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A Transposon-Mediated Gene Trap Approach Identifies Developmentally Regulated Genes in Zebrafish

Technique

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Summary

We report here development of a novel gene trap method in zebrafish using the *Tol2* transposon system. First, we established a highly efficient transgenesis method in which a plasmid DNA containing the *Tol2* transposon vector and the transposase mRNA synthesized in vitro were coinjected into one-cell stage embryos. The transposon vector inserted in the genome could be transmitted to the F1 progeny at high frequencies, and regulated gene expression by a specific promoter could be recapitulated in transgenic fish. Then we constructed a transposon-based gene trap vector containing a splice acceptor and the GFP gene, performed a pilot screen for gene trapping, and obtained fish expressing GFP in temporally and spatially restricted patterns. We confirmed the endogenous transcripts were indeed trapped by the insertions, and the insertion could interfere with expression of the trapped gene. We propose our gene trap approach should facilitate studies of vertebrate development and organogenesis.

Introduction

Zebrafish *Danio rerio* has been developed as a model animal to study vertebrate development by genetic approaches (Streisinger et al., 1981). Large-scale screens for mutants using a chemical mutagen have been performed, and hundreds of mutations affecting various processes of vertebrate development have been successfully isolated (Driever et al., 1996; Haffter et al., 1996). Cloning of the mutated genes, however, has been a laborious work since identification of the point mutations created by the chemical mutagen requires time-consuming positional cloning and/or candidate gene cloning approaches. On the other hand, an insertional mutagenesis method using a pseudotyped retrovirus, which is composed of a genome based on the Moloney murine leukemia virus and the envelope glycoprotein (G protein) of the vesicular stomatitis virus, has been developed (Gaiano et al., 1996a, 1996b; Lin et al., 1994). This method enabled a large-scale screen for insertional mutants to be performed and the mutated genes to be cloned rapidly (Amsterdam et al., 1999; Golling et al., 2002). Although these approaches have disclosed a number of genes important for vertebrate development, methodologies available for forward genetics in zebrafish have been still limited. Especially, enhancer trap or gene trap methods, which have been shown to be powerful for studying the function of developmental genes in *Drosophila* and mouse, have not yet been developed in zebrafish.

In *Drosophila*, enhancer trap methods using the P transposable element have been developed. The P element-based enhancer trap vectors contain a weak basal promoter and the *lacZ* reporter gene. When the vector is inserted in the genome near an enhancer of an endogenous gene, the *lacZ* gene is expressed in a temporally and spatially regulated fashion under the control of the endogenous enhancer (O'Kane and Gehring, 1987). In mouse, gene trap methods using either DNA constructs or retroviral constructs which contain a splice acceptor and a reporter gene have been developed (Friedrich and Soriano, 1991; Gossler et al., 1989). The gene trap constructs are first introduced in mouse embryonic stem (ES) cells either by electroporation or retroviral infection, and the gene trap events are detected as expression of a reporter gene in ES cells. Subsequently, temporally and spatially regulated expression in an organism may be analyzed. These successes in the development of the enhancer trap and the gene trap methods rely upon the transposon technology, which can create insertions in the germ lineage very efficiently, or the ES cell technology. Neither such a transposon technology nor the ES cell technology has been developed in zebrafish. Although a gene trap construct containing an exon cassette has been constructed based on the pseudotyped retrovirus (Chen et al., 2002), by using this construct the gene trap events were detected only by 5′ rapid amplification of cDNA ends (RACE) analysis of pooled embryos. If a gene trap method using a reporter gene, such as GFP, was successfully developed in zebrafish, the gene trap events could be detected by observing living embryos directly since these are clear and transparent.

The *Tol2* transposable element, which was found in the genome of a small freshwater teleost, the Japanese medaka fish *Oryzias latipes*, belongs to the hAT family of transposons which includes *hobo* of *Drosophila*, *Ac* of maize, and *Tam3* of snapdragon (Koga et al., 1996). The zebrafish genome does not contain this element. In order to develop a new transposon technology in

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zebrafish, we have been working on the *Tol2* element. We have identified an autonomous member of the *Tol2* element which encodes a gene for a fully functional transposase capable of catalyzing transposition in the zebrafish germ lineage and also in mouse ES cells (Kawakami et al., 1998, 2000; Kawakami and Shima, 1999; Kawakami and Noda, 2004). To date, *Tol2* is the only natural DNA transposable element in vertebrates from which an autonomous member has been identified. In the previous study, we injected a plasmid DNA containing a nonautonomous *Tol2* element, which lacked part of the transposase gene, and the transposase mRNA, which was synthesized in vitro by using cloned *Tol2* cDNA as a template, into fertilized eggs. The nonautonomous element transposed from the plasmid to the genome during embryonic development and the transposon insertions could be transmitted to the next generation through the germ lineage. We identified one founder fish that could transmit four transposon insertions to the F1 progeny out of eight injected fish (Kawakami et al., 2000). While these results suggested that the *Tol2* transposon system can be used for transgenesis in zebrafish, with this transgenic frequency it would not be easy to generate large numbers of transposon insertions, and the system could not be used as a tool for forward genetics.

In the present study, we aim to develop a gene trap method in zebrafish using the *Tol2* transposon system. First, we improved the transgenic frequency using the *Tol2* transposon system. The frequency of obtaining founder fish became higher, and the number of transposon insertions transmitted by the founder fish was increased. Second, we constructed a transposon-based gene trap vector, created hundreds of insertions of the gene trap construct in the genome, and demonstrated that fish expressing GFP in temporally and spatially restricted patterns could be obtained. Third, we characterized the gene trap fish lines and found that the insertions indeed trapped endogenous transcripts. We describe here how we developed this novel gene trap method in the zebrafish.

Results

Highly Efficient Germline Transmission of *Tol2* Insertions

Although we have demonstrated that the *Tol2* element can be inserted in the zebrafish genome by transposition and that the insertion can be transmitted to the next generation through the germ lineage, the previous transgenic frequency did not seem high enough to generate hundreds or thousands of transposon insertions in a laboratory of the standard size (Kawakami et al., 2000). In an effort to improve the transgenic frequency, we modified the protocol for transposition as described in Experimental Procedures. Also, in order to make transposon insertions visible, we constructed T2KXIG containing the *Xenopus* EF1 α enhancer/promoter (Johnson and Krieg, 1994), the rabbit β-globin intron, the EGFP gene, and the SV40 polyA signal (Figure 1A). The GFP gene was placed in the reverse orientation relative to the transposase transcription to minimize possible effects of the promoter activity located near the 5′ end of the *Tol2* element (Kawakami and Shima, 1999) (Figure 1A).

We coinjected circular DNA of the T2KXIG plasmid together with the transposase mRNA synthesized in vitro into fertilized eggs. Ten injected fish were raised to adulthood and mated with noninjected fish. We examined a total of 1297 F1 embryos obtained from these crosses for GFP expression under a fluorescent dissecting microscope and identified GFP-expressing embryos in the progeny from five injected fish (Table 1 and Figure 1B). The GFP expression was rather ubiquitous as has been observed in transgenic fish carrying the GFP gene under the control of the EF1 α enhancer/promoter (Amsterdam et al., 1995; Linney et al., 1999). The F1 embryos, which did not express GFP, were pooled and analyzed by PCR for the presence of the T2KXIG sequence. The GFP-negative embryos were also PCR-negative (data not shown), indicating that transgenic fish carrying the T2KXIG insertion always expressed GFP. In one extreme case, 100% (259/259) of F1 fish from the XIG-1 founder fish expressed GFP. Southern blot analysis of 14 F1 fish revealed that the XIG-1 founder fish transmitted more than 15 different insertions (Figure 1B). In the case of XIG-3, 27% of F1 fish expressed GFP and the founder fish transmitted nine insertions (Figure 1B). The number of insertions transmitted by each XIG founder fish was thus counted, and from one to more than 15 were transmitted mosaically to the F1 fish (Table 1). The same membrane filters were used for Southern blot analysis using a probe that hybridized to the backbone portion of the T2KXIG plasmid. The plasmid probe did not hybridize to most of the bands, indicating that the insertions were generated through transposition. Three bands in the XIG-1 F1 fish and one band in the XIG-3 F1 fish, however, were detected by using the plasmid probe (Table 1), indicating that these bands probably resulted from integration of the entire plasmid DNA which occurred concomitantly with transposition. Excluding these four, a total of 28 transposon insertions were transmitted by five founder fish to the F1 progeny. Thus, the frequency of obtaining founder fish per injected fish was 50% and the average number of transposon insertions transmitted by single founder fish was 5.6 in the present study.

Regulated Gene Expression by Transgenesis Using *Tol2*

An important application of transgenesis in zebrafish is to construct transgenic fish expressing GFP in a specific tissue or organ (Higashijima et al., 1997; Long et al., 1997). To test whether the *Tol2* transposon system can be utilized for this purpose, we isolated a 1.6 kb DNA fragment upstream of the *six3.2* gene, which is expressed in the anterior neural plate and the eye anlage (Kobayashi et al., 2001), and constructed T2Ksix3.2G (Figure 1A). We injected the pT2Ksix3.2G plasmid DNA and the transposase mRNA into fertilized eggs and identified two founder fish out of seven injected fish (29%). In this experiment, since the GFP-negative fish were not analyzed for the presence of the T2Ksix3.2G sequence by PCR, F1 embryos carrying the insertion but expressing GFP at very low levels might have been overlooked. Although the frequency of obtaining founder fish here, determined by the GFP expression, was a little lower

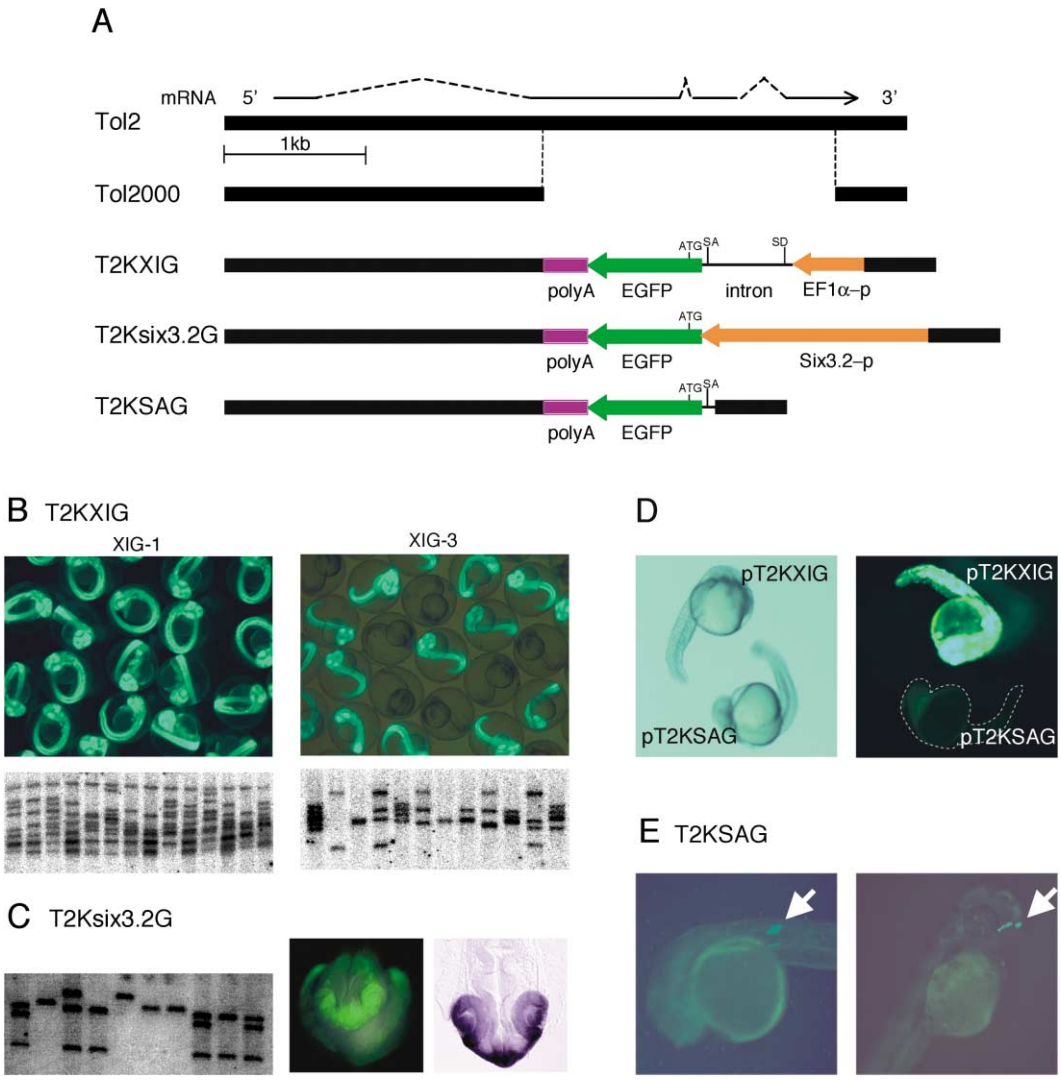


Figure 1. Transposon Constructs and Highly Efficient Transgenesis in Zebrafish

(A) Transposon constructs used in this study. Tol2, the full-length *Tol2* element and mRNA encoding the transposase; Tol2000, a modified nonautonomous *Tol2* lacking the region indicated by the dotted lines and containing multiple restriction enzyme sites; T2KXIG, the GFP expression cassette composed of the *Xenopus* EF1α enhancer/promoter, the rabbit β-globin intron, the EGFP gene, and the SV40 polyA signal, is inserted into Tol2000; SA, splice acceptor; SD, splice donor; ATG, the initiation codon for the EGFP gene; T2Ksix3.2G, the EGFP gene is placed downstream of the *six3.2* promoter; T2KSAG, the promoter and the splice donor are removed from T2KXIG.

(B) Transgenesis using T2KXIG. GFP expression in F1 embryos from XIG-1 and XIG-3 founder fish (upper panels) and Southern blot analysis of the F1 fish (lower panels) using the EGFP probe.

(C) Transgenesis using T2Ksix3.2G. Southern blot analysis of F1 transgenic fish using the GFP probe (left). GFP expression in the F2 embryo carrying a single T2Ksix3.2G insertion (center) and expression of the endogenous *six3.2* mRNA revealed by whole-mount in situ hybridization (right).

(D) GFP expression in embryos injected with the T2KXIG plasmid and the transposase mRNA and with the T2KSAG plasmid and the transposase mRNA.

(E) GFP expression in some muscle cells at day 1 (left) or some neurons at day 2 (right) in embryos injected with the T2KSAG plasmid and the transposase mRNA.

than that of the above experiment using T2KXIG, it is practically important that founder fish could be obtained by testing such a small number of the injected fish. F1 fish from one of the founder fish were analyzed by Southern blot using the GFP probe. Five different insertions were transmitted by the founder and transgenic F1 fish with a single insertion could be identified (Figure 1C). The F2 embryos with the single insertion expressed GFP in the forebrain and eyes at 24 hpf. The expression pattern was similar to that of the endogenous *six3.2* mRNA as revealed by whole-mount in situ hybridization (Figure 1C). This result indicates that the regulated gene expression can be recapitulated by transgenesis using the *Tol2* transposon system. Different levels of GFP expression were, however, observed in fish with different insertions (data not shown), suggesting that the locus

Table 1. Transgenesis Using T2KXIG

Founder Fish	GFP+/F1	T2KXIG DNA[a]	Plasmid DNA[b]
XIG-1	259/259 (100%)	>15	3
XIG-2	78/183 (43%)	4	0
XIG-3	30/111 (27%)	9	1
XIG-4	2/69 (2.9%)	1	0
XIG-5	51/102 (50%)	3	0

[a]The number of bands detected in F1 fish by Southern blot using the GFP probe.
[b]The number of bands detected in F1 fish by Southern blot using the backbone plasmid probe.

where the transposon had integrated might have affected its expression.

Construction of a Transposon-Based Gene Trap Vector

Since we established the method to generate transposon insertions in the zebrafish genome very efficiently, we then tested whether the *Tol2* transposon system could be used to develop a gene trap method. Although gene trap events are expected to occur less frequently than enhancer trap events, we chose the former since the trapped gene would be easily identified by 5′ rapid amplification of cDNA ends (RACE). We constructed T2KSAG containing a splice acceptor, a promoterless EGFP gene, and the SV40 polyA signal (Figure 1A). The GFP gene included an ATG codon that can serve either as an initiation codon or as an internal methionine, as the sequence between the splice acceptor and the ATG codon does not contain any stop codons in the same frame as GFP. Unlike the embryos injected with the T2KXIG plasmid and the transposase mRNA, embryos injected with the T2KSAG plasmid and the transposase mRNA hardly expressed GFP at 24 hpf (Figure 1D), indicating that the sequence upstream of the GFP gene in T2KSAG lacked the promoter activity. We found, however, in some embryos injected with the T2KSAG plasmid and the mRNA, that some somatic cells expressed GFP rather strongly (Figure 1E), suggesting that gene trap events might have occurred in these somatic cells. Since T2KSAG was expected to function as we designed, it was used for further studies.

A Pilot Screen for Gene Traps

In order to determine whether T2KSAG can indeed capture endogenous transcripts and to elucidate how often such gene trap events can occur, we decided to perform a pilot experiment. We injected circular DNA of the T2KSAG plasmid and the transposase mRNA into fertilized eggs and raised the injected fish to adulthood. 156 injected fish (107 males and 49 females) were used for mating. 62 fish (60 males and 2 females) were crossed with noninjected wild-type fish of the opposite sex (Table 2, hereafter referred to as WT-cross), and the remaining 94 fish (47 males and 47 females) were crossed as pairs (Table 2, hereafter referred to as P-cross). The total of 28,525 F1 embryos from these crosses were examined for GFP expression under a fluorescent dissecting microscope at 12 hr, 24 hr, 36 hr, 2 d, 3d, and 5d after fertilization. F1 embryos with detectable GFP expression were identified from 30 WT-crosses (29 males and 1 female) and 26 P-crosses. When no F1 embryo from a cross expressed GFP, at least 50 embryos from the cross were pooled, and analyzed by PCR for the presence of the T2KSAG sequence. The pools of embryos from 9 WT-crosses (9 males) and 7 P-crosses were PCR-positive, indicating that T2KSAG insertions that did not cause GFP expression were transmitted to these embryos (Table 2). In the P-crosses, it was not determined whether the founder fish was a male or a female (or both).

A variety of GFP expression patterns were observed in the T2KSAG transgenic F1 embryos; i.e., some were very weak and some were very strong, or some were quite ubiquitous, and some were temporally and spatially restricted patterns. This indicated that T2KSAG was inserted in various loci in the genome and GFP was expressed under the control of various endogenous promoters. Among these F1 embryos, we sorted out 36 GFP expression patterns as stronger than others at day 1 of development (Figure 2A). Tentatively, the places where GFP was expressed were described based on observations made with a fluorescent dissecting microscope (Table 3). These unique expression patterns were generated from 16 WT-crosses (SAG and SAGm) and 10 P-crosses (SAGp) and transmitted mosaically to the F1 progeny (from 0.6 to 45%). The same founder fish were used for mating repeatedly to collect the F1 embryos with the same GFP expression pattern. These F1 fish were raised and studied further. F1 embryos, in which very low levels of GFP expression was detected, were not kept and therefore not characterized.

The fish with the 36 unique expression patterns were analyzed by Southern blot hybridization using the GFP probe. For instance, two distinguishable patterns, SAG4A (heart) and SAG4B (forebrain), were found in F1 embryos from a WT-cross using the SAG4 founder fish. In these F1 fish, four different transposon insertions were detected by Southern blot (Figure 2B). In the case of SAG4A, 12 F1 fish with GFP expression in the heart were analyzed by Southern blot, and the transposon insertion responsible for the expression pattern and fish with a single insertion were identified (Figure 2C). This analysis also revealed an important notion that a single copy of the T2KSAG insertion could generate GFP expression at the detectable levels under a fluorescent dissecting microscope. From one to more than 25 T2KSAG insertions were detected in the F1 or F2 fish with the same expression pattern derived from each founder fish (Table 3). When a number of insertions were cotransmitted with the expression pattern, the fish were crossed with wild-type fish to obtain fish with single insertions. During such efforts, the insertions responsible for the expression patterns were identifiable by the Southern blot analysis since sibling fish with the same expression patterns always carried at least one same band in common as had been shown in SAG4A.

We found that embryos from female fish with the SAG2, SAG14, SAG20, SAG92, SAGm11A, SAGm11B, SAGm18B, SAGp4, SAGp49A, and SAGp53B expression patterns expressed GFP even at the one-cell stage (Figure 2D and Table 3), suggesting that the putative trapped genes were expressed also during oogenesis.

Table 2. A Pilot Screen for Gene Traps Using T2KSAG

	Crossed with Noninjected Fish (WT-Cross)	Crossed as a Pair (P-Cross)	Total
Injected fish mated	62 fish	47 pairs (94 fish)	156 fish
Analysis of F1 embryos			
GFP+	30 fish	26 pairs	
GFP− but PCR+	9 fish	7 pairs	
GFP− and PCR−	23 fish	14 pairs	
Founder fish identified	39 fish	33 pairs (~38–42[a] fish)	~80 fish (~51%)

The injected fish were crossed with noninjected wild type fish (WT-cross) or with injected fish of the opposite sex (P-cross). F1 embryos were first screened for GFP expression, and then GFP-negative fish were analyzed by PCR.

[a] In some of the 33 pairs, both male and female could be founders. The number of founders included in these pairs can be estimated as ~38–42 (see Discussion).

We crossed heterozygous male and female fish with the same expression pattern, both of which carried at least one transposon insertion in common or carried the same single insertion, and analyzed the offspring for defects in embryonic development until day 5. No lethal phenotype was observed by crossing fish with those 36 expression patterns.

T2KSAG Insertions Can Capture Endogenous Transcripts

From the fish with the 36 different expression patterns, we established 16 fish lines carrying single T2KSAG insertions. The DNA fragments containing the junction between genomic DNA and the T2KSAG insertion were amplified from these lines by inverse PCR, cloned, and sequenced (Table 4). In all cases, the 8 bp target sequence was duplicated at both ends of the insertion. No obvious specificity was observed in the integration sites at the DNA sequence level. The 8 bp sequences were somewhat AT-rich (70%; 90/128). In insertional mutagenesis in zebrafish using the pseudotyped retrovirus, genes responsible for mutant phenotypes have been identified in ~50% of the cases simply by analyzing the sequence obtained by the first inverse PCR by the BLAST search (Golling et al., 2002). We could not identify any genes within those inverse PCR fragments, except for SAGm18C. This may be partly due to smaller DNA fragments amplified by our inverse PCR protocol using four-base cutters (AluI, HaeIII, or MboI). Our method, however, should allow the trapped genes to be identified by 5′ RACE.

To determine whether the T2KSAG insertion indeed captured a transcript of an endogenous gene, we performed 5′ RACE on the fish with single insertions. From eight fish lines, fusion transcripts of endogenous sequences to the T2KSAG sequence were successfully identified, and the 5′ RACE products were cloned and sequenced. These sequences were fused precisely at the splice acceptor (Figure 3A). Thus, T2KSAG was shown to function as a gene trap construct in zebrafish. As for the other eight fish lines, such a fusion transcript could not be detected by the 5′ RACE analysis carried out in the same condition. This may be explained by the fact that the amount of the fusion transcript was small, or that the 5′ end was located far away. Further studies will be needed to identify the trapped genes in all of these fish lines.

Of the eight sequences identified by 5′ RACE, the trapped exons in SAGm18B and SAGm18C were part of known genes. In SAGm18B, the T2KSAG insertion was located in an internal intron of a gene for succinyl CoA: 3-oxoacid CoA-transferase (SCOT). The reading frame of the SCOT gene was maintained through the GFP gene, suggesting that a SCOT-GFP fusion protein should be synthesized in the SAGm18B fish. In SAGm18C, both the inverse PCR and the 5′ RACE analysis revealed that the T2KSAG insertion was located within a gene for the guanine nucleotide binding protein α-12 subunit. The GFP transcript was fused to the first noncoding exon of the gene and the ATG codon in the GFP gene should be used for translation initiation.

The trapped sequence in SAG20 was found to be identical to ENSDART00000014161, a predicted gene by the genome sequencing. We performed RT-PCR and found that the predicted gene was indeed transcribed in zebrafish. The SAG20 insertion was located within the first intron of the gene and trapped its first noncoding exon.

In SAGp22A, the flanking genomic sequence identified by inverse PCR was mapped on a BAC clone (BX005254) containing the *hoxc* cluster (Amores et al., 1998), between the *hoxc4a* and *hoxc5a* genes. Although the trapped exon sequence was found in the genomic sequence upstream of *hoxc5a* and *hoxc6a*, at first it had been an unknown "orphan" exon since no gene in the database included this sequence. To test whether any of the downstream *hoxc* genes contains the "orphan" exon, we constructed reverse primers located within the coding sequences of the *hoxc6a*, *hoxc5a*, *hoxc4a*, and *hoxc3a* gene, and a forward primer in the trapped sequence and performed RT-PCR. The RT-PCR product was detected only when the analysis was performed using the forward primer and the *hoxc3a* reverse primer, indicating that it was the unidentified first exon of the *hoxc3a* gene. Thus, the SAGp22A insertion was found to capture the first noncoding exon of *hoxc3a* (Figure 3B). Our gene trap approach should be useful to disclose such uncharacterized structures of transcripts in the genome.

The other four trapped exon sequences neither contained any open reading frame nor were homologous to any known gene or any EST sequence.

The T2KSAG Insertion Can Interfere with an Endogenous Transcript

In order to determine whether a T2KSAG insertion can interfere with synthesis of a normally spliced transcript

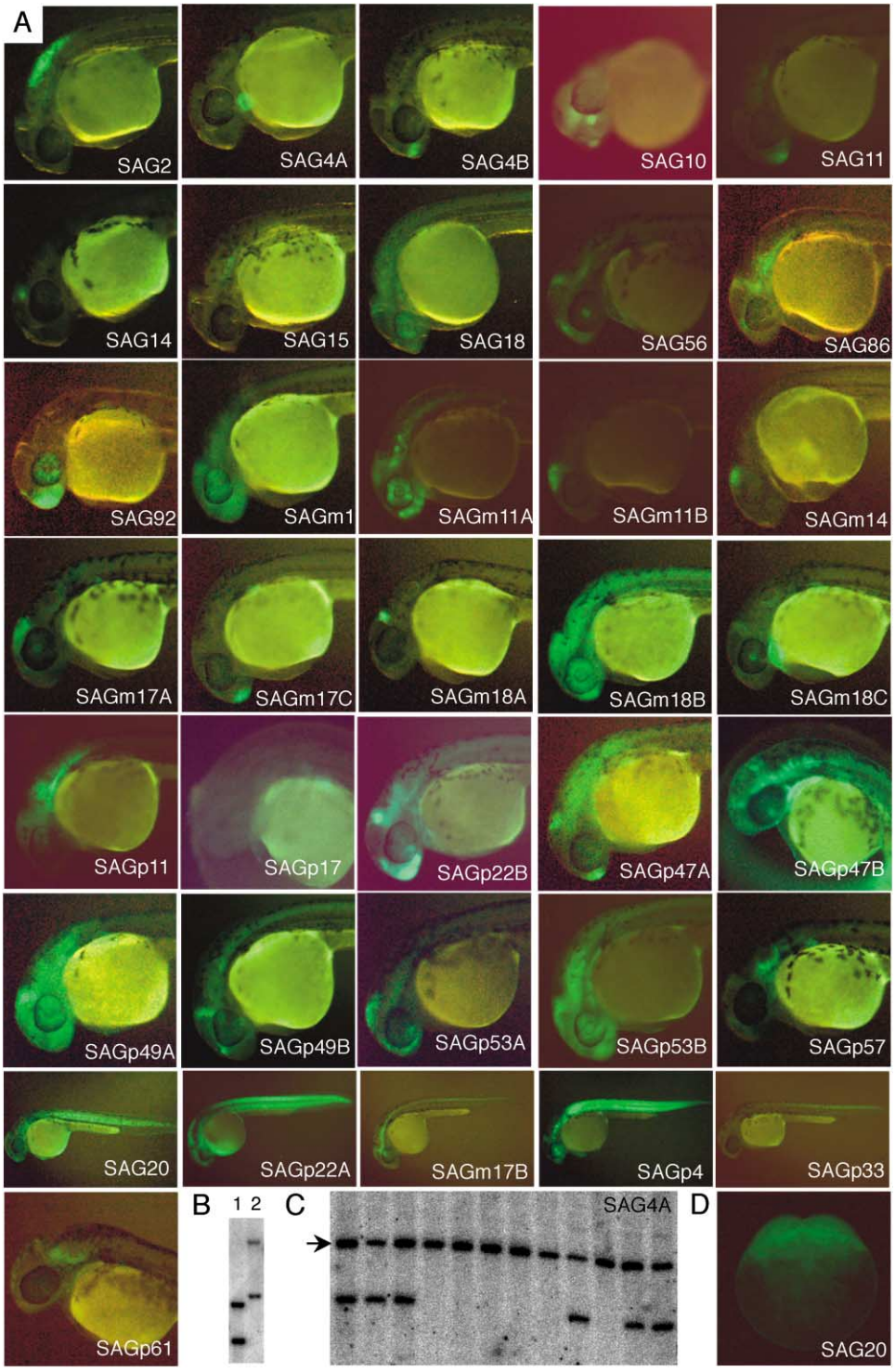


Figure 2. Unique GFP Expression Patterns Identified in Embryos Carrying the T2KSAG Insertions

(A) Various GFP expression patterns in embryos at 30–36 hpf.
(B) Southern blot analysis of the SAG4A (1) and SAG4B (2) fish.
(C) Southern blot analysis of the F1 fish with the SAG4A expression pattern. An arrow indicates the insertion responsible for the expression.
(D) GFP expression in the SAG20 embryo at 2-cell stage.

when it was inserted in an intron, we constructed a fish line homozygous for the SAGp22A insertion. The homozygous fish were viable and fertile. To obtain heterozygous and homozygous embryos, the homozygous adult fish were crossed to wild-type fish or the homozygous fish of the opposite sex. Total RNA was prepared from these embryos and RT-PCR was performed by using the forward primer in the exon1 of *hoxc3a* and a

Table 3. GFP Expression Patterns Identified in Gene Trap Lines

Pattern ID[a]	Putative Pattern	GFP+/F1[b]	Number of Insertions[c]
SAG2	hindbrain	36/429 (8.4%)	8
SAG4A[d]	heart	37/1320[e] (2.8%)	4
SAG4B[d]	forebrain	37/1274[e] (2.9%)	
SAG10	nose	20/660 (3.0%)	1
SAG11	forebrain	118/261 (45%)	3
SAG14	mhb	64/396 (16%)	1
SAG15	gut	96/370 (26%)	3
SAG18	cns	41/495 (8.3%)	1
SAG20	notochord	31/251 (12%)	2
SAG56	cns	11/204 (5.4%)	2
SAG86	cns	18/182 (9.9%)	5
SAG92	forebrain/eye	27/590 (4.6%)	3
SAGm1	head/eye	26/121 (21%)	3
SAGm11A[d]	neuron	51/355 (14%)	>25
SAGm11B[d]	midbrain	77/355 (22%)	
SAGm14	mhb	5/189 (2.6%)	1
SAGm17A[d]	midbrain	5/139[e] (3.6%)	8
SAGm17B[d]	floor plate	17/588[e] (2.9%)	
SAGm17C[d]	forebrain	36/588[e] (6.1%)	
SAGm18A[d]	mhb	15/858 (1.7%)	5
SAGm18B[d]	cns	15/858 (1.7%)	
SAGm18C[d]	heart/fin	12/858 (1.4%)	
SAGp4	somite	1/46 (2.2%)	7
SAGp11	neural crest	24/1203 (2.0%)	1
SAGp17	fin	33/439 (7.5%)	1
SAGp22A[d]	somite	24/1194 (2.0%)	2
SAGp22B[d]	forebrain/mhb	40/1194 (3.4%)	
SAGp33	neural tube	13/668 (1.9%)	1
SAGp47A[d]	forebrain/mhb	50/733 (6.8%)	4
SAGp47B[d]	neural tube	25/733 (3.4%)	
SAGp49A[d]	head	22/880 (2.5%)	5
SAGp49B[d]	forebrain/mhb	72/880 (8.2%)	
SAGp53A[d]	forebrain/mhb	2/162 (1.2%)	2
SAGp53B[d]	neural tube	1/162 (0.6%)	
SAGp57	ear	32/617 (5.2%)	1
SAGp61	neck	30/411 (7.3%)	2

[a] Founder fish identified by crosses with noninjected fish (WT-cross) and by crosses with injected fish (P-cross) are designated as SAG or SAGm, and SAGp, respectively.
[b] The numbers of embryos with the unique GFP expression pattern per the number of F1 embryos examined are shown.
[c] Southern blot analysis of two to 15 F1 or F2 fish with the unique expression patterns were carried out and the maximum numbers of T2KSAG insertions cotransmitted with the expression patterns by each founder fish were counted.
[d] Different patterns identified in F1 embryos from single founder fish or pairs are distinguished by alphabets.
[e] The different numbers of F1 embryos from crosses using the founder fish were examined to collect embryos with the expression patterns.

reverse primer in the exon4 of *hoxc3a* (Figure 3B). The *hoxc3a* mRNA was reduced in the heterozygous embryos, and more greatly in the homozygous embryos (Figure 3C). We performed RT-PCR using 2-fold serial dilutions of template cDNA prepared from wild-type embryos, and estimated that the amount of the *hoxc3a* transcript in the homozygous embryos was decreased to less than 25% of that synthesized in wild-type embryos. Thus, the T2KSAG insertion could markedly interrupt the synthesis of the normally spliced transcript. These levels of decrease should cause hypomorphic mutant phenotypes in some cases. We found, however, that the T2KSAG insertion did not abolish the wild-type transcript completely. This finding may account for the observation that we could not identify any lethal mutations in the pilot screen.

Discussion

Transgenesis Using the *Tol2* Transposon System

We established the highly efficient transgenesis method using the *Tol2* transposon system. The frequency of obtaining founder fish reached more than 50%, which is higher than the frequencies achieved by any other transgenesis methods that have been developed to express foreign genes in fish, i.e., injection of naked plasmid DNA to fertilized eggs (5% [Stuart et al., 1990], 5%–9% [Amsterdam et al., 1995]), the *Tc3* transposon system (7.5% [Raz et al., 1998]), a pseudotyped retrovirus expressing GFP (10% [Linney et al., 1999]), the *I-SceI* meganuclease system (30.5% [Thermes et al., 2002]), and the *Sleeping Beauty* transposon system (5%–31% [Davidson et al., 2003]). Transgenic zebrafish expressing GFP in specific tissues and organs have been useful to study vertebrate development (Higashijima et al., 1997; Long et al., 1997). Such studies will be speeded up by our method.

Our transgenesis method using the *Tol2* transposon system has the following features. First, transgenic fish carrying a single copy transgene can easily be isolated, while transgenic fish constructed by the plasmid DNA injection sometimes carry concatemers at a single locus (Stuart et al., 1988). Second, the transposon insertion is clean and does not cause a gross rearrangement at the

Table 4. DNA Sequences at Integration Sites Identified by Inverse PCR

Pattern/Insertion ID	DNA Sequence at the Integration Site	Accession Number
SAG4A	TTTGGCTTTG TAAATAAA ATTAGTTCAA	AB175055
SAG10	AAGCTCTGTC ACTAAAAC AAATTCCTCG	AB175056
SAG14	GGTAATACTC ACTACATG AGTACTTTTA	AB175057
SAG18	CACAGCATTA GTCAATAT AAAACACGTT	AB175058
SAG20	TTGTATTATG GAAGTAAC GTTCAAACTC	AB175059
SAG56	TTTCAAGTGT TTGCACAG CAATGTCCAA	AB175060
SAGm1	AAATTGGTTG GTTTTAGG AAAGGAAGAG	AB175061
SAGm14	CACACACACA CATACATT TAAACTCACA	AB175062
SAGm17C	ACAAAGGTTT GCCATCTG TAGCCGCAGA	AB175063
SAGm18B	ACAAATGCTA AACAAGAC TCAACTATGG	AB175064
SAGm18C	GTCAGGAAAT GTTAATTC GCTGAAAAAC	AB175065
SAGp4	ATACTAAATA CATGAATA CACCTTTAAT	AB175066
SAGp22A	ATTGGATTGT GTTATGCA TGACTAAAGT	AB175067
SAGp22B	GACTTTTTAA AGTATATA TATACAGTTG	AB175068
SAGp33	AATCTTGCCA TCTAAATC GTAAATATCC	AB175069
SAGp53A	GTGGTACCTT TTAAAAAG GTACACATTT	AB175070

Genomic DNA surrounding the T2KSAG insertion was cloned by inverse PCR from the lines carrying a single insertion. The 8 bp sequence which was duplicated upon the T2KSAG insertion is underlined.

integration locus, which is sometimes associated with an insertion created by the plasmid DNA injection (Cretekos and Grunwald, 1999). Third, expression of a transgene inserted by transposition may persist after the passage through generations. For now, the GFP expression in T2KXIG or T2Ksix3.2G transgenic fish or in T2KSAG gene trap fish can be observed consistently up to the F4 generation.

We infer that the improvement of the transgenic efficiency was achieved by modifications of the protocol for transposition. First, the transposase mRNA used in the present study contains the SV40 polyA signal in its 3′ end. Second, the concentration of the transposase mRNA used for microinjection was increased to 5-fold. Either of these changes or both may have led to an increase in the transposase activity in the injected embryos, and the transposition reaction could be stimulated. The transgenic frequency achieved by using our method has been reproducibly high in ongoing transgenic studies in our lab and also in our collaborators' labs (our unpublished data).

The Gene Trap Approach Using the *Tol2* Transposon System

In the pilot screen for gene traps, we identified 39 founder fish (38 males and 1 female) out of 62 fish crossed with noninjected fish (WT-cross), and 33 founder pairs out of 94 fish crossed as pairs (P-cross). In some of these positive P-crosses, both a male and a female could be founders. It can be estimated that these 94 fish include approximately 21 male and 21 female founder fish, i.e., $\{(47\text{-}21)/47\}^2 \fallingdotseq (47\text{-}33)/47$. Or, suppose the founder frequency in male was the same as that observed in the WT-crosses (63%; 38/60), 30 males and 8 females, i.e., $(47\text{-}30)/47 \times (47\text{-}8)/47 \fallingdotseq (47\text{-}33)/47$. If the latter were the case, a question could arise whether there may be a gender-specific effect on germline transmission of the transposon insertions. This possibility remains to be tested. The number of founder fish in the P-crosses can thus be estimated as ~38–42. Based on these assumptions, about 80 fish out of the 156 injected fish could be founders (51%). The estimated founder frequency with T2KSAG was thus similar to that observed with T2KXIG, suggesting that transposition of these two constructs could occur similarly. Suppose that the average number of insertions transmitted by single founder fish was also the same as that observed with T2KXIG, i.e., 5.6 insertions per founder fish, it can be estimated that approximately 450 T2KSAG insertions could be transmitted by 80 founder fish.

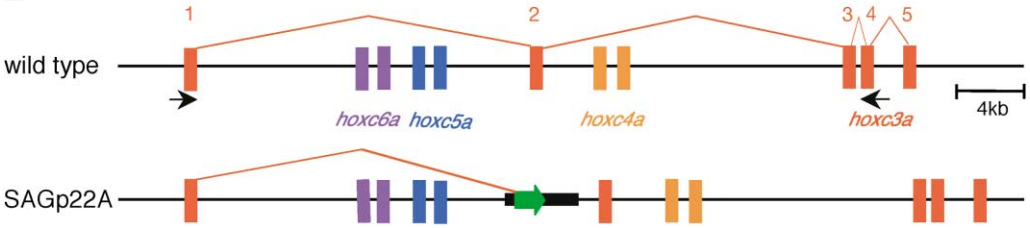
We found 36 unique GFP expression patterns by screening F1 embryos from the 156 injected fish. Since it was estimated that we screened about 450 insertions in the pilot experiment, we hypothesize about 8% (36/450) of the T2KSAG insertions in the zebrafish genome can give rise to such unique GFP expression patterns. In a gene trap approach in mouse, when the ROSAβgal retroviral construct which contained a splice acceptor and the β-galactosidase gene with an initiation ATG codon was infected to ES cells, it was estimated that 11.6% of cells carrying the proviral insertion became X-gal positive (Friedrich and Soriano, 1991). While it is difficult to compare these two different systems directly, it is interesting to note that the frequencies were comparable. It has been shown that transcription start regions are preferred targets of the retroviral integration (Wu et al., 2003). The high frequency of obtaining fish with GFP expression patterns may imply that the *Tol2* element also has such a preference for integration.

The GFP gene on the T2KSAG construct was designed to express when it is inserted either (1) upstream of the initiation codon of a gene and downstream of either a promoter or exon(s) encoding the 5′ untranslated region in the proper orientation, or (2) downstream of the initiation codon of a gene, either in an exon or an internal intron, in the proper orientation and reading frame. In the four cases where the trapped gene was identified, three were the former (SAG20, SAGm18C, and SAGp22A) and one was the latter (SAGm18B). Thus, T2KSAG can work as a gene trap construct as we designed. In the other four cases, no open reading frame was found in the trapped sequences. Further studies will be needed

A

T2KSAG sequence	...tttcctacag ctcc...cacc**atg**...	
SAG4A	...GGTGCCCATC ctcc...cacc**atg**...	unknown exon
SAG20	...ATCGCATCAG ctcc...cacc**atg**...	unknown exon (ENSDART00000014161)
SAG56	...AATACACGCG ctcc...cacc**atg**...	unknown exon
SAGm18B	...TGATCAGAGG ctcc...cacc**atg**...	3-oxoacid CoA transferase (SCOT)
SAGm18C	...GGGACTACCA ctcc...cacc**atg**...	G protein, alpha-12
SAGp4	...ACCCGAACAG ctcc...cacc**atg**...	unknown exon
SAGp22A	...GTGTCTTGTT ctcc...cacc**atg**...	unknown exon (*hoxc3a*)
SAGp53A	...GATTTATTAG ctcc...cacc**atg**...	unknown exon

B



C

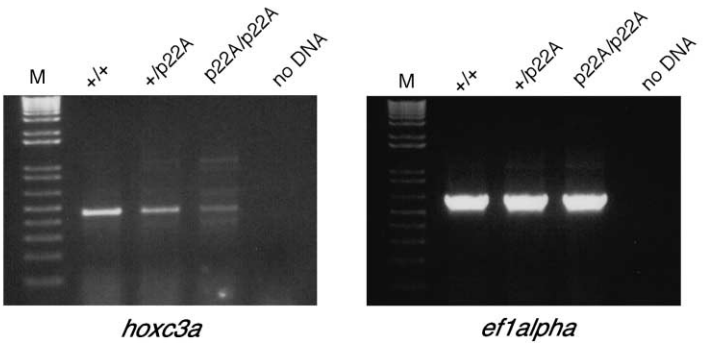


Figure 3. The T2KSAG Insertions Captured Endogenous Transcripts

(A) Fusion transcripts identified by 5′ RACE from the gene trap fish lines. The T2KSAG sequence is shown in lower case and italics. The splice acceptor is underlined. The translation initiation codon of the GFP gene is shown in bold. The endogenous exon sequence fused to the T2KSAG sequence is shown in upper case. Accession numbers: SAG4A, AB175327; SAG20, AB175328; SAG56, AB175329; SAGm18B, AB175330; SAGm18C, AB17533; SAGp4, AB175332; SAGp22A, AB175333; SAGp53A, AB175334.

(B) The structure of the *hoxc* cluster in wild-type and SAGp22A fish. The *hoxc3a* exons are numbered from the 5′ end. The arrows indicate the positions and directions of the primers used in the experiment below.

(C) RT-PCR analysis of the *hoxc3a* transcript in wild-type and SAGp22A heterozygous and homozygous embryos. The *hoxc3a* transcript is reduced to less than 25% in the SAGp22A homozygous embryos (left). RT-PCR using the primers in the EF1α transcript was carried out as a positive control.

to determine whether they also are unidentified 5′ noncoding exons.

Possible Applications of the Gene Trap Approach

Currently, construction of transgenic fish with specific GFP expression, which is useful to study organogenesis, has been carried out as follows. First, a gene expressed in a specific tissue or organ should be identified and the genomic DNA surrounding the gene should be cloned. Second, the DNA fragment containing the promoter activity should be identified and a plasmid DNA containing the promoter and the GFP gene should be constructed. Finally, transgenic fish should be generated by microinjection of the plasmid DNA. Thus, making one transgenic fish line with specific GFP expression will be a project of more than one year. We propose that our approach will be an alternative. Since one unique expression pattern can be isolated in every four or five injected fish (23%; 36 patterns out of 156 injected fish), a small lab can collect tens or hundreds of different expression patterns within one year, which may include the desired expression pattern. Further, in the future, a collaborative work by several laboratories would produce thousands of gene trap lines, which should be useful resources. A number of genes that may play important roles in vertebrate development also will be identified by analyzing those fish lines.

The *Tol2* transposon system should be applied to develop the Gal4-UAS system. In *Drosophila*, the Gal4-UAS trans-activation system has been combined with the enhancer trap system (Brand and Perrimon, 1993). It has been shown that Gal4 can activate expression of a

gene placed downstream of UAS in transgenic zebrafish (Scheer and Campos-Ortega, 1999). By replacing the GFP gene on T2KSAG with the Gal4 gene, we will be able to obtain a number of fish lines expressing Gal4 in various tissues and organs. Such Gal4 lines should allow a gene of interest to be expressed at the desired time and place. We are currently testing whether such a system is feasible.

The gene trap approach should be applied to generate insertional mutations. The number of genes essential for zebrafish embryonic development was estimated as approximately 2400 by large-scale chemical mutagenesis screens (Driever et al., 1996; Haffter et al., 1996). The fugu genome is thought to contain 38,510 gene transcripts (Ensembl, Fugu v20.2b.1 released on April 1, 2004). Suppose that the zebrafish genome contained approximately 40,000 genes; mutations in about 6% of the genes should lead to embryonic lethality (2400/40,000). Therefore, we could have obtained two embryonic recessive lethal mutations by screening the 36 gene trap lines in the pilot screen if all of the trapped genes had been disrupted by the insertions. No obvious mutant phenotype was observed. The following explanations may account for this finding. First, the 36 insertions may not have disrupted the function of 36 genes. The insertion in an intron may possibly allow a normally spliced transcript to be synthesized at low levels, as we observed in the case of SAGp22A, and, for some essential genes, such low levels of the wild-type transcript would be sufficient for homozygous embryos to survive. If this was the case, the elimination of splicing that skipped over a transposon insertion by modifying the gene trap construct would increase the mutagenic frequency. This could be achieved if we could obtain a splice acceptor and a poly A signal that work better in zebrafish. Second, the zebrafish genome may contain more than 40,000 "genes." These genes include transcriptionally active loci, not only genes encoding proteins but also noncoding RNAs. Third, the number of essential genes may be less than 2400. It should be noted that the mutagenic frequency observed in the pseudotyped retroviral insertional mutagenesis also has been low, i.e., one embryonic lethal mutation per 85 proviral insertions (Golling et al., 2002), although the retrovirus preferentially integrates in the transcription start region of genes (Wu et al., 2003). Analysis of more gene trap lines will be needed to get more insights into the mutagenic frequency with our method. Alternatively, a complete loss of the function of the trapped gene may be achieved by mobilizing the integrated transposon and inducing chromosomal deletions, as has been developed in *Drosophila* by using the P element (Cooley et al., 1990). A transposon inserted in the genome can transpose from the existing locus to a new locus when the transposase activity was supplied by microinjection of the transposase mRNA into cells (K.K., unpublished data). In this regard, it will be important to know whether deletion mutations can be induced at the excision locus efficiently. Studies are in progress along these lines.

We established here a novel transposon-mediated gene trap approach in zebrafish. This approach should facilitate studies on the function of genes involved in vertebrate development and organogenesis and provide a basis for further development of useful genetic methodologies in zebrafish.

Experimental Procedures

Plasmids

T2KXIG: The EGFP expression cassette containing the *Xenopus* EF1α enhancer/promoter (Johnson and Krieg, 1994), the rabbit β-globin intron, the EGFP gene (Clontech, Inc.), and the SV40 polyA signal was constructed by replacing the GFP gene of the XIG construct with the EGFP gene (Amsterdam et al., 1995). It was cloned between XhoI and BglII of Tol2000 (Kawakami and Noda, 2004). T2KSAG: The T2KXIG plasmid DNA was digested with ApaI and self-ligated. The EF1α enhancer/promoter and the splice donor site were removed. T2Ksix3.2G: The genomic DNA containing the *six3.2* promoter was cloned from a lambda phage genomic library (Stratagene) by plaque hybridization. A series of deletions in the promoter region was constructed and placed upstream of the GFP gene. The promoter activity was tested by microinjection of those DNA constructs into fertilized eggs and by examining GFP expression transiently in the injected embryos. A 1.6 kb HindIII fragment thus identified was placed upstream of the EGFP gene and the SV40 polyA signal, and cloned into the Tol2000 vector.

Microinjection of Plasmid DNA and Transposase mRNA

In the previous study, mRNA was synthesized using pBS-TP and T7 RNA polymerase (Kawakami et al., 2000). In the present study, the transposase cDNA (Kawakami et al., 2000) was cloned into pCS2+ (Rupp et al., 1994; Turner and Weintraub, 1994), resulting in pCS-TP, and mRNA was synthesized in vitro using mMESSAGE mMACHINE SP6 Kit (Ambion Inc.). By using this protocol, the yield of mRNA was greatly increased. Also, mRNA synthesized from pCS-TP contains the SV40 polyA signal in the 3′ region. ~1 nl of a DNA/RNA solution containing 25 ng/μl circular DNA of pT2KXIG, pT2Ksix3.2G, or pT2KSAG and 25 ng/μl transposase mRNA were injected into fertilized eggs.

GFP Expression in Embryos

GFP expression in embryos was examined using fluorescent dissecting microscopes MZ FL III and MZ 16 FA (Leica), and photos were taken using DC200 (Leica).

Southern Blot Hybridization

5 μg of genomic DNA extracted from a fin clip was digested with BglII, separated by electrophoresis on 1% agarose gel, transferred to Hybond-N+ (Amersham), and hybridized with ^{32}P-labeled probes. The ~800 bp BamHI-ClaI fragment from the T2KXIG plasmid was used as the GFP probe. Linearized pBluescript (Stratagene) DNA was used as the backbone plasmid probe.

PCR, Inverse PCR, 5′ RACE, and RT-PCR

PCR to detect the T2KXIG and T2KSAG sequence in the pooled embryos was carried out using the primers EGFP/f1 (5′-CTCCTG GGCAACGTGCTGGTT-3′) and EGFP/r1 (5′-GTGGTGCAGATGAAC TTCAG-3′). Inverse PCR was carried out as described (Kawakami et al., 2000) with some modifications. The genomic DNA was digested with MboI, HaeIII, or AluI and self-ligated. Nested primers used to amplify the 5′junctions are: first round, Tol2-5′/f1 (5′-AGTACTTTTTAC TCCTTACA-3′) and Tol2-5′/r1 (5′-GATTTTTAATTGTACTCAAG-3′); second round, Tol2-5′/f2 (5′-TACAGTCAAAAAGTACT-3′) and Tol2-5′/r2 (5′-AAGTAAAGTAAAAATCC-3′). Nested primers used to amplify the 3′junctions are: first round, Tol2-3′/f1 (5′-TTTACTCAAGTA AGATTCTAG-3′) and Tol2-3′/r1 (5′-CTCCATTAAAATTGTACT TGA-3′); second round, Tol2-3′/f2 (5′-ACTTGTACTTTCACTTGA GTA-3′) and Tol2-3′/r2 (5′-GCAAGAAAGAAAACTAGAGA-3′). 5′ RACE was carried out by using 5 μg of total RNA, the reverse primers in the GFP gene, EGFP/r2 (5′-CTTGCCGTAGGTGG CATCGCCCTC-3′), EGFP/r3 (5′-GCTGAACTTGTGGCCGTTTAC-3′) and EGFP/r4 (5′-GATGGGCACCACCCCGGTGA-3′), and 5′ RACE system (Invitrogen). The total RNA was prepared from 50 embryos (day 1) expressing GFP in unique patterns by using TRIZOL Reagent

(Invitrogen). To determine which gene contained the trapped sequence in SAGp22A, RT-PCR was carried out by using oligo dT-primed cDNA synthesized from 5 μg of total RNA from wild-type embryos and primers: hoxc3a/f1 (5′-AACAAGACACAAGGCAAGC AAC-3′), hoxc3a/r1 (5′-GTCACCAGTTTTCAGTTTTTCTG-3′), hoxc4a/r1 (5′-CTTGCTGGCGAGTGTAAGCAGT-3′), hoxc5a/r1 (5′-AACTTTGAGTCCTTCTTCCAC T-3′), and hoxc6a/r1 (5′-GGTAT CTGGAGTAAATCTGGC G-3′). To analyze the *hoxc3a* transcript, total RNA were prepared from wild-type, the SAGp22A heterozygous, and the SAGp22A homozygous embryos. The cDNA were made by using the oligo dT primer and used to perform 30 cycles of RT-PCR using primers: hoxc3a/f1 (5′-AACAAGACACAAGGCAAGCAAC-3′) and hoxc3a/r2: (5′-TCATCCAAGGGTACTTCATGGT-3′). As a control, 27 cycles of RT-PCR were carried out using primers in the zebrafish EF1α gene: EF1 α /exon3/f1 (5′-ACATTGCTCTCTGGAA ATTCGAG-3′) and EF1 α /exon6/r1 (5′-TGACCTCAGTGGTTACATT GGC-3′).

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