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Possible Applications to Aquaculture of Salmonid *Sleeping Beauty* Transposon Vectors

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ABSTRACT

The *sleeping beauty* (*SB*) vectors have been designed from transposons "sleeping" in thousands of incomplete copies in the salmon/trout genomes. The *SB* system consists of one vector containing the transposase enzyme and of second vector carrying the inverted terminal repeats flanking an exogenous gene. The *SB* dual vectors system originated from fish, but it has been mostly used in mammals that lack these transposon systems. Several human clinical trials for gene therapy are using this SB system. Some of the advantages of *SB* vectors include the introduction of a defined, non-viral sequence as a single copy with each transposition (instead of a random recombination), thus avoiding the carrier over of prokaryotic sequences. This manuscript reviews the development of *SB* transposase versions and their derived vectors for possible use in fish cultured at a commercial level. There is a potential use for the *SB* vectors for the creation of transgenic fish, for the improvement of fish DNA vaccines and for the identification of genes in fish that might be important to improve the culture of these animals. However, the presence of thousands of incomplete *SB* transposon-related copies in many fish genomes and the possible activity of their endogenous *SB* transposases might interfere with the use of *SB* vectors in fish. Those possible limitations will depend on the fish specie and will have to be investigated in each case.

Keywords: DNA vaccination, gene identification, sequence tags, transgenics, VHSV
Abbreviations: bp, base pair; **DR**, direct repeat; **EPC**, *epithelial papulosum cyprini*; **IgH**, immunoglobulin heavy-chain; **HIRRV**, hirame rhadovirus; **IHNV**, infectious hematopoietic necrosis virus; **IR**, inverted repeat; **ITR**, inverted terminal repeats; **MHC**, major histocompatibility complex; **PCR**, polymerase chain reaction; **RTG2**, rainbow trout gonad 2; ***SB***, *sleeping beauty*; **SHRV**, snake-head rhabdovirus; **SVCV**, spring viremia carp virus;**Tc1**, Transposon *Caenorhabditis* 1; **TCR**, T Cell Receptor; **Tdr1**, Transposon *Danio rerio*; **Tol2**, Transposon *Oryzias latipes* 2; **Tom1**, Transposon *Oncorhynchus mykiss* 1; **Tpp1**, Transposon *Pleuronectes platessa* 1; **Tss1**, Transposon *Salmo salar* 1*;*; **Tsn1**, Transposon *Salvelinus namaycush* 1; **Tzf1**, Transposon zebrafish 1; **VHSV**, viral hemorrhagic septicemia virus

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INTRODUCTION

Many different kinds of RNA and DNA mobile sequences or transposons are distributed throughout the genomes of most fish species (Tafalla *et al*. 2006), comprising up to 5% of their genomes in the case of salmonids (Krasnov *et al*. 2005). Active transposons would be highly deleterious, and therefore they are usually inactive in fish. Natural transposition events must be followed by inactivation of the transposition reaction usually at the translational level. Thus, although 0.5% of the transposon sequences are transcribed (as demonstrated by high-throughput sequencing of salmonid fish cDNA libraries) they are not translated (Krasnov *et al*. 2005). Many thousands of incomplete DNA transposon copies per haploid genome belong to the Tc1-related family (Tc1, Transposon *Caenorhabditis* 1). These family of transposons excise-and-transpose (cut-and-paste) DNA sequences from one genome locus to another driven by active transposase enzymes acting on sequences flanked by ~ 250 base pairs (bp) inverted repeats (IR) or inverted terminal repeats (ITR) (**Fig. 1**). Each IR is itself flanked by 2 direct repeats (DR) of ~ 30 bp which bind the transposase enzyme (the so called IR/DR structure). Autonomous active copies contain their own active transposase gene flanked by complete ITR (**Fig. 1**).

Transposon copies of 500-2500 base pairs (bp) flanked by ITR were first identified in fish by polymerase chain reaction (PCR) using a single primer derived from their ITR. They were described in zebrafish (*Danio rerio*, Tdr1), rainbow trout (*Oncorhynchus mykiss*, Tom1) and Atlantic sal-

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Invited Review

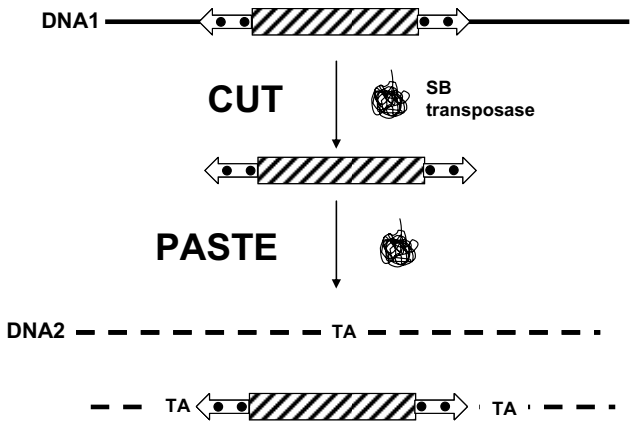

Fig. 1 Cut-and-paste transposition for an autonomous fish *SB* transposon. Fish autonomous *SB* transposons, encode an active *SB* transposase gene that once transcribed and translated catalyzes the cleavage-transfer (cut-and-paste) reactions for transposition. The transposase gene is flanked by imperfect inverted repeats (IR), which contain 2 direct repeats (DR) each (the so called IR/DR structure). Upon binding to the DRs and synapsis of the inverted terminal repeats (ITR), excises the transposon from its DNA1 site, and reintegrates it into a target DNA2 site (cut-and-paste transposition). *SB* transposase exclusively integrates into TA dinucleotides that are duplicated upon transposition. Hatched rectangle, transposase gene. Open arrows, inverted terminal repeats (ITR). •, Direct repeats (DR). TA, TA dinucleotides. Continous and dashed lines, DNA 1 and DNA2 sites.

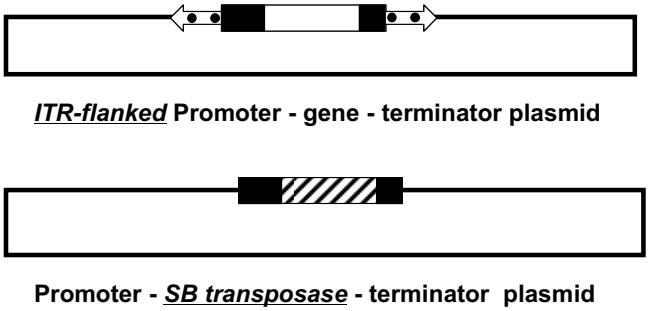

Fig. 2 Example of a *trans* configuration for *SB*/ITR vectors. For gene delivery purposes, the *SB* transposon (**Fig. 1**) is typically used as a two-component vector. An exogenous gene flanked by transcriptional control sequences (promoter and terminators) and flanked by ITRs is one of the vectors and the *SB* transposase is supplied *in trans* by a second vector (**Fig. 2**). To induce transposition, cells are cotransfected with the two vectors: for instance, 5' ITR - SV40 promoter - neomycin resistance gene - SV40 polyA terminator - 3' ITR and *SB* transposase under the cytomegalovirus CMV promoter and a terminator. Since any transfected plasmid gene can spontaneously integrate into the cell genome even without the help of a transposase, the assay to demonstrate *SB*-dependent transposition involves parallel transfections of an ITR-flanked plasmid harbouring an antibiotic resistance gene without or with the help of a *SB* transposase gene plasmid. Following antibiotic selection, the ratio between the number of antibiotic resistant colonies in the presence and in the absence of the *SB* transposase plasmid is, therefore, an estimation of the efficiency of specific transposition. Black rectangles, promoter and terminator transcriptional control sequences. Open arrows, ITR-flanking sequences. •, Direct repeats (DR). Hatched rectangle, active *SB* transposase gene. A collection of *SB* vectors are available at http://biosci.cbs.umn.edu/labs/perry.

mon (*Salmo salar*, Tss1) (Goodier and Davidson 1994; Radice *et al*. 1994). More recently they have been also identified in catfish (*Ictalurus punctatus*) (Liu *et al*. 1999; Xu *et al*. 2006; Nandi *et al*. 2007) and other salmonids (Krasnov *et al*. 2005). Copies of these transposons revealed considerable intra-individual as well as inter-specific sequence variations in their transposase gene and ITRs (Izsvak *et al*. 1995; Krasnov *et al*. 2005). Although the existence of low levels of transposase activity cannot be excluded, none of those copies seem to be usually active (with the exception of Tzf1, previously Tdr2) (Ivics *et al*. 1996a; Lam *et al*. 1996). Thus, most sequenced copies contained incomplete or nonautonomous transposons with deletions, frameshift mutations or premature termination codons in their transposase genes (Ivics *et al*. 2004).

This manuscript reviews the development of the first active Tc-1 transposase enzyme (*sleeping beauty*, *SB*), their derived vectors (*SB*/ITR vectors) and their improvements for possible applications in fish. There is a potential use for the *SB* vectors for the creation of transgenic fish, for the improvement of fish DNA vaccines and for the identification of genes in fish that might be important to improve the culture of these animals.

OVERVIEW OF THE DEVELOPMENT OF *sleeping beauty SB*/ITR VECTORS FROM SALMON AND TROUT INACTIVATED TRANSPOSON COPIES

The *SB*/ITR vectors, generally consist in two separate plasmids, one coding for the *SB* transposase and the other for an ITR-flanked gene expression cassette (**Fig. 2**). *SB* transposase mRNA (Wilber *et al*. 2006, 2007) and/or protein (Jarver *et al*. 2008) have been also used as a source of exogenous transposase enzymatic activity to avoid insertion of transposon plasmid into DNA by random recombination.

The 2 transposase binding sites (IR/DR structure) located in each of the ITR (Izsvak *et al*. 2000, 2002) are recognized and cut by active *SB* transposases with the help of cellular factor(s) (Yant and Kay 2003; Zayed *et al*. 2003). Next, the excised ITR-flanked sequences are pasted in the genome in their TA dinucleotide sites (Plasterk *et al*. 1999), present in ~200 million different locations per fish genome (Williams 2008). Although near random, the TA integration sites are preferably surrounded by some sequence-specific target domains (Liu *et al*. 2005; Yant *et al*. 2005), leading to an small bias toward genes and some upstream regulatory regions (Walisko *et al*. 2008). The amino terminal end of an active *SB* transposase contains nuclear localisation signals and α helixes with leucine-zippers that are used to bind determined DNA sequences (Ivics *et al*. 1996b; Yant *et al*. 2004). The carboxy terminal end of the protein contains a characteristic 3 amino acid sequence (aspartic, aspartic and glutamic amino acids; this sequence is found in many similar enzymes) that has a catalytic function. Other domains seem to be implicated in dimerization of the transposase (van Pouderoyen *et al*. 1997; Auge-Gouillou *et al*. 2005).

Combinations between incomplete copies of Tss1 identified in Atlantic salmon (*Salmo salar*) and Tom1 identified in rainbow trout (*Oncorhynchus mykiss*) and 32 additional mutations were included into the original Tss1 copy to obtain the first active *SB* transposase and to develop dual *SB10*/ITR plasmid vectors (Ivics *et al*. 1997) (**Table 1**). The *SB* transposase plasmid contained an expression cassette for the 1600 bp active *SB* transposase. The ITR-flanked plasmid contained an expression cassette of a defined gene flanked by ITR (5' ITR-promoter-gene-polyA terminator-3' ITR).

Between 2002 and 2004 more than 200 mutations were introduced into the *SB*10 transposase by different authors (Geurts *et al*. 2003; Yant *et al*. 2004; Zayed *et al*. 2004) to increase their transposition efficiency in human cells. Three main strategies have been applied to derive hyperactive mutants of the *SB* transposase: (1) introduction of amino acids and/or small blocks of amino acids from related active transposases (Geurts *et al*. 2003), (2) systematic substitution of alanine (Yant *et al*. 2004), and (3) rational replacement of selected amino acid residues (Zayed *et al*. 2004). These studies have found 15-20 amino-acid replacements that resulted in a relatively modest increase in transpositional activities. From these studies, the *SB*11 (Geurts *et al*. 2003), *SB*12 (Zayed *et al*. 2004) and H*SB*3 (Yant *et al*. 2004) versions of the *SB* vectors were obtained (**Table 1**). In 2005, by studying the amino acid alignment of 9 active Tc1 catalytic car-

Table 1 Development of mammalian active *SB* transposase and ITR vector versions.

Transposon	Name	Mutations	Acc. number GenBank	Relative efficiency	Reference
Tss1	*SB1*	wild type	L12206	0	Radice *et al*. 1994
Tss1	*SB2*	wild type combination	L12207	0	Ivics *et al*. 1997
Tom1	*SB3*	wild type combination	L12209	0	Ivics *et al*. 1997
	SB10[a]	32 site directed mutagenesis	--	1	Ivics *et al*. 1997
	SB11	site directed mutagenesis	--	2-3	Geurts *et al*. 2003
	SB12	site directed mutagenesis	--	3-4	Zayed *et al*. 2004
	HSB3	site directed mutagenesis	--	8	Yant *et al*. 2004
	HSB16	site directed mutagenesis	--	7	Baus *et al*. 2005
	SBx100	*In vitro* evolution	--	100	Mates *et al*. 2009
Tss1	ITR	wild type		1	Izsvak *et al*. 2000
	ITR*2*	site directed mutagenesis TA + 4 mutations		4	Cui *et al*. 2002
	ITR*3*	site directed mutagenesis 5' ITR = 3' ITR		2	Yant *et al*. 2004

[a] Mutations into a rainbow trout (*Oncorhynchus mykiss*, Tom1) and Atlantic salmon (*Salmo salar*, Tss1) hybrid transposase gene (*SB*3) first restored the reading frame and then nuclear localisation signals, DNA-binding and excision/integration activities. ITR were named as ITR2 and ITR3 to differentiate among those versions. T, transposon.

boxy terminal domains, 18 novel mutations were designed, generated and combined with previous ones. The resulting *HSB*17 version showed a 17-fold enhancement of transposition compared to the original *SB*10 transposase (Baus *et al*. 2005). Most recently, an *SB*100x (100-fold increase in specific transposase activity) has been obtained by using a high-throughput genetic screen to select for hyperactive *SB* transposases generated by an *in vitro* evolution process (Izsvak *et al*. 2009; Mates *et al*. 2009).

Mutations and changes in the initial ITR sequences (Izsvak *et al*. 2000) also allowed a ~4-fold increase in the efficiency of transposition in human cells (Cui *et al*. 2002; Yant *et al*. 2004). Some combinations of both *SB* transposase (not including the last hyperactive versions) and ITR versions in human cells have produced *SB*/ITR vectors whose transposition efficiency might be around 8-14 fold higher than the original versions. However, a comparative study among the best *SB* transposases and ITR versions and their possible combinations in fish cells has not been reported yet. It is possible that assays of those different *SB* transposase / *SB* ITR versions in fish cells could select the most active for use in every fish species.

PRESENCE OF ENDOGENOUS *SB*/ITR-RELATED SEQUENCES IN FISH CELLS

Mammals have no endogenous *SB* activity nor ITR related sequences (*SB*/ITR$^-$) present into their genomes. Therefore, insertion of exogenous ITR-flanked genes only depends on the exogenous *SB* transposase and ITR-flanked gene provided (extra-genomic transposition). Once the ITR-flanked gene is inserted into the mammalian genome, however, the rate of its excision only depends on the level of exogenous *SB* transposase supplied and the number of ITR-flanked gene copies introduced before (intra-genomic transposition).

In contrast to mammalians, the insertion of exogenous ITR-flanked genes in fish would also be affected by the presence in their genomes of endogenous *SB* transposase and/or ITR sequences. Compared to mammalians where there should be a much lower efficiency of transposition (since a transposon exogenously introduced into a cell has to traverse both the plasma and nuclear membranes plus return to the cytoplasm to express *SB* transposase activity), intra-genomic transposition of a transposon already present in the fish genome should be much higher and faster. On the other hand, because the level of *SB* transposase activity in fish must be tightly inhibited to avoid their excessive activity and lethal effects, the introduction of exogenous ITR-flanked genes in fish cells might also experience some endogenous regulation (Krasnov *et al*. 2005; Tafalla *et al*. 2006).

The finding of *SB*/ITR complete sequences proved to be a difficult task due to the presences of many copies of partial *SB*/ITR sequences in the fish genome. Thus, only in European plaice (Tpp1, *Pleuronectes platessa*) (Leaver 2001) and in lake trout (Tsn1, *Salvelinus namaycush*) (Reed 1999) active copies of Tc1 transposons were detected, most probably due to their small number. Our own efforts to detect complete genomic *SB*-related sequences (using touchdown polymerase chain reaction to bypass possible sequence variations among the different genomic copies), complete genomic *SB* transposase (using touchdown PCR) and/or transcribed *SB* transposase sequences (using RT-PCR) in both *Epithelioma papulosum cyprini* (EPC) and rainbow trout gonad (RTG2) cell lines were unsuccessful. Although sequences of the expected size could be obtained, none of the sequenced contained a complete *SB* transposase gene (unpublished work), confirming previously reported data (Ivics *et al.* 1996b). Most probably, the number of clones to be sequenced to obtain a complete *SB* transposon or *SB* transposase sequence, if there exits any, is too high to be practical. Maximal identities of only 88% could be obtained by comparing (Tafalla *et al.* 2006) some of the published *SB* transposase/ITR endogenous sequences from carp (*Cyprinus carpio*) (L48683) or rainbow trout (L12209) to the active *SB*10/ITR (Ivics *et al.* 1997). However, among the thousands of existing copies of *SB*/ITR sequences per haploid genome in both carp and rainbow trout, there could exist a few complete active copies. On the other hand, since a few complete, potentially active transposase gene(s) have been detected in some fish (Reed 1999; Leaver 2001), it seems likely that some *SB*/ITR-related sequences might still be active or at least have some residual activity in particular fish species, physiological moments and/or individual fish.

The activity of transposases in zebrafish (Tzf1) (Lam *et al*. 1996) and medaka (Tol2, *Oryzias latipes*) (Koga *et al*. 1996) were only detected during reproduction. On the other hand, a preliminary study on the effects of exogenous *SB*/ITR vectors in several fish cell lines with antibiotic genes flanked by ITR (Izsvak *et al*. 2000), showed low *SB* dependent transposition efficiencies for most fish cell lines when compared to human HeLa cells (**Table 2**). The generation of a substantial number of antibiotic resistant fish cell colonies in the absence of added exogenous *SB* transposase argued about the existence of some endogenous *SB* transposase activity. However, those low numbers could also be due to random recombination. To try to detect possible activity of endogenous *SB* transposase in EPC (*epithelioma papulosum cyprini*) and RTG2 (rainbow trout gonad) cell lines, an excision-transposition assay (Lampe *et al*. 1996) was used in our laboratory. The excision-transposition assay consisted in co-transfecting fish cells with two plasmids coding for two different prokaryotic antibiotic resistance genes: kanamycin in the ITR-flanked gene (donor) and chloramphenicol in the target plasmid. Double antibiotic resistance colonies could not be rescued when *E. coli* was transfected with the plasmids isolated from the co-transfected fish cells (unpublished). ITR-kanamycin-ITR sequences could only be detected from fish cells in the target plasmid after a first PCR amplification of the target plasmids and PCR reamplification of their ITR insert (unpublished) by using a unique ITR specific primer (Ivics *et al*. 1996b;

Table 2 Preliminary assays of *SB10*/ITR in several fish cell lines.

Fish	Cell line	Clones/10^6cells:		Fold increase	Specific integration, %
		-SB	*+SB*		
zebrafish *Danio rerio*	ZF4	7	13	1.8	?
carp *Cyprinus carpio*	EPC	54	129	2.3	?
seabream *Sparus auratus*	SAF1	9	13	1.4	?
medaka *Oryzias latipes*	OLF136	10	34	3.4	?
trout *Oncorhynchus mykiss*	RTG	4	13	3.2	?
swordtail *Xiphophorus hellerii*	A2	37	108	2.9	?
fathead minnow *Pimephales promelas*	FHM	4	104	26	90
human *Homo sapiens*	HeLa	282	8750	31	90

Cell lines were co-transfected with a plasmid containing the 5' ITR - SV40 promoter - neomycin resistance gene - SV40 polyA signal - 3' ITR ± a plasmid containing the *SB*10 transposase gene under the control of the human cytomegalovirus (CMV) promoter (**Fig. 2**) (Izsvak *et al*. 2000). Clones were then selected by resistance to the antibiotic G418. The excision-integration due to *SB* transposase (specific integration) were estimated by comparing the results with those obtained by using another marker outside the ITRs (unspecific recombination). Results were averages from at least 2 experiments. Reproduced with Dr. Plasterk's permission (Izsvak *et al*. 2000).

Izsvak *et al*. 1995). Such a method was capable of detecting *SB* activity in EPC but not in RTG2 fish cell lines. In any case there were very low levels of endogenous *SB* transposase activity. Due to the low transfection efficiency to RTG2 and other cells from commercially important fish, similar assays are difficult to interpret until more efficient methods of transfection are developed for fish cell lines.

To be active, a complete transposase has to be transcribed before translation. It is not yet known how natural fish *SB* transposases are transcribed but transposase transcripts are abundant in fish (Krasnov *et al*. 2005) and for instance, not only sense but also antisense transcripts of *SB* transposons were detected in catfish (Nandi *et al*. 2007). Most recently, it has been shown that 65 bp of the 5' untranslated region (5'-UTRs) present in some wild type *SB* transposons directs transcription in mammalian cells. Similar regulator sequences might be operative in fish but this has not been yet investigated. On the other hand, it has been shown that mammalian transcription of *SB* transposases from the 5'-UTR was upregulated by the host-encoded high-mobility group 2-like 1 (HMG2L1) protein (Walisko *et al*. 2008). The *SB* transposase protein antagonized the effect of the HMG2L1 regulator, suggesting a negative feedback mechanism on its own expression (Walisko *et al*. 2008) and confirming earlier observations that postulated the so called overexpression inhibition of transposase activity to explain some of their *in vitro* behaviours (Geurts *et al*. 2003).

Although the salmonid genome contains ~ 10^5 Tc1-like sequences, the major fraction is not transcribed. Transcription of rainbow trout transposons was activated by external stimuli, such as toxicity, stress and bacterial antigens as shown by many studies using microarrays (Krasnov *et al*. 2005). On the other hand, inactivated or incomplete copies of *SB* transposon-related sequences have been found inside some fish genes. For instance, the third intron of the rainbow trout toll receptor 9 (Omtlr9) gene, contains an insertion of 1080 bp (GenBank accession no. ACC93939) (Ortega-Villaizan *et al*. 2009), with high homology (sequence identity >85%, query coverage >65%) to the active *SB*10 transposon (Ivics *et al*. 1997). The "Omtlr9 transposon" was transcribed to yield non functional Omtlr9 gene transcripts. The "Omtlr9 transposon" high sequence similarity with other sequences present in rainbow trout IgH (Immunoglobulin heavy-chain) locus, MHC (Major Histocompatibility Complex) Class I region, ABCB2 region and IgD gene, and Atlantic salmon MHC Class I and II and TCR (T Cell Receptor) locus regions among others (Krasnov *et al*. 2005), suggest these inactive *SB* transposon copies could have some functional purposes affecting the expression of those fish genes into which they are inserted (Krasnov *et al*. 2005; Ortega-Villaizan *et al*. 2009).

POSSIBLE USE OF *SB*/ITR VECTORS TO OBTAIN TRANSGENIC FISH

The *SB*/ITR vectors have some advantages over other non viral and viral vectors to obtain transgenic fish. Using the *SB*/ITR vectors, a defined, non-viral sequence can be introduced as a single copy with each transposition into the genomes. No other type of gene vector allows a specific gene transfer without getting the prokaryotic sequences incorporated into the genome, as it has happened to most of the transgenic fish obtained to date. The main drawbacks of other fish vectors, such as the low rates of genomic integration and the concatemeric structure of the integrating transgene constructs, can be thus circumvented by using *SB*-mediated gene delivery. Single expression cassettes, resulting from *SB* transposition-mediated integration, are presumably less prone to transgene silencing and recombination than the concatemeric insertions created by more classical methods (Mates *et al*. 2009). Thus, both the frequency of transgenic founders and the frequency of germ-line integration as well as the stability of the transgenics might be increased by using *SB*/ITR vectors as shown by genes inserted into the genomes of model fish like zebrafish (Davidson *et al*. 2003) or medaka (Grabher *et al*. 2003).

Sequencing of the junctions of ITR-flanked insertions newly introduced or previously inserted by exogenous *SB* transposase (Liu *et al*. 2004), showed 10 in 10 zebrafish while only 1 in 10 of the medaka lines being the result of exogenous *SB* transposase-dependent transposition (specific transposition) (Davidson *et al*. 2003; Grabher *et al*. 2003). The concentration of the ITR plasmids, the molecular ratio between the exogenous *SB* transposase and ITR, the influence of species-specific host fish factors, the interference of endogenous *SB*/ITR-related sequences and/or other unknowns, might be some of the possible explanations for the two different extreme ratios between specific/unspecific insertions obtained in those two fish models (Wadman *et al*. 2005). In terms of influence of fish specie-specific factors to *SB*/ITR insertion dynamics, we might expect most cultured species to fall in between the zebrafish and medaka extremes. On the other hand, most of the important cultured species, such as salmonids or carps, are tetraploid or pseudotetraploid (only tetraploid in some genes). Tetraploids would have roughly twice as many *SB*/ITR-related sequences in their genomes and activation of such a number of endogenous *SB*/ITR-related sequences might avoid transgenics generation in some of those fish species. We do not yet know how this might influence the behaviour of their corresponding *SB*/ITR induced transgenics (de Boer *et al*. 2007).

POSSIBLE USE OF *SB*/ITR VECTORS TO IDENTIFY GENES IMPORTANT FOR AQUACULTURE

The identification of genes important for aquaculture production such as those affecting growth rates and/or disease resistance could be approached by ITR tagging the germ line of the target fish species. The procedure would be similar to the natural onset of albino phenotypes in medaka by the Tol2 transposon (Koga *et al*. 1996) or to the induced isolation of oncogenes in mice by using a specific *SB*/ITR

Table 3 Some of the possible applications of *SB*/ITR vectors in fish.

Cells transfected	Purpose	Results	References
Germ line	Transgenics	expression of a desired gene in transgenic zebrafish	Davidson *et al*. 2003
	Gene identification (sequence tags)	enhancer identification in vertebrates	Balciunas *et al*. 2004
		promoter identification in zebrafish	Westerfield *et al*. 1992
		polyA identification in mouse	Zambrowicz *et al*. 1998
		gene deficient vertebrate mutants	Clark *et al*. 2004
Somatic	Immunization	DNA vaccination (preliminary *in vitro* experiments)	Ruiz *et al*. 2008

Modified from Wadman *et al*. 2005, Hackett and Alvarez 2000, Coll 2001a and Tafalla *et al*. 2006.

version specifically designed for massive insertional mutagenesis (Clark *et al*. 2004; Dupuy *et al*. 2005). The inactivation of the phenotype/gene(s) by ITR insertional mutagenesis would generate an inactivated gene(s) tagged by the ITR (or other ITR-flanked sequences) that would generate a knock-out fish in that gene(s). Their altered phenotype will allow the identification of the target gene sequences by polymerase chain reaction and subsequent cloning (Collier *et al*. 2005; Dupuy *et al*. 2005). Once the tagged gene(s) have been identified, more traditional marker-assisted selection methods could be applied on fish wild type populations.

For *SB*/ITR to do massive insertional mutagenesis in mice however, either an enormous number of initial transgenics needed to be made (Keng *et al*. 2005) or specific *SB*/ITR vectors need to be used (Dupuy *et al*. 2006). Thus, for massive insertional mutagenesis in mice, two limitations of the usual *SB*/ITR vectors had to be overcome: (1) only one or two transpositions were induced per mouse born (Fischer *et al*. 2001) and (2) exogenous *SB* transposase excision of ITR genes previously incorporated into the mouse genome reintegrated at sites too close to the donor site (within 10-25 Mbp) (Dupuy *et al*. 2005). Because that transposition was too low and local for massive insertional mutagenesis, *SB*/ITR vectors of the smallest size were designed, since the smaller the ITR plasmid size (Balciunas *et al*. 2006; Walisko *et al*. 2008), the higher their insertional rate (Karsi *et al*. 2001). As a result, the size of the ITR plasmid was ~ 2000 bp smaller, approaching the optimal size for transposition (Dupuy *et al*. 2005). When the ITR plasmids previously introduced into the genome, were excised by exogenous *SB* transposase, a maximal of 37 new reintegration events could be detected (Collier *et al*. 2005), allowing the identification of more ITR tagged genes.

SB/ITR vectors have been used as gene (Clark *et al*. 2004), enhancer (Balciunas *et al*. 2006), promoter (Westerfield *et al*. 1992) and/or terminator (Zambrowicz *et al*. 1998) tags in model vertebrates including zebrafish (**Table 3**). The development of highly hyperactive SB100x, mentioned above, could serve as a useful new reagent to promote massive insertional mutagenesis (Izsvak *et al*. 2009; Mates *et al*. 2009).

SB/ITR vectors and methods like those used in mammalians might be applied to fish to identify important genes for aquaculture. An example might be the pursuing of rainbow trout strains resistant to rhabdoviral infections such as those caused by viral hemorrhagic septicemia (VHS) or infectious hematopoietic necrosis (IHN) viruses. Trout with increased resistance to waterborne infection, the natural route of infection, by rhabdoviruses has been already produced by selective breeding (Yamamoto *et al*. 1991; Dorson *et al*. 1995; Ristow *et al*. 2000; Slierendrecht *et al*. 2001; Quillet *et al*. 2007). Rhabdoviral injection killed 100% of trout of all clones, suggesting an entrance mechanism of resistance (Quillet *et al*. 2007) corroborated by the absence of rhabdoviruses in trout resistant body after a waterborne-challenge and no rhabdovirus growth on fin tissue obtained from resistant trout (Dorson and Torchy 1993; Quillet *et al*. 2001). Susceptibility to IHNV was also variable among homozygous clones (Yamamoto *et al*. 1991; Trobridge *et al*. 2000) and correlated with the susceptibility to VHSV, suggesting the existence of common non-specific mechanism(s) of resistance (Slierendrecht *et al*. 2001). While non-specific resistance mechanisms may belong either to the innate immune system or to the viral entrance pathways in the fin surfaces (Helmick *et al*. 1995; LaPatra *et al*. 1996; Harmache *et al*. 2006), the gene(s) implicated still remain to be identified.

In zebrafish, transposon activity caused an average of 3.9 new transpositions per each new progeny individual genome (Lam *et al*. 1996). Similarly, it is possible that transposon activity in rainbow trout at the moment of reproduction might be activated and be responsible for at least some of the individual variations of rhabdoviral resistance observed (Quillet *et al*. 2007). The trout clones obtained with extreme rhabdoviral-resistance phenotypes (full resistance vs. full susceptibility), could be further studied for the possible presence of transposons inactivating genes. Alternatively, they can also be used for further genetic (search for candidate genes by *SB*/ITR massive insertional mutagenesis) and physiological (gene expression profiling by microarrays) correlation studies to identify novel antiviral pathways and genes involved in resistance to rhabdoviruses.

POSSIBLE USE OF *SB*/ITR VECTORS TO IMPROVE DNA VACCINATION AGAINST AQUACULTURE PATHOGENS

The *SB*/ITR vectors are being used more and more frequently to transfect somatic mammalian cells for gene therapy purposes (Collier and Largaespada 2007; Liu and Visner 2007; Maryon 2007) in the mouse (Score *et al*. 2006; Bell *et al*. 2007; Aronovich *et al*. 2007; Belur *et al*. 2008) and avian (Kong *et al*. 2008) models as well as in human cells (Frommolt *et al*. 2006; Huang *et al*. 2008). The progresses are so important than even the first human trials are underway (Williams 2008).

Somatic transfer into mammalian models using *SB*/ITR vectors have been optimized for use in gene therapy rather than for DNA vaccination. Thus, special *SB*/ITR vectors in *cis* configuration were designed having the *SB* transposase and the ITR in the same plasmid (Mikkelsen *et al*. 2003), coding for *SB*/ITR/antibiotic resistance gene, all in the same plasmid (Harris *et al*. 2002) or using adenovirus/*SB* hybrids (Yant *et al*. 2002). Finally, site-selective integration has been engineered into some ITR plasmids by including sequence specific DNA-binding domains (Kaminski *et al*. 2002).

In fish, *SB*/ITR vectors in *trans* and/or in *cis* configurations might be used to transfect somatic cells with the purpose to improve DNA vaccination against fish pathogens. Such fish pathogens include rhabdoviruses (including the IHNV and VHSV mentioned above), the most widely distributed fish disease-causing agents producing losses not only in young but also in adult fish. Rhabdoviruses are nowadays not only world widely distributed but also affecting numerous fish species (Coll 2001b). Among other fish viruses widely distributed, the infectious pancreatic necrosis (IPN) birnavirus (Macdonald and Gower 1981; Bernard and Bremont 1995), the infectious salmon anaemia orthomyxovirus (Gregory 2002; Kibenge *et al*. 2005), and several nodaviruses (Frerichs *et al*. 1996; Sommerset *et al*. 2003; Biering *et al*. 2005) can also infect many cultured fish species (Bernard and Bremont 1995; Biering *et al*. 2005).

Although vaccination seems now to be the most adequate method to control viral diseases in cultured fish (Kurath *et al*. 2007; Kurath 2008), only a few recombinant

protein vaccines have been licensed in China and Japan against spring viremia of the carp virus (SVCV, another rhabdovirus) and red seabream iridovirus, in Chile and Norway against IPNV and a live attenuated vaccine in Germany against VHSV. More recently, in Canada the first DNA vaccine against IHNV has been approved (Salonius *et al*. 2007). Thus, while the injection of rainbow trout (*Oncorhynchus mykiss*) with recombinant rhabdoviral G proteins produced in *Escherichia coli* (Lorenzen *et al*. 1993b; Estepa *et al*. 1994), yeast (Estepa *et al*. 1994) and/or baculovirus (Koener and Leong 1990) did not obtained good protection levels against IHNV (Xu *et al*. 1991) or VSHV (Lorenzen *et al*. 1993a; Estepa *et al*. 1994), more than 95% protection were obtained by intramuscular injection of their G gene (Anderson *et al*. 1996a, 1996b; Lorenzen *et al*. 1998a, 2000; Fernandez-Alonso *et al*. 2001; LaPatra *et al*. 2001; Lorenzen *et al*. 2001; McLauchlan *et al*. 2003; Lorenzen and LaPatra 2005). DNA vaccination seems to be the way to go for viral diseases prevention in cultured fish (Kurath *et al*. 2007; Kurath 2008). At the laboratory scale, DNA vaccines to rhabdoviruses consisting in a plasmid coding for their G gene under the control of the human cytomegalovirus (CMV) promoter delivered by intramuscular injection have protected different fish species (Leong and Munn 1991; Anderson *et al*. 1996a; Lorenzen *et al*. 1998a; LaPatra *et al*. 2000) against different fish rhabdoviruses such as VHSV, IHNV, spring viremia of carp (SVCV) (Kim *et al*. 2000), hirame rhabdovirus (HIRRV) (Takano *et al*. 2004) and snaked head rhabdovirus (SHRV) (Kim *et al*. 2000). Because research is still going on, for instance to lower the amount of plasmid required for practical DNA vaccination by mass delivery methods (Fernandez-Alonso *et al*. 2001; Tafalla *et al*. 2008; Coll *et al*. 2009), the use of *SB*/ITR vectors might be an alternative to increase both their specific activity and duration. Although, there exists some possible risk of activating endogenous *SB* transposons in fish genomes, that in itself could also help to stimulate an stronger local immunological response by inducing higher cellular damage and consequent inflammation. The intramuscular injection of rainbow trout with plasmids containing expression cassettes of the VHSV G protein flanked by ITR induced higher anti-G specific antibody serum (unpublished) and interferon/Mx mRNA levels (two markers of early responses to VHSV infection) (Dr. Tafalla and Dr. Cuesta, pers. comm.) than the same expression cassette without the ITRs. It is not yet known whether or not the ITR-flanked *G* gene was incorporated into the trout cell genomes, since even low levels of endogenous *SB* transposase activity could not be detected in the rainbow trout RTG2 cell line (unpublished). If *in vivo* G gene insertion into the trout cell genomes turns out to be a requirement for the enhanced host responses, safety concerns will probably arise on the possible use of *SB*/ITR vectors for DNA vaccination on cultured species. Alternatively, the host enhanced responses could be due to promoter enhancement by the ITR. Thus, the VHSV G gene expression cassette flanked by ITR was the most effective for *in vitro* G protein expression amongst 10 different promoter-containing plasmids (Ruiz *et al*. 2008). In this model, the 5' ITR by itself had no promoter activity (Ruiz *et al*. 2008) and *cis*-enhancing properties of the 5' or 3' ITR of the *SB* transposon in mammalian cells (Moldt *et al*. 2007) did not show any enhancing effect on the promoters (unpublished). The relative levels of *SB* transposase activity and expression from the ITR-flanked gene should be fine tuned to obtain optimal expression of the desired gene, as shown in the *in vivo* mice model (best expression was obtained with less active promoters for the *SB* transposase than for the ITR-flanked gene) (Mikkelsen *et al*. 2003). Work is going on in our laboratory to further characterize the enhancement of expression of ITR-flanked G protein and their host effects while we search for most effective fish promoter alternatives.

CONCLUDING REMARKS

The applications of the *SB*/ITR vectors to human health and to basic science are growing in importance with more and more applications being developed every year (Liang *et al*. 2009; Mates *et al*. 2009; Ruf *et al*. 2009; Song *et al*. 2009), including ongoing clinical trials (Williams 2008). Still largely unexplored in commercial fish production, the *SB*/ITR vectors offer new possibilities to increase aquaculture yields (**Table 3**). Thus they can be used to insert genes into the germ line to increase production, resistance to diseases or to obtain biopharmaceuticals (Rocha *et al*. 2003). The *SB*/ITR vectors might even be tested for transgenesis of hard-to-transform species, such as shellfish where there is unknown whether endogenous *SB* transposon-related sequences do exist. They can also be used for massive insertional mutagenesis in the germ line of commercial fish species to tag and identify potentially important genes for marker-assisted selection methods. On the other hand, the *SB*/ITR vectors might also be used for somatic transfection/transposition or simply as enhancers of promoters such as it would be required to improve fish immunisation for new more potent DNA vaccination vectors (Lorenzen *et al*. 1998b; Traxler *et al*. 1999; Fernandez-Alonso *et al*. 2001; Purcell *et al*. 2004; Takano *et al*. 2004). Expectations are that the *SB*/ITR vectors could be used either to decrease their required plasmid dosages for fish mass immunisation or to increase the duration of the fish immune response.

The kind and activity of the *SB*/ITR vector required for each application would depend on the application itself. Thus, sometimes massive transpositions need to be avoided, for instance as required for stable fish transgenesis (Davidson *et al*. 2003; Grabher *et al*. 2003), while some other times the efficiencies of transposition and the number of copies need to be increased, for instance, for insertional mutagenesis to identify genes or for DNA immunization (Collier *et al*. 2005; Dupuy *et al*. 2005). Therefore and despite possible interferences with the presence of thousands of incomplete *SB* transposon-related copies and/or activity of endogenous *SB* transposases in fish genomes, there are possibilities to obtain transgenic fish, identification of genes important for aquaculture and/or improvement of fish DNA vaccines which remain largely unexplored.

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