Pineal-Specific Expression of Green Fluorescent Protein Under the Control of the Serotonin-*N*-Acetyltransferase Gene Regulatory Regions in Transgenic Zebrafish

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ABSTRACT **Zebrafish serotonin-*N*-acetyltransferase-2 (zfAANAT-2) mRNA is exclusively expressed in the pineal gland (epiphysis) at the embryonic stage. Here, we have initiated an effort to study the mechanisms underlying tissue-specific expression of this gene. DNA constructs were prepared in which green fluorescent protein (GFP) is driven by regulatory regions of the zfAANAT-2 gene. In vivo transient expression analysis in zebrafish embryos indicated that in addition to the 5′-flanking region, a regulatory sequence in the 3′-flanking region is required for pineal-specific expression. This finding led to an effort to produce transgenic lines expressing GFP under the control of the 5′ and 3′ regulatory regions of the zfAANAT-2 gene. Embryos transiently expressing GFP were raised to maturity and tested for germ cell transmission of the transgene. Three transgenic lines were produced in which GFP fluorescence in the pineal was detected starting 1 to 2 days after fertilization. One line was crossed with *mindbomb* and *floating head* mutants that cause abnormal development of the pineal and an elevation or reduction of zfAANAT-2 mRNA levels, respectively. Homozygous mutant transgenic embryos exhibited similar effects on GFP expression in the pineal gland. These observations indicate that the transgenic lines described here will be useful in studying the development of the pineal gland and the mechanisms that determine pineal-specific gene expression in the zebrafish.** Published 2002 Wiley-Liss, Inc.†

Key words: **pineal gland; zebrafish embryo; serotonin-*N*-acetyltransferase; transgenic fish; green fluorescent protein; *mindbomb*; *floating head***

INTRODUCTION

The pineal gland (epiphysis) is an unpaired brain structure, located above the third ventricle. It transduces photoperiodic information into physiological changes through rhythmic production and secretion of melatonin. In all cases, high levels of melatonin occur at night, making melatonin "the hormone of the night" (Arendt, 1995). This night/day signal is thought to synchronize other circadian rhythms and to modulate photoperiodic regulation of seasonal physiological rhythms (Arendt, 1995). Another site of melatonin production is the retina, where melatonin is thought to play a local paracrine role.

The daily rhythm in melatonin production is generated by serotonin-*N*-acetyltransferase (AANAT). The increased production of melatonin during the night reflects increased AANAT activity; rapid cessation of melatonin production by light is due to proteasomal degradation of the enzyme (Gastel et al., 1998; Falcon et al., 2001). In addition to this highly conserved post-translational mode of regulation, transcriptional regulation occurs in some species, in which AANAT mRNA levels increase 10- to >100-fold at night (Roseboom et al., 1996; Klein et al., 1997). In rodents, this process is driven by the circadian clock in the suprachiasmatic nucleus. In fish, and other nonmammalian vertebrates, the rhythm in AANAT mRNA is driven by a circadian clock located within the photoreceptor cells of the pineal gland (Bernard et al., 1997; Bégay et al., 1998). Studies in the chicken indicate this involves an E-box regulatory site (Chong et al., 2000), which is known to mediate expression of clock-controlled genes (Reppert and Weaver, 2001).

Drs. Gothilf and Toyama have contributed equally to this study.
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AANAT is encoded by a single gene in most vertebrates. However, fish contain two AANAT genes, AANAT-1 and AANAT-2, which are differentially expressed in the retina and pineal gland, respectively (Coon et al., 1999; Benyassi et al., 2000). Developmental studies in the zebrafish (*Danio rerio*, zf) embryo indicate that zfAANAT-2 mRNA is first detected in the pineal gland (epiphysis) 22 hours postfertilization (hpf). A clock-controlled, circadian rhythm in zfAANAT-2 mRNA levels begins 2 days postfertilization (dpf; Gothilf et al., 1999; Gamse et al., 2002). During the third day of development, zfAANAT-2 mRNA expression in the primordium of the parapineal can be transiently detected rostral to the pineal (Gothilf et al., 1999). At the fourth day of development, this structure disappears and a new zfAANAT-2 and opsin expression domain is situated unilaterally to the pineal (Concha et al., 2000; Gamse et al., 2002). This pattern of development of the pineal complex was first described in a salmonid more than 100 years ago (Hill, 1891) and was found in other fish species (McNutly, 1984). In contrast to the developmental pattern of expression in the pineal complex, retinal zfAANAT-2 mRNA expression is first detected on day 3 postfertilization, apparently in association with retinal photoreceptor development. These tissue-specific and temporal patterns of zfAANAT-2 expression make it an attractive model for studying the molecular basis of these characteristics of gene expression.

Here, we describe transgenic zebrafish lines that exhibit pineal gland-specific green fluorescent protein (GFP) expression under the control of the zfAANAT-2 gene regulatory regions. We demonstrate that these lines have potential utility in studying the development of pineal photoreceptor cells and identifying mutations that alter pineal development.

RESULTS

Organization of the Zebrafish AANAT-2 Gene

Two λ clones, zf6 and zf7B, were isolated from a zebrafish genomic library. Restriction enzyme mapping and nucleotide sequence analysis indicate that the two clones code for the zfAANAT-2 gene. Clone zf6 contains 1.65 kb of 5′-flanking region, the entire coding region, composed of three exons and two introns, and approximately 9 kb of 3′-flanking region. Clone zf7B contains approximately 7 kb of 5′-flanking region, the first two exons and part of the second intron (Fig. 1).

The organization of the AANAT genes isolated from chicken and mammals is conserved (Klein et al., 1997). These genes are composed of four exons and three introns. The first intron is within the 5′ untranslated region (UTR) and the other two introns interrupt the coding region at conserved locations. The zfAANAT-2 gene is composed of three exons and two introns that interrupt the coding region at the conserved sites but does not have an intron in the 5′ UTR. The absence of this intron may characterize fish AANAT genes (Coon et al., 1999).

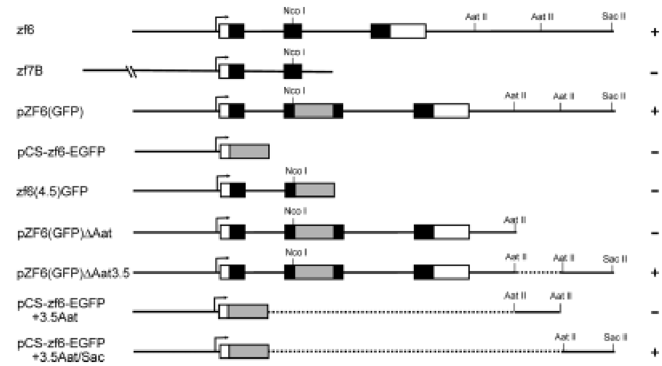

Fig. 1. Organization of zfAANAT-2 genomic clones and promoter-reporter constructs. Lines represent introns or flanking regions, white bars and black bars represent untranslated regions and coding regions, respectively. Gray boxes represent green fluorescent protein (GFP) coding regions. Dotted lines represent deletions. Clones and constructs are shown from top to bottom in the order they appear in the Results section. The plus and minus symbols on the right indicate whether the construct did (+) or did not (-) drive pineal-specific expression of the reporter.

Promoter Region

By using 5′ rapid amplification of cDNA ends (RACE), RNAse protection assay and primer extension, the transcription start site was determined to be adenosine, situated 123 bp upstream of the first ATG start codon. The zfAANAT-2 gene has a TATA-less promoter, and the transcription start site with its surrounding bases are similar to the Initiator element (Inr; Chalkley and Verrijzer, 1999) (Fig. 2). The area -215 to -15 is characteristic of a CpG island. Computer analysis of the 5′-flanking region (Fig. 2) revealed the presence of numerous sequence elements in close proximity to the start site that may bind known transcription factors and stabilize or destabilize transcription initiation. Of particular interest are the E-box centered at position -87 from the transcription start site and an adjacent DeltaE binding site centered at -94 (Yasui et al., 1998). Computer analysis also identified two binding sites for the rod cone and orthodenticle homeobox proteins (CRX/OTX) (Li et al., 1998) at positions -283 and -353 and for the ubiquitous zinc finger transcription factor SP1 (Kadonaga et al., 1987) centered at position -60. Interestingly, a novel stretch of 18 bp at position -9 to -26 is also present in the chicken AANAT promoter, upstream on the opposite strand. More distal, an imperfect cAMP-responsive element (Benbrook and Jones, 1994) is located at position -1405.

Transient Expression of Promoter-Reporter Constructs

DNA of λ clones zf6 and zf7B and mouse AANAT λ clone (Roseboom et al., 1998), as a control, were microinjected into the cytoplasm of one- or two-cell stage zebrafish embryos. Embryos were collected at 36 hpf and zfAANAT-2 mRNA was detected by whole-mount in situ hybridization (ISH). A very strong and specific

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ATTTTCTAGA CCTGGTAAGA ACTCTGGCAG CTGCATTTTG TACTAATTGA -1601
AGTTCGTTAA TTGAGGATGC TGGGCTGCCT GCAAACAGAT CATTAGAGTA -1551
GTCCAGCCTA GAAGTCATAA AAGTATTGAC TAACTTGGAG CACCATCTGC -1501
TGTTAAAATC TATCCTAGAG TGCCACGTGC TGTTAAAAAC TGGCATAGCC -1451
                                               CREBP1
TAGAGCGCCA TCTGCTGTTA AAAGCTAGCC TAGAATGCCA TTTTATGTTA -1401
AAAACTAGCC TAATGCACCA TCTGCTAGTA AAAACTAAAG TAGCCTAGAA -1351
CACCATCTGG TGGTAAAAAC TAACCTAGAC TGCCACCTTG TGTTAAAAAC -1301
TAAGGTAGTC CAGAACGCCA TCTGGTGGTA AAACCTAGTC TAGAATCTCA -1251
TTTTCTGTTA AAACTAGTCT AGAGCACCAT CTGCCAGTAA AAACTAACCT -1201
GGATTGCCAT CTGCTGTTAA AAACTATGGT GGCCTAGAGC ACCAAGTTCT -1151
GGTAAAAACT AGAGTAGCCT AGAGCGCCAT CTGCTGTTAA AAACTAACCT -1101
GGATTGCCAT CTGCTGTTAA AAACCAAGTG TAGCCTAGAG CAACAAGTTC -1051
TGGTAAAAAC TAGAGTAGCC TAGAGCGCCA TGCCCCTGCT AAAAACTAAC -1001
TTGGATTGCC ATGTGCTGTT AAAACCCGAG TGTAGCCTAG AGCAACAAGT  -951
TCTGGTAAAA ACTAGAGTAG CCTAGAGCGC CATCTGCTGT TAAAAACTAA  -901
CCTGGATTGC CATCTGCTGT TAAAAACCAG TAGCCTAGAG CAACAAGTTC  -851
TGGTAAAAAC TAGAGTAGCC TAGAGCGCCA TCTGCTGTTA AAAACTAACC  -801
TGGATTGCCA TCTGCTGTTA AAAACCAAGT GTAGCCTAGA GCAACAAGTT  -751
CTGGTAAAAA CTAGAGTAGC CTAGAGCGCC ATCTGCTGTT AAAAACTAAC  -701
CTGGATTGCC ATCTGCTGTT AAAAACCAAG TGTAGCCTAG AGCAACAAGT  -651
TCTGGTAAAA ACTAGAGTAG CCTAGAGCGC CTTCTGCTGT TAAAAACTAA  -601
CCTGGATTGC CATCTGCTGT TAAAAACCAA GTGTAGCCTA GAGCAACAAG  -551
TTCTGGTAAA AACTAGAGTA GCCTAGAGCA CCATCTGCTG TTAAAAACTA  -501
AGCTCAGAAT CAATTCAAGA GAGCAGTTTG ATATTTTTCA GAAGTGATGC  -451
AGAAGCAATT TCTCAAATGT GTATTTGTCC ACAGCTCTAA TAGATGTTTT  -401
                                                CRX/OTX
ATTTTTTGGA GCTTCATTCC GAAGTTAGGA CTATAACACT TCTGTTAATT  -351
GTACCTGTGG GTTTTATTTA TCATCTGACA ATCTCATCCA TGACCCATGA  -301
                CRX/OTX
CTAGTTCTCA CTGTCTTAAT TCTCTGCGGC TTGATGAATT TGGAAACTGC  -251
AGATATGAAA AAGCAAGCAG GACTGGCTTA ACCAGCGCAA AGCTTTCTCT  -201
GGCTGTACAA ACAGTTTCTG GTAAGTGTTT GGAGAGGCTG ATCAGCTGCG  -151
CGTTGAGTAC CACAGGCTTT CAGCTCACAG ACTTTTAGGG AATTGCTGCA  -101
    DeltaE    E-Box                          SP-1
TCTTCACCTG GCACGTGTGC GCACTTTAGG AGATCAGGGG CGGGCTGGAA   -51
AACAGACGGG ATGAGGGGAG GGTTAAAGGC GAGGAGAGGT GATAGGATTC    -1
AAACACATCA CACCACTGAG ACGAGCAGAC AAATCCACCA ACAGACAGAC   +50
TGAAGTTTCC TAAAAGTCAG GAGCTCGGGT TAGTTTGGTC AGTACAGTAT  +100
TGTCTAAAGT GTGCGCGTGT CAGATG                            +126
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Fig. 2. Zebrafish AANAT-2 promoter. Sequence of the 5′-flanking region and 5′ untranslated region. Transcription start site (+1) is in boldface and italic type. The putative initiator (Inr) sequence is underlined. Putative binding sites for transcription factors are boxed and labeled accordingly. The ATG translation start codon (+124–126) is shaded.

zfAANAT-2 signal was detected in the epiphysis after 1.5 hr of staining in approximately 30% of the embryos injected with λ clone zf6 (Fig. 3A). In contrast, embryos injected with the zf7B clone or mouse AANAT λ clone failed to generate a signal at this time (Fig. 3B,C); a weak signal representing endogenous zfAANAT-2 expression was detected after staining for 8 hr (data not shown). The large difference in the staining intensity among these different λ clone-injected embryos suggests that λ clone zf6 contains the regulatory elements required for pineal-specific expression in the zebrafish embryo.

To confirm the above findings, sequence encoding GFP was inserted in frame into exon II of the zfAANAT-2 gene. The resulting construct, pZF6(GFP) (Fig. 1), was microinjected into one- and two-cell stage zebrafish embryos, the embryos were fixed at 36 hpf, and GFP mRNA was detected by using whole-mount ISH. This strategy resulted in a specific signal in the pineal gland in ~35% of injected embryos with no extra-pineal expression in the remaining embryos (Fig. 3D), demonstrating that pZF6(GFP), like zf6 λ DNA, contains regulatory elements that confer pineal-specific expression.

Constructs containing the 5′-flanking region and 5′ UTR upstream of the β-gal or EGFP reporters—pCSzfNAT-nβgal and pCS-zf6-EGFP (Fig. 1) —were also injected. In contrast with the above results, microinjection of these did not produce a consistent pattern of expression; rather, in ~25% of the injected embryos, expression was randomly scattered throughout the body and expression was not observed in the pineal gland (data not shown). The failure of the 5′-flanking region to drive pineal-specific expression, compared with the results obtained with the entire genomic clone indicates that important regulatory elements are present in other regions of the gene, e.g., introns and/or 3′-flanking region.

The influence of the first intron on promoter activity was tested by microinjection of zf6(4.5)GFP (Fig. 1) followed by whole-mount ISH analysis for GFP mRNA. Microinjection of this construct resulted in scattered GFP mRNA expression; no GFP mRNA signal was detected in the pineal (data not shown). These results suggest that the regulatory elements that confer pineal-specific expression are downstream of the first intron.

To test the influence of 3′-flanking sequences, 7 kb of the 3′-flanking region was deleted from pZF6(GFP). Surprisingly, microinjection of the deleted construct pZF6(GFP)ΔAat did not result in GFP mRNA expression in the pineal of 2-day-old embryos; expression was inconsistent with no apparent pattern (data not shown). On the other hand, a smaller deletion of a 3.5-kb *Aat*II fragment, pZF6(GFP)ΔAat3.5 (Fig. 1), did not eradicate promoter activity. Microinjection of this construct resulted in pineal-specific GFP mRNA expression in ~30% of injected embryos, similar to the results obtained with pZF6(GFP). The addition of this deleted 3.5-kb *Aat*II fragment to the pCS-zf6-EGFP construct, giving rise to pCS-zf6-EGFP+3.5Aat (Fig. 1), did not confer pineal-specific GFP expression (data not shown). These results suggest that there are tissue-specificity regulatory elements in the downstream-most 3.5-kb fragment of the zf6 clone.

Based on the above findings, the distal 3′-*Aat*II/*Sac*II 3.5-kb fragment was recovered and cloned into pCS-zf6-EGFP. Microinjection of this construct, pCS-zf6-EGFP+3.5Aat/Sac (Fig. 1), restored promoter activity as indicated by a strong exclusive fluorescence in the pineal cells (Fig. 4A). This construct was injected into several hundred embryos in a series of experiments. Expression was consistently observed to occur in ~30% of injected embryos; extra-pineal expression was not seen in the remaining embryos. The pineal signal was detected up to 6 dpf, probably reflecting accumulation of the stable GFP rather than production of new GFP.

Generation of a zfAANAT2-GFP Transgenic Line and Its GFP Expression

The transient expression studies suggested that pCS-zf6-EGFP+3.5Aat/Sac contains sufficient regulatory elements to drive GFP expression in a tissue-

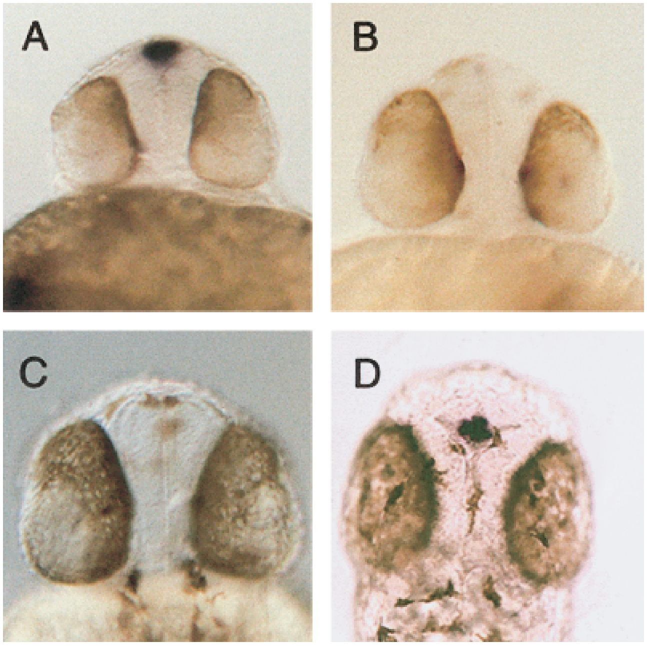

Fig. 3. zfAANAT-2 genomic clone (zf6) contains sufficient regulatory elements to drive transient pineal-specific expression of reporter gene. Whole-mount in situ hybridization (ISH) for zfAANAT-2 mRNA (A–C, frontal view) or green fluorescent protein (GFP) mRNA (D, dorsal view) in 30–36 hpf embryos after microinjection of λ genomic clones zf6 (**A**), zf7B (**B**), mouse AANAT gene (**C**), and plasmid construct pZF6(GFP) (**D**). Embryos were kept in constant darkness. ISH procedure was done as described, except that staining was performed for only 1.5 hr instead of the usual 8 hr that is done for detection of endogenous zfAANAT-2 mRNA.

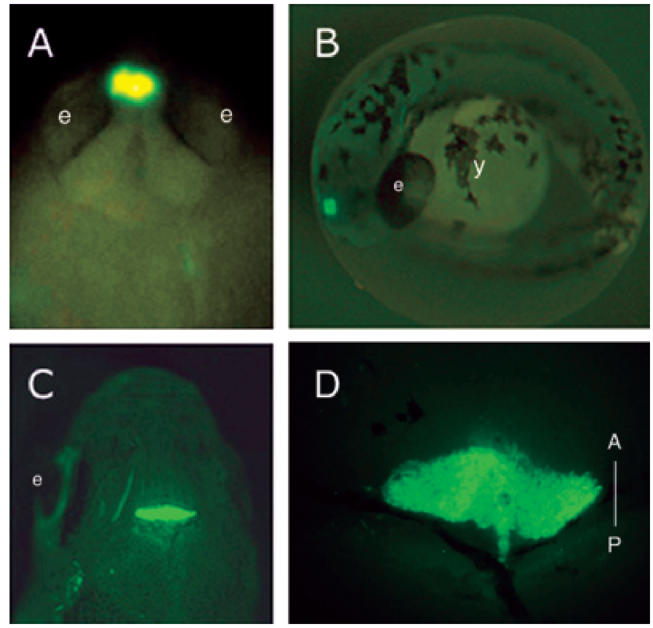

Fig. 4. Pineal-specific expression of green fluorescent protein (GFP) is driven by the 5′-flanking region and a downstream fragment. **A:** Transient expression of GFP in a 3-day-old larva after microinjection of pCS-zf6-EGFP+3.5Aat/Sac, frontal view of the head. **B:** Stable expression of GFP in a prehatched 32 hpf TG(AANAT2:EGFP)^{y8} F2 embryo inside the chorion membrane. **C:** Stable expression of GFP in 3-month-old TG(AANAT2:EGFP)^{y8} F2 adult. **D:** Higher magnification of C. Skin and brain cartilages were removed to expose the pineal gland. A-P indicates anterior-posterior orientation. e, eye; y, yolk.

specific manner and, therefore, was used in an effort to generate transgenic zebrafish lines. When this construct was injected as both linear and circular DNA, ~30% of injected embryos displayed a pineal gland-specific GFP signal. These GFP-positive larvae were raised to adulthood (founder fish).

To establish stable transgenic lines, founder fish were crossed against wild-type or crossed with each other to generate F1 embryos. Of 73 founder fish tested, two produced embryos expressing GFP in the pineal gland. With one founder, which was injected with a linearized construct, 5% of embryos expressed GFP at day 1 (12 GFP positive of 235), suggesting that the transgenic founder is a germline mosaic, common in production of transgenic zebrafish at the founder generation (Du and Dienhart, 2001). Transgenic embryos were raised to adulthood, and one female was crossed with wild-type male to produce a transgenic line, TG(AANAT2:EGFP)^{y8}. The GFP signal in this line was detectable as early as 23–24 hpf and became stronger at 32 hpf (Fig. 4B). The second founder fish, injected with the circular construct, gave rise to offspring displaying two levels of GFP expression. Among F1 progeny of this founder, 13% (89 GFP positive of 690) of embryos expressed GFP in the pineal gland at day 1 and an additional 11% (75 GFP positive of 690) were GFP positive at day 2. These GFP-positive embryos were raised to adulthood and produced two transgenic lines, TG(AANAT2:EGFP)^{y9} and TG(AANAT2:EGFP)^{y12}, respectively. In the TG(AANAT2:EGFP)^{y9} line, GFP signal was detectable at 50 hpf, whereas in the TG(AANAT2:EGFP)^{y12} line the GFP signal was detectable several hours later at 56 hpf. These results suggest that the differences in the onset of GFP expression were hereditary, possibly reflecting different integration sites or copy numbers of the injected gene. In all three lines, examined from 1 to 6 dpf, the GFP signal was observed strictly in the pineal gland; a GFP signal was not detected in the retina or the parapineal organ. The TG(AANAT2:EGFP)^{y8} line, with strong GFP expression, was used for further analysis.

Pineal gland-specific GFP expression was examined in 3-month-old transgenic progeny of TG(AANAT2:EGFP)^{y8}. The robust GFP signal was observed in the pineal gland through the skin and the skull roof (Fig. 4C). To determine the expression domain more clearly, skin and parietal bone were removed to expose the pineal gland. GFP was expressed not only in the pineal body but also in an underlying midline structure, which appears to be the pineal stalk (Gothilf et al., 1999) (Fig. 4D). An asymmetrical pattern of staining—consistent with expression in the parapineal—was not detected. Likewise, retinal GFP fluorescence was not detected. Rhythmic changes in fluorescence were not observed in the transgenic fish, presumably because the expressed GFP protein is stable. However, preliminary data indicate that TG(AANAT2:EGFP)^{y8} larvae exhibit a daily rhythm of GFP mRNA expression (data not shown).

GFP Expression Under Mutant Background

As we reported previously, the expression of the zfAANAT-2 gene is influenced by two zebrafish mutants, *floating head* (*flh*^{n1}) and *mindbomb* (*mib*^{ta52b}; Gothilf et al., 1999). zfAANAT-2 mRNA levels are reduced in the *flh* homozygote embryos due to the reduction in the number of the pineal photoreceptors (Masai et al., 1997). In contrast, zfAANAT-2 expression is upregulated in the *mib* embryos due to the overproduction of neuronal cells (Schier et al., 1996). In view of this, the expression of GFP under the control of the zfAANAT-2 promoter was examined in these mutant backgrounds to determine whether it behaved as the endogenous zfAANAT-2 gene.

Heterozygote mutant carriers (*flh*$^{+/-}$ or *mib*$^{+/-}$) were crossed with TG(AANAT2:EGFP)^{y8} heterozygote fish and only GFP-positive offspring, heterozygous for GFP, were raised to sexual maturity. The mature GFP$^{+/-}$ F1s were crossed with each other to identify heterozygote mutant carriers (TG(AANAT2:EGFP)$^{y8+/-}$/*flh*$^{+/-}$ or *mib*$^{+/-}$) and to generate homozygous mutant transgenic embryos (TG(AANAT2:EGFP)^{y8}/*flh*$^{-/-}$ or *mib*$^{-/-}$). When F1s that are heterozygous for the mutation and for the transgene are crossed, one of four offspring should be homozygous for the mutation (*flh*$^{-/-}$ or *mib*$^{-/-}$) and among them three quarters should be GFP positive.

Homozygous mutant embryos were identified by their distinct morphologic abnormalities, and GFP expression was carefully examined at several time points. As expected, among 26 *flh* homozygous embryos, 9 did not express GFP (GFP$^{-/-}$) and 17 were GFP positive (GFP$^{+/-}$ or GFP$^{+/+}$). The GFP signal in the pineal gland was significantly reduced in these 17 *flh* mutant embryos compared with that of wild-type embryos (Fig. 5A,B). Among 25 *mib* homozygous embryos, 8 did not express (GFP$^{-/-}$) and 17 were GFP positive (GFP$^{+/-}$ or GFP$^{+/+}$). A distinct pineal GFP signal was obvious a few hours earlier in the *mib* mutants as compared to wild type; and, the signal remained stronger in the mutant even after 48 hr (Fig. 5C–F).

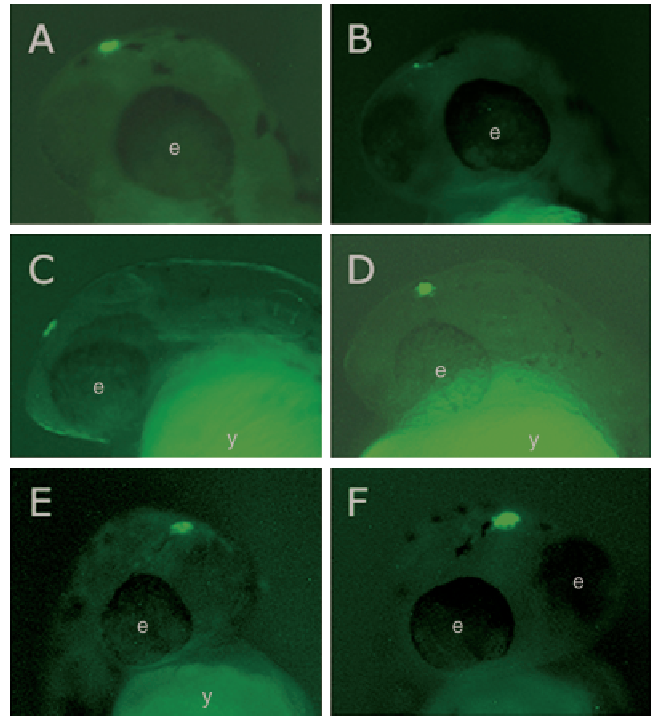

Fig. 5. Effect of mutations on green fluorescent protein (GFP) expression in transgenic fish. **A,B:** Embryos resulting from incross of *flh*$^{+/-}$; AANAT2:GFP$^{+/-}$ at 48 hours postfertilization (hpf). A: Normal sibling; B: *flh*$^{-/-}$ embryo. **C–F:** Embryos resulting from incross of *mib*$^{+/-}$;AANAT2: GFP$^{+/-}$ at 32 hpf (C,D) and 48 hpf (E,F). C and E are normal siblings; D and F are *mib*$^{-/-}$ embryos. Homozygous mutant embryos (*flh*$^{-/-}$ and *mib*$^{-/-}$) were identified by their morphology. All pictures were taken at the same light strength and exposure time.

DISCUSSION

AANAT-2 expression in zebrafish is exclusive to retinal and pineal complex (epiphysis and paraphysis) photoreceptor cells. In the zebrafish embryo, zfAANAT-2 gene expression begins at 22 hpf in the pineal primordium and at 3 dpf in the developing retina. This evidence makes the zfAANAT-2 gene an excellent marker to study the molecular basis for pineal- and retinal-specific gene expression. In this study, we have cloned and characterized the zfAANAT-2 gene and have shown by using an *in vivo* transient expression assay that both the 5′-flanking region and a 3′ fragment are needed to obtain pineal-specific expression. On the basis of this information, we produced transgenic zebrafish lines that exhibit pineal-specific expression of GFP that was affected by mutations that are known to have an effect on pineal development.

Promoter Region

As in other fish species, a clock-controlled rhythm in zfAANAT-2 mRNA levels in the pineal gland occurs in zebrafish (Bégay et al., 1998; Gothilf et al., 1999). This rhythm may be regulated by means of the E-box element at position -87; these elements are known to bind the basic-helix-loop-helix (bHLH) transcription factor heterodimer CLK:BMAL and up-regulate the expression of clock-controlled genes (Jin et al., 1999; Reppert and Weaver, 2001). E-box elements are present in other known fish AANAT promoters, including zebrafish AANAT-1 (Coon et al., unpublished results), pufferfish (Flint et al., 2001; accession no. AY016023), and a Fugu AANAT promoter (Bases 5221-8920 of scaffold number 10140, Rokshar et al., DoE Joint Genome Institute, unpublished results). Moreover, a functional E-box element has been described in the promoter of the chicken AANAT gene (Chong et al., 2000).

Recently, knockdown of zebrafish OTX-5, by microinjection of morpholino antisense oligonucleotides, was shown to decrease the amplitude of the zfAANAT-2 mRNA rhythm in the pineal gland (Gamse et al., 2002). This may occur through its binding to the CRX/OTX binding sites (also termed pineal regulatory elements; Li et al., 1998) at positions -283 and -353. This binding site is also present in the fish AANAT promoters listed above.

In the rat, the AANAT promoter is activated strongly by means of cAMP acting through a cAMP response element (CRE; Baler et al., 1997). The zfAANAT-2 promoter contains only one, imperfect, CRE site in the distal promoter region. However, the presence of this site in all other known fish AANAT promoters may indicate that this site has a role in the regulation of AANAT promoter activity.

The rhythmic activity of the zfAANAT-2 promoter may be facilitated by other putative transcription factor binding sites, including those binding SP1 and DeltaE. DeltaE is a zinc finger DNA binding protein that has been shown to repress bHLH-mediated transcription of various genes by competing with the binding of bHLH protein activators on overlapping sequence elements (Genneta et al., 1994; Sekido et al., 1994; Yasui et al., 1998). The possible function of the E-box and of the other putative transcription factor binding sites in the regulation of rhythmic expression of the zfAANAT-2 gene is yet to be tested.

Pineal Gland-Specific Expression

Although the 5′-flanking region contains regulatory elements that can supposedly drive promoter activity, the presence of a downstream fragment is required to drive pineal-specific expression of the reporter gene. Transcription factors that may be responsible for pineal photoreceptor-specific expression may be the known factors RX (Kikuchi et al., 1993), CRX (Li et al., 1998), or both (Kimura et al., 2000), or other known or novel transcription factors. The issue of the functionality of these elements in the downstream region of the gene requires further investigation.

In contrast to previous results showing zfAANAT-2 mRNA expression in photoreceptors of the pineal and retina, the genomic clones and constructs containing several kilobases of 5′- and 3′-flanking regions together did not direct expression of the reporter gene to the retina in either transient expression assays or in the transgenic lines. One explanation for this is that the elements that confer retinal expression are not present in the regions containing sequences required for pineal-specific expression; those regions responsible for retinal expression may reside elsewhere in the gene. Another possible explanation is that GFP protein does not accumulate at detectable levels in retinal photoreceptor cells, because the level of promoter activity is lower, as indicated by the low level of retinal zfAANAT-2 mRNA compared with that in pineal cells.

Characterization of Transgenic Fish

Several observations indicate that the transgenic zebrafish lines described here will be useful in analysis of the development of pineal photoreceptor cells and the molecular basis of pineal photoreceptor-specific gene expression. This belief is indicated in part by the pattern of distribution of the reporter in the adult transgenic fish, which is restricted to the pineal gland and stalk, indicating that it is expressed in photoreceptors. Moreover, mutations that cause abnormal development of the pineal (Masai et al., 1997; Schier et al., 1996), reduce (*flh*) or elevate (*mib*) zfAANAT-2 mRNA levels (Gothilf et al., 1999) and expression of GFP in the transgenic fish in a similar way.

The lines of fish described here will be useful in mass mutagenesis and screening for pineal mutations, crossing with different known mutants, and overexpression or knockdown of transcription factors that are suspected to be involved in pineal development and pineal-specific gene expression.

EXPERIMENTAL PROCEDURES

Isolation of AANAT Genomic Fragments

A zebrafish genomic library in λ FIX-II (Stratagene, La Jolla, CA) was screened at moderate stringency with a ^{32}P-labeled (Megaprime labeling kit, Amersham, Arlington Heights, IL) open reading frame (ORF) of pike AANAT-2 cDNA (Coon et al., 1999). Hybridizations were performed at 60°C in QuickHyb (Stratagene) and final washes in 0.2× standard saline citrate, 0.1% sodium dodecyl sulfate at 60°C for 30 min. Two clones (zf6 and zf7B) were identified and purified. Inserts from clones zf6 and zf7B were released by using *Not*I and subcloned into pBluescript II (Stratagene) to give pZF6 and pZF7B, respectively.

Determination of the Transcription Start Site

The transcription start site was determined by using three independent methods.

5′ RACE. First-strand cDNA was synthesized from retinal and pineal mRNA by using zf6r1 primer (5′-gtttctctttatcccagcc-3′), corresponding to nucleotides 421–439 of the zfAANAT-2 cDNA (accession no. AF124756) antisense strand. 5′ RACE was performed by using zf65 primer (5′-ttcgaaactcgctggccggt-3′), corresponding to nucleotide 225-244 of the zfAANAT-2 cDNA antisense strand, and a universal primer (5′ RACE kit, Gibco BRL). The reaction product was subcloned into pGEM-T Easy (Promega, Madison, WI) and sequenced by using an ABI automated sequencer. This clone, zf5′, contains 123 bp 5′ to the ATG translation start codon. An additional first-strand cDNA synthesis and 5′ RACE were performed by using more upstream primers; a product was obtained indicating a 5′ UTR of similar length.

RNAse protection assay. By using pZF6 as a template, a DNA fragment spanning 455 bp upstream of the ATG translation start codon was PCR amplified by using zf6f7 (5′-tcatctgacaatctcatcc-3′) and zf6r9 (5′-cgtcggatcctgacacgcgcacactttag-3′) primers. The product was subcloned into pGEM-T Easy, and the resulting clone, pF7R9, was linearized with *Nco*I for use as a template to synthesize [^{32}P]-labeled RNA probe (MAXIscript in vitro transcription kit, Ambion, Austin, TX). The probe (60 fmol; specific activity, 37,000 cpm/fmol) was hybridized with adult zebrafish eye poly(A$^+$)RNA (extracted from 20 eyes). After an overnight incubation at 42°C, unprotected fragments were degraded with a mixture of RNAse A and RNAse T1 and the protected fragment, of approximately 120 bp, was resolved on a 5% denaturing polyacrylamide gel.

Primer extension. Primer extension and sequencing reactions were performed by using px2 primer (5′-acacgcgcacaccttagacaatactg-3′), corresponding to nucleotides 95-120 of the zfAANAT-2 cDNA antisense strand. The primer was 5′-end labeled with ^{32}P by using T4 polynucleotide kinase. A mixture of 1 μg of adult zebrafish eye poly(A$^+$)RNA and 1 pmol of labeled primer was denatured at 95°C for 5 min and then allowed to hybridize at 70°C for 10 min. Reverse transcription was done at 42°C by using avian myeloblastosis virus (AMV) reverse transcriptase (Promega). After the reaction was completed, additional zebrafish eye poly(A$^+$)RNA (1 μg) was added and the mixture was denatured at 95°C for 5 min and allowed to hybridize at 70°C for 10 min; a second reverse transcription reaction was initiated with the addition of the thermophilic reverse transcriptase *Tth* DNA polymerase (Epicentre, Madison, WI) and incubated for 30 min at 70°C to disrupt secondary structures that may have inhibited AMV reverse transcriptase. The reverse transcription product was resolved on an 8% polyacrylamide gel along with a sequencing reaction of pZF6 that was performed by using the fmol sequencing system (Promega).

DNA Constructs

pCSzfNAT-nβgal. The zfAANAT-2 promoter was placed upstream of a β-galactosidase coding sequence. A 1.8-kb fragment containing 1.65 kb of 5′-flanking region and the 123-bp 5′ UTR of the zfAANAT-2 gene was PCR amplified from the λ clone zf6 as a template by using a specific primer zf6r9 containing *Bam*HI restriction site (5′-cgtcggatcctgacacgcgcacactttag-3′) and T3 primer. The product was digested with *Bam*HI and *Sal*I and ligated into *Bam*HI/*Sal*I-cut pCS-nβgal (Turner and Weintraub, 1994).

pCS-zf6-EGFP. The zfAANAT-2 promoter was placed upstream of an EGFP reporter gene. A fragment containing the 5′-flanking region and 5′ UTR of the zfAANAT-2 gene was subcloned into pCS2+EGFP vector upstream of the EGFP-coding sequence as described above for pCSzfNAT-nβgal. pCS-zf6-EGFP has another polylinker downstream of the EGFP-coding sequence for subcloning of additional regions of interest (see below).

pZF6(GFP). Sequence encoding EGFP was inserted in frame into exon II of the zfAANAT-2 gene. A GFP-coding DNA fragment was PCR amplified by using a set of GFP-specific primers containing *Nco*I restriction sites. The fragment was digested with *Nco*I and ligated into a unique *Nco*I site in exon II of pZF6 (a genomic zfAANAT-2 clone subcloned into pBluescript II, see above).

pZF6(GFP)ΔAat3.5. Part of the 3′-flanking region was deleted from pZF6(GFP). pZF6(GFP) was digested with *Aat*II and religated. This procedure resulted in a deletion of approximately 3.5 kb in the 3′-flanking region, leaving the upstream-most 2 kb and downstream-most 3.5 kb of 3′-flanking sequence.

pZF6(GFP)ΔAat. Most of the 3′-flanking region was deleted from pZF6(GFP). pZF6(GFP) was double digested with *Sac*II and *Aat*II and religated with the addition of a polylinker. This procedure resulted in a deletion of approximately 7 kb of the 3′-flanking region, leaving only 2 kb of 3′-flanking sequence.

zf6(4.5)GFP. A fragment spanning the 5′-flanking region down to exon II was placed upstream of an EGFP reporter. pZF6 was digested with *Nco*I, situated in exon II, and *Not*I, situated 5′ to the insert, and the resulting 4.5-kb fragment was inserted instead of the α-actin promoter of αp-GFP(S65A) (Higashijima et al., 1997) in frame with the GFP(S65A)-coding sequence.

pCS-zf6-EGFP+3.5Aat. Part of the 3′-flanking region was added to pCS-zf6-EGFP. pZF6 was cut with *Aat*II, and a 3.5-kb *Aat*II fragment was recovered and cloned into *Aat*II-cut pGEM-T Easy vector. The insert, a 3.5-kb *Aat*II fragment, was excised with *Apa*I and *Not*I located in the pGEM-T Easy vector and ligated into *Apa*I/*Not*I-cut pCS-zf6-EGFP downstream of the polyadenylation signal.

pCS-zf6-EGFP+3.5Aat/Sac. Downstream-most part of the 3′-flanking region was added to pCS-zf6-EGFP. pZF6 was cut with *Aat*II and *Sac*II, and the 3′-most 3.5-kb fragment was cloned into *Aat*II/*Sac*II-cut pGEM-T Easy. The insert, a 3.5-kb *Aat*II/*Sac*II fragment, was excised with *Sac*II and *Apa*I located in the pGEM-T Easy vector and subcloned into *Sac*II/*Apa*I-cut pCS-zf6-EGFP downstream of the polyadenylation signal.

Microinjection of Zebrafish Embryos and Transient Expression Assay

Plasmid DNA was purified by using a plasmid isolation kit (Qiagen, Valencia, CA). DNA from λ clones was extracted to high purity by CsCl step and equilibrium density-gradient centrifugation (Maniatis et al., 1982). The DNA constructs (Fig. 1) were diluted to a concentration of 50 ng/μl and phenol red was added to a final concentration of 0.1%. Approximately 2 nl of the diluted DNA was microinjected into the cytoplasm of one- or two-cell stage zebrafish zygotes, and development proceeded at 28°C. Injected embryos were collected at different times after fertilization, and zfAANAT-2 promoter activity was estimated in all injected embryos by one of the following methods: (1) When β-gal was used as the reporter gene, β-gal labeling was done as described (Du et al., 1997). (2) When GFP was used as the reporter gene, GFP fluorescence in live embryos was detected under a dissecting microscope (Leica MZFLIII) equipped with ultraviolet-light source and GFP filters. Alternatively, GFP mRNA was detected by whole-mount ISH (Toyama and Dawid, 1997). (3) When DNA of λ clones was injected (no reporter gene used), zfAANAT-2 mRNA levels were determined by whole-mount ISH. Each DNA construct was tested two to three times; each time, > 100 embryos were injected.

Production of Stable Transgenic Lines

To generate stable transgenic lines, pCS-zf6-EGFP+3.5Aat/Sac (300 pg) was injected into the cytoplasm of wild-type AB* zebrafish zygotes immediately after fertilization, before fertilized eggs reached the one cell stage. Both circular and *Cla*I-linearized constructs were injected. Three days after injection, embryos were examined, and GFP fluorescence-positive embryos were selected and cultured to adult stage.

Embryo and Larval Culture

Zebrafish embryos were generated by natural mating and raised at 28°C in egg water (Westerfield, 1995). Two mutant zebrafish lines, *mindbomb* (*mib*^{ta52b}; Schier et al., 1996) and *floating head* (*flh*^{n1}; Halpern et al., 1995; Talbot et al., 1995), were also studied because they exhibit abnormal pineal development (Schier et al., 1996; Masai et al., 1997) and zfAANAT-2 expression (Gothilf et al., 1999).

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