Insertional mutagenesis by the Tol2 transposon-mediated enhancer trap approach generated mutations in two developmental genes: tcf7 and synembryn-like

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Gene trap and enhancer trap methods using transposon or retrovirus have been recently described in zebrafish. However, insertional mutants using these methods have not been reported. We report here development of an enhancer trap method by using the Tol2 transposable element and identification and characterization of insertional mutants. We created 73 fish lines that carried single-copy insertions of an enhancer trap construct, which contained the zebrafish hsp70 promoter and the GFP gene, in their genome and expressed GFP in specific cells, tissues and organs, indicating that the hsp70 promoter is highly capable of responding to chromosomal enhancers. First, we analyzed genomic DNA surrounding these insertions. Fifty-one of them were mapped onto the current version of the genomic sequence and 43% (22/51) were located within transcribed regions, either exons or introns. Then, we crossed heterozygous fish carrying the same insertions and identified two insertions that caused recessive mutant phenotypes. One disrupted the tcf7 gene, which encodes a transcription factor of the Tcf/Lef family mediating Wnt signaling, and caused shorter and wavy median fin folds and pectoral fins. We knocked down Lef1, another member of the Tcf/Lef family also expressed in the fin bud, in the tcf7 mutant, and revealed functional redundancy of these factors and their essential role in establishment of the apical ectodermal ridge (AER). The other disrupted the synembryn-like gene (synbl), a homolog of the C. elegans synembryn gene, and caused embryonic lethality and small pigment spots. The pigment phenotype was rescued by application of forskolin, an activator of adenylyl cyclase, suggesting that the synbl gene activates the Gs pathway leading to activation of adenylyl cyclase. We thus demonstrated that the transposon-mediated enhancer trap approach can indeed create insertional mutations in developmental genes. Our present study provides a basis for the development of efficient transposon-mediated insertional mutagenesis in a vertebrate.

KEY WORDS: Zebrafish, tcf7, synembryn, Enhancer trapping, Insertional mutagenesis

INTRODUCTION

In zebrafish, phenotype-driven mutagenesis screens have been performed by two ways. First, chemical mutagenesis screens using ENU have identified hundreds of mutations that cause defects in various processes of embryonic development (Driever et al., 1996; Haffter et al., 1996). Identification of genes responsible for the mutant phenotypes requires positional cloning, which is still laborious (although sequencing and annotation of the genome are nearing completion). Second, a pseudotyped retrovirus has been used to mutagenize the genome (Gaiano et al., 1996). By using this approach, more than 300 recessive lethal mutants and the mutated genes have been identified (Amsterdam et al., 2004). However, a large-scale insertional mutagenesis screen using pseudotyped retrovirus is demanding in terms of labor and space because it requires raising of a large number of F2 families to identify mutant phenotypes in F3 embryos (Amsterdam et al., 1999).

Recently, gene trap and enhancer trap methods were developed in zebrafish. In gene trapping, a Tol2 transposon construct containing a splice acceptor and the GFP gene was constructed. When the construct was integrated within a gene and the splice acceptor trapped its transcript, GFP is expressed (Kawakami et al., 2004). In enhancer trapping, a Sleeping Beauty construct containing a modified EF1α promoter and the GFP gene (Balciunas et al., 2004), a Tol2 construct containing the keratin8 promoter and the GFP gene (Parinov et al., 2004), and a retroviral construct containing the gata2 promoter and the YFP gene (Ellingsen et al., 2005) were used. When the enhancer trap constructs were integrated in the genome and the minimal promoters were activated by enhancers, GFP or YFP is expressed in regulated fashions. It has been demonstrated that these methods can create transgenic fish expressing the reporter proteins in specific cells, tissues and organs, which are useful resources for developmental biology. However, it has not been reported that insertions of these gene trap or enhancer trap constructs can cause any observable mutant phenotype.

We found that the medaka fish Tol2 element encodes a fully functional transposase (Kawakami et al., 1998; Kawakami and Shima, 1999; Kawakami et al., 2000) and, since then, have been developing genetic methods in zebrafish by using Tol2 (Kawakami, 2005). Our goal is to develop an efficient transposon-mediated insertional mutagenesis method as follows. First, random integrations of a gene trap or an enhancer trap construct are created in the genome of the germ cells in the fish (F0) injected with a transposon-donor plasmid and the transposase mRNA. Second, F1 embryos exhibiting unique GFP expression patterns are collected and raised. Third, by mating male and female F1 fish that carry the patterns of different colors, F2 families are raised producing unique GFP expression patterns in different embryos. By genotyping these embryos, it will be possible to obtain the insertions of the tol2 transposon construct in the genome of F2 fish. Finally, by genotyping F2 fish, it will be possible to obtain the insertions of the tol2 transposon construct in the genome of F2 fish. Finally, by genotyping F2 fish, it will be possible to obtain the insertions of the tol2 transposon construct in the genome of F2 fish.
same insertion, F2 embryos are analyzed for the mutant phenotype. If an insertion disrupted an essential gene, homozygous embryos show a mutant phenotype. Finally, the gene responsible for the mutant phenotype can be cloned rapidly, as the locus is tagged by the transposon. Zebrafish researchers should benefit from this methodology because it will require maintenance of smaller numbers of F1 fish than chemical mutagenesis or retroviral mutagenesis, and the F2 screen will be carried out within a shorter period of time than the F3 screen.

As a first step toward this goal, it is important to demonstrate that a transposon-mediated gene trap or enhancer trap method can indeed create a mutant. In our previous gene trap screen, we created homozygous embryos by mating, but could not identify recessive phenotypes (Kawakami et al., 2004; Kotani et al., 2006). In addition, in the previous gene trap and enhancer trap screens using transposons and retrovirus, recessive mutant phenotypes have not been analyzed extensively (Balcianas et al., 2004; Ellingsen et al., 2005; Parinov et al., 2004). In the present study, we constructed an enhancer trap construct containing the zebrafish hsp70 promoter and the GFP gene, performed an enhancer trap screen, and established fish lines expressing GFP in specific cells and tissues. We then analyzed phenotypes of homozygous embryos by crossing these lines and found that insertions in the tcfl and the synembry-n-like (sybl) gene caused recessive mutant phenotypes. Tcfl is a transcription factor mediating Wnt signaling. Although the zebrafish tcfl gene was cloned previously (Veien et al., 2005), no mutation has been reported. Sybl is a zebrafish homolog of the C. elegans synembryn gene, the product of which has been shown to activate both the Goq and Gog pathway, leading to production of diacylglycerol and cyclic AMP, respectively (Miller et al., 2000; Schade et al., 2005). Previously, a mutation of sybl was identified by retroviral insertional mutagenesis (Amsterdam et al., 2004), but its phenotype has not been characterized in detail. We will describe characteristics of these mutants and demonstrate that insertions of the enhancer trap construct can indeed disrupt the function of developmental genes.

MATERIALS AND METHODS

Construction of T2KHG transgenic fish

T2KHG contains the hsp70 promoter (a kind gift from Dr J. Kuwada) (Halloran et al., 2000), the GFP gene and the polyA signal between the hsp70 promoter and hsp70 enhancer trap construct containing the zebrafish hsp70 promoter and the GFP gene, performed an enhancer trap screen, and established fish lines expressing GFP in specific cells and tissues. We then analyzed phenotypes of homozygous embryos by crossing these lines and found that insertions in the tcfl and the synembry-n-like (sybl) gene caused recessive mutant phenotypes. Tcfl is a transcription factor mediating Wnt signaling. Although the zebrafish tcfl gene was cloned previously (Veien et al., 2005), no mutation has been reported. Sybl is a zebrafish homolog of the C. elegans synembryn gene, the product of which has been shown to activate both the Goq and Gog pathway, leading to production of diacylglycerol and cyclic AMP, respectively (Miller et al., 2000; Schade et al., 2005). Previously, a mutation of sybl was identified by retroviral insertional mutagenesis (Amsterdam et al., 2004), but its phenotype has not been characterized in detail. We will describe characteristics of these mutants and demonstrate that insertions of the enhancer trap construct can indeed disrupt the function of developmental genes.

Computational analyses

The integration sites were mapped on the zebrafish genome sequence (Zv6) by BLAT (Kent, 2002). Fifty-one random insertions were created 10,000 times by using the computer system in DDBJ, NIG. The locations of mRNA and Ensemble transcripts were obtained from all_mrna.txt.gz and Ensemble transcripts were obtained from all_mrna.txt.gz (see Fig. 3A). A DNA/RNA mixture (1 nl) containing 30 ng/μl plasmid DNA harboring T2KHG and 5 ng/μl transposase mRNA synthesized in vitro was injected into fertilized eggs. F1 samples were washed in 66% formamide/2×SSCT at 65°C for 1 hour to over night. The purified with mini Quick Spin RNA Columns (Roche). Prehybridization and hybridization were performed at 65°C for 1 hour to over night. The signals were detected by using BCIP/NBT Color Development Substrate (Promega). The reaction was stopped by washing with PBS.

Southern blot hybridization and PCR analyses

Southern blot hybridization, inverse PCR, linker-mediated PCR, RT-PCR, 3’ RACE and 5’ RACE were carried out as described previously (Kawakami, 2004; Kotani et al., 2006). Primers used for these analyses are as follows.

**Southern blot hybridization**

HG2A: 5’-GAG GAG AAG AAG GGC CAT CTC ATT C-3’ (forward) and 5’-CTA CAT AAC ACT CTC GAA AAT GAT C-3’ (reverse)

HG3A: 5’-GTC CTG ACG TCA ATC TGT CAT G-3’ (forward) and 5’-CTG TAC TCG AGA ACT GTG A-3’ (reverse)

HG6A: 5’-TCC AGC ACT GAA TGA TGC AGA AAT G-3’ (forward) and 5’-TCA CAG TTG GGC AGC CAT GAA G-3’ (reverse)

HG6B: 5’-ATG TCT TCC AAC CAA GAC GCC ACC TC-3’ (forward) and 5’-GTC TAA TTC TCA CTG TAG TTC-3’ (reverse)

HG6C: 5’-AGG CGT TGT TAT GTC GGA AAA G-3’ (forward) and 5’-CTC GTG TGA GTA GTA GAG CGA-3’ (reverse)

HG6D: 5’-AGG CGT GAG TAC CAT CGT-3’ (forward) and 5’-CCT TTC CAT TCG AGA GTC T-3’ (reverse)

HG10A: 5’-CAG CGA TTG ACT GTT TTC CGC AAC-3’ (forward) and 5’-CTA CTC TGA GAT AAC AGA CTG TTG-3’ (reverse)

HG21A: 5’-GCA GAT TGA ACT CAT CAC TGC T-3’ (forward) and 5’-CAC TGA TCA GGC TTT TAT GCG AGF-3’ (reverse)

HG21B: 5’-CAG TGT GAT CCC ACG AGC TCC TTC-3’ (forward) and 5’-CTT CAT AGC ATC TAG CCC AGT AGA-3’ (reverse)

HG21C: 5’-GAC GTG TTC GAA AAG TTT GTT-3’ (tcfl-f1) and 5’-GCT TGG TCA GGT AGA CAG G-3’ (reverse)

HGn8H: 5’-GTC TGA GAC GAC TGA CAG TGT-3’ (synbl-f) and 5’-CCTC GAC GGC AGC TCA TCC TTC T-3’ (synbl-r2)

HGn43A: 5’-GTT TGA CCT GCT GCA TTA CCG A-3’ (forward) and 5’-TCA AGG GCT TTT CTG CGG GAG T-3’ (reverse)

**MO injection**

Antisense morpholino oligonucleotides (MO) (Gene Tools) against translation initiation sites of tcfl (tcfl-MO) and left (lef1-MO) and a splice donor of synbl (synbl-MO) were synthesized:

tcfl-t2: 5’-GTT TTT CCA AAC ATG TAT GAG TTT-3’

tcfl-t2: 5’-GAC TGT TTG TTA GTT TTC GGA G-3’

tcfl-t7: 5’-GAA CGA GCA GAT GAC CGG TTC-3’

**Whole-mount in situ hybridization**

Probes were synthesized with DIG RNA labeling kit (Roche), and purified with mini Quick Spin RNA Columns (Roche). Prehybridization and hybridization were performed at 65°C for 1 hour to over night. The samples were washed in 66% formamide/2×SSCT at 65°C for 30 minutes, in 33% formamide/2×SSCT at 65°C for 30 minutes, in 2×SSCT at 65°C for 15 minutes, and in 0.2×SSCT at 65°C for 30 minutes twice. The samples were then incubated in blocking solution (150 mM maleic acid, 100 mM NaCl, 5% blocking reagent (Roche), 5% new born calf serum, pH 7.5, 0.1% Tween-20) at 4°C overnight, then incubated with 1/4000-1/8000 volume of anti-digoxigenin-AP Fab-fragments (Roche) at room temperature for 4 hours or at 4°C overnight. Samples were washed in maleic acid buffer (150 mM maleic acid, 100 mM NaCl, 0.1% Tween-20, pH 7.5) at room temperature for 25 minutes three times and then overnight. The signals were detected by using BCIP/NBT Color Development Substrate (Promega). The reaction was stopped by washing with PBS.

**Pigmentation analysis and forskolin treatment**

Embryos were placed in the dark for at least 30 minutes and then observed soon after they were transferred onto the stage of a microscope. One-cell stage embryos were injected with 0.1-1 nl of 1-3 mg/ml MOs suspended in H2O using Femtotet (Eppendorf).

Embryos were then cultured for an additional 16 hours at 28°C.

**Development**

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RESULTS

An enhancer trap screen using the Tol2 enhancer trap construct

We constructed T2KHG that contained the zebrafish hsp70 promoter and the EGFP gene (see Fig. 3A). We expected that transcription from the hsp70 promoter to be activated at normal temperatures when it was influenced by chromosomal enhancers. We co-injected transposon-donor plasmid DNA containing the T2KHG construct and the transposase mRNA, crossed 77 injected fish with wild-type fish, and analyzed at least 40 embryos from each cross for GFP expression. We found that offspring (F1) from 70% (54/77) of the injected fish carried T2KHG insertions and showed more than 100 different GFP expression patterns. The GFP-positive F1 fish were raised and analyzed by Southern blot hybridization. The number of transposon insertions carried by individual F1 fish varied, from one to more than ten. Excluding fish with more than ten insertions, we analyzed 215 different transposon insertions. These fish were further outcrossed to establish fish lines with single insertions. In the course of the outcross, we observed a total of 125 unique GFP expression patterns (Table 1).

Cloning and identification of integration sites

In order to clarify relationship between the expression pattern and the insertion, we established 73 transgenic fish lines that carried single copy insertions of T2KHG (Table 1, Fig. 1) (see Table S1 in the supplementary material). This confirmed that these expression patterns were indeed caused by single T2KHG insertions. We treated some of these lines by heat shock and observed strong GFP expression throughout the body, indicating that the hsp70 promoters on T2KHG integrated in various loci were still capable of responding to heat shock (data not shown). Although these fish lines expressed GFP in temporally and spatially restricted fashions, most of them expressed GFP weakly in the heart at 24 hpf, and in the heart, skeletal muscle and lens at day 5. We performed whole-mount in situ hybridization and found that the hsp70 mRNA was detected in the same regions at normal temperatures (data not shown), indicating that these reflected the basal activity of the hsp70 promoter. We also noticed that the hsp70 promoter showed maternal expression.

We cloned and sequenced genomic DNA surrounding the 73 insertions. Fifty-one insertions were successfully mapped by BLAT against the zebrafish genome. The HG21C insertion disrupted the tcf7 gene encoding a transcription factor downstream of Wnt signaling (Fig. 1, Fig. 3B). Although the mRNA did not show such an expression pattern (ventral mesoderm; Fig. 2I,J), tcf7 (HG21C) and synbl (HGn8H) were expressed weakly in the whole body (data not shown). These were not similar to the GFP expression patterns. In these cases, the hsp70 promoter was likely to be influenced by enhancers that regulate expression of their neighboring genes. We will describe such an example in the case of HGn8H below.

<table>
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<th>Table 1. Summary of the enhancer trap screen</th>
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Expression patterns of genes at the integration sites

To compare the GFP expression patterns with expression patterns of the genes at the integration sites, we performed RT-PCR for eight genes identified in HG3A, HG6C, HG6D, HG10A, HG10B, HG21C, HGn8H and HGn43A. In all of these cases, cDNA clones were obtained, indicating that the genes were indeed transcribed. Furthermore, we performed RT-PCR for eight genes found at the integration sites in HG2A, HG6A, HG6B, HG21A, HG21B, HG21K, HGn15B and HGn54A, which are predicted genes based on either EST, GenScan or Ensembl transcripts. In five cases (HG2A, HG6A, HG6B, HG21A, and HG21B), RT-PCR products were amplified, indicating that these were transcribed. Taking these into account, 43% (22/51) of the insertions were localized within protein-coding transcribed genes (Table 1) (see Table S1 in the supplementary material).

Then, we performed whole-mount in situ hybridization using the chotl (HG2A), cyp2e2 (HG3A), ide (HG6A), soxlz (HG6B), uros (HG6C), asbl (HG6D), ripk2 (HG10B), lmo7 (HG21A), hg21b (novel gene; HG21B), tcf7 (HG21C) and synbl (HGn8H) probes. Expression of chotl (myotome; Fig. 2A,B), cyp2e2 (yolk; Fig. 2C,D), ide (myotome, Fig. 2E,F), soxlz (myotome; Fig. 2G,H), uros (ventral mesoderm; Fig. 2I,J), lmo7 (myotome; Fig. 2K,L), hg21b (otic vesicle; Fig. 2M,N) and tcf7 (median fin fold; Fig. 3B,C) recapitulated respective GFP expression patterns, at least partly, indicating that the hsp70 promoter was activated by enhancers that regulated those genes. By contrast, asbl (HG6D), synbl (HGn8H) and ripk2 (HG10B) were expressed weakly in the whole body (data not shown). These were not similar to the GFP expression patterns. In these cases, the hsp70 promoter was likely to be influenced by enhancers that regulate expression of their neighboring genes. We will describe such an example in the case of HGn8H below.

The HG21C insertion disrupted the tcf7 gene

To elucidate whether the T2KHG insertions can cause observable mutant phenotypes, we analyzed phenotypes of homozygous embryos for 54 insertions including 20 insertions mapped within transcribed regions (see Table S1 in the supplementary material). We found morphological defects in the progeny from HG21C and HGn8H heterozygous parents.

In HG21C, T2KHG was integrated within the tcf7 gene that encodes a transcription factor downstream of Wnt signaling (Fig. 3A, Fig. 5A). GFP fluorescence and gfp mRNA were detected in the dorsal retina, diencephalon, tail bud and median fin fold at 24 hpf (Fig. 1, Fig. 3B). Although tcf7 mRNA was detected in broad areas in the brain, gfp mRNA did not show such an expression pattern (Fig. 3B,C), suggesting that a putative brain enhancer of tcf7 did not influence the hsp70 promoter. We analyzed 568 embryos obtained from HG21C heterozygous parents (Fig. 3D-H). One hundred and fifty-four out of 420 GFP-positives, but none of 148 GFP-negatives, showed short and wavy median fin folds at 60-72 hpf. Then, we performed genotyping by PCR and found that all of 83 GFP-positives with the fin phenotype were homozygous and all of 139 GFP-positives without the fin phenotype were heterozygous (Fig.
These results strongly suggested that the observed fin defect is a recessive mutant phenotype caused by the transposon insertion. The length of the median fins was restored to nearly the wild-type level after day 6, but the wavy edge was observed at least until day 14 (data not shown). The homozygous fish were viable and fertile. In wild-type embryos, \\textit{tcf7} expression in the median fin fold was detectable after the 15-somite stage, was maintained through 24 hpf, then was gradually weakened at 36 hpf, and almost disappeared by 48 hpf (Fig. 3K, M). \textit{tcf7} is also expressed in the pectoral fin bud, strongly in the apical ectodermal ridge (AER) and weakly in the

**Fig. 1. GFP expression patterns in enhancer trap lines.** GFP expression patterns in 24 hpf to day 5 embryos carrying single T2KHG insertions. Numbers after HG represent individual founder fish. Letters after numbers represent distinct patterns obtained from single founder fish. The patterns and transposon integration sites are described in Table S1 in the supplementary material.
mesoderm (I,J), myotome (K,L) and otic vesicle (M,N).

were detected in myotome (A,B), yolk (C,D), myotome (E-H), ventral wild-type embryos at 24 hpf using probes indicated (top right). Signals indeed caused by a decreased Tcf7 activity, we injected 3S,T). To confirm that the observed defects in fin development were 84% of embryos injected with 0.1 ng and 0.3 ng tcf7-MO, respectively, exhibited shorter and wavy pectoral fins and median fin folds, which resembled the phenotype observed in the homozygous embryos (Fig. 3U-W).

Roles of Tcf7 and Lef1 in outgrowth of the pectoral fin

tcf7 and lef1, both members of the Lef/Tcf family of transcription factors that mediate Wnt signaling, are expressed in the fin bud and are thought to be functionally redundant (Veien et al., 2005). However, this notion has not yet been tested. Taking advantage of the tcf7 mutant, we injected lef1-MO into HG21C homozygous and heterozygous embryos to examine their possible functional redundancy in pectoral fin development. Fin outgrowth was severely reduced in 79% (45/57) of MO-injected homozygous embryos but not in MO-injected heterozygous embryos (0/74) (Fig. 4A-C), indicating that Lef1 compensated the loss of the Tcf7 activity and Tcf7 or Lef1 is required for fin outgrowth.

We then analyzed expression of tcf7 and lef1 in the pectoral fin bud at the AER induction (28 hpf) and maintenance (38 hpf) stages. The tcf7 expression was detected strongly in the AER and weakly in the mesenchyme at 28 hpf and 36 hpf (Fig. 4E,G). The unique expression of tcf7 in the AER at 36 hpf may account for the small and wavy fin phenotype observed in the HG21C homozygous embryos at later stages (Fig. 3S,T). To define the defects in lef1 and tcf7 loss-of-function embryos, we analyzed expression of mesenchymal (fgf10) and ectodermal (dlx2a, fgf24, wnt3l and fgf8) markers (Akimenko et al., 1994; Fischer et al., 2003; Norton et al., 2005; Reifers et al., 1998) in the lef1-MO-injected embryos. At 28 hpf, the mesenchymal fgf10 expression was similar in lef1-MO-injected wild-type and tcf7 mutant embryos (Fig. 4H,I). By contrast, expression of dlx2a in the AER was severely reduced in the lef1-MO-injected tcf7 mutant embryos; i.e. the expression was absent in about half of the injected embryos and detectable but very weak in the rest (n=11, Fig. 4J,K)., suggesting that AER induction was impaired in the lef1 and tcf7 loss-of-function embryos. At 38 hpf (48 hpf for fgf8), fgf10 was expressed normally in both lef1-MO-injected wild-type and tcf7 mutant embryos (Fig. 4LM), while expression of the AER markers dlx2a, fgf24, wnt3l and fgf8 was absent from the ectoderm in the MO-injected tcf7 mutants (n=6 each, Fig. 4L-U). The results obtained when wild-type or heterozygous embryos were used for MO injection were essentially indistinguishable (data not shown). From these results, we concluded that Lef7 and Tcf1 are functionally redundant during pectoral fin out growth and play essential role(s) both in the AER induction and maintenance stages.

Characterization of transcripts in the HG21C enhancer trap line

In HG21C, T2KHG was integrated in the coding region in the first exon of the tcf7 gene (Fig. 5A). To understand how the tcf7 gene was disrupted, we performed 3’ RACE using nested primers in the first exon. In the HG21C allele, the 3’ RACE products were stopped within the insertion (Fig. 5B). The longest transcript had a capacity to produce a truncated protein of 44 amino acids containing the N-terminal region of Tcf7, which is unlikely to be functional. Then, we performed RT-PCR using the f3 and r3 primers to detect possible transcripts that passed over the insertion. Two faint bands that were detected in HG21C homozygous embryos were cloned and sequenced. These bands represented abnormally spliced transcripts containing premature stop codons (Fig. 5C). As a transcript containing a wild-type sequence of the first exon could not be detected, the HG21C allele is likely to be null.

Furthermore, we analyzed how the hsp70 promoter is activated in the enhancer trap lines by 5’ RACE. Although the zebrafish hsp70 promoter has been a useful tool (Halloran et al., 2000; Uemura et al., 2005), the transcription start site has not yet been characterized. First, we prepared RNA from heat-shocked HG21C homozygous embryos, and obtained four 5’ RACE clones. Three of them contained the same A at the 5’ ends, which we designated as position +1, and the other contained A at –2 at the 5’ end (Fig. 5D). Second, we prepared RNA from HG21C homozygous embryos at normal temperatures, and sequenced two 5’ RACE clones. These contained A at +1 and +2 at their 5’ ends (Fig. 5D). Thus, the transcription start sites were nearly the same in both heat-shocked and non heat-
shocked conditions, indicating that the *hsp70* promoter on T2KHG was indeed activated by a putative tcf7 enhancer in the trap line. The 5′ RACE analysis did not amplify the longest 3′ RACE product probably because the smaller amount of transcripts that started from the tcf7 promoter. We also analyzed four 5′ RACE clones amplified from the HG2A and HG21B lines at normal temperatures. Similar to the 5′ RACE products from HG21C, three and one clones contained A at +1 and A at +2 as the 5′ ends, respectively. In the course of these analyses, we found an intron in the *hsp70* promoter fragment (Fig. 5D). We investigated EST sequences in the database, and found that the endogenous *hsp70* gene also contains an intron in the 5′ UTR.

**The HGn8H insertion disrupted the synembryn-like gene**

In HGn8H, T2KHG was integrated within the first intron of the *synembryn-like* (*synbl*) gene (Fig. 6A). We analyzed 485 embryos obtained from HGn8H heterozygous parents and found that 140
out of 370 GFP-positives, but none of 115 GFP-negatives, showed small pigment spots at day 2, developed edema at day 5, and were gradually degraded (Fig. 6B-F). Then we performed genotyping by PCR and found that all of 37 GFP-positives with the edema phenotype were homozygous and all of 42 GFP-positives without the edema phenotype were heterozygous (Fig. 6G,H), suggesting that the insertion created a recessive lethal mutation.

To determine how the insertion affected the synbl transcript, we performed RT-PCR using the synbl-f and r2 primers. In HGn8H homozygous embryos, the RT-PCR product was not detected (Fig. 6I), indicating that the HGn8H insertion interfered with the synbl expression nearly completely. To confirm that the mutant phenotype was caused by a decreased Synbl activity, we injected synbl-MO into wild-type embryos at the one-cell stage. Eighty percent (105/131) of the MO-injected embryos exhibited small pigment spots, which were similar to the HGn8H mutant phenotypes (Fig. 6S). All of the injected embryos formed edema and were gradually degraded. From these results, we concluded that the HGn8H mutant phenotype was caused by the decreased Synbl activity. In the case of HGn8H, we could not detect a transcript that stopped within the insertion either by 3′ RACE or RT-PCR (data not shown).

In HGn8H, GFP and gfp mRNA was expressed in the anterior ventral diencephalon, midbrain and spinal cord at 24 hpf (Fig. 6J,K). By contrast, the synbl mRNA was accumulated weakly throughout the body (Fig. 6L). To explain this discrepancy, we hypothesized that...
a putative enhancer that regulates a neighboring gene activated the hsp70 promoter. To test this hypothesis, we cloned cDNA of the rfx4 gene that was located ~5 kb upstream of synbl (Fig. 6A). rfx4 encodes a winged helix transcription factor RFX4 which is essential for brain morphogenesis in mice (Blackshear et al., 2003). We found that the rfx4 mRNA was accumulated in the anterior ventral diencephalons and the spinal cord (Fig. 6M), where the strong gfp expression was detected. This result suggested that an rfx4 enhancer activated the hsp70 promoter.

**Rescue of the synbl phenotype by forskolin**

In mammals, two synembryn homologs, RIC8A and RIC8B, have been identified (Klattenhoff et al., 2003; Tall et al., 2003). The zebrafish genome also contains another synembryn homolog (ric8a). A phylogenetic analysis showed the zebrafish synbl gene is closer to the mammalian RIC8B gene (Fig. 6N). We cloned the zebrafish ric8a cDNA (AB354735), analyzed its expression and found that it is expressed rather weakly (Fig. 6O,P). The stronger and broader expression of synbl may account for its essential role.
Mutagenesis by enhancer trapping

hsp70 transcription activity. We are currently dissecting the zebrafish promoter exhibited the highest enhancer trap activity. A possible shown that rat RIC8A is a GTP exchange factor for G (Miller et al., 2000; Schade et al., 2005). Biochemical studies have that the observed small pigment spot is caused by a decrease in the cAMP level (Logan et al., 2006). These prompted us to hypothesize that the high responsiveness to enhancers, may suggest that a similar mechanism also operates the zebrafish hsp70 promoter. It is interesting that, unlike Drosophila and human, the zebrafish hsp70 promoter is TATA-less as there is no TATA sequence upstream of the identified transcription start site. Based on the information about the transcription start site as well as the intron in the 5' UTR, we aim to construct improved versions of enhancer trap constructs with minimum basal activities.

We found that, in the case of HGN8H, the hsp70 promoter on T2KHG integrated in the synbl gene was probably activated by an enhancer of its neighboring gene: rfx4. A similar phenomenon has been described also in retroviral enhancer trapping (Kikuta et al., 2007). How was the hsp70 promoter affected by an rfx4 enhancer but not by a synbl enhancer? How did the rfx4 enhancer affect the hsp70 promoter while not affecting the synbl promoter in a wild-type condition? Studies on the rfx4 and synbl enhancers and promoters may illuminate an unknown mechanism that governs specificity between enhancer and promoter.

Roles of Tcf7 and Lef1 in AER formation

We found that Tcf7 and Lef1 are essential for expression of AER markers in the ectoderm in the early and late stages of the pectoral fin development. It has been shown that, in the early limb/fin induction stage, Wnt2b, which is expressed in the lateral plate mesoderm (LPM), activates expression of fgf10 in the mesenchyme of the limb/fin buds in chicken and zebrafish (Kawakami et al., 2001; Ng et al., 2002). In mouse, although Wnt2b expression was not detected in LPM, Fgf10 expression became weaker in Lef1−−;Tcf7−− embryos and it was suggested that signaling mediated by unidentified Wnt(s) is required to maintain normal levels of Fgf10 expression (Aagarwal et al., 2003). By contrast, our present study indicated that Lef1 and Tcf7 are not required for the mesenchymal fgf10 expression in zebrafish. Thus, components of Wnt signaling involved in Fgf10 induction are species specific and additional Tcf genes may compensate the loss of tcf7 and lef1 in zebrafish. In addition, our loss-of-function study suggested that the Wnt signaling in the ectoderm mediated by Lef1 and Tcf7 is essential for AER maintenance. This notion is consistent with the previous observations that Wnt3a-mediated β-catenin-dependent signaling activates expression of AER markers in the chicken limb ectoderm (Kawakami et al., 2001; Kengaku et al., 1998) and mouse Lef1−−;Tcf7−− embryos exhibit a defect in limb development (Galecer et al., 1999).

As tcf7 is exclusively expressed also in the dorsal retina, it can be speculated that Tcf7 may have a unique role also in this area (Fig. 3B,C) (Veien et al., 2005). At present, however, we have not detected any obvious defects in the dorsal retina (M. Yamaguchi, I. Masai, E. S. Veien and R. Dorsky, personal communications). It has been shown that the same factors, such as Dlx genes, fgf24 and sp9, are expressed both in the AER and the edge of the median fin fold (Abe et al., 2007). Although a mechanism that regulates outgrowth of the median fin folds is largely unknown, we noticed that outgrowth of the median fins was also impaired in the lef1 and tcf7 loss-of-function embryos (data not shown), suggesting that similar Wnt and Fgf signaling pathways regulate development of both pectoral fins and median fin folds.
The zebrafish *synembryn-like* gene activates the G<sub>G</sub>q pathway

It has been shown that *synembryn* and its mammalian homologs are involved in activation of G<sub>G</sub>q proteins (Klattehoff et al., 2003; Miller et al., 2000; Schade et al., 2005; Tall et al., 2003). Recently, in zebrafish, it was shown that dispersion of melanosomes is enhanced by activation of adenyl cyclase (Logan et al., 2006). Our study established a link between these two processes. The disruption of the *synbl* function caused aggregation of melanosomes, which could be restored by activation of adenyl cyclase. Therefore, it is reasonable to postulate that the *synbl* gene, a homolog of mammalian RIC8B, is involved in activation of the G<sub>G</sub>q pathway, leading to activation of adenyl cyclase and dispersion of melanosomes. The *synbl* gene may be involved in regulation of pigmentation in wild-type conditions, such as a physiological color change during background adaptation. However, the edema phenotype and the embryonic lethality were not rescued by the forskolin treatment. The concentration of forskolin may not be high enough to rescue those phenotypes, or alternatively, those phenotypes may be caused by failures in activation of other G proteins, such as G<sub>G</sub>q, which is known to bind to human RIC8B in vitro (Klattehoff et al., 2003).

Insertional mutagenesis by enhancer trapping

In this study, we isolated two phenotypic mutants out of 54 enhancer trap insertions. Although this frequency is not far superior to that with retroviral insertional mutagenesis in which one in 80 insertions causes embryonic lethality (Amsterdam et al., 1999), we think insertional mutagenesis by enhancer trapping should have the following merits. First, only a small number of F1 fish that show interesting GFP expression patterns need to be raised. Second, as heterozygous fish carrying same insertions can be identified in the F1 generation, it is not necessary to raise a large number of F2 fish, and, instead, homozygous phenotypes can be detected by analyzing F2 embryos. Third, as the place to be analyzed is illuminated by GFP, subtle morphological defects, such as fin phenotypes in the F2 embryos. Finally, as insertions are ‘visible’, carriers can be easily maintained without time-consuming genotyping. We demonstrated that our enhancer trap construct can be integrated within transcribed regions at a relatively high frequency. It is higher than that calculated for retroviral enhancer trap insertions, although it is not statistically different from that calculated for random integration. This finding opened a possibility that insertional mutagenesis could be performed more efficiently if an enhancer trap construct that can disrupt the function of target genes more efficiently was developed; for example, development of enhancer trap constructs carrying more elements to interfere with endogenous transcripts, such as a splice acceptor plus a polyA signal, etc. Studies along this line are in progress in our laboratory. In conclusion, our present study provided a basis for the development of efficient transposon-mediated insertional mutagenesis in a vertebrate.

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**Supplementary material**

Supplementary material for this article is available at http://dev.biologists.org/cgi/content/full/135/1/159/DC1

**References**


Mutagenesis by enhancer trapping

RESEARCH ARTICLE


