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Probing Pineal-specific Gene Expression with Transgenic Zebrafish†

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Abstract

The pineal gland of zebrafish (*Danio rerio*) contains lightsensitive photoreceptor cells and plays an important role in the neuroendocrine system. The zebrafish *exorhodopsin* gene encodes a pineal-specific photoreceptive protein, whose promoter region harbors a *cis*-acting element, pineal expression-promoting element (PIPE), directing pineal-specific gene expression. For *in vivo* genetic studies on PIPE-binding proteins and their regulatory mechanisms, we generated a transgenic zebrafish line, *Tg(P_{20-rh}/P:gfp)*, that expresses green fluorescent protein (GFP) under the control of the zebrafish *rhodopsin* promoter fused with 20 PIPE repeats. In *Tg(P_{20-rh}/P:gfp)* fish, PIPE-dependent gene expression is visualized by GFP fluorescence in the pineal gland along with PIPE-independent GFP signals in the retinal rod photoreceptors. The transgenic fish exhibit detectable and reproducible GFP fluorescence in the larval pineal gland by 5 days postfertilization. Antisense morpholino-mediated knock-down of a pineal transcription factor gene, *otx5*, suppresses pineal GFP expression in the transgenic line. In a pilot screen of *N*-ethyl-*N*-nitrosourea-treated fish of the GFP transgenic line, we isolated potential dominant mutations that cause attenuation of pineal GFP fluorescence with a marginal effect on the retinal GFP signal. The results suggest that the *Tg(P_{20-rh}/P:gfp)* line will be useful for detecting deficits in PIPE-dependent gene expression in the pineal gland.

INTRODUCTION

In many vertebrates, the pineal gland is a photosensory organ located on the dorsal surface of the brain that rhythmically produces melatonin, an indoleamine hormone involved in the regulation of biological rhythms (1–5). Pineal melatonin production is regulated by light and/or by an intrinsic circadian clock. This regulation involves pineal-specific genes coding for proteins such as aryl-alkylamine *N*-acetyl transferase (AANAT), an enzyme necessary for pineal melatonin synthesis (6), and in some animals (birds and reptiles) pinopsin, a photoreceptive protein (opsin) first found in the chicken pineal gland (7,8). A pineal-specific opsin gene found in ray-finned fish (9) codes for exorhodopsin (*exorh*) and is a duplicated form of the rhodopsin (*rho*) gene. In zebrafish, *exorh* is expressed specifically in the pineal gland, while *rho* is specific to the retinal rod photoreceptors (9).

We recently showed that the pineal-specific expression of *exorh* requires two kinds of *cis*-acting elements in its promoter (10). One is the putative binding site of a conerod homeobox protein, Crx (11,12). Protein members of this Crx/Otx gene family are involved not only in

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pineal-specific expression but also in retinal rod expression (13–16). The other *cis*-element in the *exorh* promoter is a novel 12-bp sequence, which we named pineal expression-promoting element (PIPE) (10). Importantly, introduction of PIPE into the zebrafish *rho* promoter, which contains Crx/Otx-binding sites but lacks PIPE, results in the induction of ectopic gene expression in the zebrafish pineal gland (10). These studies suggest that multiple *cis*-acting elements—Crx/Otx-binding sites and PIPE—synergistically mediate pineal-specific gene expression (10,16).

PIPE-binding transcription factor(s) should play a key role in pineal-specific gene expression, but they remain unidentified. In the present study, we established a transgenic line to visualize PIPE-dependent gene expression *in vivo* by green fluorescent protein (GFP) fluorescence in the zebrafish pineal gland. We utilized this transgenic line in the knock-down experiment of a pineal transcription factor as well as in a genetic screen of relevant mutations. The results suggest that this transgenic line will be useful for studies of the molecules and mechanisms underlying pineal-specific gene expression.

MATERIALS AND METHODS

Animals

Zebrafish (EkkWill strain) were reared on a 14/10 h light-dark cycle in fish water containing 2 g of Instant Ocean salts (Aquarium Systems, Mentor, OH) per gallon of distilled water. Embryos were raised at 28.5°C as described previously (17) and staged according to the literature (18).

Transgene construction

The *rh/P:gf*p plasmid (Fig. 1A, middle), renamed from Rh(-1084)/PIPE (10), was modified to introduce an *I-SceI* meganuclease site and multiple PIPE repeats upstream of the rhodopsin (*rho*) promoter as follows: A pair of oligonucleotides, 5'-CGTCTGACCCCAATCTGA-3' and 5'-GACGTCAGATTGGGGTCA-3', containing the PIPE sequence were annealed to each other. This double-strand oligonucleotide (1× PIPE) was then phosphorylated by T4 polynucleotide kinase and ligated by T4 DNA ligase, resulting in the formation of a mixture of DNA fragments containing variable numbers of PIPE repeats. The DNA mixture was inserted into the *SacI* site upstream of the *rho* promoter in the *rh/P:gf*p plasmid. From the resulting pool, a plasmid containing 20 PIPE repeats (20× PIPE) was selected by DNA sequencing. The plasmid was modified by a double-strand oligonucleotide insertion into the *Bss*III site to introduce an *I-SceI* site just upstream of the 20× PIPE repeats. The resulting plasmid, *P₂₀-rh/P:gf*p (Fig. 1A, bottom), was used to generate transgenic lines.

Generation of transgenic lines

Transgenic lines were established by *I-SceI*-mediated transgenesis according to the literature (19,20). Briefly, a mixture of the *P₂₀-rh/P:gf*p plasmid and *I-SceI* meganuclease (New England Biolabs, Ipswich, MA) was injected into one- or two-cell-stage zebrafish embryos, which were then raised to adulthood. From this pool, three founder (F0) fish having the transgene in their germ cells were selected by PCR-mediated genotyping of their F1 offspring. Among the three transgenic lines, the one exhibiting the strongest GFP signal in the pineal gland was used in the following analyses.

Targeted gene knock-down by antisense morpholino injection

Antisense morpholino oligonucleotides (MOs) were purchased from GeneTools, LLC (Philomath, OR) or Open Biosystems (Huntsville, AL). *otx5*-MO (5'-CATGACTAACTCTCTCTCTCTC-3') (14) was used to repress *otx5* translation. The

standard control MO (5'-CCTCTTACCTCAGTTACAATTTATA-3') was used as a negative control. One-cell-stage embryos were injected with 0.5–1.0 nL of 1.0 mg mL⁻¹ MO in 0.05% phenol red and then raised at 28.5°C. For examination of retinal GFP fluorescence, some of the larvae were raised in 0.003% 1-phenyl-2-thiourea (PTU) in fish water to prevent melanin pigment formation in the retinal pigment epithelium.

ENU mutagenesis

Adult males of homozygous *Tg(P_{20-rh}/P:gfp)* fish were mutagenized according to the literature (21,22) with some modifications: Briefly, 20 males, 5–6 months old, were treated with 3 mM *N*-ethyl-*N*-nitrosourea (ENU; Sigma, St. Louis, MO) at 21°C for 60 min four times in total (once a week) and then kept a month for recovery. Thirteen out of the 20 mutagenized fish survived. The mutagenized males were crossed with nonmutagenized (wild-type) homozygous *Tg(P_{20-rh}/P:gfp)* females to produce a F1 offspring pool. At 5 days postfertilization (dpf), the F1 larvae were subjected to microscopic selection using pineal GFP fluorescence as the screening index, while the retinal rod GFP was used as the reference index. Those exhibiting morphological abnormalities (1.9% of total F1 larvae) were excluded from the selection. Selected F1 larvae were raised to adulthood, and crossed with homozygous *Tg(P_{20-rh}/P:gfp)* fish to obtain the F2 offspring. F2 larvae were examined for pineal GFP. If ~50% of the F2 offspring from a single F1 fish showed suppressed GFP, the F1 fish was identified as a candidate for a mutant having a deficit in PIPE-dependent pineal transcription.

Microscopic observation

The transgenic larvae were anesthetized with 0.6 μM tricaine methanesulfonate (MS-222; Sigma) in fish water, and transferred to a plastic dish filled with a 0.1% agarose suspension in fish water. GFP expression in the larvae was examined under a fluorescence dissection microscope MZFLIII (Leica, Wetzlar, Germany) equipped with a digital still camera COOLPIX 4500 (Nikon, Tokyo, Japan).

RESULTS

Establishment of PIPE-dependent GFP transgenic line

We previously identified a pineal-specific *cis*-acting element, PIPE, in the zebrafish *exorh* promoter and established a transgenic line expressing GFP under the control of the 1 kb *rho* promoter inserted with a single PIPE sequence (*rh/P:gfp*, Fig. 1A) (10). This transgenic animal exhibited some GFP fluorescence in the pineal gland (Fig. 1C) in addition to retinal rod GFP (Fig. 1B). The *Tg(rh/P:gfp)* line could be useful for *in vivo* assays of PIPE-dependent gene expression, though a problematic feature of this line is that the pineal GFP fluorescence is highly variable among individuals: Some fish show distinct GFP signals in the pineal gland whereas others have extremely weak pineal GFP perhaps due to insufficient transcriptional activation by a single artificial PIPE sequence. We reasoned that this problem could be overcome by increasing the number of PIPE sequences in the transgene promoter. To this end, we established another transgenic line, designed to express GFP in the pineal gland under the control of multiple PIPE sequences (*P_{20-rh}/P:gfp*, Fig. 1A). In the new transgenic line, *Tg(P_{20-rh}/P:gfp)*, pineal GFP was usually first detectable at 3 dpf (Fig. 2C) and it became stronger and less variable at 4 and 5 dpf (Fig. 2D,E). The pineal GFP at 5 dpf in the *Tg(P_{20-rh}/P:gfp)* larvae (Fig. 1E) was much brighter in fluorescence intensity, and the number of GFP-positive cells was greater than that in the *Tg(rh/P:gfp)* line (Fig. 1C). Retinal GFP fluorescence, on the other hand, was comparable between the two lines (Fig. 1B,D). By 5 dpf, all the tested homozygous larvae had distinctly higher GFP fluorescence signals in the pineal gland, showing that multiple PIPE sequences significantly increase pineal GFP expression.

Validation of the transgenic line by knock-down experiments

Otx5, a member of the Crx/Otx subfamily of homeobox proteins, is involved in gene expression in the zebrafish pineal gland (14). It is thought to bind to the putative Crx/Otx-binding sequences in the zebrafish *exorh* promoter to enhance the pineal transcription of *exorh* in combination with PIPE (10). In fish with the transgene *P_{20-rh}/P:gfp*, as well as with the *rh/P:gfp* (10), Otx5 binding to the Crx/Otx-binding sequences in the *rho* promoter region should promote pineal GFP expression in conjunction with PIPE-mediated transcriptional activation. To test the feasibility of using the *Tg(P_{20-rh}/P:gfp)* line for the study of pineal transcription machinery, Otx5 function in transgenic larvae was inhibited by antisense morpholino-mediated knock-down. Injection of an antisense morpholino against *otx5* (*otx5*-MO) largely suppressed pineal GFP expression in *Tg(P_{20-rh}/P:gfp)* larvae at 3 dpf (Fig. 3A). A slight decrease in retinal GFP expression was also detected (Fig. 3B), being consistent with the contribution of Otx5 to retinal rod gene expression (14).

A pilot screen for genetic mutants using the *Tg(P_{20-rh}/P:gfp)* line

One approach to identify PIPE-binding transcription factor(s) is *via* a forward genetic screen seeking fish with abnormal pineal GFP signals. For such genetic screening, the *Tg(P_{20-rh}/P:gfp)* line is ideal because (1) the pineal GFP is mediated by PIPE in the transgene and (2) the retinal rod GFP can be used as a control to show that the mutation has no general effect on transcription and that the GFP transgene itself is intact. To test this strategy, the *Tg(P_{20-rh}/P:gfp)* line was used in a pilot screen for dominant pineal mutants. Twelve ENU-mutagenized *Tg(P_{20-rh}/P:gfp)* males were crossed with nonmutagenized *Tg(P_{20-rh}/P:gfp)* females to obtain an F1 offspring pool. A total of 4010 F1 larvae were microscopically examined for pineal GFP at 5 dpf, resulting in the isolation of 58 F1 larvae exhibiting significantly suppressed pineal GFP fluorescence. The F1 fish were then tested for genetic transmission of the suppressed pineal GFP signal to F2 offspring. Six of the F1 fish transmitted the phenotype (Fig. 4). We consider the six F1 fish as candidates who have mutations affecting PIPE-dependent pineal transcription.

DISCUSSION

In the present study, we established a transgenic line to visualize by *in vivo* GFP fluorescence PIPE-dependent gene expression in the zebrafish pineal gland. We utilized this transgenic line, *Tg(P_{20-rh}/P:gfp)*, for a knock-down experiment of Otx5 as well as for a pilot screen seeking mutants of PIPE-mediated pineal transcription.

One of the unanswered questions regarding pineal-specific transcription is the identity of PIPE-binding transcription factor(s). One approach to this problem is by screening appropriate protein libraries using the DNA sequence of PIPE as bait (e.g. yeast one-hybrid screening). This *in vitro* approach is highly sensitive, but often yields many false positives. Candidate proteins thus need to be validated in appropriate physiologic tests. The *Tg(P_{20-rh}/P:gfp)* line could be useful for such *in vivo* validation in combination with the knock-down of candidate proteins. An alternative approach is by marker-assisted screening of genetic mutants by using GFP-transgenic animals to visualize protein–DNA interactions. Zebrafish forward-genetics has been a powerful tool for studies of a variety of biologic phenomena (23,24), and could also be useful for analyzing the biochemical mechanisms underlying tissue-specific gene expression. In this study, we adopted marker-assisted forward genetics utilizing the GFP transgenic line in order to detect biochemical DNA–protein interactions. In our pilot mutant screen, we successfully identified potential carriers of dominant mutations that attenuate GFP fluorescence in the pineal gland. A problem with this approach is that identification of the mutated genes can be time-consuming and labor-intensive, but this

potentially could be overcome by a combination of the current strategy with transposon-mediated insertional mutagenesis recently developed in zebrafish (25).

In conclusion, the current study suggests that the transgenic line, *Tg(P_{20-rh}/P:gfp)*, will be useful for the detection of deficits in PIPE-dependent gene expression and thus could contribute to studies of the molecular machinery underlying pineal-specific gene expression.

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Figure 1.

GFP transgenic lines exhibiting PIPE-dependent pineal fluorescence. (A) DNA constructs to generate GFP transgenic lines. The 1 kb promoter of the zebrafish *rhodopsin* gene (red, upper) was modified so as to generate a single PIPE sequence (blue) in the *rh/P:gfp* construct (middle) (10). The *rh/P:gfp* was further modified by the addition of 20 repeats of PIPE to generate the *P₂₀-rh/P:gfp* used in this study (lower). (B–G) Comparison of pineal GFP expression between GFP transgenic lines. Fluorescent images of a *Tg(rh/P:gfp)* larva (B, C), a *Tg(P₂₀-rh/P:gfp)* larva (D, E) and a nontransgenic larva (F, G) from the lateral (B, D, F) or dorsal (C, E, G) views. Magnified images of fluorescence in the pineal glands of the transgenic larvae are shown in the insets (C, E).

Figure 2.

Developmental change in GFP expression in a *Tg(P₂₀-rh/P:gfp)* larva. Bright-field and fluorescent images of a typical *Tg(P₂₀-rh/P:gfp)* larva (A–E) and a nontransgenic larva (F–J) were obtained by lateral views at 1 dpf (A, F), 2 dpf (B, G), 3 dpf (C, H), 4 dpf (D, I) and 5 dpf (E, H). These larvae were also photographed dorsally at 2–5 dpf (B'–E', G'–J'). Note that the pineal GFP fluorescence in the transgenic animal (arrowheads) was first detected at 3 dpf and increased in intensity and in number of stained cells up to 5 dpf. GFP fluorescence in the retinal rod photoreceptors (arrows) was detected through the ocular lens *via* a lateral view. Scale bars, 500 μ m.

Figure 3.

Knock-down experiment of a pineal transcription factor in the *Tg(P_{20-rh}/P:gfp)* line. The transgenic embryos were injected with *otx5*-MO (A and B, 0.5 ng) or control MO (C and D, 0.5 ng) at the one-cell stage and examined for GFP fluorescence in the pineal gland (A' and C', arrowheads) and retina (B' and D', arrows) at 3 dpf. The embryos shown in panels (B) and (D) were raised in 1-phenyl-2-thiourea to visualize better retinal GFP fluorescence by inhibiting the pigment formation.

Figure 4.

Suppressed GFP fluorescence in the pineal gland of the mutant line. (A, B) GFP fluorescence in the pineal gland was dorsally viewed in an F2 larva from one of the mutant lines (A) and in a control larva (B) at 5 dpf with the anterior to the top. (C) Intensity of the pineal GFP was measured in each of 20 F2 larvae of the mutant line (red dots) and 20 control larvae (blue dots). The fluorescence intensity was normalized to the averaged value of the control larvae. Note that a significant number of the F2 larvae from the mutant line (red) have lower fluorescence intensity in the pineal gland than the control larvae (blue). F2 larvae from the other five mutant lines also showed a similar phenotype in the pineal GFP fluorescence.