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Transposon-mediated enhancer trapping in medaka

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Abstract

We tested the *Sleeping Beauty* transposable element for its ability to efficiently insert transgenes into the genome of medaka (*Oryzias latipes*), an important model system for vertebrate development. We show that the SB transposon efficiently mediates integration of a reporter gene into the fish germ line. In pilot experiments, we established 174 transgenic lines with a transgenesis efficiency of 32%. Transgenes are stably transmitted to, and expressed in, subsequent generations. Interestingly, the transgenic lines show novel expression patterns with temporal and spatial specificity at a rate of 12% (21/174), likely due to both, enhancing and silencing position effects. Furthermore, promoter-dependent GFP expression in injected fish embryos is tightly correlated with germ line transmission, facilitating easy selection of founder fish. Thus, the SB transposon/transposase system provides a highly efficient tool for transgenesis in general and for the generation of novel reporter gene expression patterns in particular.

Keywords: Transgenesis; Sleeping Beauty; Teleost; Microinjection

1. Introduction

Medaka (*Oryzias latipes*) and zebrafish are popular model systems for vertebrate development and disease due to their short generation times, transparency of embryos and well-established procedures in genetics and experimental embryology. Transgenesis in fish, combined with the use of in vivo markers such as GFP (Chalfie et al., 1994), provides a key tool to study developmental processes through the analysis of gene function or by identification of novel genes required during embryonic development by insertional mutagenesis (Jaenisch, 1988; Westerfield et al., 1992; Hackett, 1993; Gaiano et al., 1996). However, techniques widely used in mouse and *Drosophila* such as enhancer detection or gene trapping (Rubin and Spradling, 1982; O'Kane and Gehring, 1987), although attempted with limited success in zebrafish (Bayer and Campos-Ortega, 1992) and frog (Bronchain et al., 1999), have rarely been used due to low frequency of vector integration into the fish genome. Several protocols to improve transgenesis frequency and transgene expression have been developed in recent years, including plasmid microinjection (Stuart et al., 1988), retroviral infection (Lin et al., 1994) and others (Holt et al., 1990; Inoue et al., 1990; Klein and Fitzpatrick-McElligott, 1993). Microinjection appears to be the method of choice, although major drawbacks, e.g. mosaic transgene expression in G0, low insertion frequency and mosaic germ line distribution have not yet been overcome. Application of transposons (Gibbs et al., 1994; Raz et al., 1998; Kawakami et al., 2000), inverted terminal repeats (ITR) of viruses (Fu et al., 1998; Hsiao et al., 2001) and meganuclease-mediated insertion (Thermes et al., 2002) improved transgenesis in fish substantially in one or several aspect(s).

The systematic use of transposable elements, namely P-element-mediated enhancer trapping and transgenesis, is a key technique facilitating the successful analysis of *Drosophila* development (Rubin and Spradling, 1982; O'Kane and Gehring, 1987). In non-drosophilid insects, zebrafish and mammalian cells P-elements do not transpose (Handler et al., 1993; Gibbs et al., 1994), suggesting host factor

Abbreviations: AAV, adeno-associated virus; cDNA, DNA complementary to RNA; CSKA, cyotskeletal-actin; FACS, Fluorescence activated cell sorting; F1, 2, . . ., filial generation 1, 2, . . .; G0, generation 0; GFP, green fluorescent protein; IR/DR, inverted/direct repeats; ITR, inverted terminal repeats; pA, polyadenylation signal; PCR, polymerase chain reaction; SB, sleeping beauty; SV40, simian virus 40; TcE, Tc1-like elements.

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requirements. In contrast, the transposase of Tc1-like elements (TcEs) is sufficient for TcE transposition (Vos et al., 1996). *Sleeping Beauty* (SB), an artificially reconstructed member of the TcE family, mediates reporter construct integration in a wide range of vertebrate cells in tissue culture (Ivics et al., 1997) and in the mouse in vivo (Dupuy et al., 2001; Fischer et al., 2001; Horie et al., 2001). Here we show that the Sleeping Beauty (SB) system (Ivics et al., 1997) efficiently generates transgenic medaka lines with a high proportion of lines exhibiting novel, spatially and temporally restricted GFP expression patterns. In addition, transient, promoter-dependent expression of the transgene was strongly enhanced using the SB transposon alone or in combination with transposase (SB10).

2. Materials and methods

2.1. Plasmids

For control injections, an expression cassette containing the cytoskeletal-actin promoter (cska) of *X. borealis* (Thermes et al., 2002), GFP and a SV40 pA was cloned into a pBS KS backbone resulting in a 4.8-kb plasmid (p294). For application of the SB system, the same expression cassette was cloned between the IR/DRs of SB (Ivics et al., 1997) in a pBS KS backbone resulting in a 5.6-kb plasmid (transposon) (p381). SB10 mRNA was transcribed in vitro from the plasmid pBSSK/SB10 (kind gift of Z. Ivics) using the mMessage mMachine kit (Ambion).

2.2. Injections

Medaka embryos and adults of the Cab inbred strain were used in all experiments (Loosli et al., 2001). Fertilized eggs were collected immediately after spawning (at the onset of light) and placed in pre-chilled Yamamoto's embryo rearing medium (Yamamoto, 1975). For injection, one-cell stage embryos were transferred to 4 °C to slow down development. In all experiments, a pressure injector (FemtoJet, Eppendorf, Germany) was used with borosilicate glass capillaries (GC100T(F), Clark Electromedical Instruments, UK). Capillaries were backfilled with the injection solution (DNA: 50–100 ng/μl; Yamamoto buffer: 1×; SB mRNA: 100 ng/μl). To test for functional endogenous SB recognition sequences (IR/DRs), SB10 mRNA was injected in concentrations up to 400 ng/μl. DNA was prepared using a Qiagen Maxiprep kit (Qiagen, USA) and dialysed using nitrocellulose filters ((#VSW01300), Millipore, USA). DNA was injected through the chorion into the cytoplasm of the one-cell stage embryos. Embryos were raised to sexual maturity; transgenic carriers were identified by out crossing to wild type fish.

2.3. Epifluorescence microscopy

Embryos were observed and scored using a MZFLIII dissecting microscope (Leica) with a 370- to 420-nm excitation filter and a 455-nm LP emission filter.

2.4. Southern blotting

Genomic DNA (8 μg) or 200 pg plasmid DNA (p294 or p381) were digested with 100 units of restriction enzyme (*Sty*I or *Bam*HI) overnight. The fragments were separated by gel electrophoresis on a 0.8% gel in TAE buffer and blotted onto a Genescreen Plus hybridisation transfer membrane (NEN Life Science Products). Hybridisation and probe labelling was performed using the AlkPhos Direct labelling and detection kit (Amersham Life Science) according to protocol. The insert probes consist of a 750-bp fragment containing the GFP open reading frame (probe 2) or a 2-kb *Sac*I/*Apa*I fragment of the control plasmid containing the cska promoter, the GFP open reading frame and the SV40 polyadenylation signal (probe 1).

2.5. Isolation of flanking genomic sequences

Genomic regions flanking the insertions were isolated by splinkerette PCR as described (Devon et al., 1995). In brief, genomic DNA from transgenic lines was digested with *Xho*I. Nested PCR was performed (primary PCR: spl/left-IR/DR and spl/right-IR/DR primers, secondary PCR spl-nest/left-IR/DR-nest and spl-nest/right-IR/DR-nest primers; 1 μl was transferred from primary to secondary PCR) 95 °C 30 s; 95 °C 15 s, 71 °C 1 min −2 °C per cycle, 72 °C 2 min (5 cycles); 95 °C 15 s, 61 °C 2 min, 72 °C 2 min + 9 s per cycle (28 cycles).

Spl: cgaatcgtaaccgttcgtacgagaa, spl-nest: tcgtacgagaatcgctgtcctctcc, left-IR/DR: tttactcggattaaatgtcaggaattg, left-IR/DR-nest: tgagtttaaatgtatttggctaaggtg, right-IR/DR: agtgtatgtaaacttctgacccactgg, right-IR/DR-nest: cttgtgtcatgcacaaagtagatgtcc.

2.6. Accession number

Accession number of genomic insertion in SV line: AJ404849.

3. Results

3.1. Application of the SB system results in increased numbers of G0 embryos uniformly expressing GFP

To efficiently use an animal model system for transposon-mediated transgenesis, it needs to be free of (cryptic) endogenous elements that could be mobilized by the newly introduced transposase. The absence of such sequences in

the medaka genome was verified functionally by over-expression of the SB transposase in wild type medaka upon injection of SB10 mRNA into embryos. Even at the highest concentration, no effects on embryonic survival or development were observed (data not shown). In addition, low stringency Southern blot analysis using the inverted terminal repeats (IR/DRs) that serve as recognition elements for the SB transposase as a probe did not yield a signal, indicating that the medaka genome is free of endogenous elements capable of transposition by the SB transposase.

To address the potential of the SB transposon and the SB10 transposase for transgenesis, early one-cell stage embryos were co-injected with a reporter vector (transposon) and mRNA encoding the SB transposase (Fig. 1). The reporter contained an expression cassette with the cytoskeletal-actin promoter/enhancer (cska) of *X. borealis* to drive moderate, ubiquitous expression of GFP. This expression cassette was flanked by the terminal inverted repeats of SB containing the binding sites of the transposase (p381). Injections were performed with or without SB10 mRNA. A similar vector, lacking SB recognition sequences (control construct), was used for control injections (p294).

In contrast to mosaic expression due to transient transcription of non-integrated plasmids (Winkler et al., 1991), an early integration event leads to the transmission of the transgene to all of the daughter cells, and thus results in GFP expression in large clones of cells (Fig. 1). To distinguish between these two possibilities, embryos were screened for GFP expression at day 3 of development.

The injected embryos were scored and grouped according to the degree of mosaicism in GFP expression: (A) no GFP expression, (B) mosaic expression only and (C) ubiquitous GFP expression (Figs. 1B,C and 2A). Upon injection of control construct that lacks SB recognition sequences and in the absence of transposase, only 13% of surviving embryos expressed GFP uniformly in the entire body. Almost 40% did not show any fluorescence and about one half expressed GFP in mosaic cell clones of variable size, in accordance with results previously reported for DNA microinjection (Chou et al., 2001).

Conversely, when SB IR/DRs were included, uniform, promoter-dependent expression was the predominant effect. This was observed in 45% of the surviving embryos co-injected with the transposon and SB10 mRNA and in 42% injected with the transposon only (Fig. 2A). Thus, the presence of IR/DRs strongly enhanced promoter-dependent transgene expression in G0, indicating that SB IR/DRs, similar to the ITRs of adeno-associated virus (AAV) (Chou et al., 2001), significantly enhance transient transgene expression.

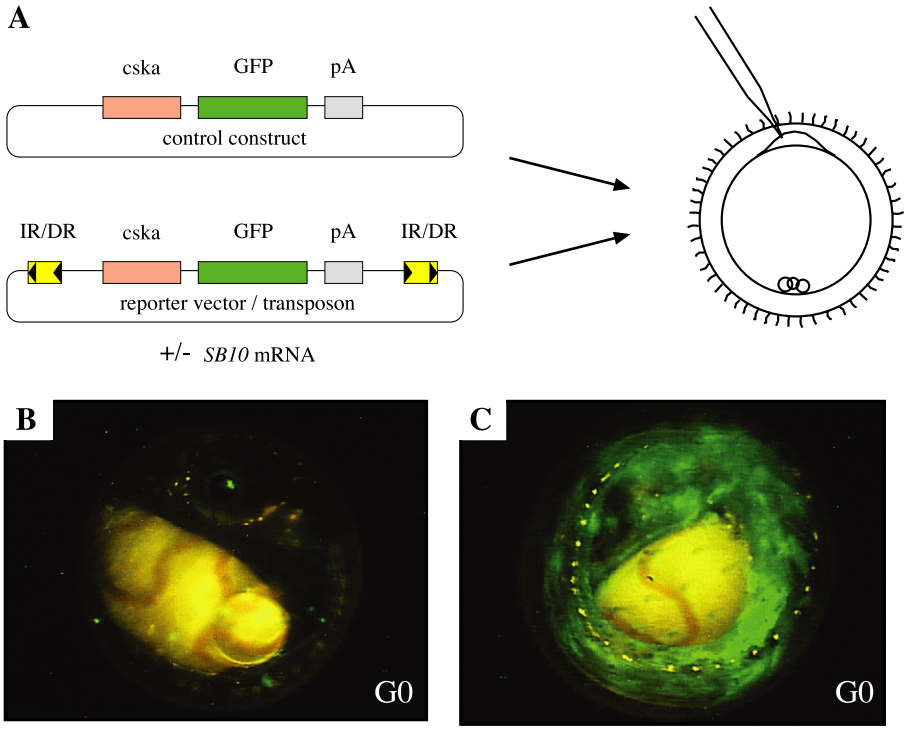

Fig. 1. Generation of founder fish by microinjection of circular DNA into one-cell stage medaka embryos. (A) Circular plasmid DNA containing an expression cassette with the cytoskeletal-actin promoter (pink box) of *X. borealis* driving GFP (green box), and a SV40 poly A signal (grey box) was injected into one-cell stage medaka embryos as control construct. To apply the SB transposon system, this expression cassette was flanked by SB recognition sequences (inverted direct repeats, IR/DR, transposon, yellow box) and injected with or without SB10 transposase mRNA. (B) Medaka embryo 3 days after injection showing mosaic expression of GFP. (C) Medaka embryo 3 days after injection showing promoter dependent ubiquitous expression of GFP.

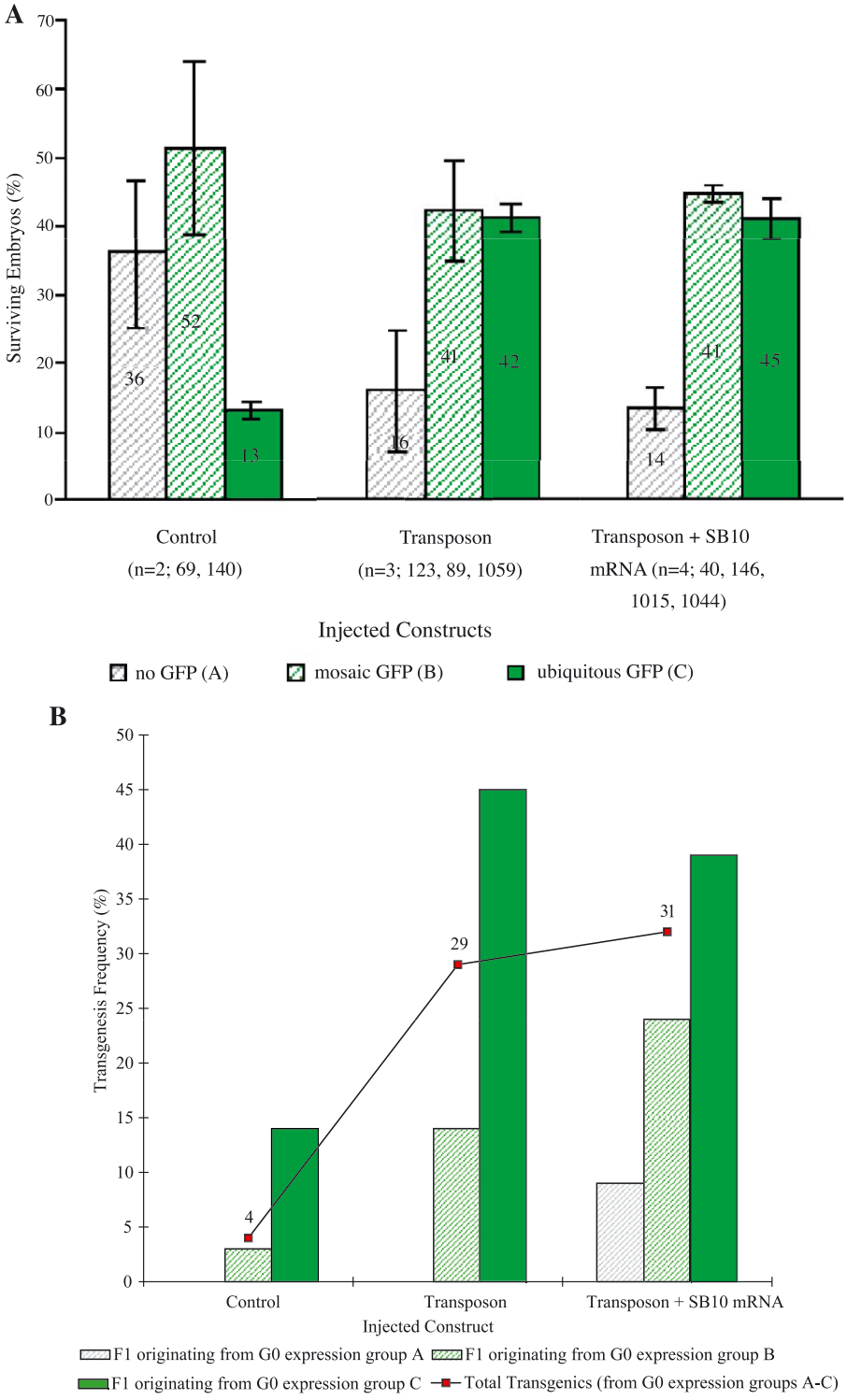

Fig. 2. The SB transposon system enhances transient expression and transgenesis frequency. (A) GFP expression was monitored in G0 upon injection of control construct (Control) or reporter vector (Transposon) and upon co-injection of transposon with SB10 mRNA (Transposon + SB10). After 3 days of development, embryos were grouped in expression groups according to the level of transgene expression (A–C). Percentages of surviving embryos exhibiting GFP expression are indicated within the bars. *n*, number of experiments (amount of embryos per experiment is indicated within brackets). (B) Bars indicate transgenesis frequencies of each G0 expression group (A–C). Line indicates the average total transgenesis frequency, taken together all screened G0 fish from all G0 expression groups.

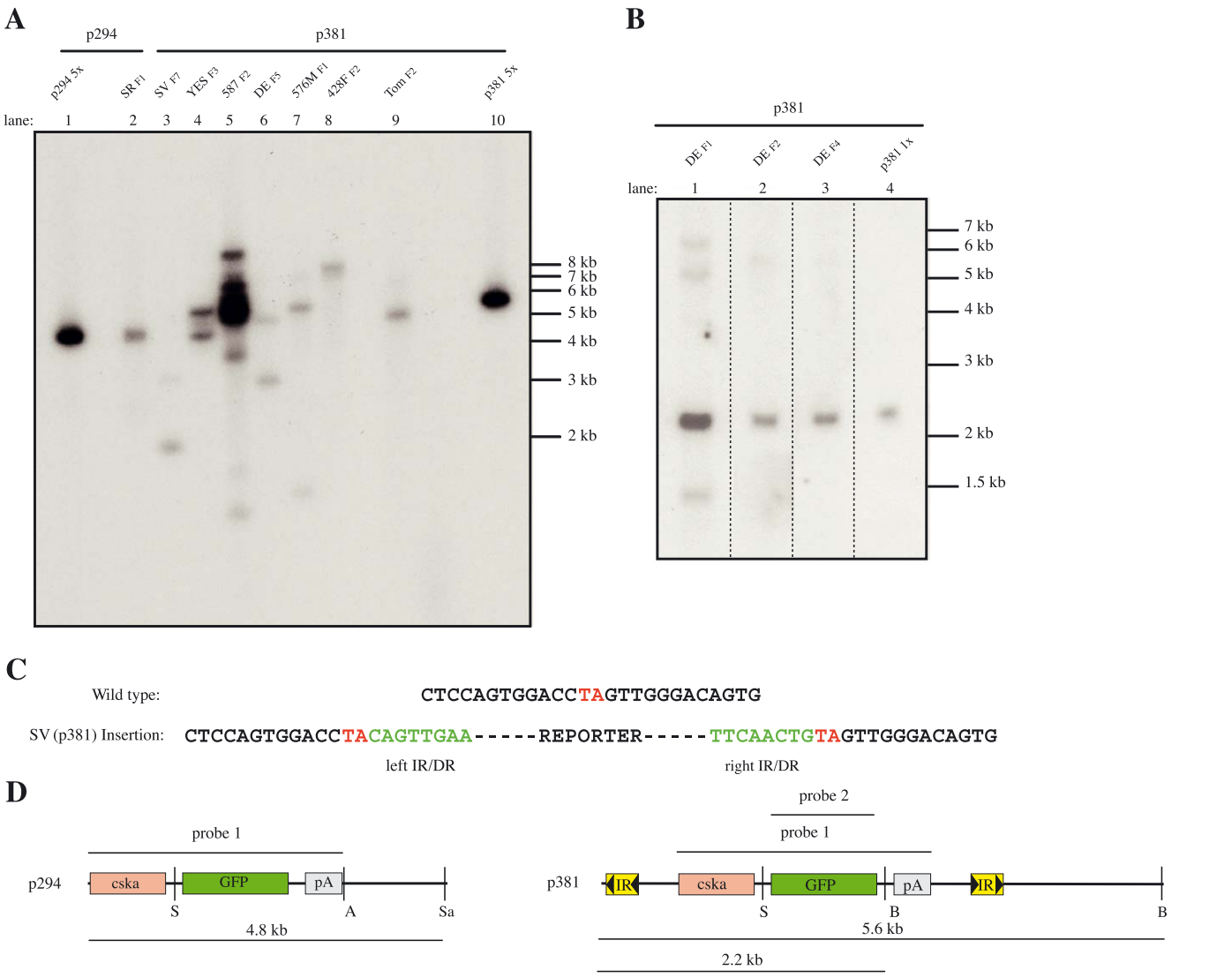

Fig. 3. Southern analysis of transgenic lines and genomic locus of SB10-mediated insertion in transgenic line SV. (A) Southern blot on offspring of eight independent transgenic lines. Genomic DNA and plasmid DNA (p294 and p381) was digested with *Sty*I. Genomic DNA (8 μg) and 200 pg of plasmid DNA (P, corresponding to five copies) were loaded and hybridised with a *Sac*I/*Apa*I insert fragment (probe 1). Filial generations are indicated. Lane 1, plasmid 294; lane 2, Transgenic line SR shows a signal at 4.8-kb corresponding to the entire linearised plasmid and two weak junction fragments at 2.8-kb and 4.9-kb, indicative for a random plasmid insertion in a tandem array; lane 3, Transgenic SV exhibits two junction fragments of 2.1-kb and 3.3-kb, respectively, in agreement with an integration of insert only mediated by SB10; lane 4, Transgenic YES shows hybridisation signals at 5.6-kb (entire linearised plasmid) and two junctions at 4.8-kb and 5.2-kb, possibly due to illegitimate SB10-mediated integration of a concatemer: lane 5, Transgenic 587, several signals are revealed, including a 5.6-kb plasmid signal and several junctions, indicative of more than one independent insertions. The two lowest fragments do not yield a signal when probed with vector sequence only (data not shown) indicating the possibility of illegitimate SB10-mediated integration in at least one of the cases; lane 6, Transgenic DE shows two junction fragments of 5.3-kb and 3.2-kb, respectively (both also hybridising with vector sequence, not shown), indicating random plasmid insertion; lane 7, Transgenic 576M yields the 5.6-kb fragment for the entire linearised fragment and two junctions at 1.5-kb and 8-kb, indicative for random plasmid insertion; lane 8, Transgenic 428F exhibits two junction signals at 8-kb and 8.5-kb due to random plasmid insertion; lane 9, Transgenic Tom shows two junction fragments at 5.2-kb and 7-kb due to random insertion; lane 10, plasmid 381. (B) Segregation of multiple independent insertions was analysed with transgenic line DE. Genomic DNA was digested with *Bam*HI and 8 μg was loaded. At least four independent insertions including a 2.2-kb signal expected for a *Bam*HI digest of the insert, a 1.4-kb, 5-kb and 6.5-kb fragment hybridising with the GFP probe (probe 2) in F1 (lane 1) were reduced leaving only the 2.2-kb fragment (including GFP, cska promoter and left IR/DR) in generations F2 (lane 2) and F4 (lane 3) while specific GFP expression was retained. Copy number was determined in comparison to 40 pg plasmid standard (corresponding to one copy) (lane 4); GFP coding sequence was used as probe (probe 2). (C) Wild type locus (WT) of the insertion of transgenic line SV. Target site of SB10 transposase (TA dinucleotide) is marked in red. SB10-mediated insertion of a single copy of the expression cassette flanked by IR/DRs (green) of transgenic line SV leads to the predicted duplication of the TA target site (red). (D) Schematic drawings of plasmids p294 and p381, restriction sites and probes used are indicated; S, *Sty*I; B, *Bam*HI, A, *Apa*I; Sa, *Sac*I.

3.2. Establishment of stable transgenic lines using the SB system

To further examine to what extent GFP expression in G0 correlates with germ line transmission of the transgene, we analysed the F1 offspring of all three expression groups for their ability to found transgenic lines. G0 fish were mated to wild type and the F1 offspring was screened for GFP expression (Fig. 2B).

This analysis showed that G0 expression was a reliable indicator for the efficient selection of transgenic founders. Application of the SB transposon alone or in combination with SB10 mRNA enhanced total transgenesis frequencies to 31% (174/560) compared to 4% (3/70) resulting from control construct injections. Strongly expressing G0 fish injected with the SB transposon (group C) transmitted GFP expression in 39% (118/305) or 45% (33/74) with or without transposase, respectively. On the other hand, of the fish injected with the control construct and showing uniform G0 expression (13% of injected survivors, expression group C), 14% (2/14) stably transmitted GFP to the next generation.

From the mosaic G0 fish (expression group B), 14% (4/28) or 24% (12/50) founded stable transgenics with transposon only or in combination with SB10 mRNA, respectively, while only 3% (1/34) transmitted a functional transgene to the F1 generation when the control construct had been injected.

GFP negative G0 fish (expression group A) transmitted a GFP expressing transgene to the next generation in 9% (7/77) of the analysed fish only upon injection of the complete SB system.

These numbers indicate a good correlation between the expression of the transgene in G0 and the frequency of stable transgenics using the complete SB system or the SB transposon alone. Embryos that exhibit a uniform GFP expression in G0 result in the highest yield of transgenic fish. This greatly facilitates an easy selection of putative founder fish for medium- to large-scale approaches.

3.3. Genomic integration of single or multiple copies

To investigate the molecular nature of the integrations, we performed Southern blot analysis on GFP positive fish. Eight lines are shown, including seven transgenics with novel expression patterns (Fig. 3A; lanes 2–9; Table 1), and one line expressing GFP in a promoter-dependent manner (Fig. 3A; lane 3). The copy numbers of the integrated transgenes were determined to range from a single copy (lanes 3, 6–9) to more than 10 copies (lane 5 and data not shown). The transgenic line SV (lane 3) revealed a banding pattern consistent with a single copy insertion mediated by SB10. In order to verify the proposed mechanism of SB10-mediated transposition in medaka, we analysed the SV insertion in more detail by cloning and sequencing flanking genomic regions (GenBank accession no. AJ404849). Comparison to the wild type locus revealed TA target site duplications flanking the inserted SB transposon, a molecular hallmark of transposition of Tc1-like elements (Ivics et

Table 1
Summarised features of transgenic lines presented in Figs. 3 and 4

Transgenic line	Injection	Germline transmission frequency	Type of insertion	Integration mechanism	GFP expression pattern
SV (p381)	Transposon + SB10	nd	Insert only	SB10	Ubiquitous
YES (p381)	Transposon + SB10	nd	Complete tandem array	Illegitimate SB10	Heart ventricles
587 (p381)	Transposon + SB10	nd	Complete tandem array	Illegitimate SB10	Lens, habenulae, pineal gland, posterior tectum, central row of cells in ventral hindbrain
DE (p381)	Transposon	8.1%	Complete tandem array	Random plasmid integration	Olfactory pits, diencephalon, epihysis
576M (p381)	Transposon + SB10	27.7%	Partial array	Random plasmid integration	Somites, muscles
428F (p381)	Transposon + SB10	55.5%	Partial array	Random plasmid integration	Yolk sac
Tom (p381)	Transposon + SB10	10.3%	Partial array	Random plasmid integration	Anterior neural tube, posterior notochord
MH	Transposon + SB10	23.7%	nd	nd	Telencephalon, mid-hindbrain boundary, CNS
Hoi	Transposon + SB10	6.1%	nd	nd	Otic vesicle
SR (p294)	Control	15.8%	Complete tandem array	Random plasmid integration	Retina

The enhancer trap lines presented are summarised here. Type of injection, germ line transmission frequency, type of insertion, the proposed integration mechanism and a description of the GFP expression pattern are described where determined. nd, not determined.

al., 1997) (Fig. 3C). Most of the other lines revealed complete or partial plasmid tandem head-to-tail insertions (Iyengar et al., 1996) either at a single locus or at multiple independent sites (Fig. 3A and data not shown). This is likely due to the strong DNA ligation and replication activity in early fish embryos (Hackett, 1993). Several transgenics (Fig. 3A, lanes 2, 4, 5, 7) show a signal at 4.8-kb or 5.6-kb, respectively, which is indicative for an entire plasmid insertion, in addition to flanking fragments (Fig. 3A, B, D). We thus cannot exclude the possibility that transgene concatemers can be inserted into the genome by the proposed mechanism of the SB10 transposase. Other lines (lanes 6, 8, 9) do not show this entire plasmid signal but instead show two flanking fragments of various sizes, similar to the SV line. However, in contrast to SV, most insertions in these transgenics also exhibit signals when hybridised with a probe containing only vector sequence indicative for integration of partial tandem arrays as a consequence of random plasmid insertion (not shown).

We find stable expression of the transgenes in tandem arrays over so far up to seven generations. This hints at a transgene activity-stabilizing function of SB IR/DR sequences. Recently, similar results have been reported by Noma et al. (2001) who identified inverted repeats, shielding euchro-

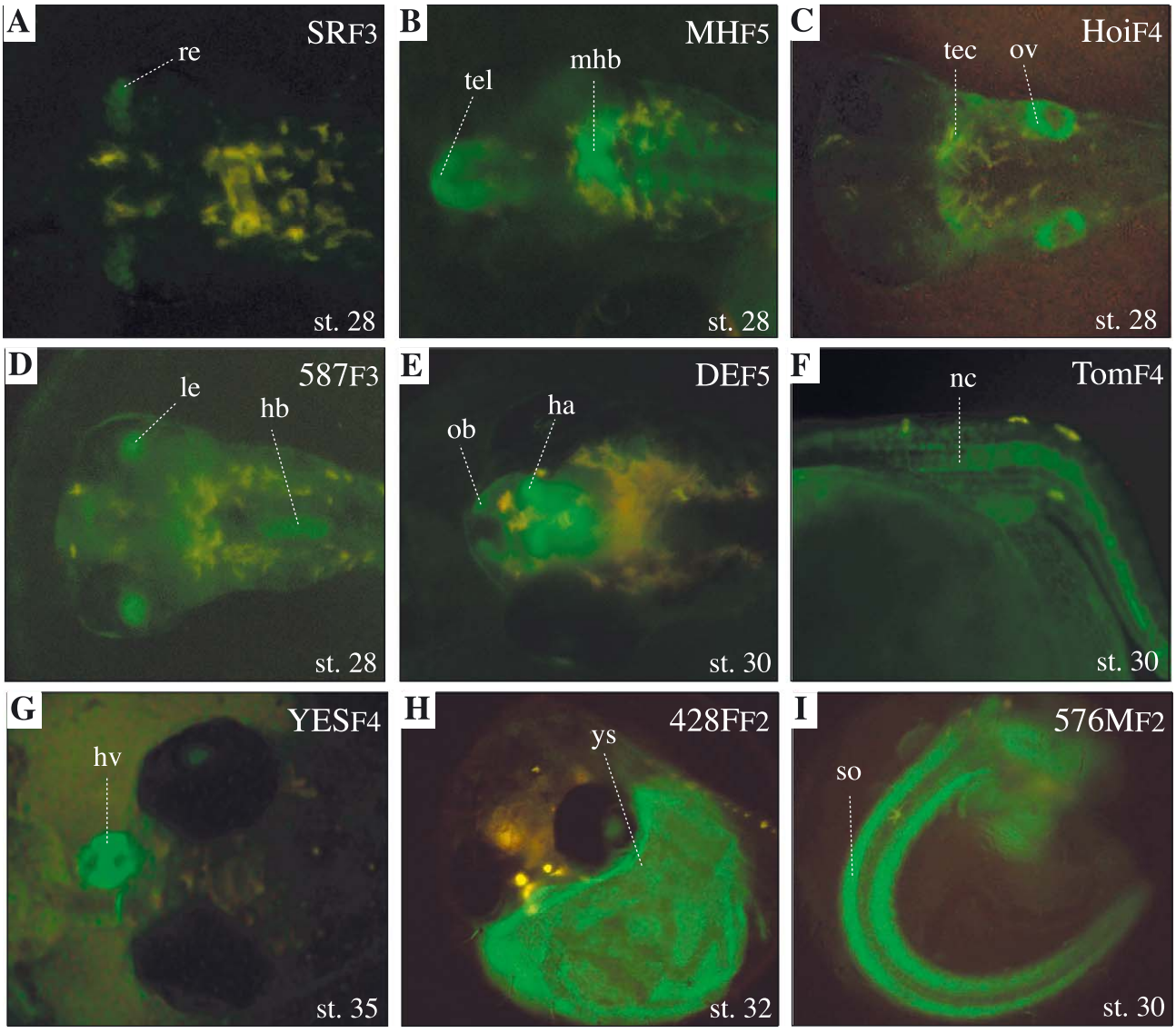

Fig. 4. Stable transgenic lines with spatially and temporally restricted GFP expression patterns. Collection of nine specific transgenics (A–I) showing expression of GFP in various tissues or organs; generation and developmental stages are indicated; anterior is to the left (A–G). (A) SR, GFP expression is enhanced in anterior cells of the retina. (B) MH, strong GFP expression in telencephalon, mid-hindbrain boundary and along the spinal chord. (C) Houichi (Hoi), otic vesicles and tectum show expression of GFP. (D) 587, GFP is enhanced in lens and a row of cells in the ventral hindbrain. (E) DE, olfactory bulbs, olfactory neurons and habenulae exhibit GFP expression. (F) Tomoe (Tom), cells of the notochord express GFP. (G) YES, ventricles of the heart are GFP positive. (H) 428F, GFP expression in epidermis of the yolk, frontal view. (I) 576M, strong GFP signal in somites, dorsal view. ha, habenulae; hb, hindbrain; hv, heart ventricles; le, lens; mhb, mid-hindbrain boundary; nc, notochord; ob, olfactory bulbs; ov, otic vesicles; re, retina; so, somites; tel, telencephalon; tec, tectum; ys, yolk.

matic regions of the mating-type locus of the fission yeast, thereby acting as barriers for heterochromatin spreading.

We segregated multiple insertions by selective screening in subsequent generations. Already in siblings of the F2 generation, the number of independent insertions in F1 (at least 4; Fig. 3B lane 1) of line DE was reduced (lane 2–3). In addition, we observed a reduction of ubiquitous GFP expression in some specific transgenic lines upon selective screening over several generations, likely due the decrease of multiple independent insertions.

The degree of mosaicism of the germ line is indicative of the time point of transgene integration. A single integration event at the one-cell stage results in a non-mosaic heterozygous fish that transmits the transgene to 50% of its offspring, while greater fractions indicate multiple independent insertions. We analysed the frequency of germ line transmission from identified G0 founders to their F1 progeny. Between 8% and 60% of the offspring showed GFP expression indicating single or multiple insertion events between 1 and 8 cell stages (Table 1). The transgenes were subsequently inherited in a Mendelian fashion over many generations without any alteration of expression level or pattern.

3.4. High frequency generation of spatially and temporally restricted expression patterns in F1 embryos

We screened 560 fish that were injected with the SB transposon only (128) or in combination with the SB10 transposase (432) and established 174 independent transgenic fish lines expressing GFP. F1 offspring of the majority of the founders (153/174) exhibited ubiquitous GFP expression according to the activity of the cska promoter. Strikingly, however, 12% (21/174) of the transgenics featured typical characteristics of ‘enhancer-trap’ lines, i.e. spatially and/or temporally restricted transgene expression due to regulation imposed by sequences near the insertion site. We showed previously that the relatively weak cska promoter/enhancer element of *X. borealis* may be used to generate novel patterns of expression (Thermes et al., 2002). Accordingly, we found one novel pattern among 70 fish injected with the control construct (Fig. 3, lane 2, Fig. 4A, and Table 1). Injections of transposon without transposase resulted in ~ 2% (2/128) novel patterns. Noticeably, SB10 transposase augmented pattern formation almost three-fold: 19 out of 432 screened fish injected with the complete SB system exhibit novel GFP expression patterns (Fig 3, lanes 4–5, 7–9, Fig. 4B–D, F–I and Table 1).

Among those 21 lines with novel expression patterns, we found a variety of different patterns ranging from single cell types to whole organs (Fig. 4A–I). A number of lines expressed GFP in ectodermally derived organs, but we also identified specific expression in tissues derived from other germ layers. For several lines, inheritance of appropriate transgene expression has been observed up to the 7th generation. Thus, among the transgenic lines we have established up to now, novel GFP patterns are generated in an unbiased fashion. We found lines specifically expressing GFP in the anterior retina (Fig. 4A), telencephalon and mid-hindbrain boundary (Fig. 4B), otic vesicles (Fig. 4C) or in lens and specific cells of the hindbrain (Fig. 4D). Among others, we identified transgenic lines with GFP expression in olfactory bulbs, olfactory neurons and habenulae (Fig. 4E), along the notochord (Fig. 4F), in ventricles of the heart (Fig. 4G), the yolk (Fig. 4H), somites and trunk muscles (Fig. 4I).

4. Discussion

Here we report the use of the Sleeping Beauty system as a highly efficient tool for the generation of transgenic medaka fish. Transgenesis is enhanced several fold by the presence of the SB recognition sites alone (transposon), even in the absence of SB10 transposase. Furthermore, our results reveal an intriguing potential to generate novel, random patterns of GFP expression. Other than in ‘‘conventional’’ transgenesis, the expression of the transgene is stable in subsequent generations even if it integrated in tandem arrays.

In contrast to control injections, the mosaicism of reporter gene expression was greatly reduced in G0 fish injected with the SB transposon. Efficient promoter-dependent expression in G0 depends on the presence of IR/DRs. Although ~ 45% of these G0 fish are transgenic founders, the other half was not transmitting GFP, indicating that ubiquitous expression in G0 was not due to early integration. Thus, widespread GFP expression in G0 appears to be due to an equal distribution of extrachromosomal plasmid DNA. A similar mechanism, the direction of the injected DNA to the nucleus, has been suggested for the ITRs of AAV in *Xenopus* and zebrafish (Weitzman et al., 1996). This allows an even segregation of transient, non-integrated reporter DNA and eventually later integration. Consequently, the SB transposon also provides a useful tool for transient expression studies upon injection of G0 embryos.

Furthermore, the uniform expression of the transgene in G0 embryos injected with transposon only or the complete SB system is a reliable marker for the efficient selection of transgenic founders, limiting a time- and space-consuming screening effort in F1. We found that SB10 acts with the proposed mechanism integrating a single copy insert sequence into a TA dinucleotide in the transgenic line SV. However, transgenesis frequencies using the transposon are comparable with or without transposase (31% or 29%, respectively) and thus appear relatively independent of the SB10 transposase. Do IR/DRs influence transgene integration of inserted DNA? For AAV-ITRs, palindromic sequences that fold into hairpin structures and function as origins of replication, it has been suggested that they also play a crucial role in the process of integration (Balague et al., 1997). Mammalian cell culture experiments revealed an improvement of integration frequency when reporter genes were flanked by AAV-ITR sequences over control reporters

(Balague et al., 1997). The IR/DRs of SB may perform similar functions, although such structural properties remain to be shown.

Strikingly, in the presence of SB10 transposase, novel expression patterns were found at high frequency, indicating a preference of integration near regulatory sequences, in contrast to random plasmid insertion. These patterns range from single cell types to larger tissues or organs (Fig. 4), and do not show a bias for any germ layer. Among these transgenic fish, we observed classical enhancer traps showing enhanced GFP expression at a restricted area but still exhibiting weak ubiquitous GFP expression controlled by the cska promoter fragment. In other transgenic lines, the ubiquitous reporter gene expression appeared to be specifically silenced in all but the enhanced tissues. Thus, application of promoter fragments of weak to moderate strength allows detection of both enhancing and silencing properties of regulatory elements. SB10 transposase co-injection uniquely increases the generation of novel random patterns of reporter gene expression almost three-fold compared to injections of control constructs or transposon in the absence of transposase. This result is not only due to the high integration frequency, because techniques such as the meganuclease approach, which reaches comparable transgenesis efficiencies, do not result in a comparable trapping activity (Thermes et al., 2002). Approaches using viral constructs in fish, although leading to efficient integration and mutagenesis, have not been shown to be useful as enhancer trapping tools so far.

Thus, similar to the P-element of *Drosophila* (Tsubota et al., 1985), SB integration in medaka might be favoured in the vicinity of transcriptional regulatory elements. The target site for SB transposition is part of a palindromic AT-rich sequence (ataTAtat) (Vigdal et al., 2002). Transgene insertion may be directed to AT-rich scaffold attachment regions or matrix-attached regions by the SB10 transposase even if the integration event itself is not mediated by the transposase, that could account for the increased frequency of novel expression patterns obtained by transposon/transposase co-injections. These regions are frequently comprised of control elements that maintain independent realms of gene activity (Vigdal et al., 2002). Some of these might also cohabit with transcriptional enhancers or silencers (Boulikas, 1995). The finding that mutant phenotypes were not observed so far when crossed to homozygousity further supports the view of preferential integration into non-coding regions.

For the rapid isolation of genomic DNA flanking the insertion, splinkerette PCR (Devon et al., 1995) was successfully applied for the transgenic line SV. However, independent insertions need to be segregated by successive out-crossing prior to cloning attempts, which is possible within one generation as shown for the transgenic line DE. Problems with tandem arrays can be overcome by preselection of the flanking fragments after Southern blotting. The ongoing sequencing of the medaka genome and genomic resources available is crucial prerequisites for the isolation and identification of DNA sequences in the vicinity of insertion sites on a larger scale and for the characterisation of the genes controlled by the trapped regulatory elements in vivo.

We are currently exploiting some of the specifically expressing GFP lines for analyses of mutants established in recent medaka mutagenesis screens. The dissociation of entire embryos allows for easy sorting of labelled cells/tissue by fluorescence-activated cell sorting (FACS), which in turn provides a resource for specific cell cultures, cDNA libraries and expression profiling. By simply changing the reporter construct, the method can be used to generate Gal4 driver or gene trap lines. The enhancement of transgenesis frequencies combined with the random generation of novel expression patterns using the SB transposon system allows the fast and simple generation of a wide range of diverse GFP expression patterns. For an average sized lab, 5000 injections per month are a feasible goal and, enhanced by the SB system, could give rise to more than 900 transgenic lines, of which 110 are expected to show a differential expression pattern. Thus, a set of transgenic lines expressing GFP in developmentally important structures/organs can be established and used without devoting a major effort to the isolation and characterization of promoter elements.

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