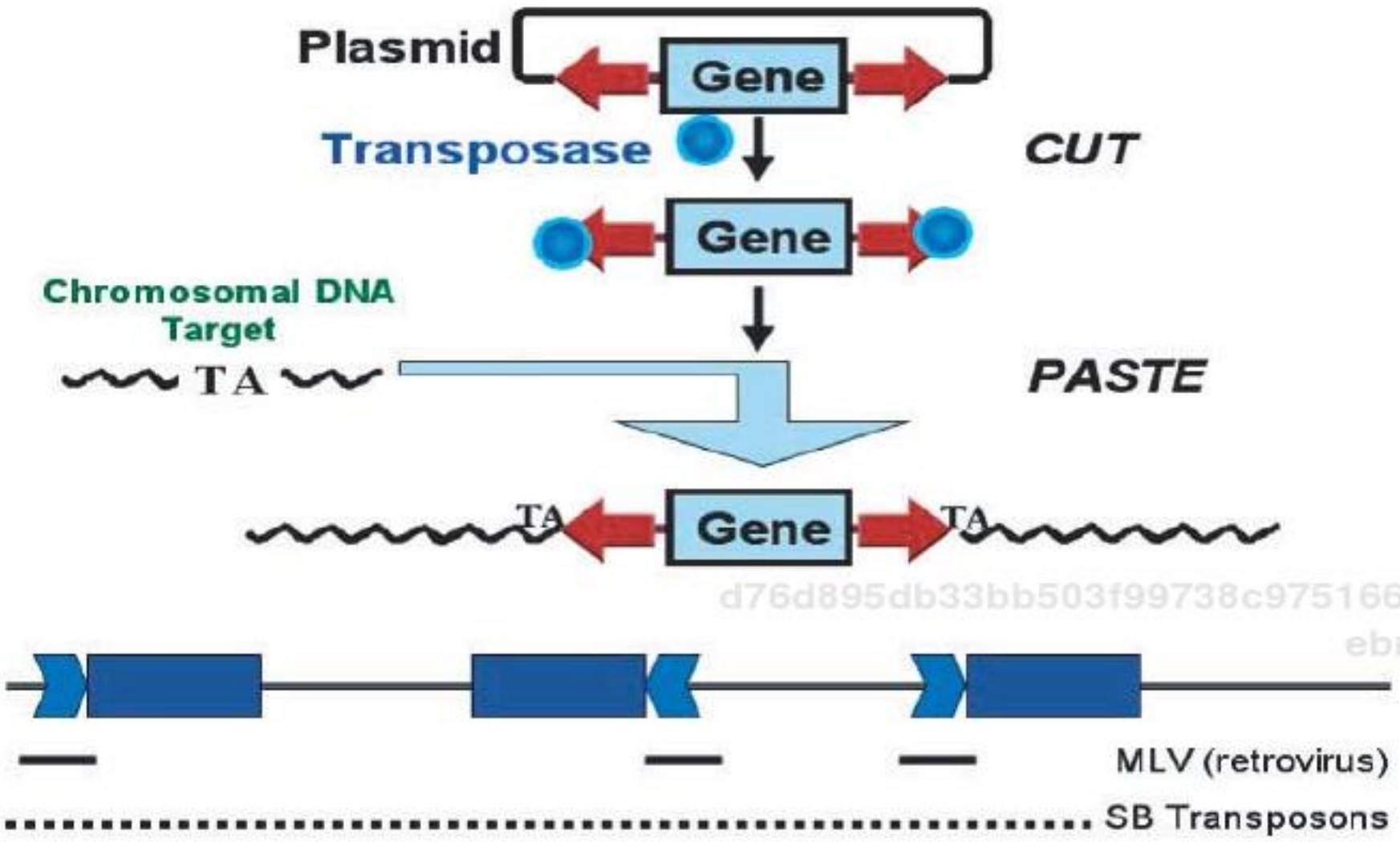

Fig. 2 The cut-and-paste mechanism of transposition. The transposon contains a gene with an appropriate transcriptional regulator. Two transposase molecules bind to each inverted terminal repeat. In experiments using transposons as a delivery vehicle, the transposase is supplied from another source, either from a plasmid carrying the transposase gene or from an mRNA co-delivered with the transposon. The schematic at the bottom illustrates the integration site preferences for retrovirus (promoter-proximal regions) compared to *Tc1/mariner* transposons (nearly random). Promoter regions are shown as arrowheads and genes are shown as blocks. The long horizontal lines show the integration pattern of retroviruses and the dotted line shows the more random pattern of integration by transposons.

TA dinucleotide basepair for an integration site, which is duplicated during the integration process (described further in Fig. 3). The excised DNA is flanked by inverted terminal repeats to which the appropriate transposase molecule binds. Emphasizing this simplicity of action, *Tc1/mariner*-type transposable elements are ubiquitous in animal genomes, suggesting that they require few, if any, species-specific host factors. Indeed, both the *Tc1* transposon from *Caenorhabditis elegans*[85] and the *Mos* transposon from *Drosophila mauritiana*[86] were shown to transpose in cell-free systems in the presence of their respective transposase enzymes made in *E. coli*. Consequently, transposons carrying a gene-of-interest could be mobilized by transposase provided in *trans*.

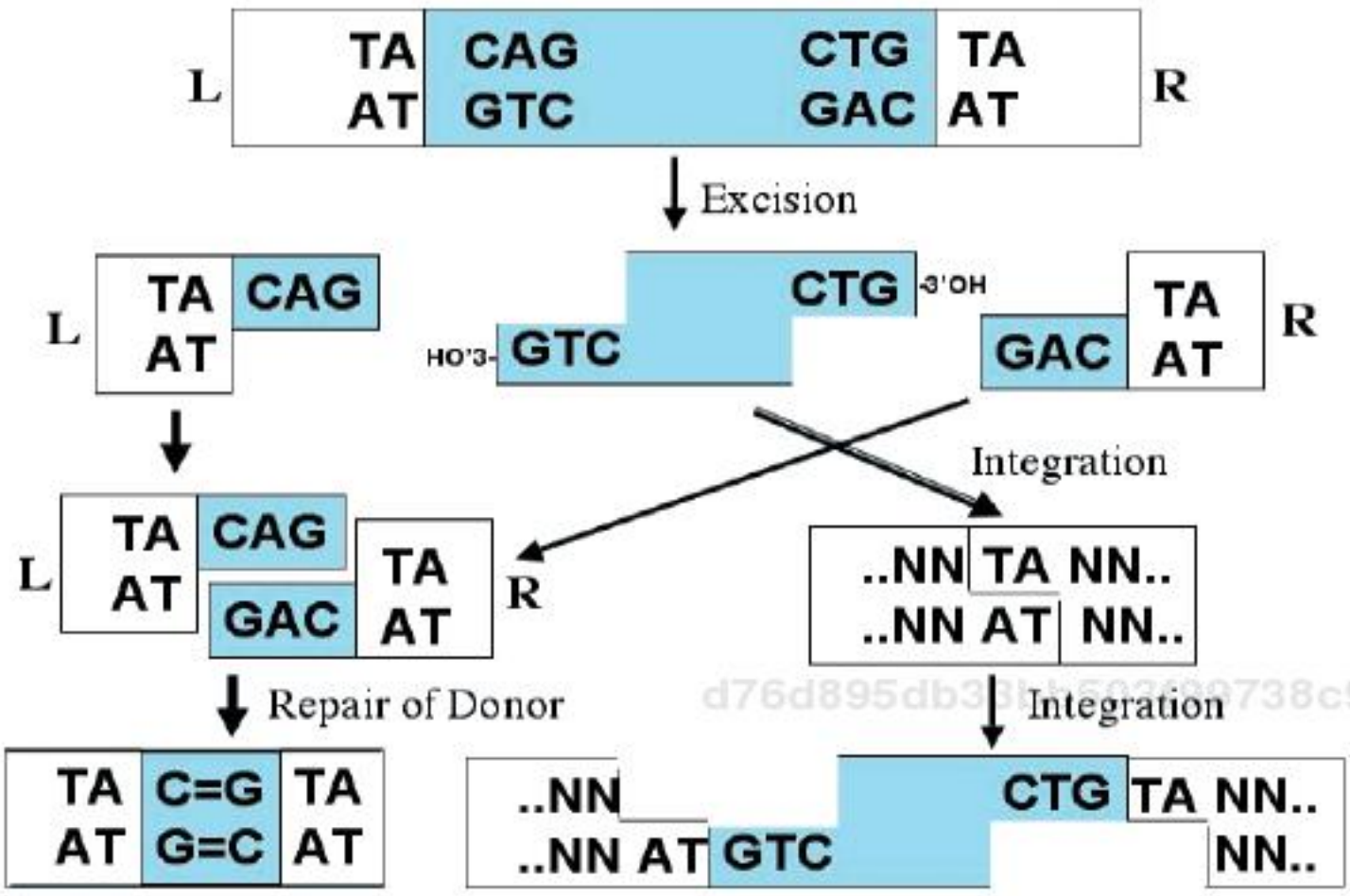

Fig. 3 *Tc1/mariner*-type transposition. The transposon is shown as a shaded sequence. The excision step is shown on the second line. Integration follows to the right and repair of the transposon donor plasmid on the left. The last step of the integration process is repair of the gaps (lower right corner) by DNA repair enzymes. In the lower left block, the stacked = signs represent an A–T or T–A basepair. The excision and integration steps are coordinated, with the 3' hydroxyl groups of the excised transposon attacking the TA dinucleotide basepairs in the target DNA sequence.

Unlike retroviruses, *Tc1/mariner*-type transposable elements integrate into TA sequences nearly at random (more on this later).

A more detailed schematic of the excision-integration process, with concomitant repair of the "donor" DNA molecule whence the transposon was excised is shown in Fig. 3. TA basepairs flank *Tc1/mariner*-type transposons. In the excision step transposase cleaves the transposon in a staggered manner such that three bases, CTG, overhang at each 3' end of the transposon (top two lines in the Fig. 3). The 3' ends of the excised transposon invade the target DNA molecule (indicated by the .. NNTANN .. sequence) at a TA sequence by cutting it in a staggered manner to expose two 5' ends with overhanging TA nucleotides (third line in Fig. 3). Integration is completed by repair of the 5-base gaps on both strands. Note that the original TA basepair target sequence is duplicated on both flanks of the transposon following

integration. This is referred to as target site duplication. The donor DNA's left and right ends (indicated by L and R) that have extending CAG overhangs on their 3' ends, are brought together with a single A–A mispairing in the center. Repair enzymes then correct one of the two bases to produce an A–T basepair. The result is that upon remobilization, a "footprint" is left in the original site such that the initial sequence of TA is changed to TAC(A or T)GTA; that is, a five-basepair canonical sequence is inserted in a site into which a *Tc1/mariner*-type transposon entered and then left. Imprecise repair can alter the canonical footprint. Overall, the excision-integration-repair process is a series of breaking and joining of phosphodiester bonds that is highly coordinated by transposase. Thus, transposases are not like restriction enzymes that release a product that is free to wander to the next reaction site. Rather the excision and integration reactions are coordinated events.[84,87]

The theory looks simple in Figs. 2 and 3. However, when we tested the activity of *C. elegans Tc1* transposons in zebrafish and cultures of human HeLa cells, we found no indication of transposition; the levels of integration were roughly equal whether the transposase was present or not.[88] These results were similar to those obtained for another DNA transposon, the Drosophila *P*-element that has specific requirements for host cofactors.[88,89] In a search to find endogenous elements that could circumvent these difficulties, the Emmons lab showed evidence of transposable elements belonging to the *Tc1/mariner* family in salmonids and zebrafish.[90] The report led us to search for *Tc1/mariner*-like elements in many species of fish, but all of the sequences we isolated had many mutations and gaps in the transposase genes and inverted repeats of the transposon vector.[91,92] The search uncovered two families of *Tc1/mariner*-like elements in zebrafish, *Tdr1* and *Tdr2* that evolved from a common ancestor.[92] Others have found similar incomplete elements in various species of fish.[91,93–95] All of the reported elements are defective, with gaps, stop codons, and frame-shift mutations in the putative transposase-coding sequences.

Despite the frustrating failure to find an active transposase gene in a large number of inactive transposase sequences, it was possible to

derive a theoretical sequence for an active transposase enzyme from phylogenetic principles. Based on the theoretical sequence, an active transposase was assembled in a 10-step process of site-specific mutagenesis of a salmonid transposase gene that entered an evolutionary sleep more than 10 million years ago. The awakened transposase was named *Sleeping Beauty* (SB).[75] We refer to the transposon and transposase as the SB transposon system. The SB transposase was able to improve integration from 20- to 40-fold in mammalian cells[75] and about 20-fold in zebrafish embryos.[76] This represents about an order of magnitude higher rate of integration than that from two heterologous transposon systems, *Tc3* from the nematode *C. elegans*[96] and *mariner* from insects.[97] However, it should be noted that *mariner* transposons can also transpose at low rates into chicken germline cells[98] and human cells.[99] In a head-to-head competition, Fischer *et al.*[100] compared the rates of transposition by the SB transposon system and a variety of transposons from nematodes and flies and found that the SB system was about 10-fold more effective in delivering a neomycin phosphotransferase (*neo*) gene to chromosomes in cultured human HeLa cells than the others. In these experiments as with all others reviewed here, *non-autonomous* transposons were used, i.e. transposons in which the transposase gene was replaced with alternative genetic cargo. The transposase is generally supplied by another plasmid carrying the transposase gene or an mRNA encoding the transposase.

A non-*Tc1/mariner*-type, active mobile element, *Tol2*, was identified in an albino medaka after it transposed into and inactivated a pigment gene.[101–103] The *Tol2* transposon belongs to the Ac/Ds family of transposable elements with sequence similarities to *hobo* in Drosophila and the *Tam3* transposon found in snapdragons.[104,105] It encodes a transposase with excision[105,106] and integration[107,108] activity. The transposon is active in mammalian cells as well as fish.[109,174] Unlike the transposase genes of *Tc1/mariner*-like elements, the *Tol2* transposase gene is divided into three exons.[110] The structure of the *Tol2* element is compared to that of various *Tc1/mariner* transposons in Fig. 4. Note that there are two sub-categories of *Tc1/mariner*-like transposons, those with two repeats within each inverted terminal repeat (called IR-DRs for

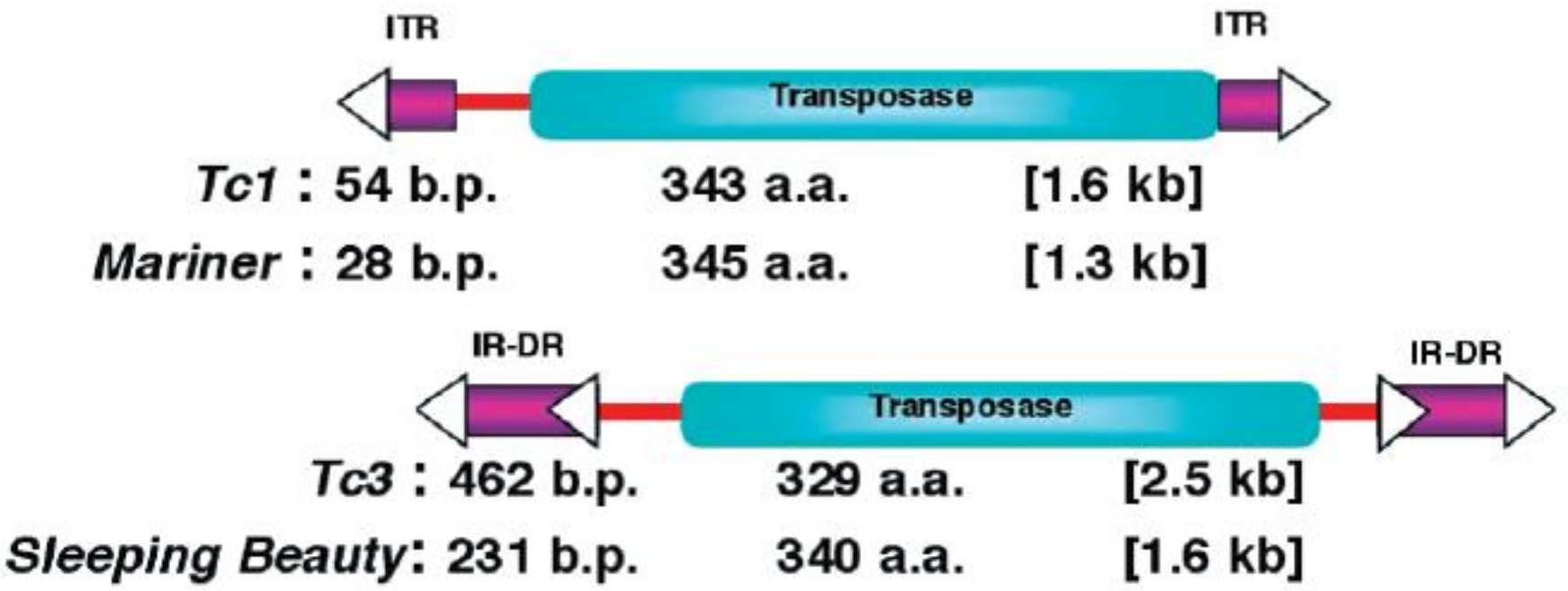

Fig. 4 Comparative structures of *Tc1/mariner*-like transposons and the *Ac*-like *Tol2* transposon. Each of the DR sequences in the SB transposon is 31 bp. The schematic of the SB transposon is hypothetical. An active, autonomous element has not been produced since it could have uncontrolled transposition activity in vertebrate cells. ITR, inverted terminal repeat sequence; IR-DR, inverted repeat containing direct repeated sequences.

inverted repeats containing direct repeats) and those with a single inverted repeats. The two inner DRs vary from the outer DRs.[111]

2.2. Advantages of Transposon-Mediated Gene Transfer

Transgenesis by transposition has several subtle benefits. First, the transposition reaction delivers a defined DNA sequence to a chromosome. Cutting and pasting is precise to the basepair on both ends. Prokaryotic sequences that might contain CpG-rich, methylation-sensitive sequences that could lead to silencing of the transgenes do not accompany the integrated transposon. Second, only single copies of transposons integrate at a given transgenic locus. In contrast, concatemers of transgenes often enter chromosomes by gene transfer via random integration of plasmids.[112,113] Concatemerization has been associated with gene silencing.[114–116] For these reasons, and maybe others, expression of genes delivered in transposons is stable. Third, studies

from gene transfer into cells of non-dividing tissues of mammals, e.g. liver and lung, suggests that the SB transposon system can pass through nuclear membrane to the DNA in the nucleus.[117,118,156] This may be the result of the binding of four transposase proteins, each of which has a nuclear localization motif, to the inverted terminal repeats of the transposon. These proteins may be sufficient to facilitate transport of the transposon, presumably still in the donor plasmid, through a nuclear membrane.[119] Fourth, compared to viruses, transposons are easy to construct and prepare. Moreover, the transposon system has two components, an active transposase and the DNA transposon that is mobilized. This binary requirement renders the SB system relatively safe when the source of transposase is either a short-lived mRNA or an unintegrated gene. Lastly, compared with viruses that have limitations on the sizes of the genomes that can be transduced, transposons do not have an *a priori* upper limit beyond which transposition does not occur.

While size limitations are not expected for transposition, studies have shown that the efficiency of integration of transposons decreases with size. Three studies of this effect have been done in tissue culture using transposons that carried an antibiotic resistance gene so that cells carrying an integrated transposon could be quantified. Two groups[120,121] compared the sizes of transposons in which sequences from lambda virus were inserted into the transposons and found that enhanced colony formation was essentially lost when transposons exceeded 6–8 kilobase pairs (kbp). In the third study, the "stuffer" DNA was from the salmon beta-actin gene and different size transposons containing different antibiotic resistance genes were co-transfected into cells. In this case, elevated gene transfer to chromosomes was evident even in transposons larger than 10 kbp.[122] Figure 5 shows a comparison of two of the sets of results. The difference in outcomes for the larger transposons could be due to the nature of the assay. If the prokaryotic lambda sequences induced silencing of the selective marker gene, then those transposition events would not have been recorded. If so, then the probability of silencing would increase as the length of the lambda stuffer sequence increased. Alternatively, plasmids of increased length might have greater difficulty traversing either or both the plasma and nuclear membranes of the transfected cells, and prokaryotic sequences might exacerbate difficulties

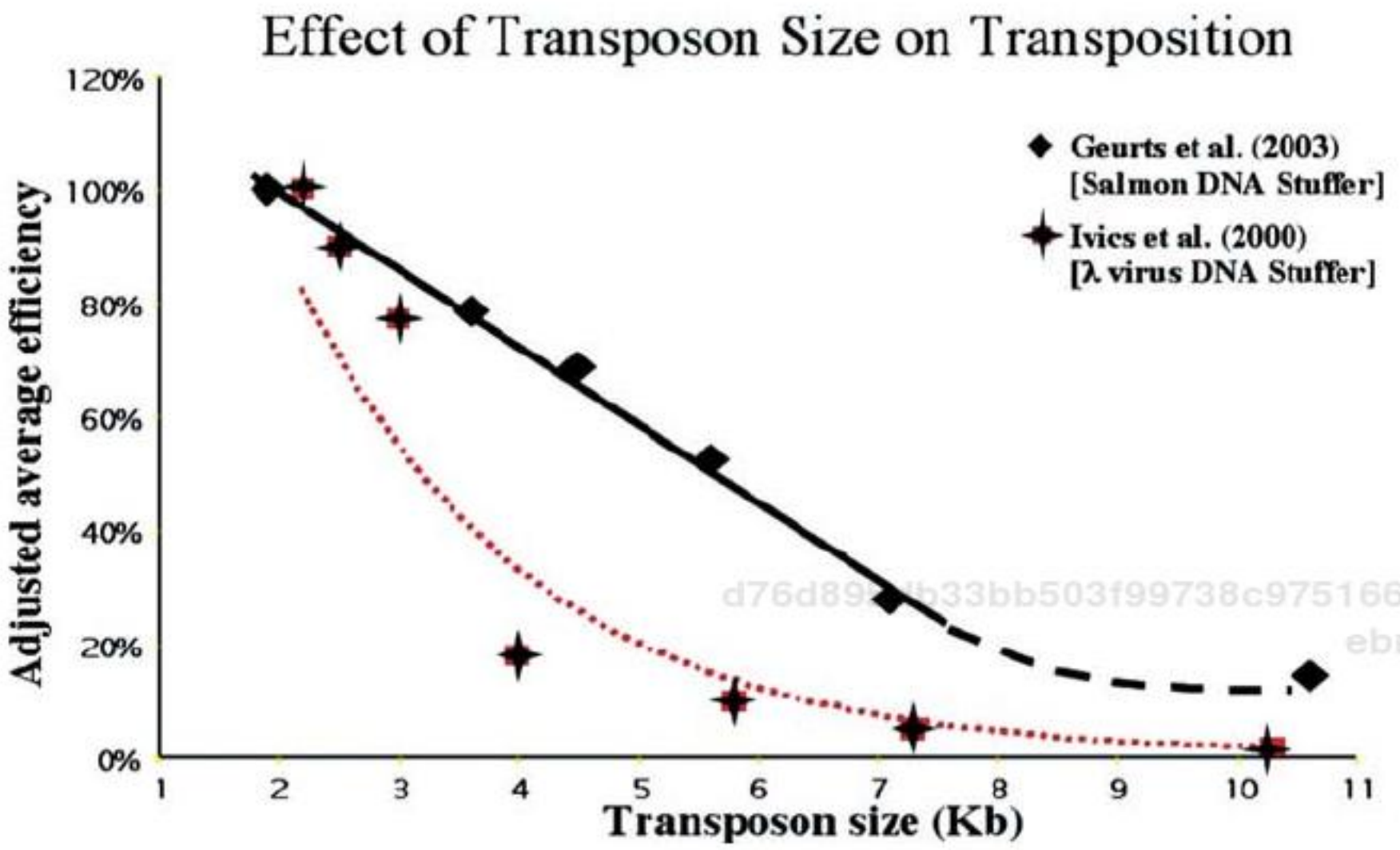

Fig. 5 Gene transfer in SB transposons as a function of transposon length in HeLa cells. [Data from Ivics *et al.*[120] and Geurts *et al.*[122]]

in membrane passage. Regardless of the reason, it appears that transposons up to 5–6 kbp, which would accommodate an estimated 80% of vertebrate cDNAs,[123,124] can efficiently deliver genes to chromosomes.

3. Applications of Transposons in Fish

Transposon systems are powerful vectors for integrating genes into chromosomes. But, first they must be introduced into cells. Active transposons require a transposase, whose activity can be delivered by the transposase gene, an mRNA copy, or presumably the encoded enzyme. For most applications in fish, the method of delivery of a transposon system is similar to that of any DNA. The next steps include analyzing whether integration occurred, whether it was by transposition, and, in cases where the sequence of the insertion site is of interest. Determining the integration status, defined as the exact sequence inserted and the locus into which it integrated, is essential for most genetic analyses. There are several methods for gene transfer and analysis of integration.

3.1. Delivery of Transposon Systems into Fish Embryos

Several methods of DNA delivery to fish embryos have been reported. They are reviewed in the following sections. Only microinjection has been reported for delivery of transposon systems in fish. While methods for DNA delivery other than microinjection have not been efficient for integration and expression of plasmid DNA, these alternative methods may be more useful for effective delivery of transposons. For example, it may be that earlier introduction of DNA and transposase into the cell will lead to transposition at the one- or two-cell stage of embryogenesis. This consideration is discussed further at the end of the review in Section 4.4.

3.1.1. Microinjection

Microinjection is the most popular form of gene transfer into fish embryos because of its reliability.[6] One reason is that fish eggs are about 1000–30,000 times larger than mammalian eggs, which makes injection into the embryo or yolk fairly easy — an experienced person can inject more than 1000 embryos per hour. However, injection into fish pronuclei is very difficult because they make up only about 0.001% of the one-cell embryo compared with about 5% in a mammalian embryo. As a result, pronuclear injections are often impractical, although in some species such as medaka this technique can be used. Moreover, soon after fertilization the chorion hardens so penetration by the injection needles becomes harder with time. Injection of DNA into oocytes has been examined[125,126] but the technique has gained little interest owing to the difficulties in harvesting both the oocytes and sperm for fertilization afterinjection. For certain types of fish embryos the chorion is removed prior to gene delivery (e.g., Culp *et al.*;[127] Müller *et al.*[128]). However, the procedure is labor-intensive, slow, and embryo survival and transgene integration rates do not appear to be better than those obtained by microinjection though the chorion.

In most cases, about 10^6 to 10^7 molecules of DNA in an aqueous, buffered volume of 1 to 2 nl, about 20 to 100 times the average nuclear

volume of fish nuclei, are injected into embryos or fertilized eggs.[6,31] It appears that most of the microinjected DNA remains as a distinct aggregate in zebrafish embryos as visualized by labeling with ethidium bromide.[129] Of this large number of molecules, in general less than 100 will integrate; an efficiency of about 0.01%. This is nearly 100-fold less than that achieved using the pseudotyped viruses. This aggregation explains in part the relatively poor integration results. The conclusions from hundreds of microinjection experiments in fish, using a wide variety of genes, promoters, and methods of assay, are that (1) embryonic survival decreases to less than about 10% when more than 100 pg of DNA were injected; (2) only a portion of the injected embryos express the transgenic DNA in a mosaic fashion; and (3) only a proportion of fish that develop from injected embryos are able to pass on the transgenic construct as an integrant in a fish chromosome.[6] However, this is generally sufficient because hundreds of embryos can be injected in a single day. For this reason, microinjection is the most popular method of gene transfer and for standard gene transfer in fish. Nevertheless, microinjection is perceived to be tedious and inefficient. Consequently, other methods for gene transfer have been developed and reported, mainly in zebrafish and medaka.

3.1.2. *Electroporation*

Electroporation is used to depolarize cellular membranes to allow entry of DNA through the plasma membrane.[130] Electroporation of DNA has been tried in several species of fish embryos following removal of their chorions.[125,126,128,131–137] However, as noted above, this is a time-consuming process that defeats the objective of mass transfer of DNA without treatment of each embryo. Many variations of conditions have been reported, but the bottom line is that electroporation has not caught on with most labs and few labs use it routinely.

3.1.3. *Sperm-mediated DNA delivery*

Sperm-coated with DNA was first reported to work in mice with relatively high frequency and no damage to the embryo,[138] but Brinster *et al.*[139]

reported their inability to reproduce the results. DNA does bind to sperm[140–142] and can be taken up into fish embryos,[134,143–145] but the genes are not expressed. A new method has been tried, intra-cytoplasmic injection of detergent-disrupted sperm and sperm-heads.[146] Up to 20% of transgenic mice produced offspring that expressed transgenes. The sperm heads were "considered dead due to disrupted membranes" but still supported full development. This method still requires microinjection and thus cannot be considered a mass transfer procedure that is substantially different from standard microinjection.

Electroporation of sperm with transgenic DNA prior to fertilization has been reported.[147–150] However most of the DNA that is brought into the egg remains on the exterior surface, as evidenced by its susceptibility to DNases.[145,151] The efficiencies for integration into chromosomes of DNA constructs brought into fish in this way is not known. At this point, the difficulties in the procedure appear to be greater than the benefits. Gene transfer into zebrafish by sperm nuclear transplantation has been tried as well.[152]

3.1.4. *Biolistics*

"Shock and awe" is sometimes employed when subtle means fail. Bombardment of cells with DNA-coated particles, *bio*logical bal*listics*, has been tried with fertilized loach, zebrafish and rainbow trout eggs, resulting in high mortality and low expression rates.[153–155] As an alternative, electrospray delivery has been attempted in zebrafish. For this, a fine mist of water containing transgenic DNA was electrostatically propelled onto a plate containing hundreds of embryos. Expression of the transgenic constructs was never detected, even at voltages that killed the embryos.

3.1.5. *Lipofection*

Nucleic acids encapsulated in synthetic lipid vesicles can be taken up into cells and tissues of animals where they can be expressed (e.g. Kren *et al.*[157]). In fish, several cocktails of DNA and proteins have been mixed

and used to deliver transgene constructs to dechorionated zygotes at the 2–16 cell stage.[158,159] As with the sperm methods, the DNA could get into the embryos, but it was soon lost and its expression was transitory.

Together, the results of all of the transgenic procedures emphasize that delivery of transgene DNA is only half of the problem of transgenesis. The second half is integration into chromosomes for passage to subsequent generations and stable expression of the genetic material. For this, the DNA must penetrate the nuclear membrane and insert into a chromosome for genetic studies. The *Sleeping Beauty* transposon is designed to be catalytically inserted into chromosomes and SB transposases with their nuclear localization motifs may assist in conveying transposons through the nuclear membrane.

3.1.6. *Remobilization of transposons*

A genetic method of introducing transposons into new loci is to remobilize transposons already occupying a position a genome. This is the way transposons naturally spread from a single invading species to multi-copy numbers in genomes. This is very apparent with the several thousand-fold amplification in numbers over millennia that have occurred for most *Tc1/mariner*-type transposons in vertebrate genomes. Remobilization has been successful in mice using the *Sleeping Beauty* transposon system discussed in later Section 4.4. Three laboratories have reported efficiencies that range from an average of 0.2 to 2.0 remobilizations per newborn mouse pup following a mating between mice with SB transposons and mice expressing a SB transposase gene.[100,160,161] The higher rates of remobilization were obtained from animals in which transposons were remobilized from concatemers of transposons that entered the genome by random recombination rather than transposition.[160,161] There is also evidence for remobilization in zebrafish. Using probes to the endogenous transposon *Tzf* (also named *Tdr2*[91,92]), Lam *et al.*[162] found evidence of movement of *Tzf* sequences based on two-dimensional gel electrophoresis analysis. Although they found evidence for excision of transposons, they did not detect any new integration events, which would have been expected. However,

the rate of the observed excision events correlates with the background mutation rate of 8×10^{-5} per gene per generation in zebrafish.[163] Additional evidence for the potential of element remobilization comes from the observation that *Sleeping Beauty* transposons can be excised from one integration site in transgenic zebrafish following injection of a transposase source into embryos.[164] Together, these data support the possibility that remobilization of transposons could be an effective way of delivering sequences to new sites in fish genomes.

3.2. Analysis for Integration of Transgenes into Chromosomes

About 1 to 10 million plasmids are microinjected into a single fish embryo in most experiments. Yet at best only a very few genomes take up even a single copy of DNA, an efficiency of integration of less than 10^{-4} percent. There are two important consequences of so few transgenic DNAs making their way into chromosomes. First, the integrating copies of transgenic DNA are diluted more than a million-fold with unintegrated DNA of identical sequence, making analysis of the integrated DNA exceedingly difficult. The unintegrated DNA can recombine, at variable rates that depend on the input conformation of the transgenic DNA, to form concatemers (reviewed in Hackett,[27] Iyengar *et al.*[112]). Second, after microinjection virtually all transgenic F_0 fish are mosaic.[129] As a result, screening for transgenic fish is quite labor-intensive, requiring the raising of all embryos subjected to transgenic DNA until gametes can be accurately assayed for the presence of integrated foreign DNA. In the early years of fish transgenesis, several assays were used to indicate integration had occurred, including dot-blotting or PCR amplification, expression of the transgene after larval development, Southern blotting to show that the size of the transgenic DNA changed as a result of its integration into chromosomes, and detection of transgenic DNA or its expression in F_1 progeny. None of these assays was sufficient. Because of the enormous amounts of unintegrated DNA that can recombine with itself, assays such as Southern blotting and PCR amplification that examined transgenic DNA

size often gave misleading results. Stuart *et al.*[165,166] showed that unintegrated transgenic DNA was passed in an episomal state into F_1 progeny. The problems associated with the persistent presence of unintegrated transgenic DNA in fish have been reviewed.[6]

Three methods are reliable indicators of integration, chromosome *in situ* hybridization of tagged probes to metaphase chromosomes,[167] mendelian segregation of the transgenes (and their expression) in F_2 and subsequent progeny, and linker-mediated PCR or inverse PCR[164,168] to determine the sequence of the locus into which the transgenes integrated. The last method is essential in order to verify transposition of specific sequences rather than uptake of the transgenic DNA by random recombination.[75] In tissue culture, the initial SB transposon system using pT transposons and the SB10 transposase is 30- to 40-fold more efficient in directing integration of genes into chromosomes of HeLa cells than by random recombination[75] and about 10-fold more active than *Tc1*, *Tc3*, and various *mariner*-type transposon systems from invertebrates.[100] Since then both the pT transposon, pT2,[111] and the SB transposase, SB11[122] have been improved to now deliver transposition rates in cells more than 100-fold above random integration. In the mouse, the complete SB transposon system delivers expressing genes to liver[117,169] and lungs[118] in adult mice. In all of these studies, transposition was assessed by isolation and sequencing of the transposon junctions to verify that the increased gene transfer was due to transposition.

3.3. Transposition of Transgenes into Chromosomes of Fish

Several transposon systems have been used for gene transfer into zebrafish and medaka fish chromosomes, including the *Drosophila P* element[89,170] and *mariner* transposon,[97] the nematode *Tc3* transposon[96] that resembles the *Sleeping Beauty* transposon in that it has long IR-DR inverted terminal repeats, the medaka *Tol2*[105,107] and the salmonid-based SB transposon.[164,171] The invertebrate transposons have little or no activity above injection of plasmids and so are not discussed further.

3.3.1. *Transposition in fish using the SB transposon system*

The Hackett and Ekker labs have done a number of studies of using the SB transposon system in zebrafish in which the initial pT transposons were supplied on plasmids and an mRNA encoding SB10 transposase synthesized *in vitro* were co-microinjected into 1- to 4-cell stage embryos. The plasmid pSBRNAX was designed to generate ^{7m}G-capped SB10 mRNA by T7 RNA polymerase using the mMessage (Ambion) Machine transcription kit.[164,171] The embryos were raised to adulthood and examined for gene expression (made easy by the use of fluorescent protein markers). Fish that expressed a marker gene contained within the transposon in at least one cell were crossed with wild-type to test for transmission of the transposon. In these fishes, we found 5–10% of the population had germlines that produced at least one transgenic gamete. This suggested that the germline preferentially took up transgenic DNA and that most somatic tissues took up the transgenic DNA at a late stage of development. In most cases, we found that expressing fish in the F_1 generation would pass on the gene in a mendelian manner.[171,172]

This low rate of integration was disappointing, especially in light of the success of the transposon in delivering genes to chromosomes of somatic tissues of mice (cited above). This led to the use of the more advanced pT2 transposons and SB11 transposase. Davidson *et al.*[171] found that SB transposase enhanced the transgenesis and expression rate six-fold, from about 5% to more than 30%. This doubled the total number of tagged chromosomes over standard, plasmid injection-based transgenesis methods. In their report, they demonstrated that ubiquitous tissue-specific promoters, such as the lens γ-crystallin promoter could be used for reproducible and multi-generational gene expression.[55] Figure 6 summarizes the procedure and efficiencies for integration of transgenes in zebrafish chromosomes using SB transposons. The figure shows that the transposase is supplied via injection of mRNA encoding the enzyme rather than delivery of the SB transposase gene or the protein itself. By injecting the mRNA, there is a brief, transient presence of the transposase that declines as both the mRNA and the translated products decay. There

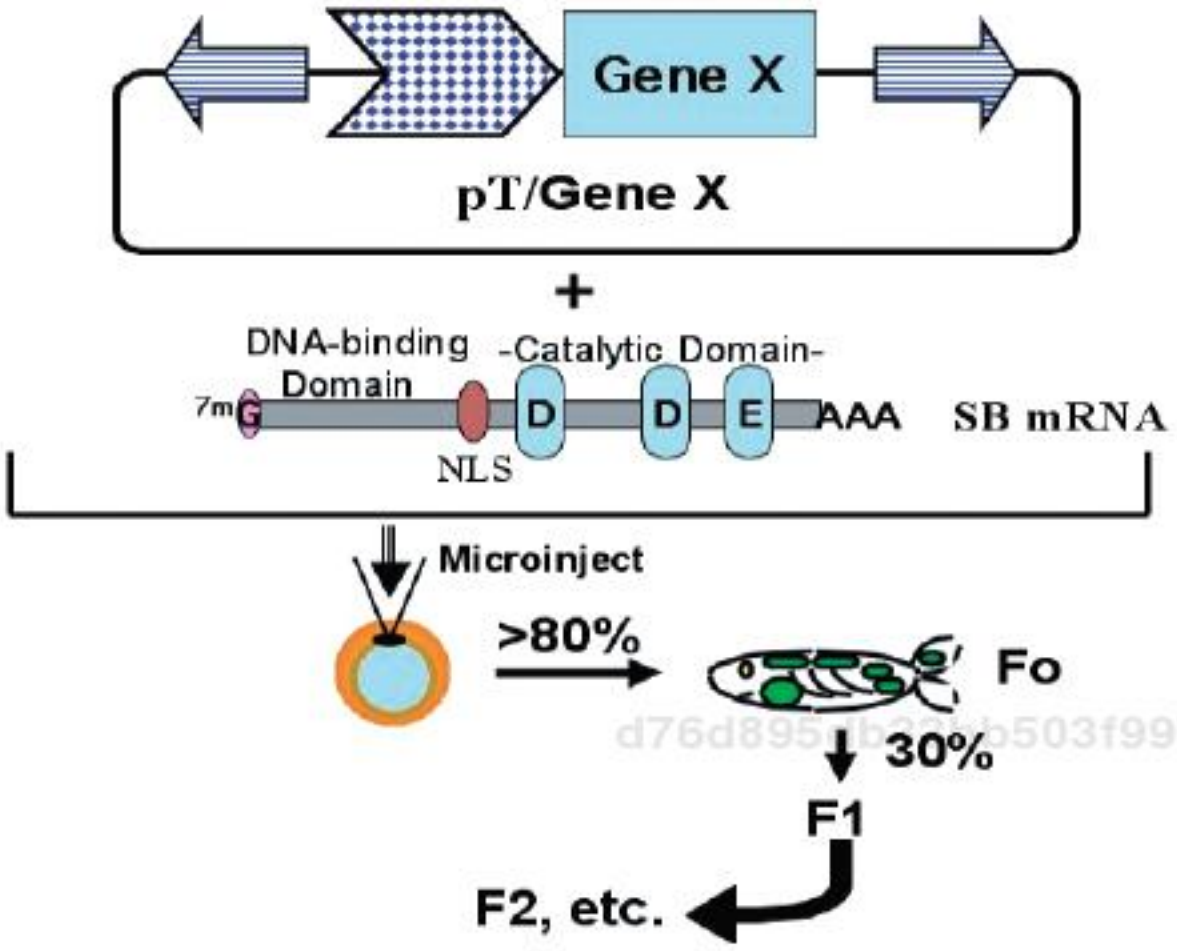

Fig. 6 Gene transfer of SB transposons into zebrafish embryos. The SB transposon comprises the IR-DRs (inverted arrows) and an expression cassette composed of a promoter (dotted arrowhead) and a gene (X). About 5×10^6 transposons and 10^8 mRNAs encoding SB transposase are injected per 1–4 cell embryo. About 80% of the injected embryos will express the transgene (filled "cells" in the fish). About 30% of the adults will have at least some gametes that are transgenic. When bred, these proceed to pass the transgene in a mendelian manner.

appears to be an optimal ratio of SB transposase protein to transposon DNA from studies done in tissue-cultured cells[122] that still needs to be determined for microinjection of zebrafish embryos. This may further lead to higher rates of insertion.

Although the rates of transposition look low following microinjection into fish embryos, the same is seen in other organisms. In insects, only about one plasmid per thousand injected will result in an excision event even though the transposition reaction can be mimicked *in vitro*.[173] Similarly, only one in a million transposons enters a mouse chromosome following microinjection of pronuclei.[161]

An important finding has been that generally expression of transgenes is maintained over several generations when introduced in a transposon[171] compared to that from plasmids where expression is often lost or altered.[175] This is probably due to the methylation of prokaryotic sequences in plasmids that accompany random integration.[6,176] Alternatively, injections of plasmids can lead to integration of concatemers

and rearranged recombination products from which gene expression may be silenced or unstable.[127,165,166,177] For genetic studies, reliable, multi-generation expression is essential. Transposition removes all plasmid sequences and inserts single units in a given locus, which explains the reliable expression from transposon-mediated transgenic fish.

3.3.2. *Transposition of trap vectors in fish using the SB transposon system*

The various genome projects for vertebrates[123,124] have identified approximately 35,000 genes in vertebrates, most of which have functions whose physiological significance is unknown. Finding the "bottom line" functions of genes is important both for basic research as well as pharmaceutical development.[178] As noted earlier, finding the functions of genes can be accomplished by various mutagenesis screens using chemicals, but identifying the loci by positional mapping has been a problem — the procedure is extremely labor-intensive and slow (reviews by Driever *et al.*,[17] Haffter *et al.*,[18] Eisen[179]). While the total number of mutants recovered by insertional mutagenesis using retroviruses by the Hopkins lab is lower, these screens have been responsible for identifying as many of the corresponding mutated genes as in all the chemical mutagenesis screens in a large number of laboratories combined.[71] While the retroviruses served as little more than insertional "tags" that allowed interrupted genes to be identified in these screens, the large number of identified genes underscores the utility of an insertional mutagenesis approach. A more powerful insertional tag would be one that could be followed easily followed by its expression, e.g. a fluorescent protein. As demonstrated by Davidson *et al.*,[171] transposons harboring expressible fluorescent protein genes are eminently feasible.

Transposons have been used for insertional mutagenesis genome-scans of many species from viruses to mammals.[180–191] These transposons operate similar to the pseudotype retroviruses discussed earlier. A better way of investigating genes and their activities is to use "trap" vectors that express easily detectable reporter molecules when they insert in a transcriptional unit or in the vicinity of a transcriptional regulatory motif.[192] There are four basic types of trap vectors that differ in their

requirements for activation of the reporter gene: enhancer traps,[193–195] promoter traps,[196–198] gene traps,[198–202] and poly(A) traps.[203] Gene traps and poly(A) traps can be designed as a single unit.[204] By scanning for activation of the traps, genes with specific responses to environmental, stress, or developmental cues can be discerned under various genetic conditions. For these reasons, insertional mutagenesis has become an important complement to the genome projects.[188,205–209]

With the above in mind, Clark and his colleagues initiated a project to produce panoply of trap vectors in *Sleeping Beauty* transposons. These vectors are illustrated in Fig. 7. Clark *et al.*[210] have compared the efficiencies of expression of SB transposons with a complete expression cassette, pT/SV40-Neo that has a neomycin phosphotransferase II gene (with poly(A) sequence) under the regulation of an SV40 promoter, with an equivalent SB transposon with a Neo gene trap in which the IRES-Neo gene cannot be expressed unless it integrates into an active transcriptional unit in the correct orientation, i.e. the gene is oriented in the same direction as the promoter behind which it inserts. Using tissue-cultured HeLa cells, they found that about 1 in 15 integrations resulted in activation of the gene trap, which corresponds to about 4 in 15 integrations into transcriptional units when orientation and percentage of the genes expressed is taken into account. This rate is close to the 1 in 4 rate expected for random integration into a mammalian genome.[210] Thus, the functional assay is consistent to other findings with the findings that SB transposons integrate nearly randomly in vertebrate genomes.[164,212–214]

One problem with trap vectors that use a reporter gene directly for activation is that most genes are expressed at relatively low levels.[215] Consequently, the intensity of a fluorescent protein trap might be below detection. This situation would be aggravated if short-half-life versions of fluorescent proteins were used to improve resolution of when and where trapped genes are expressed. Consequently, Clark *et al.*[210] replaced the reporter gene with one encoding a transcriptional enhancer-binding protein. When this gene is activated, it serves to direct expression of a fluorescent protein behind a strong promoter, thereby amplifying the signal up to 100-fold.

In summary, the best trap vectors are those that insert in a very random manner and allow assessment of a mutagenized locus. Hence,

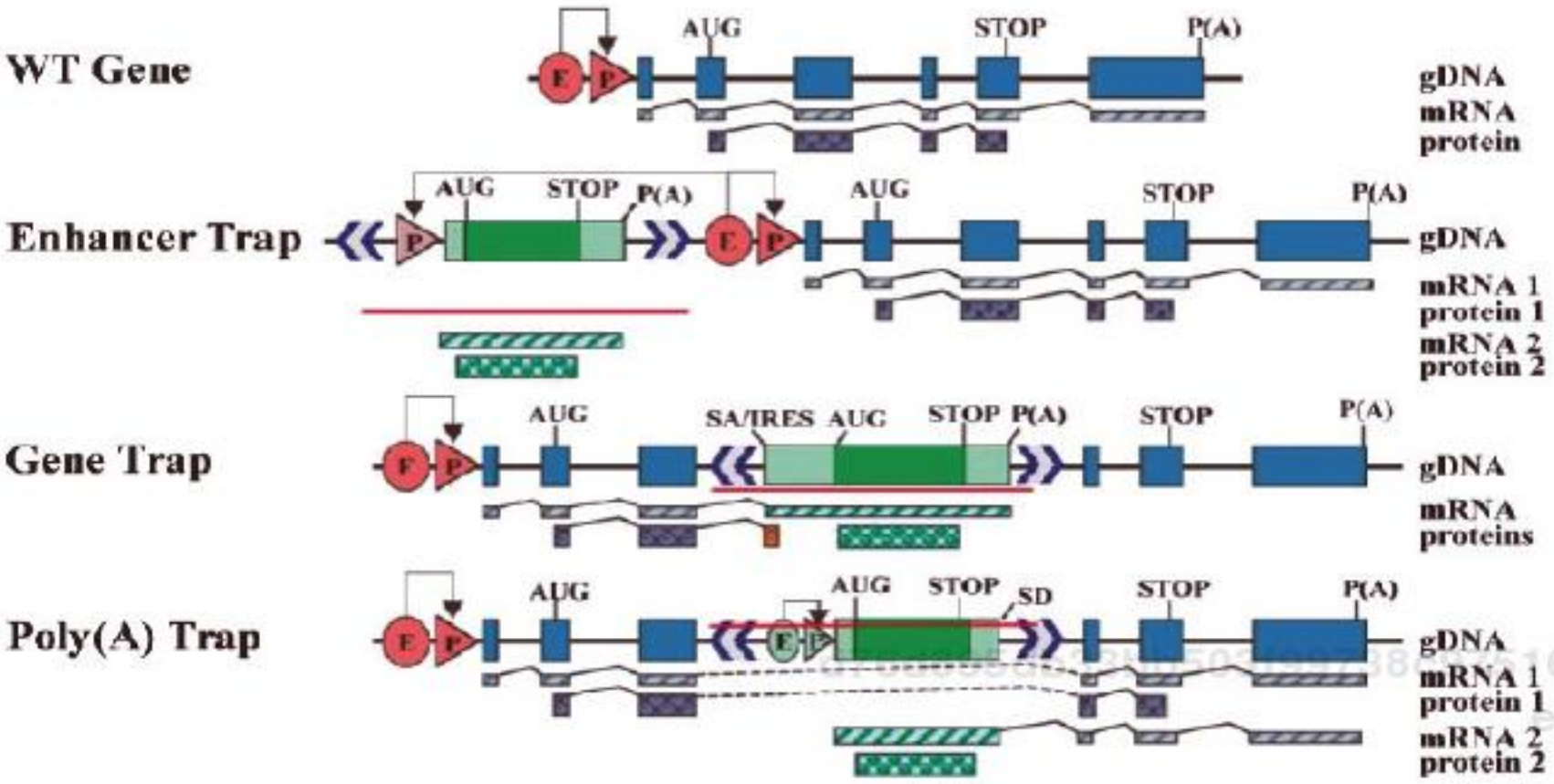

Fig. 7 SB transposon-based trap vectors for functional genomic studies in vertebrates. A genetic locus (WT Gene) is shown on the top line with exons as boxes, introns as lines, transcriptional enhancers as circles (E) that activates (arrow) a promoter (P). RNA transcripts with introns are indicated below the gene and the encoded proteins are shown at the bottom. The activities of enhancer, gene and poly(A) traps are shown in the three examples where the transposon is indicated by the blue, inverted, double arrowheads. Enhancer traps use the WT enhancer element to activate a minimal promoter (triangle with a "P" in the transposon) to activate a marker gene (e.g. GFP shown in green). When a transposon with a gene trap inserts in either a transcribed exon *or* intron, the marker gene is expressed from an Internal Ribosome Entry Site (IRES) that can activate protein synthesis at the normal initiation site for the marker protein. The splice acceptor (SA) site ensures that the marker sequences are spliced into the mRNA when the transposon integrates into an intron; the SA site may not be used if the transposon integrates into an exon. Poly(A) traps have both a splice acceptor and splice donor site. Poly(A) trap vectors must integrate into a transcriptional unit so that the marker gene can acquire a poly(A) sequence for stability. To ensure inclusion of the poly(A) sequence should the transposon integrate into an intron, the poly(A) trap vector has a splice-donor (SD) site at its 3' end. This vector has a strong enhancer/promoter driver so it does not need an SA site. Should the poly(A) trap integrate into an intron, the normal message may be formed (with splicing out of the poly(A) trap) along with the marker transcript that includes the same 3' poly(A) as the interrupted gene (adapted from Clark[211]).

it is likely that the SB transposon traps will integrate more randomly, and therefore detect more genes, than retrovirus vectors.

3.3.3. *Transposition in fish using the Tol2 transposon system*

The *Tol2* transposon is a member of the *hAT* family of transposons, so named because of the relatedness of the *hobo* element in flies, the *Activator* (*Ac*) transposon in maize, and the *Tam3* mobile element in snapdragons. The *Tol2* transposon was originally identified by its insertion into the tyrosinase gene that reduced coloration in certain medaka fish.[102] This element is active — it is found in different genomic positions, even in closed populations of fish.[216] The transposase of *Tol2* has four open reading frames that appear to be arranged in two spliced genes,[102,110] a more complicated arrangement than that of the *Sleeping Beauty* transposon. About 10 to 30 copies exist in medaka, and most appear to be autonomous, but *hAT* transposons have not been found in zebrafish.[105] The *Tol2* system is active in zebrafish[105–107] and in mammalian cells[109] and has been used as a gene transfer vector.[217] The integration site preferences of the *Tol2* transposase gene have not been identified, but *Tol2* transposons cause an 8-basepair duplication at the insertion site, compared with the TA duplication for SB transposons. The relative activities of the *Tol2* transposon and the SB transposon have not been compared side-by-side. These two transposon systems may have complementary features for functional genomic studies in fish. The identification of *Tol2*, *hAT*-type, and the SB, *Tc1/mariner*-type, transposons may just be the initial entries into synthetic and natural mobile elements that will be used for gene transfer in fish as well as other vertebrates. Further development of the *Tol2* system, along with more refinements in the available transposons and perhaps other natural and synthetic transposons, should lead to more versatility in precise gene-transfer in fish.

4. Future Directions

Transposons allow transfer of precise, single-copies of genetic material to chromosomes. At present, only transposons derived from vertebrates

have high activities in vertebrates. The reasons for this are unclear and finding them is the focus of future directions. For transgenesis in fish, a major goal is to achieve integration of transgenes in the chromosomes of one-cell stage embryos. By doing this, effects of transgenesis can be elucidated in a single generation. Currently, the mosaicism in the founder populations of transgenic fish demands at least two generations before genetic studies can be conducted. Solutions to this problem may come from more complete understanding of the transposition process and the factors involved, or in the development of new delivery systems.

4.1. Parameters Affecting Transposition

Parameters that affect transposition are illustrated in Fig. 8. Several of these parameters are under investigation for the *Sleeping Beauty* transposon system, including sequences of the inverted terminal repeats and the direct repeats (DRs) that comprise each IR. Indeed, the DRs are not identical in the outer and inner positions[75] and it is crucial that DRs with weaker DNA-binding ability exist in the outer position.[111,217] The reason for this is unknown but probably reflects the dynamic nature of binding and release of transposase during the transposition reaction. A second component of the SB transposon system certainly can be improved — the transposase itself. Initial refinements have been reported[122] and more are sure to follow. The initial transposase and the modifications are largely based on phylogenetic consensus sequences.[90,92] However, DNA-based transposons exist in nature because they transpose very infrequently over evolutionary time. Hence, improvement in transposase activity under experimental conditions should be possible using appropriate screening following random mutagenesis. Other factors that affect transposition are the ratio of transposase to transposon and the lengths of the transposon.[220] The excision assay,[219,221] shown at the lower left corner of Fig. 8, is a relatively quick assay that has been developed to facilitate assays on the efficiency of transposition. The assay is based on the precise excision of the transposon from a plasmid and the subsequent repair of donor (Fig. 3; note the ambiguity in the central basepair of the canonical

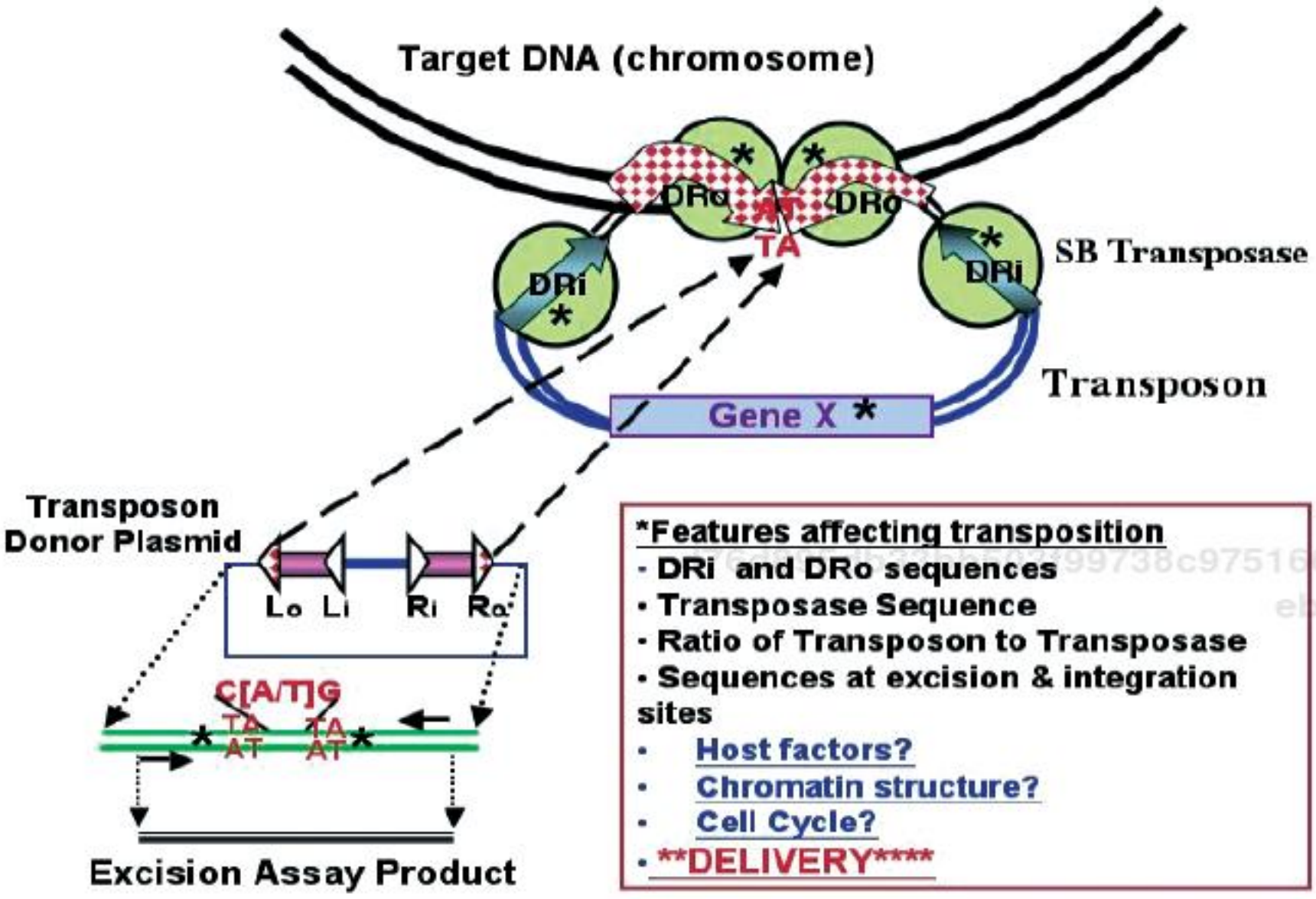

Fig. 8 Summary of parameters that can be altered to improve transposition. The schematic shows a transposon, represented by inverted terminal repeats composed of two DRs labeled Lo and Li for the left outer and inner DRs and Ri and Ro for the right inner and outer DRs on a plasmid (lower left). The efficiency of transposition (dashed lines) from a donor plasmid into a TA-target site on a chromosome is affected by the sequences surrounding the donor site (bottom left) as well as the conformation of the integration site. The differences in sequences of the outer and inner DRs are reflected by the different shadings. SB transposase is shown as a circle. Asterisks indicate components and motifs that affect transposition.

footprint) that defines a PCR product from primers on either side of the donor transposon (Fig. 8, bottom left corner). Several of these parameters that affect transposition are discussed in more detail in the following sections.

4.2. Identification of Host Factors Associated with Transposition

A major objective is to achieve integration in early-stage embryos at the one- to two-cell stage. With microinjection the amounts of DNA

delivered to the average embryo can be better quantified than with the alternative procedures. As noted earlier, although about 1 to 10 million molecules are delivered to each embryo, the genomes of only a few cells incorporate a transposon. One physiological phenomenon in zebrafish and medaka that surely affects transposition is the speed of DNA replication. Owing to the rapidity of the early cleavage cycles, there must be at least 100 times as many DNA polymerases per unit length of fish chromatin as for mammalian chromatin, resulting in a situation where most of the chromatin is probably not in a form that can allow recombination.[6] DNA replication apparently does not depend on specific DNA sequences (*ori* sequences) that are required for DNA synthesis later on[222] because injected plasmids are replicated.[165] In zebrafish, transcription begins after the midblastula transition (ca. 1000-cell stage, Kane and Kimmel,[223] Kimmel *et al.*[224]). At this time replication of chromatin slows and the DNA apparently becomes available for enzymes that mediate integration. This is the basis for the late injection of pseudotype retroviral vectors for insertional mutagenesis in zebrafish. DNA conformation and chromatin binding factors also appear to influence transposition of SB transposons.[225–227] Presumably there are factors that, if co-injected into newly fertilized embryos with the transposon components, would lead to transient relaxation of chromatin at the one-cell stage to permit transposition. Identifying these factors is an important goal for fish transgenesis regardless of vector or method of introduction. It could be that introducing the transposon system into gametes prior to fertilization would allow access of transposons to chromatin before the onset of chromatin condensation and rapid rates of DNA replication.

4.3. Regulation of the Ratio of Transposase to Transposons

Transposition of SB transposons requires four transposase molecules per transposon. Binding of one to three transposase molecules might be able to facilitate nuclear import as a result of the nuclear localization motifs on transposase but not transposition. This hypothesis is supported by studies where one or more of the transposase binding sites were

deleted in the IR/DR region of a transposon.[111,120] On the other hand, overexpression of transposase interferes with transposition.[122] Consequently, the initial source of transposase is important in transposition-mediated transgenesis. Studies in mice wherein plasmids harboring transposase genes have been injected indicate that the ratio of transposon plasmid to transposase has an effect.[117,118] A systematic investigation of the effects of different levels of DNA or mRNA encoding transposase has not been reported, nor has the injection of purified transposase with transposons.

Rapid achievement of optimal levels of transposase to transposon in early-stage embryos would facilitate early integration. The method of delivery of the components of the transposon system may have an effect on the ratio of transposase to transposon. For instance, if only a few molecules with a transposon and a few with a transposase gene are delivered to a cell, then the ratio of transposase to transposon will vary from cell to cell. Alternatively, if hundreds or more molecules are delivered to a cell, e.g. via microinjection, then the ratios of the two molecules will be similar from cell to cell.

4.4. Transposase-Expressing Lines of Fish

Functional genomics in mice using the SB transposon system has been facilitated by lines of mice that express SB transposase.[100,160,161,164] This allows remobilization of transposons in subsequent animals of up to an average of one to two new insertions per offspring. The SB-expressing lines of mice appear to have normal phenotypes with no indications of endogenous transposons being mobilized by the transposase.[230] Realizing the benefits of using fish that express fluorescent proteins when in various transposon trap vectors, attempts to develop lines of zebrafish that express SB transposase are being made (D. Balciunas, pers. comm.).

4.5. Site-Specific Integration

Transposition of the DNA transposons discussed in this review is characterized by relatively random integration throughout vertebrate

genomes. This is the rule for transposons. Nevertheless, there is considerable interest in developing transposases with site-specific integration capability. The DNA-binding motif in the N-terminal third of the SB transposase is known to interact with the DR sequences of the transposon. However, the DNA-interacting motif for TA-dinucleotide basepairs at the target site resides somewhere in the catalytic domain in the carboxyl-terminal half of the transposase; it is not identified. As a result, directed mutagenesis to alter the specificity of integration has not been accomplished.

Site-specific integration has a broader implication than use in fish. The SB transposon system is an efficacious means of inserting defined sequences of DNA into mammalian chromosomes without using viruses. The SB system has been used to deliver genes for long-term expression to livers[117,231] and lungs[118] of mice. The system looks feasible for use in human gene therapy once delivery methods are better defined. The ability to direct specifically transposons to a given site in chromosomes would increase the safety of this method of delivery. Thus, methods that were initiated to improve transgenesis in fish are being adapted for use in humans. Truly, fish are an excellent model system for more than merely finding the functions of genes and their interactions.

Acknowledgments

We are grateful for all of the input from our laboratory colleagues over the years. Our academic laboratories are supported by grants from the Arnold and Mabel Beckman Center for Transposon Research and NIH to PBH and SCE.

References

1. Detrich HW, Westerfield M and Zon LI (eds.) (1999a). *The Zebrafish: Biology. Meth. Cell Biol.* vol 59. Academic Press, San Diego.
2. Detrich HW, Westerfield M and Zon LI (eds.) (1999b). *The Zebrafish: Genetics and Genomics. Meth. Cell Biol.* vol 60. Academic Press, San Diego.

3. Dodd A, Curtis PM, Williams LC and Love DR (2002). Zebrafish: bridging the gap between development and disease. *Hum. Mol. Genet.* **9**: 2443–2449.
4. Ekker SC (2000). Morphants: a new systematic vertebrate functional genomics approach. *Yeast* **17**: 302–306.
5. Drummond IA (2000). The zebrafish pronephros: a genetic system for studies of kidney development. *Pediatr. Nephrol.* **14**: 428–435.
6. Hackett PB and Alvarez MC (2000). The molecular genetics of transgenic fish. *Recent Adv. Mar. Biotech.* **4**: 77–145.
7. Fishman MC (2001). The genomic cosmos. *Nature* **410**: 1033.
8. Goldman D, Hankin M, Li Z, Dai X and Ding J (2001). Transgenic zebrafish for studying nervous system development and regeneration. *Transgen. Res.* **10**: 21–33.
9. Nasevicius A and Ekker SC (2000). Effective targeted gene 'knockdown' in zebrafish. *Nat. Genet.* **26**: 216–220.
10. Peterson RT, Link BA, Dowling JE and Schreiber SL (2000). Small molecule developmental screens reveal the logic and timing of vertebrate development. *Proc. Natl. Acad. Sci. USA* **97**: 12965–12969.
11. Shin JT and Fishman MC (2002). From zebrafish to human: modular medical models. *Ann. Rev. Genom. Hum. Genet.* **3**: 311–340.
12. Sehnert AJ and Stainier DYR (2002). A window to the heart: can zebrafish mutants help us understand heart disease in humans? *Trends Genet.* **18**: 491–494.
13. Streisinger G, Walker C, Dower N, Knauber D and Singer F (1981). Production of clones of homozygous diploid zebra fish (*Brachydanio rerio*). *Nature* **291**: 293–296.
14. Postlethwait J, Amores A, Force A and Yan Y-L (1999). The zebrafish genome. In: Detrich HW, Westerfield M and Zon LI (eds.), *The Zebrafish: Genetics and Genomics. Meth. Cell Biol.* **60**: 149–163.
15. Langheinrich U (2002). Zebrafish: a new model on the pharmaceutical catwalk. *BioEssays* **25**: 904–912.
16. Chakrabarti IS, Streisinger G, Singer F and Walker C (1983). Frequency of γ-ray induced specific locus and recessive lethal mutations in mature germ cells of the zebrafish, *Brachydanio rerio*. *Genetics* **103**: 109–122.
17. Driever W, Solnica-Krezel L, Schier AF, Neuhaus SCF, Malicki J, Stemple DL, Stanier DYR, Zwartkruis F, Abdelilah S, Rangini Z, Belak J

and Boggs C (1996). A genetic screen for mutation affecting embryogenesis in zebrafish. *Development* **123**: 37–46.

18. Haffter P, Granato M, Brand M, Mullins MC, Hammerschmidt M, Kane DA, Odenthal J, van Eeden FJM, Jiang Y-J, Heisenberg C-P, Kelsh RN, Furutani–Seiki M, Vogelsang E, Beuchle D, Schach U, Fabian C and Nüsslein–Volhard C (1996). The identification of genes with unique and essential functions in the development of the zebrafish, *Danio rerio*. *Development* **123**: 1–36.
19. Amsterdam A, Burgess S, Golling G, Chen W, Sun Z, Townsend K, Farrington S, Haldi M and Hopkins N (1999a). A large-scale insertional mutagenesis screen in zebrafish. *Genes Dev.* **13**: 2713–2724.
20. Amsterdam A and Hopkins N (1999b). Retrovirus-mediated insertional mutagenesis in zebrafish. In: Detrich HW, Westerfield M and Zon LI (eds.), *The Zebrafish: Genetics and Genomics. Meth. Cell Biol.* **60**: 87–98.
21. Beattie CE, Raible DW, Henion PD and Eisen JS (1999). Early pressure screens. In: Detrich HW, Westerfield M and Zon LI (eds.), *The Zebrafish: Genetics and Genomics. Meth. Cell Biol.* **60**: 71–86.
22. Pelegri F and Schulte–Merker S (1999). A gynogenesis-based screen for maternal-effect genes in the zebrafish. In: Detrich HW, Westerfield M and Zon LI (eds.), *The Zebrafish: Genetics and Genomics. Meth. Cell Biol.* **60**: 1–21.
23. van Eeden FJ, Granato M, Odenthal J and Haffter P (1999). Developmental mutant screens in the zebrafish. In: Detrich HW, Westerfield M and Zon LI (eds.), *The Zebrafish: Genetics and Genomics. Meth. Cell Biol.* **60**: 22–41.
24. Walker C (1999). Haploid screens and gamma-ray mutagenesis. In: Detrich HW, Westerfield M and Zon LI (eds.), *The Zebrafish: Genetics and Genomics. Meth. Cell Biol.* **60**: 44–70.
25. Darland T and Dowling JE (2001). Behavioral screening for cocaine sensitivity in mutagenized zebrafish. *Proc. Natl. Acad. Sci. USA* **98**: 116591–11696.
26. Crosier PS, Bardsley A, Horsfield JA, Krassowska AK, Lavallie ER, Collins–Racie LA, Postlethwait JH, Yan Y-L, McCoy JM and Crosier KE (2001). *In situ* hybridization screen in zebrafish for the selection of genes encoding secreted proteins. *Dev. Dynam.* **222**: 637–644.

27. Hackett PB (1993). The molecular biology of transgenic fish. In: Hochachka PW and Mommsen TM (eds.), *Biochemistry and Molecular Biology of Fishes*, Vol. 2. Elsevier, Amsterdam. pp. 207–240.
28. Gong Z and Hew CL (1995). Transgenic fish in aquaculture and developmental biology. *Curr. Top. Dev. Biol.* **30**: 177–214.
29. Maclean N (1988). Regulation and exploitation of transgenes in fish. *Mut. Res.* **399**: 255–266.
30. Hyatt TM and Ekker SC (1999). Vectors and techniques for ectopic gene expression in zebrafish. *Meth. Cell Biol.* **59**: 117–126.
31. Meng A, Jessen JR and Lin S (1999). Transgenesis. In: Detrich HW, Westerfield M and Zon LI (eds.), *The Zebrafish: Genetics and Genomics. Meth. Cell Biol.* **60**: 133–148.
32. Zbikowska HM (2003). Fish can be first — advances in fish transgenesis for commercial applications. *Transgen. Res.* **12**: 379–389.
33. Ishikawa Y, Hyodo–Taguchi Y and Tatsumi K (1997). Medaka fish for mutant screens. *Nature* **386**: 234.
34. Warren KS and Fishman MC (1998). "Physiological genomics": mutant screens in zebrafish. *Am. J. Physiol.* **275** (*Heart Circ. Physiol.* **44**): H1–H7.
35. Rubin EM and Tall A (2000). Perspectives for vascular genomics. *Nature* **407**: 265–269.
36. Talbot WS and Hopkins N (2002). Zebrafish mutations and functional analysis of the vertebrate genome. *Genes Dev.* **14**: 755–762.
37. Strehlow D, Heinrich G and Gilbert W (1994). The fates of the blastomeres of the 16-cell zebrafish embryo. *Development* **120**: 1791–1798.
38. Lee K-Y, Huang H, Ju B, Yang Z and Lin S (2002). Cloned zebrafish by nuclear transfer from long-term-cultured cells. *Nat. Biotech.* **20**: 795–799.
39. Chalfie M, Tu Y, Euskirchen G, Ward WW and Prasher DC (1994). Green fluorescent protein as a marker for gene expression. *Science* **263**: 802–805.
40. Amsterdam A, Lin S and Hopkins N (1995). The *Aequorea victoria* green fluorescent protein can be used as a reporter in live zebrafish embryos. *Dev. Biol.* **171**: 123–129.
41. Moss JB, Price AL, Raz E, Driever W and Rosenthal N (1999). Green fluorescent protein marks skeletal muscle in murine cell lines and zebrafish. *Gene* **173**: 89–98.

42. Long Q, Meng A, Wang H, Jessen JR, Farrell MJ and Lin S (1997). GATA-1 expression pattern can be recapitulated in living transgenic zebrafish using GFP reporter gene. *Development* **124**: 4105–4111.
43. Higashijima S, Okamoto H, Ueno N, Hotta Y and Eguchi G (1997). High-frequency generation of transgenic zebrafish which reliably express GFP in whole muscles or the whole body by using promoters of zebrafish origin. *Dev. Biol.* **192**: 289–299.
44. Ju B, Xu Y, He J, Liao J, Yan T, Hew CL, Lam TJ and Gong Z (1999). Faithful expression of green fluorescent protein (GFP) in transgenic zebrafish embryos under control of zebrafish gene promoters. *Dev. Genet.* **25**: 158–167.
45. Gibbs PDL and Scmale MC (1999). GFP as a genetic marker scorable through the lifecycle of transgenic zebrafish. *Mar. Biotech.* **2**: 107–125.
46. Fahrenkrug SC, Clark K and Hackett PB (1999). Dicistronic gene expression in developing zebrafish. *Mar. Biotech.* **1**: 552–561.
47. Halloran MC, Sato–Maeda M, Warren JT, Su F, Lele Z, Krone PH, Kuwada JY and Shoji W (2000). Laser-induced gene expression in specific cells of transgenic zebrafish. *Development* **127**: 1953–1960.
48. Finley K, Davidson A and Ekker SC (2001). Three color imaging using fluorescent proteins in living zebrafish embryos. *Biotechmiques* **31**: 66–72.
49. Gong Z, Wan H, Tay TL, Wang H, Chen M and Yan T (2003). Development of transgenic fish for ornamental and bioreactor by strong expression of fluorescent proteins in the skeletal muscle. *Biochem. Biophys. Res. Commun.* **308**: 58–63.
50. Yoshizaki G, Takeuchi Y, Sakatani S and Takeuchi T (2000). Germ cell-specific expression of green fluorescent protein in transgenic rainbow trout under control of the rainbow trout vasa-like gene promoter. *Int. J. Dev. Biol.* **44**: 323–326.
51. Wakamatsu Y, Ju B, Pristyaznhyuk I, Niwa K, Ladygina T, Kinoshita M, Araki K and Ozato K (2001). Fertile and diploid nuclear transplants derived from embryonic cells of a small laboratory fish, medaka (*Oryzias latipes*). *Proc. Natl. Acad. Sci. USA* **98**: 1071–1076.
52. Wan H, He H, Ju B, Yan T, Lam YJ and Gong Z (2002). Generation of two-color transgenic zebrafish using the green and red fluorescent protein reporter genes *gfp* and *rfp*. *Mar. Biotech.* **4**: 146–154.

53. Peters KG, Ro PS, Bell BS and Kindman LA (1995). Green fluorescent fusion proteins: powerful tools for monitoring protein expression in live zebrafish embryos. *Dev. Biol.* **171**: 252–257.
54. Essner JJ, Laing JG, Beyer EC, Johnson RG and Hackett PB (1996). Expression of zebrafish *connexin43.4* in the notochord and tail bud of wild-type and *no tail* mutant embryos. *Dev. Biol.* **177**: 449–462.
55. Inoue K, Ozato K, Kondoh H, Iwamatsu T, Wakamatsu Y, Fujita T and Okada TS (1989). Stage-dependent expression of the chicken δ-crystallin gene in transgenic fish embryos. *Cell Diff. Dev.* **27**: 57–68.
56. Du SJ, Frenkel V, Kindschi G and Zohar Y (2001). Visualizing normal and defective bone development in zebrafish embryos using the fluorescent chromophore calcein. *Dev. Biol.* **238**: 239–246.
57. Farber SA, Pack M, Ho S-Y, Johnson ID, Wagner DS, Dorsch R, Mullins MC, Hendrickson HS, Hendrickson EK and Halpern ME (2001). Genetic analysis of digestive physiology using fluorescent phospholipid reporters. *Science* **292**: 1385–1388.
58. Vielkind J, Vielkind U, von Grotthuss E and Anders F (1971). Uptake of bacterial H3-DNA into fish embryos. *Experientia* **27**: 347–348.
59. Schwab M, Vielkind J and Anders F (1976). An approach to genetic transformation in the Xiphophorine fish. *Mol. Gen. Genet.* **144**: 151–158.
60. Vielkind J, Haas–Abdekam H, Vielkind U and Anders F (1982). The induction of a specific pigment cell type by total genomic DNA injected into the neural crest region of fish embryos of geneus *Xiphophorus*. *Mol. Gen. Genet.* **185**: 379–389.
61. Zhu Z, Xu Hm, Li G, Xie Y and He L (1986). Biological effects of human growth hormone gene microinjected into the fertilized eggs of loach *Misgurnus anguillicaudatus* (Cantor). *Kexue Tongbao* **31**: 988–990.
62. Burns JC, Friedmann T, Driever W, Burrascano M and Yee JK (1993). Vesicular stomatitis virus G glycoprotein pseudotyped retroviral vectors: concentration to very high titer and efficient gene transfer into mammalian and nonmammalian cells. *Proc. Natl. Acad. Sci. USA* **90:** 8033–8037.
63. Lundstrom K (2003). Latest development in viral vectors for gene therapy. *Trends Biotech.* **21**: 117–122.
64. Martineau D, Bowser PR, Renshaw RR and Casey JW (1992). Molecular characterization of a unique retrovirus associated with a fish tumor. *J. Virol.* **66**: 596–599.

65. Holzschu DL, Martineau D, Fodor SK, Vogt VM, Bowser P and Casey JW (1995). Nucleotide sequence and protein analysis of a complex retrovirus, walleye dermal sarcoma virus. *J. Virol.* **69**: 5320–5331.
66. Hart D, Frerichs GN, Rambaut A and Onions DE (1996). Complete nucleotide sequence and transcriptional analysis of the snakehead fish retrovirus. *J. Virol.* **70**: 3606–3616.
67. Lin S, Gaiano N, Culp P, Burns JC, Friedman T, Yee JK and Hopkins N (1994). Integration and germ-line transmission of a pseudotyped retroviral vector in zebrafish. *Science* **265**: 666–669.
68. Gaiano N, Allende M, Amsterdam A, Kawakami K and Hopkins N (1996a). Highly efficient germ-line transmission of proviral insertions in zebrafish. *Proc. Natl. Acad. Sci. USA* **93**: 7777–7782.
69. Gaiano N and Hopkins N (1996b). Insertional mutagenesis and rapid cloning of essential genes in zebrafish. *Nature* **383**: 829–832.
70. Allende ML, Amsterdam A, Becker T, Kawakami K, Gaiano N and Hopkins N (1996). Insertional mutagenesis in zebrafish identifies two novel genes, pescadillo and dead eye, essential for embryonic development. *Genes Dev.* **10**: 3141–3155.
71. Golling G, Amsterdam A, Sun Z, Antonelli M, Maldonado E, Chen W, Burgess S, Haldi M, Artzt K, Farrington S, Lin SY, Nissen RM and Hopkins N (2002). Insertional mutagenesis in zebrafish rapidly identifies genes essential for early vertebrate development. *Nat. Genet.* **31**: 135–140.
72. Linney E, Hardison NL, Lonze BE, Lyons S and DiNapoli L (1999). Transgene expression in zebrafish: a comparison of retroviral-vector and DNA-injection approaches. *Dev. Biol.* **213**: 207–216.
73. Wu X, Li Y, Crise B and Burgess SM (2003). Transcription start regions in human genome are favored targets for MLV integration. Science **300**: 1749–1751.
74. Smith KT, Shepard AJ, Boyd JE and Lees GM (1996). Gene delivery systems for use in gene therapy: an overview of quality assurance and safety issues. *Gene Therapy* **3**: 190–200.
75. Ivics Z, Izsvák Zs and Hackett PB (1997). Molecular reconstruction of *Sleeping Beauty*, a *Tc1*-like transposon from fish, and its transposition in human cells. *Cell* **91**: 501–510.
76. Izsvák Zs, Ivics Z and Hackett PB (1997). Repetitive elements and their genetic applications in zebrafish. *Biochem. Cell Biol.* **75**: 507–523.

77. Prack ETL and Kazian HH Jr (2002). Mobile elements and the human genome. *Nat. Genet. Rev.* **1**: 134–144.
78. Kazian HH Jr and Goodier JL (2002). LIKNE drive: retrotransposition and genome instability. *Cell* **110**: 277–280.
79. Izsvák Zs, Ivics Z, Garcia–Estefania D, Fahrenkrug SC and Hackett PB (1996). DANA elements: a family of composite, short interspersed, DNA elements associated with mutational activities in zebrafish (*Danio rerio*). *Proc. Natl. Acad. Sci. USA* **93**: 1044–1048.
80. Shimoda N, Chrevrette M, Ekker M, Kikkuuchi Y, Hotta Y and Okamoto H (1996). *Mermaid*: a family of short interspersed repetitive elements widespread in vertebrates. *Biochem. Biophys. Res. Commun.* **220**: 226–232.
81. San Miguel P *et al.* (1996). Nested retrotransposons in the intergenic regions of the maize genome. *Science* **274**: 765–768.
82. Takasaki N, Murata S, Saitoh M, Kobayashi T, Park L and Okada N (1994). Species-specific amplification of tRNA-derived short interspersed repetitive elements (SINES) by retroposition; a process of parasitization of entire genomes during the evolution of salmonids. *Proc. Natl. Acad. Sci. USA* **91**: 10153–10157.
83. Ivics Z, Izsvák Z and Hackett PB (1999). Genetic applications of transposons and other repetitive elements in zebrafish. In: Detrich HW, Westerfield M and Zon LI (eds.), *The Zebrafish: Genetics and Genomics. Meth. Cell Biol.* **60**: 99–131.
84. Plasterk RH (1993). Molecular mechanisms of transposition and its control. *Cell* **74**: 781–786.
85. Vos JC, De Baere I and Plasterk RH (1996). Transposase is the only nematode protein required for *in vitro* transposition of Tc1. *Genes Dev.* **10**: 755–761.
86. Lampe DJ, Churchill ME and Robertson HM (1996). A purified mariner transposase is sufficient to mediate transposition *in vitro*. *EMBO J.* **15**: 5470–5479.
87. Plasterk RHA, Izsvak Z and Ivics Z (1999). Resident aliens: the Tc1 mariner superfamily of transposable elements. *Trends Genet.* **15**: 326–332.
88. Ivics Z, Izsvák Z and Hackett PB unpub.
89. Gibbs PDL, Gray A and Thorgaard G (1994). Inheritance of *P* element and reporter gene sequences in zebrafish. *Mol. Mar. Biol. Biotech.* **3**: 317–326.

90. Radice AD, Bugaj B, Fitch D and Emmons SW (1994). Widespread occurrence of the Tc1 transposon family: Tc1-like transposons from teleost fish. *Mol. Gen. Genet.* **244**: 606–612.
91. Izsvák Zs, Ivics Z and Hackett PB (1995). Characterization of a Tc1-like trans-posable element in zebrafish (*Danio rerio*). *Mol. Gen. Genet.* **247**: 312–322.
92. Ivics Z, Izsvák Zs, Minter A and Hackett PB (1996). Identification of functional domains and evolution of Tc1 family of transposable elements. *Proc. Natl. Acad. Sci. USA* **93**: 5008–5013.
93. Reed KM (1999). Tc1-like transposable elements in the genome of lake trout (*Salvelinus namaycush*). *Mar. Biotech.* **1**: 60–67.
94. Liu Z, Li P, Kucuktas H and Dunham R (1999). Characterization of nonautonomous *Tc1*-like transposable elements of channel catfish (*Ictalurus punctatus*). *Fish Physiol. Biochem.* **21**: 65–72.
95. Leaver MJ (2001). A family of *Tc1*-like transposons from the genomes of fishes and frogs: evidence for horizontal transmission. *Gene* **271**: 203–214.
96. Raz E, van Luenen HGAM, Schaerringer B, Plasterk RHA and Driever W (1998). Transposition of the nematode *Caenorhabditis elegans Tc3* element in the zebrafish *Danio rerio*. *Curr. Biol.* **8**: 82–88.
97. Fadool JM, Hartl DL and Dowling JE (1998). Transposition of the *mariner* element from *Drosophila mauritiana* in zebrafish. *Proc. Natl. Acad. Sci. USA* **95**: 5182–5186.
98. Sherman A, Dawson A, Mather C, Gilhooley H, Li Y, Mitchell R, Finnegan D and Sang H (1998). Transposition of the *Drosophila* element *mariner* into the chicken germ line. *Nat. Biotech.* **16**: 1050–1053.
99. Zhang L, Sankar U, Lampe DJ, Robertson HM and Graham FL (1998). The *Himar1* mariner transposase cloned in a recombinant adenovirus vector is functional in mammalian cells. *Nucl. Acids Res.* **26**: 3687–3693.
100. Fischer SEJ, Wienholds E and Plasterk RHA (2001). Regulated transposition of a fish transposon in the mouse germ line. *Proc. Natl. Acad. Sci. USA* **98**: 6759–6754.
101. Inagaki H, Bessho Y, Koga A and Hori H (1994). Expression of the tyrosinase-encoding gene in a colorless melanophore mutant of the medaka fish (*Oryzias latipes*). *Gene* **150**: 319–324.
102. Koga A, Suzuki M, Inagaki H, Bessho Y and Hori H (1996). Transposable element in fish. *Nature* **383**: 30.

103. Koga A and Hori H (1997). Albinism due to transposable element insertion in fish. *Pigment Cell Res.* **10**: 377–381.
104. Hori H, Suzuki M, Inagaki H, Oshima T and Koga A (1998). An active Ac-like transposable element in teleost fish. *J. Mar. Biotech.* **6**: 206–207.
105. Kawakami K, Koga A, Hori H and Shima A (1998). Identification of a functional transposase of the *Tol2* element, an Ac-like element from the Japanese medaka fish, O*ryzias latipes*, in zebrafish, *Danio rerio*. *Gene* **225**: 17–22.
106. Kawakami K and Shima A (1999). Identification of the Tol2 transposase of the medaka fish *Oryzias latipes* that catalyzes excision of a nonautonomous *Tol2* element in zebrafish *Danio rerio*. *Gene* **240**: 239–244.
107. Kawakami K, Koga A, Hori H and Shima A (2000). Excision of the *Tol2* transposable element of the medaka fish and its transposition in the zebrafish germline. *Proc. Natl. Acad. Sci. USA* **97**: 11403–11408.
108. Koga A and Hori H (2000). Detection of *de novo* insertion of the medaka fish transposable element *Tol2*. *Genetics* **156**: 1243–1247.
109. Koga A, Iida A, Kamiya M, Hayashi R, Hori H, Ishikawa Y and Tachibana A (2003). The medaka fish *Tol2* transposable element can undergo excision in human and mouse cells. *J. Hum. Genet.* **48**: 231–235.
110. Koga A, Suzuki M, Maruyama Y, Tsutsumi M and Hori H (1999). Amino acid sequence of a putative transposase protein of the medaka fish transposable element *Tol2* deduced from mRNA nucleotide sequences. *FEBS Lett.* **461**: 295–298.
111. Cui Z, Geurts AM, Liu G, Kaufman CD and Hackett PB (2002). Structure-Function analysis of the inverted terminal repeats of the *Sleeping Beauty* transposon. *J. Mol. Biol.* **318**: 1221–1235.
112. Iyengar A, Müller F and Maclean N (1996). Expression and regulation of transgenes in fish. *Transgen. Res.* **5**: 147–165.
113. Caldovic L, Agalliu D and Hackett PB (1999). Position-independent expression of transgenes in zebrafish. *Transgen. Res.* **8**: 321–334.
114. Dorer DR (1997). Do transgene arrays form heterochromatin in vertebrates? *Transgen. Res.* **6**: 3–10.
115. Garrick D, Fiering S, Martin DIK and Whitlaw E (1998). Repeat-induced gene silencing in mammals. *Nat. Genet.* **18**: 56–59.

116. Henikoff S (1998). Conspiracy of silence among repeated transgenes. *BioEssays* **20**: 532–534.
117. Yant SR, Meuse L, Chiu W, Ivics Z, Izsvak Z and Kay MA (2000). Somatic integration and long-term transgene expression in normal and haemophilic mice using a DNA transposon system. *Nat. Genet.* **25**: 35–41.
118. Belur L, Frandsen J, Dupuy A, Largaespada DA, Hackett PB and McIvor RS (2003). Gene insertion and long-term expression in lung mediated by the *Sleeping Beauty* transposon system. *Mol. Ther.* **8**: 501–507.
119. Zanta AM, Belguise–Valladier P and Behr J-P (1999). Gene delivery: a single nuclear localization signal peptide is sufficient to carry DNA to the cell nucleus. *Proc. Natl. Acad. Sci. USA* **96**: 91–96.
120. Izsvak Z, Ivics Z and Plasterk RHA (2000). *Sleeping beauty*, a wide host-range transposon vector for genetic transformation in vertebrates. *J. Mol. Biol.* **302**: 93–102.
121. Karsi' A, Moav B, Hackett PB and Liu Z (2001). Effects of insert size on transposition efficiency of the synthetic transposon *Sleeping Beauty* in mouse cells. *Mar. Biotech.* **3**: 241–245.
122. Geurts AM, Yang Y, Clark KJ, Cui Z, Dupuy AJ, Largaespada DA and Hackett PB (2003). Gene transfer into genomes of human cells by the *Sleeping Beauty* transposon system. *Mol. Ther.* **8**: 108–117.
123. Lander ES *et al.* (2001). Initial sequencing and analysis of the human genome. *Nature* **409**: 860–921.
124. Venter JC *et al.* (2001). The sequence of the human genome. *Science* **291**: 1304–1351.
125. Inoue K (1992). Expression of reporter genes introduced by microinjection and electroporation in fish embryos and fry. *Mol. Mar. Biol. Biotech.* **1**: 266–270.
126. Zhao X, Zhang PJ and Wong TK (1993). Application of Baekonization: a new approach to produce transgenic fish. *Mol. Mar. Biol. Biotech.* **2**: 63–69.
127. Culp P, Nüsslein–Volhard C and Hopkins N (1991). High-frequency germ-line transmission of plasmid DNA sequences injected into fertilized zebrafish eggs. *Proc. Natl. Acad. Sci. USA* **88**: 7953–7957.
128. Müller F, Lele Z, Váradi L, Menczel L and Orbán L (1993). Efficient transient expression system based on square pulse electroporation and *in vivo* luciferase assay of fertilized fish eggs. *FEBS Lett.* **324**: 27–32.

129. Westerfield M, Wegner J, Jegalian BG, DeRobertis EM and Püschel AW (1992). Specific activation of mammalian *Hox* promoters in mosaic transgenic zebrafish. *Genes Dev.* **6**: 591–598.
130. Neumann E, Schaefer–Ridder M, Wang Y and Hofschneider PH (1982). Gene transfer into mouse myloma cells by electroporation in high electric fields. *EMBO J.* **1**: 841–845.
131. Xie Y, Liu D, Zou J, Lie G and Zhu Z (1989). Novel gene transfer in the fertilized eggs of loach via electroporation. *Acta Hydrobiologica Sinica* **13**: 387–389.
132. Inoue K, Yamashita S, Hata J, Kabeno S, Asada S, Nagahisa E and Fujita T (1990). Electroporation as a new technique for producing transgenic fish. *Cell Differ. Dev.* **29**: 123–128.
133. Buono RJ and Linzer PJ (1992). Transient expression of RSVCAT in transgenic zebrafish made by electroporation. *Mol. Mar. Biol. Biotech.* **1**: 271–275.
134. Lu J-K, Fu B-H, Wu J-L and Chen TT (2002). Production of transgenic silver sea bream (*Sparus sarba*) by different gene transfer methods. *Mar. Biotech.* **4**: 328–337.
135. Powers DA, Hereford L, Cole T, Chen TT, Lin CM, Kight K, Creech K and Dunham R (1992b). Electroporation: a method for transferring genes into genes of zebrafish (*Brachydanio rerio*), channel catfish (*Ictalurus punctatus*) and common carp (*Cyprinus carpio*). *Mol. Mar. Biol. Biotech.* **1**: 301–308.
136. Murakami, Y, Motoshashi K, Yano K, Ikebukuro K, Yokoyama K, Tamiya E and Karube I (1994). Micromachined electroporation system for transgenic fish. *J. Biotechnol.* **34**: 35–42.
137. Ono H, Hirose E, Miyazaki K, Yamamoto H and Matsumoto J (1997). Transgenic medaka fish bearing the mouse tyrosinase gene: expression and transmission of the transgene following electroporation of the orange-colored variant. *Pigment Cell Res.* **10**: 168–175.
138. Lavitrano M, Camaioni A, Fazio VM, Dolci S, Farace MG and Spadafora C (1989). Sperm cells as vectors for introducing foreign DNA into eggs: genetic transformation in mice. *Cell* **57**: 717–723.
139. Brinster RL, Sandgren EP, Behringer RR and Palmiter RD (1989). No simple solution for making transgenic mice. *Cell* **59**: 239–241.
140. Atkinson PW, Hines ER, Beaton S, Matthaei KI, Reed KC and Bradley MP (1991). Association of exogenous DNA with cattle and insect spermatozoa *in vitro*. *Mol. Reprod. Dev.* **29**: 1–5.

141. Arezzo F (1989). Sea urchin sperm as a vector of foreign genetic information. *Cell. Biol. Int. Rep.* **13**: 391–404.
142. Castro FO, Hernandez O, Uliver C, Solano R, Milanes C, Aguilar A, Perez A, de Armas R, Herrea L and de la Fuente J (1991). Introduction of foreign DNA into the spermatozoa of farm animals. *Theriogenology* **36**: 1099–1110.
143. Khoo H-W, Ang L-H, Lim H-B and Wong K-Y (1992). Sperm cells as vectors for introducing foreign DNA into zebrafish. *Aquaculture* **107**: 1–19.
144. Chourrout D and Perrot E (1992). No transgenic rainbow trout produced with sperm incubated with linear DNA. *Mol. Mar. Biol. Biotech.* **1**: 282–285.
145. Patil JG and Khoo HW (1996). Nuclear internalization of foreign DNA by zebrafish spermatozoa and its enhancement by electroporation. *J. Exp. Zool.* **274**: 121–129.
146. Perry ACF, Wakayama T, Kishikawa H, Kasai T, Okabe M, Toyada Y and Yanagimachi R (1999). Mammalian transgenesis by intracytoplasmic sperm injection. *Science* **284**: 1180–1183.
147. Müller F, Ivics Z, Erdélyi F, Papp T, Váradi L, Horváth L, Maclean N and Orban L (1992). Introducing foreign genes into fish eggs by electroporated sperm as a carrier. *Mol. Mar. Biol. Biotech.* **1**: 276–281.
148. Sin FYT, Bartley AL, Walker SP, Sin IL, Symonds JE, Hawke L and Hopkins CL (1993). Gene transfer in chinook salmon (*Oncorhynchus tshawytscha*) by electroporating sperm in the presence of pRSVlacZ DNA. *Aquaculture* **117**: 57–69.
149. Tsai H-J, Tseng FS and Liao IC (1995). Electroporation of sperm to introduce foreign DNA into genome of loach (*Misgurnus anguillicaudatus*). *Canad. J. Fish. Aquat. Sci.* **52**: 776–787.
150. Kang J-H, Yoshizaki G, Homma O, Strussmann CA and Takashima F (1999). Effect of an osmostic differential on the efficiency of gene transfer by electroporation of fish spermatozoa. *Aquaculture* **173**: 297–307.
151. Symonds JE, Walker SP and Sin FYT (1994). Electroporation of salmon sperm with plasmid DNA: evidence for enhanced sperm/DNA association. *Aquaculture* **119**: 313–327.
152. Jesuthasan S and Subburaju S (2002). Gene transfer into zebrafish by sperm nuclear transplantation. *Dev. Biol.* **242**: 88–95.
153. Klein TM, Wolf ED, Wu R and Sanford JC (1987). High-velocity micro-projectiles for delivering nucleic acids into living cells. *Nature* **327**: 70–73.

154. Zelenin AV, Alimov AA, Barmintzev VA, Beniumov AO, Zelenina IA, Krasnov AM and Kolesnikov VA (1991). The delivery of foreign genes into fertilized fish eggs using high-velocity microprojectiles. *FEBS Lett.* **287**: 118–120.
155. Torgersen J, Collas P and Alestrom P (2000). Gene-gun-mediated transfer of reporter genes to somatic zebrafish (*Danio rerio*) tissues. *Mar. Biotech.* **2**: 293–300.
156. Montini E, Held PK, Noll M, Morcinek N, Al-Dhalimy M, Finegold M, Yant SR, Kay MA and Grompe M (2002). *In vivo* correction of murine tyrosinemia type I by DNA-mediated transposition. *Mol. Ther.* **6**: 759–769.
157. Kren BT, Bandyopadhyay P and Steer CJ (1998). *In vivo* site-directed mutagenesis of the *factor IX* gene by chimeric RNA/DNA oligonucleotides. *Nat. Med.* **4**: 285–290.
158. Szelei J and Duda E (1989). Entrapment of high-molecular-mass DNA molecules in liposomes for the genetic transformation of animal cells. *Biochem. J.* **259**: 249–553.
159. Szelei J, Váradi L, Müller F, Erdélyi F, Orbán L, Horváth L and Duda E (1994). Liposome mediated gene transfer in fish embryos. *Transgen. Res.* **3**: 116–119.
160. Horie K, Kuroiwa A, Ikawa M, Okabe M, Kondoh G, Matsuda Y *et al.* (2001). Efficient chromosomal transposition of a Tc1/mariner-like transposon Sleeping Beauty in mice. *Proc. Nat. Acad. Sci. USA* **98**: 9191–9196.
161. Dupuy AJ, Fritz S and Largaespada DA (2001). Transposition and gene disruption in the male germline of the mouse. *Genesis* **30**: 82–88.
162. Lam WL, Lee T-S and Gilbert W (1966). Active transposition in zebrafish. *Proc. Natl. Acad. Sci. USA* **93**: 10870–10875.
163. Mullins MC, Hammerschmidt M, Haffter P and Nüsslein–Volhardt C (1994). Large-scale mutagenesis in the zebrafish: in search of genes controlling development in a vertebrate. *Curr. Biol.* **4**: 189–202.
164. Dupuy AJ, Clark K, Carlson C, Fritz S, Finley K, Ekker S, Hackett PB, Horn S and Largaespada DA (2002). Mammalian germline transgenesis by transposition. *Proc. Natl. Acad. Sci. USA* **99**: 4495–4499.
165. Stuart GW, McMurray JV and Westerfield M (1988). Replication, integration and stable germ-line transmission of foreign sequences injected into early zebrafish embryos. *Development* **103**: 403–412.

166. Stuart GW, Vielkind JR, McMurray JV and Westerfield M (1990). Stable lines of transgenic zebrafish exhibit reproduction patterns of transgene expression. *Development* **109**: 577–584.
167. Tewari R, Michard–Vahee C, Parrot E and Chourrout D (1992). Mendelian transmission, structure and expression of transgenes following their injection into the cytoplasm of trout eggs. *Transgen. Res.* **1**: 250–260.
168. Ivics Z, Izsvák Zs and Hackett PB (1993). Enhanced incorporation of transgenic DNA into zebrafish chromosomes by a retroviral integration protein. *Mol. Mar. Biol. Biotech.* **2**: 162–173.
169. Score P, Frandsen JL, Jeske J, Hackett PB, Largaespada DA and McIvor RS (2003). Molecular evidence for *Sleeping Beauty*-mediated transposition and long-term expression *in vivo*. *Mol. Ther.* 7: 59.
170. Ivics *et al.*, unpub.
171. Davidson AE, Balciunas D, Mohn D, Shaffer J, Hermanson S, Sivasubbu S, Hackett PB and Ekker SC (2003). Efficient gene delivery and expression in zebrafish using *Sleeping Beauty*. *Dev. Biol.* **263**: 191–202.
172. Clark KJ, Geurts AM, Bell J and Hackett PB (2004). Transposon vectors for gene-trap insertional mutagenesis in vertebrates. *Genesis* (in press).
173. Atkinson PW, Pinkerton AC and O'Brochta DA (2001). Genetic transformation systems in insects. *Ann. Rev. Entomol.* **46**: 317–346.
174. Koga A and Hori H (2001). The *Tol2* transposable element of the medaka fish: an active DNA-based element naturally occurring in a vertebrate genome. *Genes Genet. Syst.* **76**: 1–8.
175. Udvadia AJ and Linney E (2003). Windows into development: historic, current, and future perspectives on transgenic zebrafish. *Dev. Biol.* **256**: 1–17.
176. Chen Z-Y, He V-Y, Ehrhardt A and Kay MA (2003). Minicircle DNA vectors devoid of bacterial DNA result in persistent and high-level transgene expression *in vivo*. *Mol. Ther.* **8**: 495–500.
177. Cretekos CJ and Grunwald DJ (1999). Alyron, an insertional mutation affecting early neural crest development in zebrafish. *Dev. Biol.* **210**: 322–338.
178. Zambrowicz BP and Sands AT (2003). Knockouts model the 100 best-selling drugs — will they model the next 100? *Nat. Rev. Drug. Disc.* **2**: 38–51.
179. Eisen JS (1996). Zebrafish make a big splash. *Cell* **87**: 969–977.
180. Cooley L, Kelley R and Spradling A (1988). Insertional mutagenesis of the *Drosphila* genome with single P elements. *Science* **239**: 1121–1128.

181. Zwaal RR, Broeks A, van Meurs J, Groenen JTM and Plasterk RHA (1993). Target-selected gene inactivation in *Caenorhabditis elegans* by using a frozen transposon insertion mutant bank. *Proc. Natl. Acad. Sci. USA* **90**: 7431–7435.
182. Zhang P and Spradling AC (1994). Insertional mutagenesis of *Drosophila* heterochromatin with single P elements. *Proc. Natl. Acad. Sci. USA* **91**: 3539–3543.
183. Sundaresan V, Springer P, Volpe T, Haward S, Jones JDG, Dean C, Ma H and Martienssen R (1995). Patterns of gene action in plant development revealed by enhancer trap and gene trap transposable elements. *Genes Dev.* **9**: 1797–1810.
184. Korswagen HC, Durbin RM, Smits MT and Plasterk RHA (1996). Transposon Tc1-derived, sequence-tagged sites in *Caenorhabditis elegans* as markers for gene mapping. *Proc. Natl. Acad. Sci. USA* **93**: 14680–14685.
185. Martienssen R (1998). Functional genomics: Probing plant gene function and expression with transposons. *Proc. Natl. Acad. Sci. USA* **95**: 2021–2026.
186. Akerley BJ, Rubin EJ, Camilli A, Lampe DJ, Robertson HM and Mekalanos JJ (1998). Systematic identification of essential genes by *in vitro mariner* mutagenesis. *Proc. Natl. Acad. Sci. USA* **95**: 8927–8932.
187. Ross–McDonald P, Coelho PSR, Roemer T, Agarwal S, Kumar A, Jansen R, Cheung K-H, Sheehan A, Symoniatis D, Umansky L, Heidtman M, Nelson FK, Iwasaki H, Hager K, Gerstein M, Miller P, Roeder GS and Snyder M (1999). Large-scale analysis of the yeast genome by transposon tagging and gene disruption. *Nature* **402**: 413–418.
188. Brune W, Menard C, Hobom U, Odenbreit S, Messerle M and Koszinowski UH (1999). Rapid identification of essential and nonessential herpesvirus genes by direct transposon mutagenesis. *Nat. Biotechmol.* **17**: 360–364.
189. Hutchison III CA, Peterson SN, Gill SR, Cline RT, White O, Fraser CM, Smith HO and Venter JC (1999). Global transposon mutagenesis and a minimal mycoplasma genome. *Science* **286**: 2165–2169.
190. Klinakis AG, Zagoraiou L, Vassilatis DK and Savakis C (2000). Genome-wide insertional mutagenesis in human cells by the *Drosophila* mobile element *Minos*. *EMBO Rep.* **1**: 416–421.
191. Hoffman LM, Jendrisak JJ, Meis JRJ, Goryshin IY and Reznikof SW (1999). Transposon insertional mutagenesis and direct sequencing of microbial genomes. *Genetica* **108**: 19–24.

192. Evans MJ, Carlton MBL and Russ AP (1997). Gene trapping and functional genomics. *Trends Genet.* **13**: 370–374.
193. Hamada K, Tamaki K, Sasado T, Watai Y, Kani S, Wakamatsu Y, Ozato K, Kinoshitqa M, Kohno R, Takagi S and Kimura M (1998). Usefulness of the medaka β-actin promoter investigation using a mutant GFP reporter gene in transgenic medaka (*Oryzias latipes*). *Mol. Mar. Biol. Biotech.* 7: 173–180.
194. O'Kane CJ and Gehring WJ (1987). Detection *in situ* of genomic regulatory elements in *Drosophila*. *Proc. Natl. Acad. Sci. USA* **84**: 9123–9127.
195. Newfeld SJ and Takaesu NT (2002). An analysis using the *hobo* genetic system reveals that combinatorial signaling by the *Dpp* and *Wg* pathways regulates dpp expression in leading edge cells of the dorsal ectoderm in *Drosophila melanogaster*. *Genetics* **161**: 685–692.
196. von Melcher and Ruley HE (1989). Identification of cellular promoters by using a retrovirus promoter trap. *J. Virol.* **63**: 3227–3233.
197. von Melcher H, Reddy S and Ruley HE (1990). Isolation of cellular promoters by using a retrovirus promoter trap. *Proc. Natl. Acad. Sci. USA* **87**: 3733–3737.
198. Fridrich G and Soriano P (1991). Promoter traps in embryonic stem cells: a genetic screen to identify and mutate developmental genes in mice. *Genes Dev.* **5**: 1513–1523.
199. Gossler A, Joyner AL, Rossant J and Skarnes WC (1989). Mouse embryonic stem cells and reporter constructs to detect developmentally regulated genes. *Science* **244**: 463–465.
200. Skarnes WC, Auerbach BA and Joyner AL (1992). A gene trap approach in mouse embryonic stem cells: the *lacZ* reporter is activated by splicing, reflects endogenous gene expression, and is mutagenic in mice. *Genes Dev.* **6**: 903–917.
201. Niwa H, Araki K, Kimura S, Taniguchi S, Wakasugi S and Yamamura K (1993). An efficient gene-trap method using poly(A) trap vectors and characterization of gene-trap events. *J. Biochem.* (Tokyo) **113**: 343–349.
202. Mitchell KJ, Pinson KI, Kelly OG, Brennan J, Zupicich J, Scherz P, Leighton PA, Goodrich LV, Lu X, Avery BJ, Tate P, Dill K, Pangilinan E, Wakenight P, Tessier–Lavigne M and Skarnes WC (2001). Functional analysis of secreted and transmembrane proteins critical to mouse development. *Nat. Genet.* **28**: 241–249.

203. Yoshida M, Yagi T, Furuta Y, Takayanagi K, Kominami R, Takeda N, Tokunaga T, Chiba J, Ikawa Y and Aizawa S (1995). A new strategy of gene trapping in ES cells using 3'RACE. *Transgen. Res.* **4**: 277–287.
204. Zambrowicz BP, Friedrich GA, Buxton EC, Lilleberg SL, Person C and Sands AT (1998). Disruption and sequence identification of 2,000 genes in mouse embryonic stem cells. *Nature* **392**: 608–611.
205. Spradling AC, Stern DM, Kiss I, Roote J, Laverty T and Rubin GM (1995). Gene disruptions using *P* transposable elements: an integral component of the *Drosophila* genome project. *Proc. Natl. Acad. Sci. USA* **92**: 10824–10830.
206. Zhang JK, Prichett MA, Lampe DJ, Robertson HM and Metcalf WW (2000). *In vivo* transposon mutagenesis of the methanogenic archeon *Methanosarcina activorans* C2A using a modified version of the insect *mariner*-family transposable element *Hima1*. *Proc. Natl. Acad. Sci. USA* **97**: 9665–9670.
207. Judson N and Mikalanos JJ (2000). Transposon-based approaches to identify essential bacterial genes. *Trends Microbiol.* **8**: 521–526.
208. Parimov S and Sundaresan V (2000). Functional genomics in aradbidopsis: large-scale insertional mutagenesis complements the genome sequencing project. *Curr. Opin. Biotechmol.* **11**: 157–161.
209. Vidan S and Snyder M (2001). Large-scale mutagenesis: yeast genetics in the genome era. *Curr. Opin. Biotechmol.* **12**: 28–34.
210. Clark KJ, Geurts AM, Bell JB and Hackett PB (2004). Identification of genes using gene traps in Sleeping Beauty transposons. *Genesis* (in press).
211. Clark KJ (2003). The development of Sleeping Beauty gene-trap transposons for insertional mutagenesis of vertebrates. Ph.D Dissertation, University of Minnesota.
212. Vigdal TJ, Kaufman CD, Izsvak Z, Voytas DF and Ivics Z (2002). Common physical properties of DNA affecting target site selection of *Sleeping Beauty* and other Tc1/mariner transposable elements. *J. Mol. Biol.* **323**: 441–452.
213. Roberg–Perez K, Carlson CM and Largaespada DA (2003). MTID: a database of Sleeping Beauty transposon insertions in mice. *Nucl. Acids Res.* **31**: 78–81.
214. Carlson C, Dupuy A, Fritz S, Roberg–Perez K, Fletcher CF and Largaespada DA (2003). Transposon mutagenesis of the mouse germline. *Genetics* (in press).

215. Hastie ND and Bishop JO (1976). The expression of three abundance classes of messenger RNA in mouse tissues. *Cell* **9**: 761–774.
216. Koga A and Hori H (1999). Homogeneity in the stucture of the medaka fish transposable element Tol2. *Genet. Res.* **73**: 7–14.
217. Koga A, Hori H and Sakaizumi M (2002). Gene transfer and cloning of flanking chromosomal regions using the medaka fish *Tol2* transposable element. *Mar. Biotech.* **4**: 6–11.
218. Hackett PB, Izsvák Zs, Ivics Z and Caldovic L (1999). Development of genetic tools for transgenic animals. In: Murray JD, Anderson GB, Oberbauer AM and McGlouoghlin MM (eds.), *Transgenic Animals in Agriculture*. CABI Publishing, Wallingford, UK, pp. 19–35.
219. Liu G, Cui Z, Aronovich EL, Whitley CB and Hackett PB (2003). Transposon-donor junction nucleotides are important for excision by *Sleeping Beauty* transposase. *J. Gene Med.* **6**: 574–583.
221. Koga A, Lida A, Kamiya M, Hayashi R, Hori H, Ishikawa Y and Tachibana A (2003). The medaka fish Tol2 transposable element can undergo excision in human and mouse cells. *J. Hum. Genet.* **48**: 231–235.
222. Hyrien O, Maric Ch and Mechali M (1995). Transition in specification of embryonic metazoan DNA replication origins. *Science* **70**: 994–997.
223. Kane DA and Kimmel CB (1993). The zebrafish midblastula transition. *Development* **119**: 447–456.
224. Kimmel CB, Ballard WW, Kimmel SR, Ullman B and Schilling TF (1995). Stages of embryonic development of the zebrafish. *Dev. Dyn.* **203**: 253–310.
225. Vigdal TJ, Kaufman CD, Izsvak Z, Voytas DF and Ivics Z (2002). Common physical properties of DNA affecting target site selection of *Sleeping Beauty* and other Tc1/*mariner* transposable elements. *J. Mol. Biol.* **323**: 441–452.
226. Zayed H, Izsvak Z, Khare D, Heinemann U and Ivics Z (2003). The DNA-bending protein HMGB1 is a cellular cofactor of *Sleeping Beauty* transposition. *Nucl. Acids Res.* **31**: 2313–2322.
227. Liu G, Geurts AM, Yae K, Srinivassan AR, Fahrenkrug SC, Olson WK, Takeda J, Horie K and Hackett PB. Target-site preference for *Sleeping Beauty* transposons. *Nucl. Acids Res.* (submitted).
228. Takeuchi Y, Yoshizaki G and Takeuchi T (1999). Green fluorescent protein as a cell-labeling tool and a reporter of gene expression in transgenic rainbow trout. *Mar. Biotech.* **1**: 448–457.

229. Largaespada D and Takeda J, personal communications (pg. 560).
230. Stainier DYR, Fouquet B, Chen J-N, Warren KS, Weinstein BM, Meiler SE, Mohideen M-APK, Neuhauss SCF, Solnica-Krezel L, Schier AF, Zwartkruis F, Stemple DL, Malicki J, Driever W and Fishman MC (1996). Mutations affecting the formation and function of the cardiovascular system in the zebrafish embryo. *Development* **123**: 285–292.
231. Amsterdam A, Lin S, Moss LG and Hopkins N (1996). Requirements for green fluorescent protein detection in transgenic zebrafish embryos. *Gene* **173**: 99–103.
232. Meng A, Tang H, Ong BA, Farrell MJ and Lin S (1997). Promoter analysis in living zebrafish embryos indentifies a cis-acting motif required for neuronal expression of GATA-2. *Proc. Natl. Acad. Sci. USA* **94**: 6267–6272.