Identification of cis-Acting Elements Repressing Blue Opsin Expression in Zebrafish UV Cones and Pineal Cells

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Opsin genes are expressed in a cell type-specific manner in the retina and the pineal organ for visual and nonvisual photoreceptive purposes, but the regulatory mechanism behind the tissue and cell selectivity is not well understood. In this study, we focus on the expression regulation of the blue-sensitive opsin gene SWS2 of zebrafish by taking a transgenic approach using the green fluorescence protein as an expression reporter. The zebrafish SWS2 is a single-copy gene and is expressed specifically in the "long single cones" in the retina. We found the following. 1) A 0.3-kb region between 0.6 and 0.3 kb 5’ of the SWS2 initiation codon, encompassing four cone-rod homeobox-binding sites (OTX sequences), contains the region necessary and sufficient to drive gene expression in long single cones. 2) A 15-bp portion (−341 to −327) in the 0.3-kb region represses the gene expression in the "short single cones," which are dedicated to the UV-sensitive opsin gene SWS1. 3) An 11-bp sequence TAACTGCCAGT (−441 to −431) in the 0.3-kb region, with its adjacent OTX element, also works as a repressor for gene expression in the pineal cells. 4) Finally, this OTX site is necessary for expression repression in the bipolar cells in the retina. These findings open a way for understanding the complex interaction of positive and negative regulatory factors that govern the cell type specificity of the opsin gene expression in the photoreceptive cells in the retina and the pineal organ. We termed the novel 11-bp sequence as the pineal negative regulatory element, PINE.

Vertebrate retina contains two types of visual photoreceptor cells, rods and cones, the former responsible for dim light vision and the latter for bright light and color vision. The vertebrate visual opsins are classified into the following five phylogenetic types that originated before the vertebrate radiation: one produced in rod cells (RH1 or rhodopsins) and the other four produced in cone cells with different spectral sensitivity, red (M/LWS), green (RH2), blue (SWS2), and UV types (SWS1) (1). The combined output from the different spectral types of the cones allows an animal to perceive color. Although the selective expression of an opsin gene in a given cone cell is a basis of forming color vision, its regulatory mechanism achieving the cell type specificity remains less understood compared with the mechanism of rod-specific expression studied for the RH1 type opsin gene (2, 3).

Non-mammalian vertebrates have multiple extra-ocular photosensors, mainly localized in the pineal complex (4) that secretes melatonin under a regular day/night cycle, the phase of which can be shifted upon its photoreception (5). Several non-visual opsin genes have been found expressed in pineal cells of non-mammalian vertebrates, such as pinopsin (6), exo-rhodopsin (exrho) (7), and parapinopsin (8). It has been found, however, that some visual opsin genes are also expressed in these pineal cells (9−11). A physiological study has shown that the M/LWS opsin is indeed involved in melatonin suppression in zebrafish pineal organ (12). Both the retina and the pineal organ are ontogenetically derived from the diencephalon and, in non-mammalian vertebrates, display many similarities in tissue and cellular morphology and biochemical properties (13). Thus, study of expression regulation of visual opsins, especially of cone opsins, is important for understanding not only the visual system but also the general photoreception system in vertebrates.

The cone-rod homeobox (Crx) protein, a member of the Otx family of the paired-like homeodomain proteins, binds the OTX core consensus sequence (GATTA) and is one of a key trans-acting regulatory factor responsible for the gene expression in the retina and pineal organ (14, 15). The mammalian Crx is produced predominantly in both the retinal photoreceptors and pineal cells and regulates expression of retinal photoreceptor-specific genes and of pineal organ-specific genes (14−17). In zebrafish, Otx5, a paralog of Crx, is produced in the retina and pineal organ and regulates genes that show circadian expression in the pineal organ (18). Although important, the Crx/Otx family alone is not sufficient for establishing the tissue- and cell-selective expression in the retina and pineal organ and presumably requires complex interactions with its variations of binding sites and other transcription factors (19−21).

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The abbreviations used are: RH1, rod opsin; GFP, green fluorescence protein; LSC, long single cones; Crx, cone-rod homeobox; SSC, short single cones; PINE, pineal negative regulatory element; M/LWS, red-sensitive cone opsin; RH2, green-sensitive cone opsin; SWS2, blue-sensitive cone opsin; SWS1, UV-sensitive cone opsin; dpf, days post fertilization; LCR, locus control region.
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The zebrafish is an ideal animal model for the study of tissue and cell selectivity of the opsin gene expression because it has a total of eight cone opsin genes with distinct spectral sensitivity in addition to the rod-specific RH1 and the pineal organ-specific exorh: the UV-sensitive SWS1, the blue-sensitive SWS2, the four green-sensitive RH2 subtypes (RH2-1, RH2-2, RH2-3, and RH2-4), and the two red-sensitive M/LWS subtypes (LWS-1 and LWS-2) (7, 22). Each cone opsin type is produced in a morphologically distinct type of cone cells in the retina as follows: SWS1 in the short single cones (SSCs), SWS2 in the long single cones (LSCs), the four RH2 subtypes in the short members of double cones, and the two M/LWS subtypes in the long members of double cones (23–25). The regulatory region of the opsin genes has been intensively studied on zebrafish with its feasibility to employ transgenic technology using a living color reporter such as the green fluorescent protein (GFP) (26–31). But core elementary sequences remain to be specified in the cone opsin regulatory regions identified thus far, unlike the case of RH1 and exorh. Regarding exorh, a cis-acting element, designated PIPE, has been identified as a pineal organ-specific expression-promoting element, together with three Crx/Otx-binding sites, in its proximal 147-bp promoter (28).

In this study, we focused on the zebrafish blue-sensitive opsin gene, SWS2, which is a single-copy gene expressed in the LSCs in the retina. By taking the transgenic approach using the GFP reporter, we identified for the first time the cis-acting elements specific for a cone visual opsin gene and also found these elements to work repressively in other cell types in the retina, i.e., SSCs and bipolar cells, and in pineal cells, thus achieving tissue and cell specificity of the cone opsin expression.

EXPERIMENTAL PROCEDURES

PAC Clone—Through the screening service of the RZPD German Resource Center for Genome Research of a zebrafish PAC library (number 706, originally created by C. Amemiya), a clone DNA (BUSMP706EI9271Q9), designated SWS2-LWS-PAC-E here, was obtained using the LWS-2 cDNA as a probe (22), which encompasses the SWS2, LWS-1, and LWS-2 in its ~87-kb insert. The nucleotide sequence of the SWS2-LWS-PAC-E corresponds to the nucleotide position 22684531–22771857 of the chromosome 11 in the Ensembl zebrafish genomic assembly version 7.

GFP Expression Constructs and Microinjection—The pEGFP-1 plasmid (Clontech) was used as the source of the GFP gene to assemble GFP expression constructs. The 5’-flanking sequences of the zebrafish SWS2 were obtained by PCR from the SWS2-LWS-PAC-E clone in the cases of ~5.2-, ~3.5-, and ~1.4-kb and ~404-bp constructs (Fig. 1A). The ~582- and the ~300-bp constructs (Fig. 1A) were prepared by PCR from the ~3.5-kb construct. The PCR primers were designed on the basis of the 5.2-kb 5’ sequence of the zebrafish SWS2 (30) (GenBank™ accession number AY512504) and are listed in Table S1. The ~1.1-kb construct (Fig. 1A) was prepared by digesting the 3.5-kb construct with EcoRI. The coinjection DNA fragments in Fig. 1F were PCRRed from the SWS2-LWS-PAC-E clone using the primers listed in Table S2. The SWS2up1.4kb:EGFP (Fig. 1F) was generated by digesting the GFP construct with the 5.5-kb 5’-flanking region of the zebrafish SWS1 (29) with HindIII. The noncomplementary and transversion mutations were introduced into the SWS2up1.4kb:EGFP (Figs. 2 and 5A) by the site-directed mutagenesis using the QuikChange kit (Stratagene, Tokyo, Japan). All the mutated sequences were sequenced to confirm that no spurious mutation was incorporated to the insert DNA or to the GFP coding region. The deletions were introduced by PCR using the circular form of the SWS2up1.4kb:EGFP (Fig. 3A) and SWS2up5.2kb:EGFP (Fig. 4A) constructs as the template (see Table S3 and Table S4, respectively, for PCR primers) and were confirmed by DNA sequencing. The deletion constructs using the SWS2up1.4kb:EGFP were sequenced further to confirm the absence of spurious mutations in the insert DNA and the GFP coding region. The deletion constructs using the SWS2up5.2kb:EGFP were made twice by independent PCRs and the two PCR products were mixed to compensate for potential PCR errors. The EXRHOup147bp and PINE-OTX-EXRHOup147bp (Fig. 5B) were obtained by PCR from the zebrafish genomic DNA using primers listed in Table S5.

The constructs were linearized with a restriction enzyme at one site in the vector backbone and were purified by agarose gel electrophoresis. The final concentration and the amount of the DNA constructs was adjusted to 50 ng/μl and 0.5–1 nl/embryo. The DNA constructs were microinjected to the cytoplasm of 1–2 cell stage of zebrafish embryos. At 7–8 days post-fertilization (dpf), the percentage of the eyes with GFP signal among all the eyes examined was taken as a measure of the efficiency of the transgene expression. The GFP-positive eyes were further distinguished into three levels by the number of GFP-positive cells per eye as follows: + + + (+50 cells), + + (5–50 cells), and + (1–4 cells), as in previous studies (30, 31). Whereas the + + + and + + were taken as a significant sign of the expression induction, the + was not. The selection of transgenic lines in the subsequent generations was performed as described previously (29).

Immunohistochemistry—Immunostaining was carried out for whole-mount retinas of 5–8 dpf larvae or adult retinal sections by following Luo et al. (30). Antibodies against zebrafish opsin raised in rabbits were provided by T. Vihmel and D. Hyde (University of Notre Dame, South Bend, IN) (24) and were used to stain cone photoreceptor cells. The Cy3-conjugated anti-rabbit IgG was used as a secondary antibody. Images of GFP and Cy3 fluorescence were captured using a Zeiss 510 laser-scanning confocal microscope (Zeiss, Thornwood, NY).

RESULTS

Genomic Region between 0.6 and 0.3 kb 5’ of Zebrafish Blue Opsin Gene SWS2 Contains the Necessary and Sufficient Regulatory Sequences for its LSC-Specific Expression—A series of GFP reporters was constructed containing 5.2 kb to 300 bp 5’ of the zebrafish SWS2 gene from the initiation codon (Fig. 1A) and were subjected to microinjection. As shown in Fig. 1A, the injection of the GFP constructs with the 5.2-kb to 582-bp upstream regions resulted in a high level of GFP expression in the retina, with a tendency, however, that the number of posi-
The OTX sites are indicated with region surrounding the region at 7–8 dpf in the zebrafish retina. A physical map of the genomic untranslated regions are indicated with EcoRI; and HincII for each of the constructs is indicated to the positive eyes graded into the three levels. The total number of eyes examined for each of the constructs is indicated to the right histogram.

The immunostaining of the embryos at 7–8 dpf using the antibody against the zebrafish SWS2 opsin (24) revealed that the cells with the GFP signals were indeed the LSCs that produce the SWS2 opsin (Fig. 1B). The specificity of the GFP expression to the LSCs in the retina was confirmed in four lines of transgenic zebrafish carrying the 5′ regions of 5.2-kb (Tg(zfSWS2–5.2A:EGFP)), 3.5-kb (Tg(zfSWS2–3.5A:EGFP)), 1.7-kb (Tg(zfSWS2–1.7A:EGFP)), and 1.1-kb (Tg(zfSWS2–1.1A:EGFP)). In these transgenic fish, seemingly all LSCs in the retina expressed GFP (Fig. 1, C–E).

To test if the 0.3-kb region contains a sufficient regulatory region for gene expression in the LSCs, a coinjection protocol using mixed concatamers of separate DNA fragments formed upon integration into the genome (32) was employed. The −595/−282 and −497/−282 fragments of the zebrafish SWS2 upstream region were injected, respectively, together with a GFP construct to which the 853 bp 5′ of the zebrafish UV opsin gene SWS1 was attached (SWS1up853bp:EGFP) (Fig. 1F). The 853 bp contains the region necessary for SSC-specific expression (30). Consistently, our microinjection of the SWS1up853bp:EGFP alone resulted in appearance of GFP-positive cells in the retina, among which only less than 1% (3/335) appeared overlapped with the LSCs immunostained with the antibody against the zebrafish SWS2 opsin (Fig. 1F). The addition of the −595/−282 and −497/−282 fragments increased the proportion to 24% (93/382) and 15% (43/286), respectively. These results indicate that the 0.3-kb region between 0.6 and 0.3 kb 5′ of the SWS2 contains the region necessary and sufficient for its expression in LSCs.

**OTX Sequences in the SWS2 Promoter Are Involved in the LSC-specific Gene Expression**—The 5.2-kb 5′ sequence of the zebrafish SWS2 contains 15 OTX sequences (GATTA or its reverse complement TAATC) (Fig. 1A), among which four (at −455, −430, −400, and −316) are clustered in the proximal 0.2-kb portion of the 0.3-kb region discussed above (Fig. 2). To examine if the four OTX sites are relevant to the LSC-specific gene expression, the OTX sequence GATTA was replaced with a reverse complement TAATC (Fig. 1B). The specificity of the GFP expression to the LSCs in the retina was confirmed in four lines of transgenic zebrafish carrying the 5′ regions of 5.2-kb (Tg(zfSWS2–5.2A:EGFP)), 3.5-kb (Tg(zfSWS2–3.5A:EGFP)), 1.7-kb (Tg(zfSWS2–1.7A:EGFP)), and 1.1-kb (Tg(zfSWS2–1.1A:EGFP)). In the transgenic fish, seemingly all LSCs in the retina expressed GFP (Fig. 1, C–E).

When the mutation was introduced to a single OTX site three levels. The right histogram shows the percentage of the LSCs among the GFP-positive cells in the retina evaluated with the immunostaining using the anti-SWS2 antibody. The total number of eyes examined for each of the constructs (n) and the number of LSCs/the number of GFP-positive cells are indicated to the right of the histograms.
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among the four (OTXmut-1 to -4 in Fig. 2), expression level did not seem to be affected. When the mutation was introduced to two of the four sites (OTXmut-5 to -10), the GFP expression was nearly abolished in the cases where the most distal site (−455) was involved (OTXmut-7, -9, and -10) but not in the other three cases. When the mutation was introduced to three of the four sites (OTXmut-11 to -14), the GFP expression was nearly absent in all cases. We confirmed that all the GFP-expressing cells were the LSCs by immunostaining using the antibody against zebrafish SWS2 opsin (data not shown). These results suggest that the four OTX sites exert individually minor but synergistically significant promoting effects on the gene expression in the LSCs, with the distal-most site at −455 being most effective in the synergy.

We introduced a series of deletions into the 0.3-kb region between 0.6 and 0.3 kb 5’ of SWS2 in the SWS2up1.4kb:EGFP (del-1 to -6 in Fig. 3A), injected the deletion constructs to zebrafish embryos, and scored the GFP expression. Consistent with the injection experiment using the OTX mutations, the deletion of the proximal 0.2-kb portion (Δ−497/−282; del-2 in Fig. 3A), containing all the four OTX sites, resulted in nearly complete loss of GFP expression. Also consistent with the result using the OTXmut-10 (Fig. 2) where the GFP expression was abolished, the deletions, including the distal two OTX sites (del-1, -2, -4, and -6) resulted in considerable reduction of GFP expression. On the other hand, deletion of the distal 100-bp portion (Δ−595/−498; del-3) had a less severe effect on the expression induction. These results suggest that the central 0.1-kb portion in the 0.3-kb region, containing distal two of the four OTX sites, plays a major role in promoting gene expression in the LSCs.

SWS2 Promoter Contains the Expression Repressor for the UV-sensitive SSCs—Unexpectedly, deletion of the proximal 0.1-kb portion (Δ−404/−282; del-5 in Fig. 3A), containing the proximal two of the four OTX sites, of the 0.3-kb region resulted in not a decrease but a significant increase of GFP
expression in the retina. The intensity of the fluorescence per cell also seemed higher than that by the control construct (data not shown). The immunostaining by the antibodies against the zebrafish opsins revealed that the GFP signals were not only from the LSCs but also from SSCs producing the SWS1 (UV) opsin (Fig. 3B). The GFP signal was not found in the other photoreceptor types, i.e. the rods, long members of double cones and short members of double cones producing the RH1 (rod), M/LWS (red), and RH2 (green) opsins, respectively. When five embryos were examined at 7–8 dpf, to which the del-5 construct was injected, 47% (335/715) of the GFP-expressing cells were LSCs in total, ranging from 38 to 53% among the five embryos (Fig. 3C). Another five embryos were examined for GFP expression in SSCs, and 62% (185/298) of the GFP-positive cells were SSCs, ranging from 53 to 84% among the five (Fig. 3C).

A similar deletion ($\Delta = 370/-282$; del-8) also resulted in the GFP expression in SSCs but not the overall increase of the expression unlike the del-5 case (Fig. 3A). When seven embryos were examined to which the del-8 construct was injected, 45% (83/184) of the GFP-expressing cells were LSCs in total, ranging from 0 to 62% among the seven (Fig. 3C). Four more embryos were examined for GFP expression in SSCs, and a high proportion (74% (84/114)) of the GFP-positive cells were SSCs, ranging from 52 to 83% among the four (Fig. 3C). These results suggest that the repressive sequence(s) for SSC expression is located between $-370$ and $-282$. The results could also suggest that the repressive sequence(s) for LSC expression may lie between $-404$ and $-370$, but the deletion $-425/-371$ (del-7) did not result in an increase of overall expression, obscuring its location possibly because the effect of the repressor loss may be compromised by the concomitant elimination of one activating OTX site. The location of the possible repressive region for LSC expression was not explored further.

To localize the repressive element(s) for the gene expression in SSCs, we further introduced a series of deletions in the 89-bp region between $-370$ and $-282$ (del-9 to -15 in Fig. 3A). The GFP expression in the SSCs was observed in the cases using the del-9 ($\Delta = 370/-334$), del-10 ($\Delta = 333/-310$), and del-14 ($\Delta = 341/-327$) (Fig. 3A). However, the proportion of the SSCs in the GFP-positive cells was much lower than the cases of del-5 and del-8; in 14–18 embryos examined, the proportion was 17% in del-9, 12% in del-10, and 21% in del-14 (Fig. 3C). On the other hand, the proportion of the LSCs in the GFP-positive cells, examined in the other 20–42 embryos, were much higher as follows: 83% (del-9), 92% (del-10), and 87% (del-14) (Fig. 3C). These results suggest that the minimum of 15-bp portion (AGTCAGGTTTGGGTG) between $-341$ and $-327$ is necessary to repress gene expression in SSCs and its surrounding region between $-370$ and $-282$ enhances its repressive ability.

**SWS2 Promoter Contains Expression Repressors for Pineal Cells and Retinal Bipolar Cells**—During the experiments thus far, we noticed that when deletions are involved in the 0.2-kb region between 0.6 and 0.4 kb 5’ of SWS2 in the SWS2up1.4kb:EGFP, the GFP expression was observed in pineal cells or retinal bipolar cells. To identify the possible repressive sequence(s) for the SWS2 expression in pineal cells or bipolar cells, a series of deletions was introduced to the 0.2-kb region in the GFP construct that a longer upstream region 5.2-kb was used (SWS2up5.2kb:EGFP). These constructs were injected to the zebrafish embryos as described above, and the GFP expression in the pineal gland and retinal bipolar cells was evaluated at 5–7 dpf (Fig. 4A). Whereas the expression level of GFP in the bipolar cells was graded by the number of GFP-positive cells per eye as in the cone cells, that in pineal cells was not distinguished by the numbers because the pineal organ is much smaller than the retina. The bipolar cells with GFP signal were identified by their morphology and location in the retina relative to the photoreceptors.

In the pineal cells, expression of GFP was prominent when the proximal 0.1-kb portion of the 0.2-kb region was deleted ($\Delta = 497/-405$; del-c in Fig. 4A) compared with the cases of no deletion (SWS2up5.2kb:EGFP), of entire 0.2-kb deletion ($\Delta = 595/-405$; del-a), of the distal 0.1-kb deletion ($\Delta = 595/-498$; del-b), and of the central 0.1-kb deletion ($\Delta = 549/-451$; del-d), suggesting that the repressive element for the pineal cells is located in the proximal 0.1-kb portion of the 0.2-kb region. The repressive region for the pineal expression was explored further by deletions e–h (Fig. 4A) and was confined to the 24-bp region between $-450$ and $-427$ by del-g (see
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**A**

![Diagram A](image)

**B**

![Diagram B](image)

**FIGURE 5. Identification of PINE by site-directed mutagenesis.** A, expression levels of the SWS2up1.4kb:EGFP reporter with mutations introduced to the del-g region in the zebrafish pineal and bipolar cells at 5 dpf. The OTX sites are indicated with ovals in the structural map of the SWS2up1.4kb:EGFP. The nucleotide sequence of the del-g region in the intact construct and the altered nucleotides from it in the mutagenized constructs are shown. The names of the mutation constructs are indicated to the left of the chart. Histograms are as shown in Fig. 4A. B, expression of the EXRHOup147bp:EGFP reporter with the PINE and the OTX sequences in the 5-dpf zebrafish pineal cells. The histogram is shown as in Fig. 4A.

Fig. 4B for the fluorescent image). These results also suggest the distal 0.1-kb portion contains some activating sequence for the pineal expression although this was not explored further.

In the bipolar cells, a significant level of GFP expression was observed only when the del-g construct was used (Fig. 4A; see Fig. 4C for the fluorescent image). In contrast, injection of the del-f construct resulted in only a low level of bipolar expression although the deletion in the construct encompassed that of the del-g (Fig. 4A). These results suggest that the 24-bp region between −450 and −427 region contains the repressive element not only for the pineal expression but also for the bipolar expression and that the region between −426 and −405 may contain some activating sequence for the bipolar expression.

An 11-bp Sequence and an OTX Sequence Repress the SWS2 Expression in the Pineal Organ—To locate the repressive elements in the −450/−427 region for the pineal expression and the bipolar expression, consecutive 3-bp mutations were introduced to this region in the SWS2up1.4kb:EGFP construct (Fig. 5A). The mutation constructs were injected to the zebrafish embryos and the GFP expression was evaluated at 5 dpf.

In the pineal cells, all mutations introduced in the region between −441 and −431, and its adjacent OTX. In bipolar cells, prominent expression of GFP was observed only when the mut-8 and, in a lower degree, the mut-7 were used where the central three nucleotides and the 5’-most nucleotide of the OTX sequence were mutated, respectively (Fig. 5A). This suggests the involvement of the OTX sequence at this position in repression of gene expression in the bipolar cells.

To test if the 16-bp sequence between −441 and −426 is sufficient for repressing the gene expression in the pineal cells, the 16 bp was attached to a GFP expression construct in which the 147 bp 5’ of zebrafish exrho is conjugated to the GFP reporter (EXRHOup147bp:EGFP) (Fig. 5B). The 147 bp contains the region necessary for pineal organ-specific gene expression (28). The construct was introduced to the zebrafish embryos as above, and the GFP expression in the pineal was evaluated at 5 dpf. Whereas injection of the EXRHOup147bp:EGFP resulted in GFP expression in 33% of the observed larvae, addition of the 16-bp sequence decreased the proportion to 1.4% (Fig. 5B), demonstrating the repressive ability of this region comprising one OTX and the adjacent 11-bp sequences in the pineal expression. We thus named the 11-bp portion as the pineal negative regulatory element (PINE).

**DISCUSSION**

In this study we showed that a 0.3-kb region between 0.6 and 0.3 kb 5’ of the SWS2 initiation codon, encompassing four OTX sites, contains the region necessary and sufficient to drive gene expression in the long single cones (LSCs) in the retina and that its central 0.1-kb portion plays a major role in the expression. We also showed that the 0.3-kb region contains repressive sequences for the SWS2 expression in other ontogenetically related cell types, i.e. the UV-sensitive short single cones (SSCs) and the bipolar cells in the retina and the pineal cells (Fig. 6). For a cone opsin gene of any vertebrate, this is the first identifica-
tion of a cis-acting regulatory region with such dual functions identified. This is also the first report of elemental sequences responsible for the cell type-specific expression of a cone opsin gene in a cis-regulatory region.

The SWS2 opsin gene is physically linked 5’ adjacent to the M/LWS opsin gene (33–35). A locus control region (LCR) was identified as the regulatory region of the Old World primate M/LWS opsin gene array at its 5’-flanking region and enables the mutually exclusive expression of the L and M opsin genes in a single cone cell (36, 37). The M/LWS LCR is highly conserved among mammals, including marsupials and monotremes (38), in an ~60-bp region containing the OTX sequences. Because of its location between the SWS2 and M/LWS opsin genes, the M/LWS LCR is suggested to function also as a bidirectional enhancer to control the SWS2 gene expression (38). However, we showed here that the expression of the zebrafish SWS2 is controlled by its own regulatory region. In zebrafish the mammalian M/LWS LCR is not traceable between SWS2 and LWS-1, and the bidirectional control hypothesis of the M/LWS LCR is not substantiated.

The deletions in the reporter constructs in our study affected its expression in only the LSCs and the SSCs among all types of photoreceptor cells. This suggests that the SWS2 shares a regulatory mechanism with SWSJ in zebrafish. Indeed, exposure of zebrafish embryos to exogenous retinoic acid decreases SWS1 and SWS2 opsin genes. suggesting also an interrelated expression regulation between the M/LWS and the SWS2 gene expression together (39). In rainbow trout, a class of cone cells switches expression from the UV opsin (SWS1) gene to the blue opsin (SWS2) gene at the juvenile stage (40), suggesting also an interrelated expression regulation between the SWS1 and SWS2 opsin genes.

We showed that the 15-bp portion at −341 to −327 of the zebrafish SWS2 initiation codon was repressive for the gene expression in SSCs by introducing a series of deletions in the GFP reporter construct (Fig. 3A). Even when a portion of the 15 bp is deleted (del-9 and del-10 in Fig. 3A), the reporter expression was induced in the SSCs. This suggests the importance of its nucleotide sequence itself but not of its position, although the importance of the sequence remains to be tested by the mutagenesis experiment. It also needs to be examined if the 15-bp portion can repress the gene expression when it is attached to the promoter of SWSJ that is expressed in SSCs. Regarding the expression repression in the bipolar cells, it is apparent that the location of the OTX sequence is important because the same OTX sequence in another position had no effect (e.g. Fig. 4A). On the other hand, regarding the expression repression in the pineal cells, we showed that the nucleotide sequence of the 11-bp region, PINE, is directly involved; mutations in the PINE allowed the reporter gene expression in the pineal cells (Fig. 5A), and the attachment of PINE and an OTX sequence to the pineal organ-specific exorh promoter abolished the reporter gene expression in pineal cells (Fig. 5B).

Recent studies using zebrafish have shown that pineal organ-specific gene expression is regulated by the cis-acting elements associated with the OTX sequence, e.g. an E-box and OTX sequences in the arylalkylamine N-acetyltransferase gene as targets of the BMAL/CLOCK and the OTX5, respectively (41), and the PIPE sequence with its surrounding OTX sequences in exrho (28). In this study, we showed that the sequences necessary to repress gene expression in the pineal cells also have a similar composition, a cis-acting element, PINE, with an OTX sequence, suggesting an intricate involvement of the Crx/Otx family of transcription factors in both activation and repression of gene expression in pineal cells. The findings of the regulatory elements in this study would facilitate further study of the complex interaction of positive and negative regulatory factors that govern the cell type specificity of the opsin gene expression in the photoreceptive cells in the retina and the pineal organ.

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